ADVANCES IN CANCER RESEARCH

VOLUME 66

ACADEMIC PRESS

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ADVANCES IN CANCER RESEARCH

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Volume 66

ACADEMIC PRESS

San Diego New York Boston
London Sydney Tokyo Toronto

This book is printed on acid-free paper. ∞

Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

International Standard Serial Number: 0065-230X

International Standard Book Number: 0-12-006666-1

PRINTED IN THE UNITED STATES OF AMERICA
95 96 97 98 99 00 BB 9 8 7 6 5 4 3 2 1

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FOUNDATIONS IN CANCER RESEARCH

THE ADVENTURES OF A BIOLOGIST: PRENATAL DIAGNOSIS, HEMATOPOIESIS, LEUKEMIA, CARCINOGENESIS, AND TUMOR SUPPRESSION

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I. Studies in England and Move to Israel: Amniocentesis and Prenatal Diagnosis on Amniotic Fluid Cells

I was born in Leipzig, Germany in 1924 and in 1933 was fortunately taken by my parents to England where I grew up. Some years after my schooling I decided to go to Israel. My original intention was to help found a kibbutz and to prepare myself for this I worked in England as a farm laborer, mainly milking cows, and went on to obtain a degree in agriculture and agricultural botany at the University of Wales in Bangor, North Wales. I then obtained a Ph.D. in plant genetics, mainly cytogenetics, at Cambridge University (Sachs, 1952a, 1953a,b; Bell and Sachs, 1953) and decided to change to work on cytogenetics and development in mammals including humans (Sachs, 1952b, 1953c,d,e, 1954a). I was able to do this in the very stimulating atmosphere of the John Innes Institute in England whose director at that time was Dr. Cyril

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Darlington, one of the early pioneers of plant cytogenetics, who gave me an appointment as a research scientist to work on mammalian chromosomes. One year after obtaining my Ph.D. I went to Israel and joined a "scientific kibbutz" in Rehovot.

When I arrived at the Weizmann Institute in Rehovot toward the end of 1952 biology was just beginning there. Since I had obtained a Ph.D. in plant genetics and spent a year at the John Innes Institute in England, working on mammalian chromosomes, I was labeled a geneticist. I was given a position to initiate research on genetics and development by Dr. Isaac Berenblum who had arrived 2 years earlier to establish a Department of Experimental Biology. The building for the department was not yet ready, so I was given a bench and a microscope in the Polymer Research Department. Because I had no other equipment I had to find something that could be done within these limitations. Since I had worked on mammalian, including human, sex chromosomes (Sachs 1953c, 1954b), I decided to determine whether the spot of condensed chromatin in the nucleus, called a chromocenter, that can be used to diagnose sex in nondividing adult human somatic cells (reviewed in Moore and Barr, 1954) was expressed during different stages of human fetal development so that it would be possible to make a prenatal diagnosis of sex. My question was whether cells in the amniotic fluid were sufficiently well preserved to be used for prenatal diagnosis of sex and for a general prenatal diagnosis of human diseases. Human amniotic fluid cells from fetuses of different ages were collected by amniocentesis. The results showed that there were well-preserved fetal cells in the amniotic fluid which could be used for prenatal diagnosis of sex (Serr *et al.*, 1955; Sachs *et al.*, 1956a,b; Sachs and Danon, 1956) and also of blood group antigens of the fetus (Sachs *et al.*, 1956c). As was suggested at the time (Sachs and Danon, 1956) this method can be applied to prenatal diagnosis of other genetic properties of the fetus. These studies using amniotic fluid cells collected by amniocentesis provided the first method that could be used for prenatal diagnosis of human diseases. This method is now widely used for prenatal diagnosis in pregnant women. My research in human genetics also included a study on fingerprint patterns in Jewish populations in Israel (Sachs and Bat-Miriam, 1957).

When the building for the Department of Experimental Biology was finally ready Dr. Berenblum, who encouraged me to go ahead and find my own way, also ensured that a supply of mice would be provided so that I could expand my activities. I first carried out some experiments on the chromosomes (Sachs and Gallily, 1955, 1956a,b) and immunological properties (Sachs and Gallily, 1956b; Feldman and Sachs, 1958; Sachs and Feldman, 1958) of tumors with different degrees of transplan-

tability. Since my main interest was the control of normal and abnormal development, I then decided to embark on some different adventures in this area. I realized that one question leads to another and that it would be advisable to choose a broad approach. I therefore decided to try and develop some new experimental systems that could be used to study the controls that regulate normal and abnormal development.

II. Hematopoiesis and Leukemia

It became clear to me that in order to analyze the development of normal and tumor cells it was necessary to develop cell culture systems in which cells could be cloned and made to develop to different cell lineages. For this I decided to use hematopoietic cells that give rise to blood cells of different lineages throughout the life of the individual. Mouse leukemias from different cell lineages induced by viruses and other agents could also be obtained, so it became possible to study *in vitro* the development in clones of normal and leukemic hematopoietic cells. I could also study the effects of infection of normal cells with leukemia viruses. The first viruses I used were the Moloney and Rauscher viruses. Since neither the normal hematopoietic nor the leukemic cells survived in the standard cell culture medium, I used feeder layers of different types of mouse cells as possible sources of factors that the hematopoietic cells may require and this worked both for the leukemic (Ginsburg and Sachs, 1961a,b, 1962) and the normal hematopoietic cells (Ginsburg and Sachs, 1963; Sachs, 1964; Pluznik and Sachs, 1965). My analysis of the molecular control of hematopoiesis therefore began with the development of a cell culture system for the cloning and clonal differentiation of different types of normal hematopoietic cells (Ginsburg and Sachs, 1963). This cell culture system made it possible to discover a family of cytokines that regulate cell viability, multiplication, and differentiation of different hematopoietic cell lineages, to analyze the origin of some hematological diseases, and to identify ways of treating these diseases with normal cytokines. I will mainly discuss cells of the myeloid cell lineages which have been used as a model system.

A. *In Vitro* Clonal Development of Normal Hematopoietic Cells

In the cell culture system that was developed, normal cells from blood-forming tissues from mice were first cultured with feeder layers of other cell types such as normal embryo fibroblasts. These other cell types were chosen as possible candidates for cells that produce the regulatory

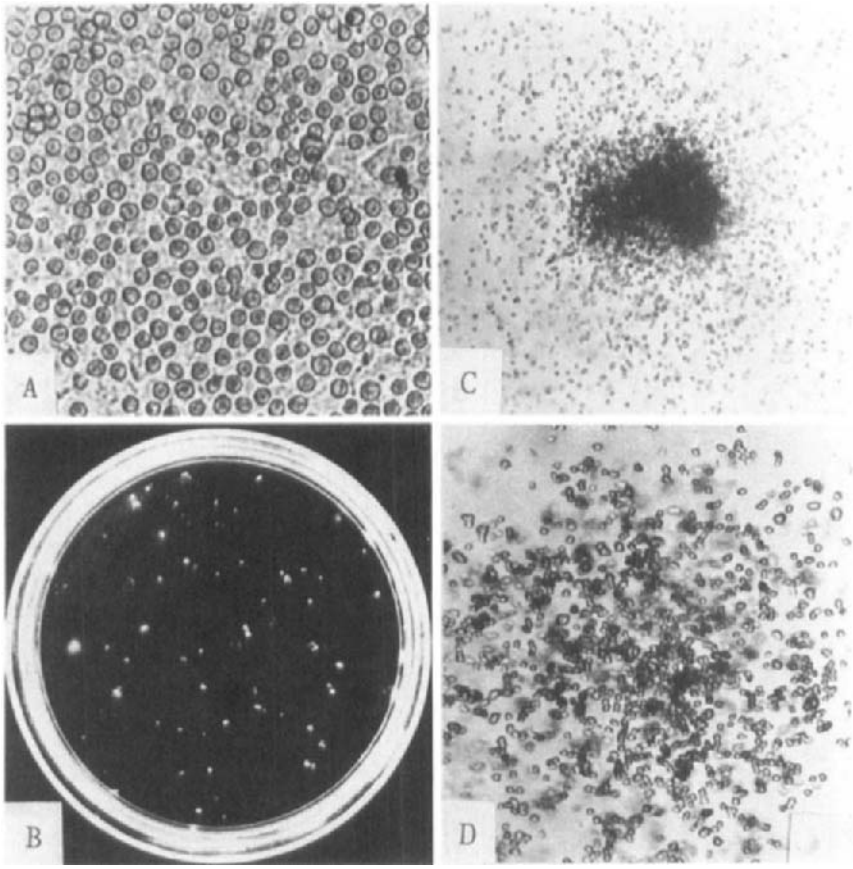

FIG. 1. Cell culture system for cloning and clonal differentiation of normal hematopoietic cells. (A) Culture of mouse mast cells that have multiplied and differentiated on a feeder layer of mouse embryo cells (Ginsburg and Sachs, 1963) (B–D) Clones of macrophages and granulocytes in cultures of normal hematopoietic cell precursors incubated with the appropriate inducer in semisolid medium containing agar. (B) Petri dish with clones (Pluznik and Sachs, 1965), (C) granulocyte clone, and (D) macrophage clone (Ichikawa *et al.*, 1966).

molecules required for the cloning and differentiation of different hematopoietic cell lineages. The first such system, published in 1963 (Ginsburg and Sachs, 1963; Sachs, 1964), using cells cultured in liquid medium (Fig. 1, Table 1), showed that by this procedure it was possible to obtain clones containing mast cells or granulocytes in various stages of differentiation. The cultures also showed differentiation to macrophages. To formulate my basic idea I wrote as the concluding sentence of this 1963 paper "The described cultures thus seem to offer a useful

TABLE I

ESTABLISHMENT OF THE CELL CULTURE SYSTEM FOR CLONING AND CLONAL DIFFERENTIATION OF NORMAL HEMATOPOIETIC CELLS AND THE USE OF THESE CULTURES TO DISCOVER COLONY-STIMULATING FACTORS

Cloning and differentiation in liquid medium (Ginsburg and Sachs, 1963)
Cloning and differentiation in agar (Pluznik and Sachs, 1965; Ichikawa *et al.*, 1966; Bradley and Metcalf, 1966)
Cloning and differentiation in methylcellulose (Ichikawa *et al.*, 1966)
Inducers for cloning and differentiation secreted by cells (Pluznik and Sachs, 1965)
Inducers for cloning and differentiation in cell culture supernatants (Pluznik and Sachs, 1966; Ichikawa *et al.*, 1966)

system for a quantitative kinetic approach to hematopoietic cell formation and for experimental studies on the mechanism and regulation of hematopoietic cell differentiation" (Ginsburg and Sachs, 1963). This 1963 paper led to such an approach.

The mast cell clones described above were obtained from cultures of thymus and the granulocyte clones from cultures of spleen. Infection of the thymus with Moloney virus and of the spleen with Rauscher virus increased the number of mast cells and granulocytes, respectively, indicating that this virus infection induced increased cell multiplication or survival (Ginsburg and Sachs, 1963). *In vivo* experiments with the Rauscher virus showed that the virus that infected nucleated erythroid cells could be assayed and cloned by a spleen colony-forming assay (Pluznik and Sachs, 1964). The culture system using feeder layers was then applied to the cloning of different cell lineages in semisolid medium containing agar (Pluznik and Sachs, 1965) which made it simpler to distinguish and isolate separate clones. This system in agar was then also used by Bradley and Metcalf (1966). Analysis of the first types of clones obtained in agar with these feeder layers showed clones containing macrophages, granuloyctes, or both macrophages and granulocytes in various stages of differentiation. The macrophage clones in agar contained many metachromatic granules giving them an apparent morphological resemblance to mast cells (Pluznik and Sachs, 1965; Ichikawa *et al.*, 1966; Bradley and Metcalf, 1966). However, these granules were not present when the cells were cloned in methylcellulose (Ichikawa *et al.*, 1966), and electron microscopy also demonstrated that these cells in agar were really macrophages that had phagocytosed agar (Lagunoff *et al.*, 1966). The experiments also showed that hematopoietic cell colonies *in vitro* (Ginsburg and Sachs, 1963; Pluznik and Sachs, 1965; Ichikawa *et al.*, 1966; Bradley and Metcalf, 1966) can originate from single cells

(Ginsburg and Sachs, 1963; Pluznik and Sachs, 1966; Paran and Sachs, 1969) and are therefore clones. This assay in agar (Pluznik and Sachs, 1965; Ichikawa *et al.*, 1966; Bradley and Metcalf, 1966) or methylcellulose (Ichikawa *et al.*, 1966)(Fig. 1, Table I) was then applied to cloning and clonal differentiation of normal human macrophages and granulocytes (Paran *et al.*, 1970; Pike and Robinson, 1970) and to the cloning of all the other blood cell lineages including erythroid cells (Stephenson *et al.*, 1971), B lymphocytes (Metcalf *et al.*, 1975), and T lymphocytes (Gerassi and Sachs, 1976). Embryo cell feeder layers were also found useful for the study of lymphocyte differentiation in a homograft response (Ginsburg and Sachs, 1965). My research on the development of cells in culture also included the *in vitro* establishment of a line of mouse myeloma cells (reviewed in Melchers *et al.*, 1978; Milstein, 1980); and studies on the formation of bone tissue from isolated bone cells (Binderman *et al.*, 1974), the development of parthenogenetic embryos (Kaufman and Sachs, 1976), and the development of neuroblastoma cells (Simantov and Sachs, 1973, 1975).

B. Discovery of Colony-Stimulating Factors

When hematopoietic cells were cloned in a semisolid substrate, such as agar, another more solid agar layer was placed between cells of the feeder layer and the hematopoietic cells seeded for cloning. This showed us that the inducer(s) required for the formation of macrophage and granulocyte clones were secreted by the feeder layer cells and can diffuse through agar (Pluznik and Sachs, 1965). This finding led to the discovery (Table I) that the inducers required for the formation of macrophage and granulocyte clones are present in conditioned medium produced by the feeder cells (Pluznik and Sachs, 1966; Ichikawa *et al.*, 1966). These inducers were found in the conditioned medium from different types of normal and malignant cells (reviewed in Paran *et al.*, 1968; Sachs, 1970, 1974a). These media were then used to purify the inducers (Landau and Sachs, 1971; Burgess *et al.*, 1977; Stanley and Heard, 1977; Lipton and Sachs, 1981; Ihle *et al.*, 1982). A similar approach was later used to identify the protein inducers for cloning of T lymphocytes (Mier and Gallo, 1980) and B lymphocytes (reviewed in Hirano *et al.*, 1990). When cells were washed at various times after initiating the induction of clones there was no further development of either macrophage or granulocyte clones unless the inducer was added again (Paran and Sachs, 1968). The development of clones with differentiated cells thus requires both an initial and a continued supply of inducer.

In cells belonging to the myeloid cell lineages, four different proteins

that induce cell multiplication and can thus induce the formation of clones (colony-inducing proteins) have been identified (reviewed in Sachs, 1986, 1987a,b, 1990, 1992, 1993). The same proteins have been given different names. After they were first discovered in cell culture supernatant fluids (Pluznik and Sachs, 1966; Ichikawa *et al.*, 1966), the first inducer identified was called *mashran gm* from the Hebrew word meaning to send forth with the initials for granulocytes and macrophages (Ichikawa *et al.*, 1967). This and other colony-inducing proteins were then renamed with different names including macrophage and granulocyte inducers (MGI) (Landau and Sachs, 1971) and MGI-type 1 (MGI-1). They are now called colony-stimulating factors (CSF) (reviewed in Metcalf, 1985; Sachs, 1987b), and one protein is called interleukin-3 (IL-3) (Ihle *et al.*, 1982) (Table II). Of these four CSFs, one (M-CSF), induces the development of clones with macrophages, another (G-CSF), clones with granulocytes, the third (GM-CSF), clones with granulocytes, macrophages, or both macrophages and granulocytes, and the fourth (IL-3), clones with macrophages, granulocytes, eosinophils, mast cells, erythroid cells, or megakaryocytes (Fig. 2, Table II). The CSFs induce cell viability and cell multiplication (reviewed in Sachs, 1987b, 1990, 1992, 1993; Lotem *et al.*, 1991a; Sachs and Lotem, 1993) and enhance the functional activity of mature cells (reviewed in Metcalf, 1985). Cloning of genes from mice and humans for IL-3, GM-CSF, M-CSF, and G-CSF have shown that these proteins are coded by different genes (reviewed in Clark and Kamen, 1987). Since the discovery of these CSFs, other cytokines have been found, including various ILs and stem cell factor (Witte, 1990), some of which can synergize with different CSFs.

It seemed unlikely that a CSF that induces cell multiplication is also a differentiation inducer whose action includes stopping cell multiplication in mature cells. I therefore looked for a protein that acts as a myeloid cell-differentiation inducer and does not have colony-stimulating activity. When we found this protein we called it macrophage and granulocyte inducer-type 2 (MGI-2) (reviewed in Sachs, 1987a,b, 1990). We showed by studies on amino acid sequence and myeloid cell differentiation-inducing activity of recombinant protein that MGI-2 is interleukin 6 (IL-6) (reviewed in Sachs, 1990) and there are presumably other normal hematopoietic cell-differentiation inducers. Studies on myeloid leukemic cells have identified other differentiation-inducing proteins called D-factor and differentiation-inducing factor (DIF) (reviewed in Sachs, 1990). D-factor was identified as a protein that has also been called HILDA and LIF, and DIF was found to be a form of tumor necrosis factor (TNF). IL-6 can induce viability and differentiation of normal myeloid precursors, but LIF and TNF which induce differentiation in

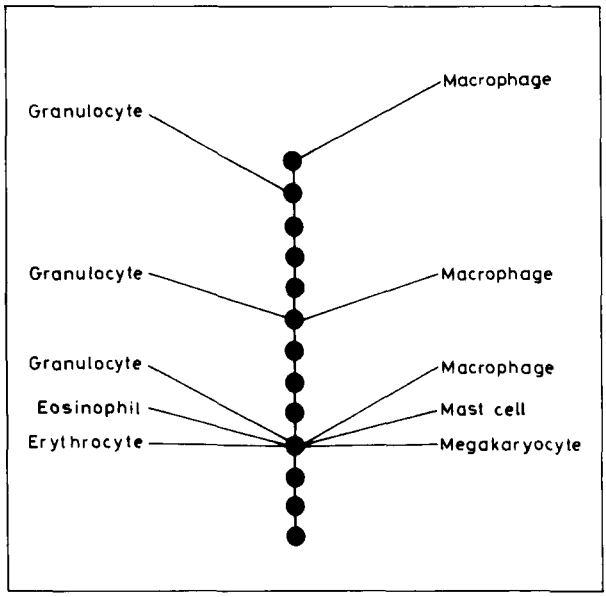

FIG. 2. Myeloid hematopoietic precursor cells can be induced to form colonies by four different colony-stimulating factors (CSFs). One (IL-3) induces the development of colonies in precursors that can develop into six cell types; the second (GM-CSF), the development of colonies in precursors that develop into two cell types; and the third (G-CSF) and fourth (M-CSF) in precursors that develop into one cell type.

certain clones of myeloid leukemic cells do not induce viability or differentiation of normal myeloid cells (reviewed in Sachs, 1990) (Table II). The existence of a multigene family of hematopoietic cytokines raised the question of whether these cytokines interact with each other.

C. HEMATOPOIETIC CYTOKINE NETWORK

We found that all four CSFs can induce the production of IL-6 which does not induce the formation of colonies but can induce myeloid precursor cells to differentiate to macrophages, granulocytes, or megakaryocytes (reviewed in Sachs, 1986, 1987a,b, 1990; Lotem *et al.*, 1989). In a colony with differentiated cells, induction of a colony by the CSFs is thus followed by production of another cytokine, IL-6, which can induce differentiation of different cell lineages. This induction of a differentiation factor by a growth factor serves as an effective mechanism to couple growth and differentiation (Fig. 3). IL-6 may switch on other, so far unidentified, factors that are required to determine the specificity of the

TABLE II

INDUCTION OF GROWTH AND DIFFERENTIATION OF NORMAL MYELOID PRECURSOR CELLS BY DIFFERENT HEMATOPOIETIC CYTOKINES

Nomenclature	Location on chromosome		Induction of colonies[a]	Induction of differentiation	
	Mouse	Human		Direct	Indirect[b]
MGI-1M = CSF-1 = M-CSF	3	5	+ (M)	–	+
MGI-1G = G-CSF	11	17	+ (G)	–	+
MGI-1GM = GM-CSF	11	5	+ (G, M)	–	+
IL-3	11	5	+ (G, M, others)	–	+
MGI-2 = IL-6	5	7	–	+ (G, M, Meg)	–
IL-1	2	2	–	–	+ (G, M, Meg)
D-factor = HILDA = LIF	11	22	–	CD	CD
DIF = TNF	17	6	–	CD	CD

[a] Colonies with macrophages (M), granulocytes (G), granulocytes and macrophages (G, M), granulocytes, macrophages, eosinophils, mast cells, megakaryocytes, or erythroid cells (G, M, others), and megakaryocytes (Meg).

[b] The four CSFs, including IL-3, and IL-1 induce production of Il-6. CD, cell death. (References in Sachs, 1990.)

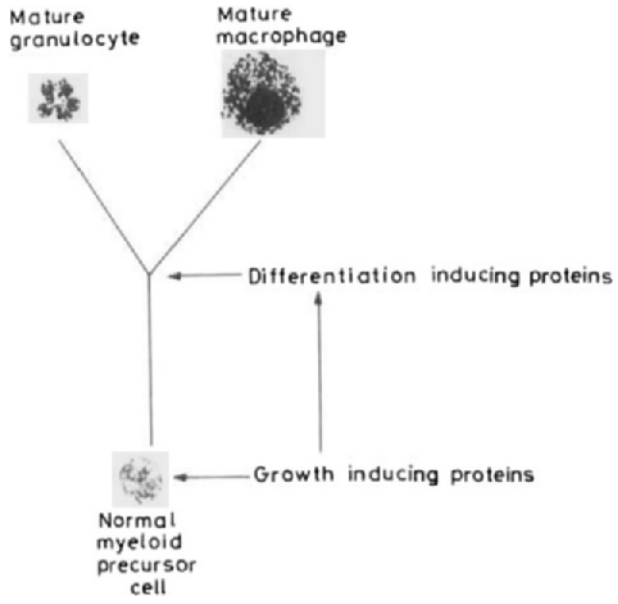

FIG. 3. The four CSFs can induce growth of normal myeloid precursor cells to form colonies. They also induce in these cells production of another cytokine, IL-6, that induces differentiation. The induction of a differentiation inducer by the CSFs provides a mechanism to couple the multiplication of normal precursor cells and their differentiation.

final cell type. This and other experiments showed that the hematopoietic cytokines function in a network of interactions (Lotem and Sachs, 1986, Lotem *et al.*, 1991b; reviewed in Sachs, 1990, 1991, 1993). This cytokine network is one of the concepts that has emerged from these studies on hematopoiesis.

Production of specific cell types has to be induced when new cells are required and has to stop when sufficient cells have been produced. This requires an appropriate balance between inducers and inhibitors of development. The network of interactions between hematopoietic cytokines (Fig. 4) therefore also includes cytokines that can function as inhibitors such as TNF. Another inhibitory cytokine, transforming growth-factor β1 (TGFβ1), which is also part of this network, can selectively inhibit the activity of some CSFs and ILs. It can also inhibit the production of some of these cytokines (Lotem and Sachs, 1990).

Parts of this network were found to function not only within the hematopoietic cell system but also in some nonhematopoietic cell types. For example, in endothelial cells that make blood vessels there is an

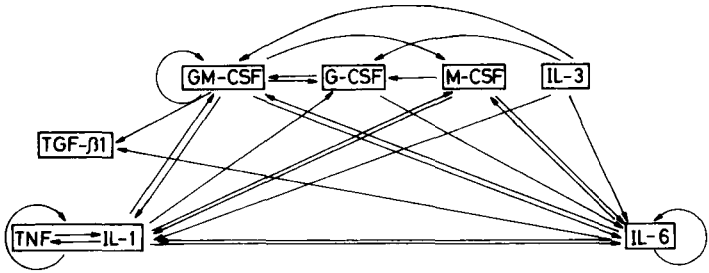

FIG. 4. The network of interactions between hematopoietic cytokines.

induction of IL-6 when new blood vessels are being formed, angiogenesis, and the production of IL-6 is switched off when angiogenesis has been completed (Motro *et al.*, 1990). The transient expression of IL-6 in the endothelial cells indicates a role for IL-6 in angiogenesis in addition to its role in regulating the development of myeloid and lymphoid hematopoietic cells. IL-6 can also induce the production of acute phase proteins in liver cells (Hirano *et al.*, 1990). The pleiotropic effects of a cytokine such as IL-6 raise the question of whether these effects on different cell types are direct or are indirectly mediated by IL-6 switching on production of other regulators that vary in the different cell types. Interpretation of experimental data on the effect of each cytokine therefore has to take into account that the regulator functions in a network of interactions, so as to avoid an incorrect assignment of a specific effect to a direct action of a particular cytokine. This network also has to be taken into account in the clinical use of these cytokines. What can be therapeutically useful may be due to the direct action of an injected cytokine or to an indirect effect due to other cytokines that are switched on *in vivo*.

A network of interactions allows considerable flexibility depending on which part of the network is activated. It also allows a ready amplification of response to a particular stimulus such as bacterial lipopolysaccharide (reviewed in Sachs, 1990, 1991). We showed that this amplification can occur by autoregulation and by transregulation of genes for the hematopoietic cytokines (reviewed in Sachs, 1990). There is also a transregulation by these cytokines of receptors for other cytokines (Lotem and Sachs, 1986, 1989). In addition to the flexibility of this network both for the response to present-day infections and to different types of infections that may develop in the future, a network may also be necessary to stabilize the whole system. Hematopoietic cytokines induce during differentiation sustained levels of transcription factors that can

regulate and maintain gene expression in the differentiation program (Shabo *et al.*, 1990). Interactions between the network of hematopoietic cytokines and transcription factors can thus ensure the production of specific cell types and stability of the differentiated state. The next questions I asked were to what extent can be the cytokines that control normal hematopoiesis also control the behavior of leukemic cells and whether there are other compounds that may have effects similar to these cytokines.

D. Normal Cytokines and Other Compounds That Control the Development of Leukemia

The first question was can myeloid leukemic cells still be induced to differentiate to mature nondividing cells by cytokines that induce differentiation in normal myeloid cells? This question has been answered by showing that there are myeloid leukemic cells (Paran *et al.*, 1970; Fibach *et al.*, 1972) that can be induced to differentiate to mature cells by a normal cytokine. The clones that can be induced to differentiate to mature macrophages or granulocytes through the normal sequence of gene expression by incubation with a normal myeloid differentiation-inducing cytokine (Fig. 5) are called D^+ clones (D for differentiation) (Fibach *et al.*, 1973). The mature cells, which can be formed from all the cells of a leukemic clone, then stop multiplying like normal mature cells (Fibach, *et al.*, 1973; Fibach and Sachs, 1974, 1975; Lotem and Sachs, 1977a) and are no longer malignant *in vivo* (Fibach and Sachs, 1975). In addition to D^+ clones that can be induced to differentiate by IL-6 which can also be induced to partially differentiate with G-CSF, we found D^+ clones from another myeloid leukemia that can be induced to differentiate with GM-CSF or IL-3 but not with IL-6 or G-CSF (Table III) (reviewed in Sachs, 1987a,b, 1990). In clones that respond to CSFs these inducers presumably induce production of an appropriate differentiation inducer. D^+ leukemic cells that respond to IL-6 can also be induced to differentiate by IL-1α and IL-1β (Table III), and this is mediated by the endogenous production of IL-6 (reviewed in Sachs, 1990).

Studies *in vivo* have shown that normal differentiation of D^+ myeloid leukemic cells to mature nondividing cells can be induced not only in culture but also *in vivo*. These leukemias, therefore, grow progressively when there are too many leukemic cells for the amount of differentiation inducer in the body. We showed that the development of leukemia can be inhibited in mice with these D^+ leukemic cells by increasing the amount of differentiation-inducing protein, either by injecting it or by injecting a compound that increases its production by cells in the body (Lotem and Sachs, 1978, 1981, 1984, 1988; reviewed in Sachs, 1987a,b,

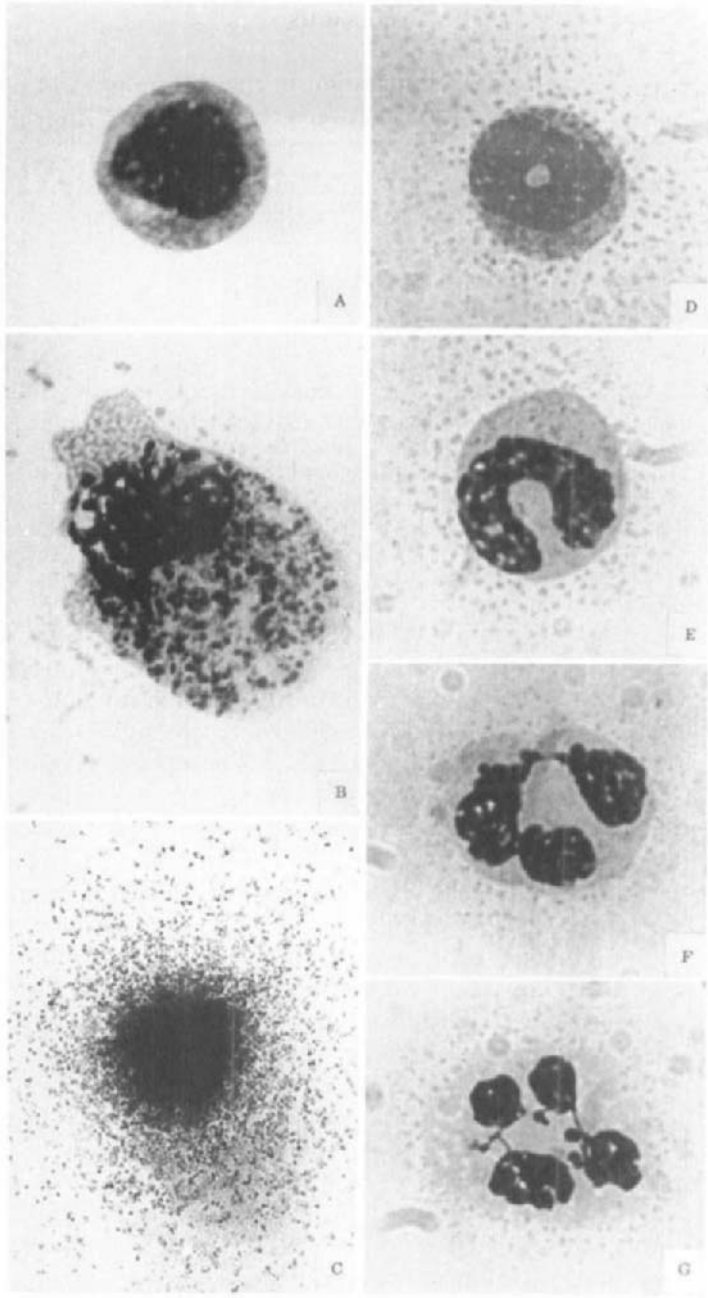

FIG. 5. Differentiation of myeloid leukemic cells to nonmalignant mature macrophages or granulocytes by normal myeloid differentiation-inducing protein IL-6. (A) Leukemic cell, (B) macrophage, (C) colony of cells with macrophages, (D–G) stages in differentiation to granulocytes (Fibach *et al.*, 1972).

TABLE III
DIFFERENTIATION OF MYELOID LEUKEMIC CELLS BY DIFFERENT HEMATOPOIETIC CYTOKINES

Myeloid leukemia	Differentiation after culture with							
	IL-6	IL-1	D-factor/LIF	TNF	IL-3	GM-CSF	G-CSF	M-CSF
M1-clone 11	+	+	−	−	−	−	±	−
M1-clone T22	+	+	+	−	−	−	±	−
7-M12	−	−	−	−	+	+	−	−
WEHI-3B	−	−	−	−	−	−	+	−
HL-60	−	−	−	+	−	−	±	−

Note. IL-1 induces differentiation indirectly in clone 11. It is suggested that the induction of differentiation in WEHI-3B by G-CSF, in clone 7-M12 by GM-CSF or IL-3, in clone T22 by D-factor/LIF, in HL-60 by TNF, and partial differentiation by G-CSF in some clones may also be indirect. +, Induced to differentiate; −, not induced to differentiate; ±, partial differentiation. (References in Sachs, 1990.)

1990, 1993). Induction of differentiation *in vivo,* like *in vitro,* can occur directly or by an indirect mechanism that involves induction of the appropriate differentiation-inducing protein either by the same cells or by other cells in the body. After injection of myeloid leukemic cells into fetuses, D^+ leukemic cells could participate in normal hematopoietic cell differentiation to mature granulocytes and macrophages in apparently healthy adult animals (Gootwine *et al.,* 1982; Webb *et al.,* 1984).

Does differentiation revert leukemic cells to a normal diploid chromosome complement? The D^+ myeloid leukemic cells have an abnormal chromosome composition (Fibach *et al.,* 1973; Hayashi *et al.,* 1974; Azumi and Sachs, 1977), and suppression of malignancy in these cells was not associated with chromosome changes. It was obtained by induction of the normal sequence of cell differentiation by a normal myeloid regulatory protein but the differentiating leukemic cells did not regain a normal diploid chromosome complement. In this suppression, the stopping of cell multiplication by inducing differentiation to mature cells thus bypasses genetic changes that produced the malignant phenotype (Sachs, 1987c) such as changes in the requirement for a normal cytokine for viability and growth and a block in the ability of growth inducer to induce differentiation inducer.

The study of different clones of myeloid leukemic cells has shown that in addition to D^+ clones there are differentiation-defective clones (Paran *et al.,* 1970; Fibach *et al.,* 1973). These are called D^- clones (Fibach *et al.,* 1973). A variety of differentiation-defective clones have been used to genetically dissect the differentiation program (Fibach *et al.,* 1973; Lotem and Sachs, 1974, 1977b; Krystosek and Sachs, 1976; Vlodavsky *et al.,* 1976; Liebermann and Sachs, 1977, 1978; Maeda and

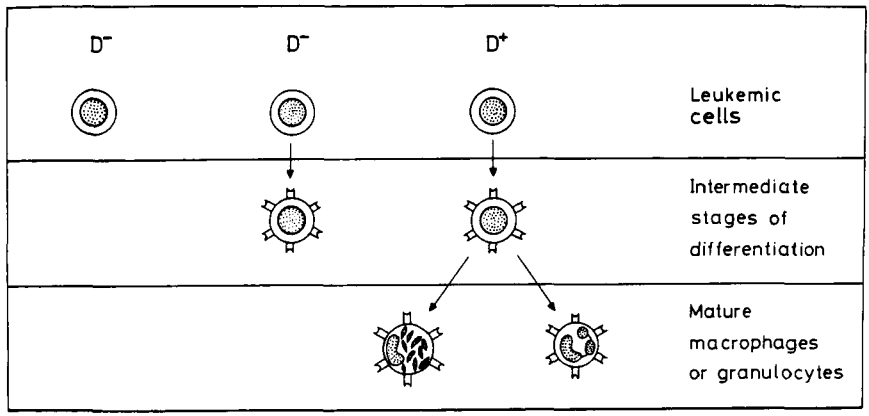

FIG. 6. Classification of different clones of myeloid leukemic cells according to their ability to be induced to differentiate by normal hematopoietic cytokines. Some differentiation-defective (D^-) clones can be induced by these cytokines to intermediate stages by differentiation, whereas other D^- clones are not induced to differentiate by these cytokines even to an intermediate stage.

Sachs, 1978; Simantov and Sachs, 1978; Hoffman-Liebermann and Sachs, 1978; Symonds and Sachs, 1979; Liebermann *et al.*, 1980; Simantov *et al.*, 1980; Hoffman-Liebermann *et al.*, 1981; Blatt *et al.*, 1992). Some D^- clones are induced by a normal myeloid differentiation-inducing cytokine to an intermediate stage of differentiation which then slows down the growth of the cells, and others could not be induced to differentiate even to this intermediate stage (Fig. 6). Since a normal differentiation inducer can induce differentiation to mature nondividing cells in the D^+ clones, I have suggested that D^+ clones are the early stages of leukemia and that the formation of D^- clones may be later stages in the further progression of malignancy (reviewed in Sachs, 1987a,b, 1990). Genetic changes which make cells defective in their ability to be induced to differentiate by a normal differentiation inducer thus occur in the evolution of myeloid leukemia. We showed that even these D^- cells can be induced to differentiate by other compounds, either singly or in combination, that can induce the differentiation program by alternative pathways. The stopping of cell multiplication by inducing differentiation by these alternative pathways bypasses the genetic changes that inhibit response to the normal differentiation inducer (reviewed in Sachs, 1987a,b, 1990). Studies on the genetic changes in D^- clones of myeloid leukemias have shown that differentiation defectiveness may be due to changes in homeobox genes. These include rearrangement of the *Hox-2.4* homeobox gene which results in abnormal

expression of this gene in the leukemic cells (Blatt *et al.*, 1988; Ben-David *et al.*, 1991). This abnormal expression inhibits specific pathways of myeloid cell differentiation (Blatt *et al.*, 1992). In other leukemias with a deletion in one chromosome 2 (Azumi and Sachs, 1977) there is a deletion of one copy of *Hox4.1* (Blatt and Sachs, 1988).

In studies with a variety of chemicals other than normal hematopoietic cytokines we found that many compounds can induce differentiation in D^+ clones of myeloid leukemic cells. These include certain steroid hormones, chemicals such as cytosine arabinoside, adriamycin, methotrexate, and other chemicals that are used today in cancer chemotherapy, and irradiation. At high doses, irradiation and these compounds used in cancer chemotherapy kill cells by inducing apoptosis, whereas at low doses they can induce differentiation. Not all these compounds are equally active on the same leukemic clone (reviewed in Sachs, 1978, 1982). A variety of chemicals can also induce differentiation in clones that are not induced to differentiate by a normal hematopoietic cytokine, and in some D^- clones induction of differentiation requires combined treatment with different compounds (reviewed in Sachs, 1982). In addition to certain steroids and chemicals now used in chemotherapy and radiation therapy, other compounds that can induce differentiation in myeloid leukemic cells include insulin, bacterial lipopolysaccharide, certain plant lectins, tumor-promoting phorbol esters (reviewed in Sachs, 1978, 1982, 1987a), and retinoic acid (reviewed in Degos, 1992). In addition to the normal myeloid cytokines, the steroid hormones, insulin, and retinoic acid are physiological compounds that can induce differentiation. It is possible that all myeloid leukemic cells which are no longer susceptible to the normal hematopoietic cytokines by themselves can be induced to differentiate by the appropriate combination of compounds. The experiments with myeloid leukemic cells have shown that there are different pathways of gene expression for inducing differentiation, and that genetic changes which suppress induction of differentiation by one compound need not affect differentiation by another compound by alternative pathways (Cohen and Sachs, 1981; reviewed in Sachs, 1982, 1987a). These results show that there is considerable flexibility in the myeloid differentiation program, and this is a concept that presumably also applies to other cell types.

E. Cytokine and Gene Control of Programmed Cell Death (Apoptosis)

Another question is what keeps normal and leukemic cells alive? Normal myeloid precursor cells depend on hematopoietic cytokines for viability, multiplication, and differentiation (reviewed in Sachs, 1987a,b,

1990; Sachs and Lotem, 1993). Withdrawal of these cytokines leads to death by a suicide program called programmed cell death which has also been called apoptosis (reviewed in Arends and Wyllie, 1991; Williams *et al.*, 1990). Although viability factors such as the CSFs also induce growth, viability and growth are separately regulated (reviewed in Sachs and Lotem, 1993, 1995). It will be interesting to determine whether viability factors such as the CSFs that can also induce cell growth induce growth directly or do so indirectly by switching on production of other factors that induce growth. There is no evidence that cancer cells are immortal. Tumor cell lines some of which have been subcultured for years contain cells that die by apoptosis. These lines can be subcultured for long periods not because the tumor cells are immortal, but because more cells survive for some time and multiply than die. As long as this balance is maintained in favor of the multiplying cells, a tumor cell line can be subcultured. Cancer cells still maintain the suicide program for cell death which can be activated by different agents and suppressed by viability factors. This has been clearly shown in experiments with myeloid leukemia (reviewed in Sachs and Lotem, 1993, 1995).

Certain myeloid leukemic cells are viability and growth factor independent and do not require an exogenously added cytokine for cell viability and growth. However, the program for cell death in these leukemic cells is not lost and can be activated in different ways. Induction of differentiation in these leukemic cells with IL-6 induces in the differentiating cells a viability factor-dependent state so that the cells lose viability by apoptosis following withdrawal of IL-6 (Fibach and Sachs, 1976; Lotem and Sachs, 1982, 1983, 1989; Lotem *et al.*, 1991a). This induction of the program for cell death occurs before terminal differentiation, and the differentiating cells can be rescued from apoptosis and continue to multiply by reading IL-6 or by adding IL-3, M-CSF, G-CSF, or IL-1 (Lotem and Sachs, 1989). The differentiating leukemic cells can also be rescued from apoptosis by the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) but not by the nonpromoting isomer 4-α-TPA (Lotem *et al.*, 1991a). TPA rescues the differentiating cells from apoptosis by a different pathway than rescue with these cytokines. TPA can thus act as a tumor promoter by inhibiting apoptosis (Lotem *et al.*, 1991a). The program for cell death is present in normal myeloid precursor cells and in more differentiated cells including mature granulocytes and macrophages. Induction of apoptosis in myeloid leukemic cells is thus a normal physiological process that can be used to suppress leukemia.

Apoptosis can also be induced in myeloid leukemic cells without inducing differentiation. Wild-type p53 protein is a product of a tumor suppressor gene which is no longer expressed in many types of tumors

TABLE IV
CONTROL OF APOPTOSIS (PROGRAMMED CELL DEATH) BY A TUMOR SUPPRESSOR GENE AND ONCOGENES

Deregulated expression of	Apoptosis		
	Induction	Enhancement	Suppression
Wild-type p53	+	−	−
c-*myc*	−	+	−
Mutant p53	−	−	+[a]
bcl-2	−	−	+

[a] Mutant p53 suppresses the enhancement of apoptosis by deregulated c-*myc* (Lotem and Sachs, 1993b). Other references in Sachs and Lotem (1993).

including myeloid leukemias (reviewed in Levine *et al.*, 1991). There is a clone of myeloid leukemic cells that completely lacks expression of p53 protein and mRNA (Yonish-Rouach *et al.*, 1991). This p53-negative clone of myeloid leukemic cells was transfected with DNA encoding a temperature-sensitive p53 mutant (Ala to Val change at position 135). The Val 135 mutant behaves like other p53 mutants at 37.5°C but behaves like wild-type p53 at 32.5° C. There was no change in the behavior of the transfected cells at 37.5°C but activation of the wild-type p53 protein at 32.5°C resulted in apoptotic cell death. This induction of apoptosis was not associated with differentiation (Yonish-Rouach *et al.*, 1991). Apoptosis can, therefore, be induced in myeloid leukemic cells not only by a differentiation-associated process, but also by expression of wild-type p53 (Table IV), in undifferentiated leukemic cells. This induction of apoptosis by wild-type p53 was inhibited by IL-6 (Yonish-Rouach *et al.*, 1991). These results indicate that wild-type p53-mediated apoptosis in these myeloid leukemic cells is a physiological process. Experiments with p53 knockout mice have shown that wild-type p53 is also involved in mediating apoptosis in normal myeloid precursors deprived of the appropriate cytokine concentration required for cell viability (Lotem and Sachs, 1993a). Experiments with p53 knockout mice have also shown that there are wild-type p53-dependent and p53-independent pathways of inducing apoptosis (Lotem and Sachs, 1993a; Lowe *et al.*, 1993; Clarke *et al.*, 1993).

The induction of apoptosis in myeloid leukemic cells by various cytotoxic agents, including cytotoxic compounds used in cancer therapy (Lotem and Sachs, 1992a), can be enhanced by deregulated expression of c-*myc* (Lotem and Sachs, 1993b). The oncogene mutant p53 (Lotem and Sachs, 1993b) and *bcl*-2 (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992;

reviewed in Korsmeyer, 1992) (Table IV) suppress the enhancing effect of deregulated c-*myc* on cell death and thus allow induction of cell proliferation and inhibition of differentiation (Lotem and Sachs, 1993b) which are other biological effects of deregulated c-*myc*. The suppression of cell death by mutant p53 and *bcl*-2 increases the probability of developing tumors, and the suppression of apoptosis by mutant p53 (Lotem and Sachs, 1993b) can explain the high frequency of mutant p53 in many types of tumors. Treatments that downregulate the expression or activity of mutant p53 and *bcl*-2 in tumor cells can be useful to increase susceptibility to cytotoxic cancer therapy (Sachs and Lotem, 1993; Lotem and Sachs, 1994). The expression of *bcl*-2 can be down-regulated in myeloid leukemic cells by cytokines such as IL-6 and G-CSF and the steroid dexamethasone (Lotem and Sachs, 1993b, 1994) and in promyelocytic leukemic cells by all-trans-retinoic acid (Chomienne *et al.*, 1992). Down-regulation of *bcl*-2 by the cytokines increased cell susceptibility to induction of apoptosis by cytotoxic compounds used in cancer therapy. But there was no such increased susceptibility after down-regulation of *bcl*-2 by dexamethasone (Lotem and Sachs, 1994), owing to up-regulation by dexamethasone of another apoptosis suppressing gene *bcl*-X_L (Lotem and Sachs, to be published). To increase cancer cell susceptibility to induction of apoptosis it will thus be necessary to select the appropriate compound for down-regulation of apoptosis suppressing genes. The experiments on the regulation of apoptosis have shown that there are alternative pathways to apoptotic cell death (Sachs and Lotem, 1993, 1995). Alternative pathways to regulate apoptosis can be useful to selectively control cell viability. These studies have shown that there are cytokine and gene controls of apoptosis in both normal and leukemic cells. The existence of alternative pathways to apoptosis shows that there is flexibility in the program for cell death which presumably also applies to other cell types.

F. Therapeutic Use of Hematopoietic Cytokines

Identification of the myeloid cell regulatory cytokines has suggested novel possibilities for therapy (reviewed in Sachs, 1978, 1986, 1987a,b, 1990, 1993; Demetri and Griffin, 1991; Sakamoto and Gasson, 1991; Oster and Schulz, 1991). The concentration of these proteins can be increased *in vivo* either by injecting one of these cytokines or by injecting a compound that induces their production (Fibach and Sachs, 1974; Lotem and Sachs, 1978, 1981, 1984; reviewed in Sachs, 1990). Injection of a CSF, such as G-CSF or GM-CSF, stimulates myelopoiesis in normal circumstances and after suppression of normal myelopoiesis *in vivo* by

compounds used in chemotherapy and irradiation therapy and compounds used for immune depression such as cyclosporine A. These treatments destroy cells that produce these cytokines. Myeloid regulatory cytokines were shown to be clinically useful in restoring the normal number and function of the myeloid blood cell population in such myeloid-suppressed cancer patients after cytotoxic cancer therapy and after immune suppression before bone marrow transplantation. Addition of these cytokines *in vitro* to bone marrow cells before grafting and/or their injection *in vivo* can increase the success of bone marrow transplants and increase survival in patients deficient in these cytokines and the cells that produce them.

Hematopoietic cytokines can inhibit induction of apoptosis induced by cancer chemotherapy compounds and irradiation (Lotem and Sachs, 1992a; Sachs and Lotem, 1993). The clinical use of cytokines to correct therapy-associated suppression of normal hematopoiesis should therefore be carefully timed to avoid protection of the malignant cells from the cytotoxic action of therapeutic compounds (Lotem and Sachs, 1992a). Because of the important functions of mature cells, such as granulocytes and other myeloid cells, the increased survival and function of mature cells induced by CSFs can also be clinically helpful to patients with deficiencies in myeloid cell production and functions (reviewed in Sachs, 1990). The finding that apparently normal granulocyte development can be induced in culture with cells from patients with infantile genetic agranulocytosis (Paran *et al.*, 1970; Barak *et al.*, 1971) has led to promising clinical results with G-CSF in children with this genetic disease. The existence of normal colony-forming cells in patients with genetic neutropenia (Mintz and Sachs, 1973) should also be clinically useful. It has been shown that injection of erythropoietin, which stimulates the development of erythroid cells, can correct the anemia in patients with chronic renal failure (Eschbach *et al.*, 1987).

The studies I described under Section D have shown that CSFs and interleukins, such as IL-1 and IL-6, can control the abnormal growth of certain types of leukemic cells and suppress malignancy by inducing differentiation (reviewed in Sachs, 1978, 1987a,b, 1990, 1993). Induction of apoptosis in leukemic cells either with (Lotem *et al.*, 1991a; Sachs and Lotem, 1993) or without (Yonish-Rouach *et al.*, 1991; Lotem and Sachs, 1992a; Sachs and Lotem, 1993) inducing differentiation is also a process that can be used to suppress malignancy. Induction of a requirement for one of these cytokines to maintain viability of the leukemic cells followed by withdrawal of the cytokine (Fibach and Sachs, 1976; Lotem and Sachs, 1982, 1983, 1989; Lotem *et al.*, 1991a) causes apoptotic death of the leukemic cells, and this would also be useful for therapy. The existence of the cytokine network has to be taken into account in the

clinical use of cytokines (reviewed in Sachs, 1990, 1991; Lotem *et al.*, 1991b; Lotem and Sachs, 1992b).

In conclusion, the development of cell culture assays (Ginsburg and Sachs, 1963; Sachs, 1964; Pluznik and Sachs, 1965; Ichikawa *et al.*, 1966; Bradley and Metcalf, 1966) for the clonal development of hematopoietic cells and discovery of the normal myeloid hematopoietic cytokines in cell culture supernatants (Pluznik and Sachs, 1966; Ichikawa *et al.*, 1966) has led to their successful clinical use for therapy of hemaotological disorders. My idea of developing these cultures to study the basic biology of hematopoiesis (Ginsburg and Sachs, 1963) has thus also turned out to be clinically useful.

III. Carcinogenesis: Tumor Viruses, Chemical Carcinogens, and Irradiation

In the experiments on the effect of *in vitro* virus infection on the development of hematopoietic cells I also wanted to test the Gross leukemia virus, and I obtained from Dr. Ludwig Gross in New York some mice carrying this virus. When hematopoietic cells from these mice were cultured on the mouse feeder layers, the feeder layers were destroyed and this destructive effect could be further passaged with supernatants from the destroyed feeder layer. It turned out that Dr. Gross's mice contained another virus which was later called polyoma virus (Eddy *et al.*, 1959). This virus was cytopathic for the mouse feeder layer cells and produced various types of tumors in different species, including mice, hamsters, and rats (Eddy *et al.*, 1959), even with plaque-purified virus (Winocour and Sachs, 1961). I had at this time taken on some more students in my laboratory, and decided to continue working with polyoma virus on nonhematopoietic cells in order to obtain a more general understanding of the mechanism of tumorigenesis in different cell types. The development of a plaque assay for polyoma virus (Winocour and Sachs, 1959; Sachs *et al.*, 1959; Dulbecco and Freeman, 1959) and *in vitro* transformation of normal cells by polyoma virus (Vogt and Dulbecco, 1960; Sachs and Medina, 1961) were used to study the lytic interaction and tumor-inducing properties of the virus in cultured cells.

The experiments on cell virus interactions with polyoma virus included studies on the lytic interaction (Winocour and Sachs, 1960), the cell-transforming interaction (Sachs and Medina, 1961; Sachs *et al.*, 1962; Medina and Sachs, 1961, 1963, 1965; Manor *et al.*, 1973), the properties of polyoma virus mutants (Sachs and Medina, 1960; Medina and Sachs, 1963; Gershon and Sachs, 1965), and the mechanism of polyoma virus-induced synthesis of cellular DNA (Gershon *et al.*, 1965; Ossovski and Sachs, 1967) which was also studied with simian virus 40

(SV40) (Gershon *et al.*, 1966). SV40 was also used to characterize SV40-specific RNA in virus-yielding and -transformed cells (Aloni *et al.*, 1968) to show that cellular DNA sequences can be integrated into SV40 DNA (Aloni *et al.*, 1969), and that SV40 integrated into cellular DNA can be activated by transfer of isolated chromosomes (Shani *et al.*, 1974, 1976).

The *in vitro* transformation by polyoma virus of cells from different species, including golden hamster cells (Vogt and Dulbecco, 1960; Sachs and Medina, 1961, Sachs *et al.*, 1962), which are particularly good for chromosome analysis (Sachs, 1952), raised the question of whether these cells could also be transformed *in vitro* by environmental carcinogens such as chemical carcinogens and irradiation. A culture system using normal hamster embryo cells was therefore developed which showed that these cells can be transformed *in vitro* from normal into malignant cells by various chemical carcinogens (Berwald and Sachs, 1963, 1965) and by X-irradiation (Borek and Sachs, 1966a). This provided a new approach to the study of carcinogenesis by environmental carcinogens and showed that an increased cellular life span is a step in carcinogenesis which is separate from induction of malignancy *in vivo* (Huberman *et al.*, 1968). Carcinogenic hydrocarbons have to be metabolized into active compounds. An *in vitro* system was also used to determine the metabolism and to identify the active metabolites that are required for transformation and mutagenesis (Huberman and Sachs, 1966, 1973, 1974, 1976; Gelboin *et al.*, 1969; Yamasaki *et al.*, 1975; Huberman *et al.*, 1976a,b).

The transformed hamster embryo cells can be distinguished from normal cells *in vitro* by a change in the cell surface control of cell replication, so that the transformed cells do not contact inhibit other transformed cells of the same type. The normal cells grow as a flat layer, whereas the transformed cells can pile up. This makes it possible to distinguish single normal from single transformed cells by the growth pattern of the colonies derived from these cells and to use the difference in this growth pattern for the quantitative assay of transformed cells (Stoker and MacPherson, 1961; Sachs, *et al.*, 1962). But transformation does not show a complete loss of the ability to respond to contact inhibition. Thus, although the transformed cells do not show contact inhibition with other transformed cells of the same type, cells transformed by polyoma virus, simian virus 40, carcinogenic hydrocarbons, and X-irradiation can still be contact inhibited by normal cells (Borek and Sachs, 1966b). Cells transformed by one carcinogen can also be contact inhibited by cells transformed by another carcinogen. For example, cells transformed by polyoma virus were not inhibited by other cells transformed by polyoma virus, cells transformed by SV40 were not inhibited by other cells transformed by SV40, but cells transformed by polyoma virus were able to inhibit and to be inhibited by cells transformed by

SV40. The specificity of the cell type induced by the transforming virus can thus be recognized by contact inhibition between transformed cells, and this specificity also applied to cells transformed by carcinogenic hydrocarbons or X-irradiation (Borek and Sachs, 1966b). We also showed that the lectin concanavalin A can be used to study some common membrane changes in cells transformed by different carcinogens (Inbar and Sachs, 1969; Rutishauser and Sachs, 1974; reviewed in Sachs, 1974a,b). A variety of morphologically different colonies have been observed after transformation of mixed populations of normal hamster embryo cells. Results obtained with clones of single cells transformed by polyoma virus or by the carcinogenic hydrocarbon benzo[*a*]pyrene have indicated that the type of normal cell from which the transformed cell is derived is a major factor in determining the morphology of the transformed cell colony (Medina and Sachs, 1965; Huberman and Sachs, 1966).

The percentage of transformed colonies has been determined after treatment of normal hamster embryo cells with different types of carcinogens. About 2% of transformed colonies was obtained after infection with polyoma virus (Sachs *et al.*, 1962; Medina and Sachs, 1963, 1965), about 3–20% after treatment with carcinogenic hydrocarbons (Berwald and Sachs, 1963, 1965; Huberman and Sachs, 1966), and about 0.5–2% after X-irradiation with 300 R (Borek and Sachs, 1966a, 1967). No such transformed colonies were observed in control cultures of untreated hamster embryo cells. The absence of transformed colonies in the controls, the little or no decrease in cloning efficiency of cells treated with the dose of carcinogen used, and the high frequency of transformation in the treated cells have indicated that with the three types of carcinogenic agents used the transformed colonies were directly induced by the carcinogens and were not merely the results of a selection of spontaneously occurring transformed cells (Sachs, 1964; Berwald and Sachs, 1965; Borek and Sachs, 1966a). The frequency of transformation observed with all three types of carcinogens was also much higher than that expected for a randomly occurring mutation.

The relationship between the percentage of transformed colonies and the dose of carcinogen has been determined for the carcinogenic hydrocarbon benzo[*a*]pyrene (Huberman and Sachs, 1966) and for polyoma virus (MacPherson and Montagnier, 1964), and a "one hit" dose–responsive curve for transformation has been obtained with both carcinogens. Experiments with seven hydrocarbons (Berwald and Sachs, 1965) have indicated a specificity for cell transformation *in vitro* only to the compounds known to be carcinogenic *in vivo*. Carcinogenic hydrocarbons can be cytotoxic. Treatment of single cell clones has shown that the cytotoxic and transforming activities of a carcinogenic hydrocarbon

are different events. Normal hamster cell clones can be either susceptible or resistant to the cytotoxic action of benzo[*a*]pyrene, and in clones susceptible to transformation transformed colonies were obtained after treatment of both types of clones (Huberman and Sachs, 1966).

The development of a transformed colony requires a change in the control mechanism for cell replication and the fixation of this change so that it can be transmitted as a hereditary property of the transformed cell. Competence can be expressed at either of these stages, so that in order to further analyze the nature of competence it was necessary to examine the requirements for fixation of the transformed state. Studies with X-irradiation-induced transformation showed that about the same frequency of transformed colonies was obtained after irradiating cells 1 day after they had been plated for cloning, sparse cultures, or confluent cultures cloned immediately after irradiation when the irradiated cells were able to undergo cell replication soon after irradiation. But no transformed colonies were obtained when confluent cultures were irradiated and the cells allowed to replicate only 3–5 days later (Borek and Sachs, 1967). Cells in a confluent culture of normal cells are inhibited from replicating. Inhibition of cell replication for 3–5 days after irradiation thus prevented expression of the transformed state. It can therefore be assumed for X-irradiation-induced transformation that a process associated with cell replication is required for expression of the transformed state. The results further indicate that fixation of the transformed state as a hereditary property of the cell requires that this process occurs soon after treatment with the carcinogen. This also applies to transformation by chemical carcinogens (Berwald and Sachs, 1965). These and other results described in this section have shown that the cell culture system using normal hamster embryo cells made it possible to use the same normal cells to study carcinogenesis *in vitro* with different types of carcinogens.

IV. Chromosome Changes and Tumor Suppression

The normal hamster cells had been changed into malignant sarcoma cells by different types of carcinogens. The next questions were can the malignancy of these sarcoma cells again be suppressed and what are the chromosome changes associated with such tumor suppression? It was found possible to reverse the malignant to a nonmalignant phenotype with a high frequency under certain culture conditions in cloned sarcoma cells in which malignancy had been induced by chemical carcinogens, X-irradiation, or by a tumor-inducing virus (Fig. 7) (Rabinowitz and Sachs, 1968, 1969, 1970a,b,c). In sarcomas induced after transfor-

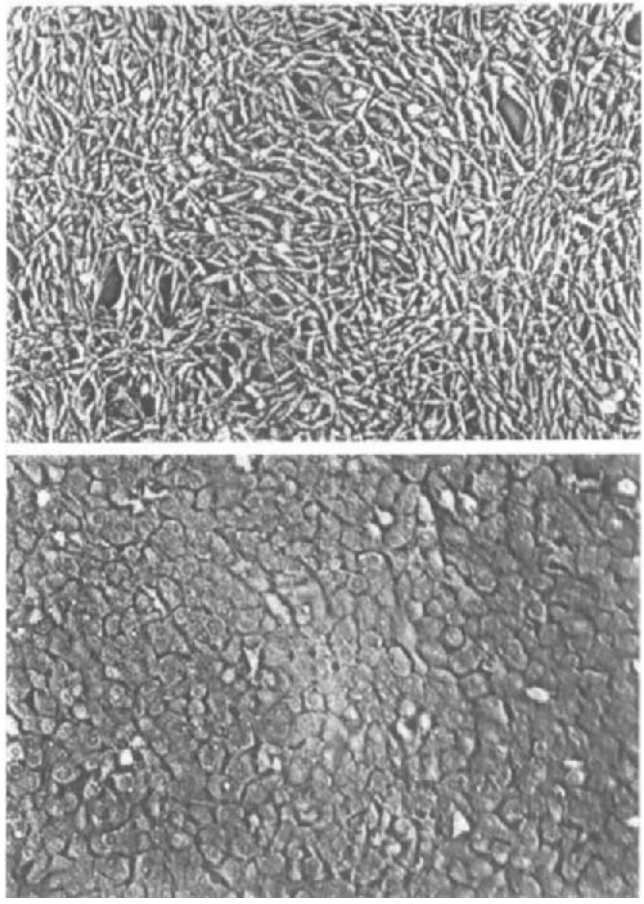

FIG. 7. Cultures of hamster sarcoma cells (top) and a nonmalignant revertant (bottom) (Rabinowitz and Sachs, 1968).

mation of normal hamster embryo cells in culture with chemical carcinogens (Berwald and Sachs, 1963) or X-irradiation (Borek and Sachs, 1966a), this reversibility of malignancy included reversion to the limited life span found with normal cells (Rabinowitz and Sachs, 1970c). Chromosome studies showed that the revertants did not revert to a normal diploid chromosome complement. The chromosome studies on normal hamster embryo cells, sarcomas derived from these cells, revertants from sarcomas which had regained a nonmalignant phenotype, and rerevertants also showed that the difference between these malignant and nonmalignant cells is controlled by the balance between genes located on

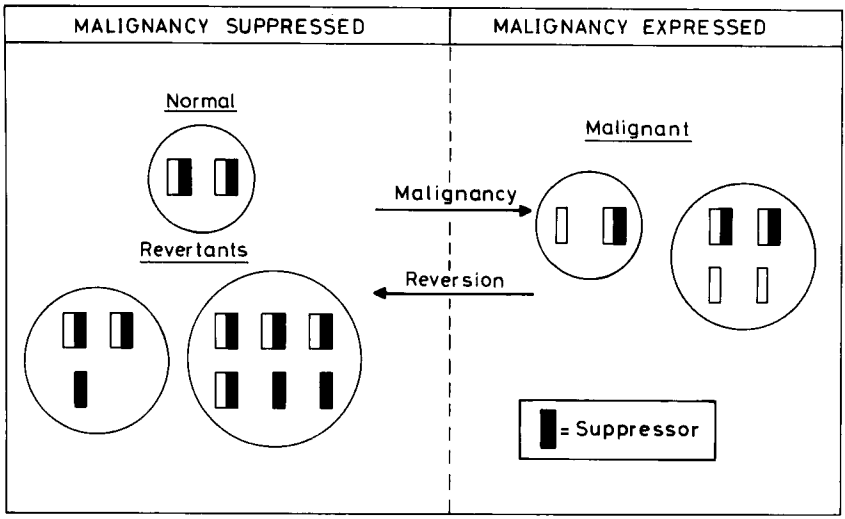

FIG. 8. The expression and suppression of malignancy by chromosome segregation resulting in a change in gene dosage owing to a change in the balance of specific chromosomes. From experiments with normal hamster embryo fibroblasts, hamster sarcoma cells, and their nonmalignant revertants. Genes for expression (□) and suppression (■) of malignancy (Rabinowitz and Sachs, 1970a).

specific chromosomes that at that time were called genes for expression (E) and genes for suppression (S) of malignancy (Fig. 8) (Rabinowitz and Sachs, 1970a; Hitotsumachi *et al.*, 1971, 1972; Yamamoto *et al.*, 1973; Sachs 1974a; Bloch-Shtacher and Sachs, 1976). When there is enough S to neutralize E malignancy is suppressed, and when the amount of S is not sufficient to neutralize E malignancy is expressed. These early experiments on golden hamster cells have shown (Rabinowitz and Sachs, 1970a; Hitotsumachi *et al.*, 1971, 1972; Yamamoto *et al.*, 1973; reviewed in Sachs, 1974, 1987c) that in addition to genes on specific chromosomes for expression of malignancy (E genes) (oncogenes), there are other genes on specific chromosomes, S genes (tumor suppressor genes), that can suppress the action of oncogenes. A chromosome that controls malignancy was also identified in Chinese hamster cells (Bloch-Shtacher and Sachs, 1977). Among the activities of these genes is the ability of a tumor suppressor gene wild-type *p53* to activate apoptosis (Yonish-Rouach *et al.*, 1991) and the ability of an oncogene, such as mutant *p53*, to suppress apoptosis (Lotem and Sachs, 1993b).

The suppression of malignancy in sarcomas in our experiments (reviewed in Sachs, 1974a) was obtained by chromosome segregation, re-

sulting in a change in gene dosage due to a change in the balance of specific chromosomes. This suppression of malignancy by chromosome segregation, with a return to the gene balance required for suppression of the malignant phenotype, occurred without hybridization between different types of cells. The nonmalignant cells were thus derived from the malignant cells by genetic segregation. Suppression of malignancy associated with chromosome changes including changes in gene balance has also been found after hybridization between different types of cells (Klein, 1981; Evans *et al.*, 1982; Kitchin *et al.*, 1982; Stanbridge *et al.*, 1982; Benedict *et al.*, 1984). These studies on cell hybrids have led to similar conclusions to those obtained from the reversal of malignancy in sarcomas without hybridization between different cell types. Hybridization between myeloid leukemic cells and nondividing normal macrophages suppressed malignancy *in vivo* and cell multiplication *in vitro* (Shkolnik and Sachs, 1978). Cell hybrids can express histocompatibility antigens of both parents (Gershon and Sachs, 1963). Changes in the balance of genes for these antigens may be responsible for some cases of suppression of malignancy *in vivo* in hybrid cells.

The D^+ myeloid leukemic cells have an abnormal chromosome composition (Fibach *et al.*, 1973; Hayashi *et al.*, 1974; Azumi and Sachs, 1977); but suppression of malignancy in these cells, which also occurred in certain clones with a high frequency, was not associated with chromosome changes. Suppression of malignancy in these D^+ leukemic cells was obtained by induction of the normal sequence of cell differentiation by a normal myeloid regulatory cytokine. In this suppression of the malignant phenotype, the stopping of cell multiplication by inducing differentiation to mature cells bypasses the genetic changes, such as the loss of requirement for the normal growth inducer and a block in the ability of growth inducer to induce differentiation inducer, that produced the malignant phenotype. Genetic changes which make cells defective in their ability to be induced to differentiate by the normal differentiation inducer occur in the evolution of myeloid leukemia. But even these D^- cells can be induced to differentiate by other compounds, either singly or in combination, that can induce the differentiation program by alternative pathways. Also in these cases, the stopping of cell multiplication by inducing differentiation by these alternative pathways bypasses the genetic changes that inhibit response to the normal differentiation inducer. This bypassing of genetic defects is presumably also the mechanism for the suppression of malignancy by inducing differentiation in other types of tumors.

Studies on the chromosomes of myeloid leukemic cells have shown that the change from D^- to D^+ and *vice versa*, that is the ability to be

induced to differentiate to mature nondividing cells by a normal myeloid regulatory cytokine, is controlled by the balance between genes that allow induction of differentiation and genes that suppress differentiation (Azumi and Sachs, 1977). It was then also shown in hybrids between different cell types that chromosome changes can suppress malignancy by restoring the ability of the cells to be induced to differentiate to nondividing cells *in vivo* in a location in the body where the cells are exposed to what is presumably the normal differentiation inducer (reviewed in Stanbridge, 1984; Harris, 1985). The appropriate chromosome changes also change these hybrid cells from D^- to D^+. Chromosome changes can thus change tumor cells from D^- to D^+, so that the cells can then be induced to differentiate when exposed to normal differentiation inducer.

It can, therefore, be concluded from studies on the molecular regulators of growth and differentiation in normal development, changes in the normal development program in tumor cells, and suppression of malignancy in myeloid leukemia and sarcomas that (A) malignancy can be suppressed by inducing differentiation either with or without genetic changes in the malignant cells, (B) this suppression of malignancy does not have to restore the normal diploid chromosome complement and all the normal controls, and (C) genetic defects that give rise to malignancy can be bypassed and their effects nullified by inducing differentiation and apoptosis.

V. Concluding Remarks

We are the consequences of biological evolution. We are both what the body needs today and what it may find useful for future evolution. The system that controls production of different types of hematopoietic cells with a limited life span during normal hematopoiesis and in crises, such as infections, wound healing, and various diseases, requires considerable flexibility. As shown in hematopoiesis, the multigene family of different interacting cytokines that has arisen during evolution is more easily adaptable and responsive to change than only single cytokines with high specificity where a lack of flexibility would be lethal. The evolution of a family of cytokines, some of which have overlapping functions, is a necessary safeguard so that if one cytokine does not function effectively under certain conditions another can take over. Safeguards can thus be achieved by providing alternative pathways. In order to analyze possible alternatives I therefore chose a broad approach to the basic biological problems and their possible clinical applications.

Since my main interest was the control of normal and abnormal development, I decided to develop new experimental systems to analyze the mechanisms that control development in various types of normal and cancer cells using cell cultures. The cell culture systems that were established were also chosen to try and find some general concepts regarding the behavior of normal and cancer cells. The establishment of a cell culture system for the clonal development of hematopoietic cells made it possible to discover the proteins, including cytokines now called CSFs and ILs, that regulate cell viability, multiplication, and differentiation of different hematopoietic cell lineages, the molecular basis of normal development in blood-forming tissues, and the changes in this development that produce leukemia. The cell culture system for induction of transformation in the same cells with a tumor virus, chemical carcinogens, and irradiation made it possible to compare *in vitro* the mechanisms of carcinogenesis by different types of carcinogens. The cell culture studies on suppression of malignancy by inducing differentiation in myeloid leukemia and specific chromosome changes in sarcomas have identified different ways of suppressing malignancy.

I suggest that there are also some general concepts that can be derived from these studies. These include (A) the value of a multigene cytokine network in regulating the viability, number, and development of different cell types; (B) the existence of alternative pathways that give flexibility to development in both normal and cancer cells, (C) the response of some cancer cells to normal regulators of development, (D) suppression of myeloid leukemia by inducing differentiation which can bypass genetic defects that give rise to the malignancy, (E) identification of chromosomes that control tumor suppression, (F) that a tumor suppressor gene such as wild-type *p53* can suppress malignancy by inducing apoptosis and that the oncogene mutant *p53* can suppress apoptosis, and (G) that hematopoietic cytokines can control apoptosis in both normal and leukemic cells. It is satisfying that the findings on prenatal diagnosis, hematopoietic regulators such as the CSFs, and the suppression of malignancy by inducing differentiation are also now clinically useful.

It was an adventure in which one question led to another and each answer again led to more questions. When I reflect that I started in Rehovot with a bench, a microscope, and some amniotic fluid, and was then involved in attempting to understand the complexity by which nature tries to ensure our survival, I can only describe this as the greatest adventure which I personally can visualize. Like all adventures, there have been disappointments as well as moments of intense pleasure and excitement, and I am grateful for the luck of being part of the scientific community in all its diversity. I have enjoyed all of it.

VI. Short Curriculum Vitae

Date of Birth:	October 14, 1924
1948:	B.Sc. 1st Class Honors, University of Wales, Bangor, N. Wales
1951:	Ph.D., University of Cambridge, England
1951–1952:	Research Scientist in Genetics, John Innes Institute, England
Since 1952:	Research Scientist, Weizmann Institute of Science, Rehovot, Israel
1960:	Established Department of Genetics and Virology
1962:	Full Professor
1962–1989:	Head, Department of Genetics
1974–1979:	Dean, Faculty of Biology
Present Position:	Otto Meyerhof Professor of Biology

Honors

1967:	Hoffman–La Roche Lecture, Rutgers University
1968:	Otto Meyerhof Professor of Biology
1971:	Karl August Forster Lecture, University of Mainz
1972:	Israel Prize for Natural Sciences
1972:	Fogarty Scholar, U.S.A. National Institutes of Health, Bethesda, Maryland
1972:	Harvey Lecture, Rockefeller University, New York
1975:	Member, Israel Academy of Sciences and Humanities
1977:	Rothschild Prize in the Biological Sciences
1979:	Samuel Rudin Visiting Professor, College of Physicians and Surgeons, Columbia University, New York
1980:	Wolf Prize in Medicine (with J. L. Gowans and C. Milstein)
1981:	Charles B. Smith Visiting Research Professor, Memorial Sloan–Kettering Cancer Center, New York
1983:	Bristol–Myers Award for Distinguished Achievement in Cancer Research, New York
1985:	Doctor Honoris Causa, University of Bordeaux
1986:	The Royal Society Wellcome Foundation Prize, London (with D. Metcalf)
1986:	R. E. Bob Smith Lecture, University of Texas System Cancer Center, Houston
1986:	General Motors Cancer Research Foundation Lecture, Walter and Eliza Hall Institute, Melbourne
1987:	Karl Beyer Lecture, University of Wisconsin, Madison
1987:	Jan Waldenström Lecture, Swedish Society of Medicine, Stockholm

1989: Alfred P. Sloan Prize, General Motors Cancer Research Foundation, New York (with D. Metcalf)
1992: Harden Lecture, Biochemical Society, England

Acknowledgments

I especially acknowledge those who have worked with me in this research as shown in the list of references. I also thank the National Cancer Institute, NIH Bethesda, Maryland, the National Foundation for Cancer Research, Bethesda, Maryland, the Catherine Lewis Foundation and David Lewis and Neil Davis family interests, the Ebner Family Biomedical Research Foundation at the Weizmann Institute of Science in Memory of Alfred and Dolfi Ebner, the Herman de Stern Foundation, and the Jerome A. and Estelle R. Newman Assistance Fund for their support.

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FOUNDATIONS IN CANCER RESEARCH

FROM ARISTOTLE, THROUGH SPEMANN, TO TUMOR IMMUNOLOGY: A PERSONAL EXPERIENCE

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The following pages do not aim at reviewing the present state of tumor immunology, neither are they an historical exposure of a field which has experienced dark and bright days in an amazing frequency. Quite a number of reviews of immunology of cancer have appeared during the past few years, and the astronomic number of references which many of them include reflect the intellectual horizons and impressive knowledge which characterize their writers. My aim here is significantly more modest in terms of the spectrum of subjects and somewhat more pretentious. This is more of an autobiographical outline of some of my own experiences in the life science and in tumor immunology, reflecting personal inclinations and subjective priorities. However subjective, they may elicit some thoughts on how gaps of past knowledge

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were transformed to present realities of scientific observations and how these may pave ways to future achievements.

I. Dreams

Leonardo da Vinci was starting a new painting when a friend entered his studio and asked "What are you actually painting?" "I am painting God," replied Leonardo. "But nobody knows how does God look like" commented his friend. "True. Yet by the time I complete the painting *everybody* will know how does God look like," said Leonardo. It appears that that particular painting was never completed. Hence, our ignorance with regard to the nature of God will continue to prevail. We have then to confine ourselves to the nature of nature.

In fact, one of the most basic questions regarding the nature of life was first formulated 1,800 years prior to Leonardo. Aristotle was observing the sequential stages of the developing chick embryo. Science historians have been focusing on and admiring the descriptive aspects of Aristotle's observations. Without proper magnifying lenses, let alone a microscope, Aristotle provided us with amazing descriptions of the sequential development of the embryonic organs, yet Aristotle was not intellectually satisfied with what he observed. What actually triggered his restless curiosity, as a result of his observations, he formulated in a single question: "Since the development of organ A is followed by organ B, and B is followed by C, does B appear *because* A has preceded it, and, does C appear because B has preceded it or alternatively does B appear independently of A, and C appears independently of B?" ("The Generation of Animals," by Aristotle.) In contemporary formulation: are the sequential stages of embryogenesis causally related? Aristotle's question remained unanswered for 2,300 years until the early days of the twentieth century when Hans Spemann in Germany carried out experiments yielding dramatic implications. Spemann experimented with early stages of the amphibian embryos, at the early gastrula stage. He removed by microsurgery the dorsal lip of the blastopore, or the embryonic tissue deriving from the blastopore dorsal lip, and transplanted them to other gastrulae. He demonstrated that the transplanted cells induced in the ectoderm of the recipient embryo the formation of a neural plate resulting in embryos or tadpoles manifesting two brains (Spemann, 1938). The eye cups of the brain once formed induced the formation of the lens, and the embryonic lens induced the formation of the cornea. Aristotle's question was thus clearly answered: in embryogenesis cells of stage A induced stage B, etc. Yet, the purification and characterization of the embryonic inducers, particularly of the neural inducer, the so-called

embryonic "organizer," was attempted following Spemann's discoveries by three generations of developmental biologists, ending up in a long series of frustrations. These seemed to have ended in October, 1993, when Richard Harland and his group reported the isolation and characterization of an embryonic protein called "noggin" which acted as a neural inducer in the frog embryo (Lamb, 1993). The protein expressed in the embryonic organizer was identified by exploring a collection of mRNAs and was demonstrated to induce neural tissue by direct action on embryonic ectoderm in the absence of mesodermal cells. The noggin thus appears to represent the endogenous embryonic neural-inducing signal. It induced anterior brain markers, but not posterior brain or spinal cord. Since high concentrations of noggin were required for the experimental morphogenetic induction, hesitations might be raised on whether noggin does in fact represent the actual "Spemann" organizer. Yet, once embryos with knockout noggin gene are generated, and the receptor for noggin on the ectodermal cells is characterized, the molecular nature of the primary embryonic induced will finally be elucidated. The recent discovery of noggin represents the transformation of the dreams of three generations of developmental biologists to actual reality.

The excitement elicited by Spemann's discoveries of embryonic induction explained my decision in 1953 to choose the Institute of Animal Genetics in Edinburgh, and its director Professor E. H. Waddington, as a site for my postdoctoral training. Yet it may also explain how the lack of adequate molecular technologies created the disappointment of those days due to the limited capacity to translate to molecular terms the dramatic observations of the cellular inductive cell interactions controlling morphogenesis. I carried out studies showing specific cell–cell recognition, i.e., tissue-specific aggregation of cells which had been dissociated from amphibian late gastrulae and neurulae cells resulting in reformation in culture of intact embryonic tissues (Feldman, 1955); it was impossible in those days to trace the molecular basis of the specific cell adhesion molecules operating in this amazing phenomenon of tissue-specific cell–cell recognition. In parallel, I studied the effects of purin and pyrimidin analogs in causing developmental malformations in the chick embryo hoping to identify genes controlling morphogenesis which were impaired by the analogues. Malformations I observed (Feldman and Waddington, 1955; Waddington *et al.*, 1955), yet they left me ignorant with regard to the identification of genes or proteins which are responsible for the morphogenetic events which were impaired by the analogues. In other experiments I detected in the eye retina a gradient of lens proteins which was correlated with the gradient of the capacity of retinal cells to regenerate a lens (Clayton and Feldman, 1955). Yet it was

impossible to determine the nature of the molecules involved in the regenerating potency. Thus, the dramatic morphogenesis of an organism raised intriguing questions regarding its molecular basis, which in those days could hardly be approached experimentally. At the early phases of these studies my naive intellectual ambition was limitless and it led to daring experiments. Thus, I was extremely impressed by the existence of two types of fertilized eggs among the different groups of animals: In some groups (i.e., amphibians), the fertilized eggs, following the first cleavage, generated two cells (blastomers) that, if separated from each other, each generated a whole intact organism. Such eggs were categorized as "regulation eggs." Other groups of animals had "mosaic eggs," i.e., eggs in which the first cleavage formed two cells, that when separated generated each half of an organism. It appeared therefore that in mosaic eggs there is a spatial distribution of precursor molecules controlling the formation of distinct organs, and each blastomer is thus predetermined to generate either the right or the left side of an organism. I assumed naively that some organ-specific antigens might be distributed within the fertilized eggs in a defined pattern so that if I^{131}-labeled antibodies to tissue-specific molecules are applied to mosaic eggs, autoradiography may reveal their intracellular location. The mosaic embryos I intended to study were a marine prevertebrate chordates ("Tunicates"). To collect their eggs I traveled from Edinburgh to the beautiful Millport island, off the western shore of Scotland, where one of the most active marine biological stations was located. The frequent visits to Millport turned out to be the main rewarding outcome of this particular project because the rabbit antibodies I generated against different tissues were hardly tissue specific; hence, no significant localization could be expected, let alone found. The disillusion in getting closer to the understanding of the molecular basis of some of the most dramatic processes in morphogenesis, despite some rewarding observations in cellular embryogenesis, was counteracted by my acquiring experience in immunological methods: The generation of antibodies, labeling them with radioactive atoms, and detecting tissue antigens by autoradiography was a useful lesson.

II. Initial Experiments: Tumor Allografts

A couple of years after my arrival in Edinburgh, I was informed that my mother, in Israel, was diagnosed for cancer. I went back home to discover that the capability of performing a successful surgery of a local esophageal carcinoma was not, in those days, significantly more advanced than the molecular methods for analyzing embryogenesis. Ex-

posing myself, following my mother's illness and death, to the field of cancer research of those days, and accepting the invitation to join the Weizmann Institute of Science in Rehovot, it became evident to me that normal developmental processes, which have intrigued my scientific interest ever since, are much more complicated than neoplasia: normal developmental phenomena include an initial phase of cell proliferation, followed by differentiation, ending in a state of terminal differentiation characterized by cessation of growth; cancer seemed a simpler system involving principally just cell proliferation. In fact, it is the arrest of differentiation which characterized the transformation of a normal to a cancerous cell. The lower level of complexity of the cancerous cell compared to that of the normal differentiating cell was, for a young experimentalist, an attractive component.

Searching for differences between normal and cancerous cells, intrigued by my exposure in Edinburgh to immunology and its techniques while studying developmental systems, I was impressed by the following observations: allografts of normal tissues were always rejected by their genetically foreign recipients, whereas many allografts of murine tumors, particularly from lines established following serial transplantations, were accepted by their allogeneic murine recipients. The prevailing notion was that such tumors may have lost alloantigens and consequently did not elicit in the mouse recipients an allograft reaction. I did not accept this notion. I performed experiments that showed that allografts of transplantable tumors that we studied grew progressively while evoking an allogeneic immune response because they manifested the capacity to resist the immune response they elicited (Feldman and Sachs, 1957, 1958; Sachs and Feldman, 1958). In fact, the transplantable tumors we studied elicited a stronger allograft response than the tumors of an identical major histocompatibility complex (MHC) which were immunologically rejected. The capacity of tumors to resist their own immunogenic effects possibly by shedding off alloantigens seemed of relevance a few years later when we investigated tumors in syngeneic recipients questioning whether tumors possess tumor-specific antigens and whether such antigens could be applied as targets for cancer immunotherapy.

With regard to immunological properties of tumor cells we made an observation in those early days which years later turned out to constitute a basis for my long involvement in the biology of cancer metastasis. I studied with David Yafe, my first Ph.D. student, bone marrow transplantation in animals that had been exposed to lethal doses of total body irradiation. I was under the illusion that we were the only ones who considered the possibility of graft versus host response (GVH) operating

in the lethal effects of parental bone marrow inoculated into irradiated F1 hybrid recipients. Although we designed some elegant experiments to prove the GVH (Feldman and Yafe, 1958), Morten Simonsen preceded us in publishing the first experimental evidence for the GVH reaction. Yet, we were only partly disappointed, since in our experiments on radiation chimeras we studied the behavior of tumors grafted onto chimeras which had been generated by inoculating bone marrow or fetal haemopoietic cells to lethally irradiated mice. We observed that tumors which were nonmetastatic in normal syngeneic recipients did generate metastasis in the recipients which had been exposed to lethal doses of total body irradiation and were protected by fetal haemopoietic cells (Feldman and Yafe, 1959b; Yafe and Feldman, 1959; Feldman and Yafe, 1962). The formation of metastasis in these radiation chimeras suggested that the function of the immune system in controlling metastatic growth was distinct from the control of growth of the local tumor, a phenomenon which in later years constituted a central focus in my scientific work.

III. Initial Realities: Tumor-Specific Transplantation Antigen

The question of the possible expression of tumor-specific antigens is as old as the entire field of cancer research. Yet all attempts, prior to 1956, to demonstrate antigens specific to cancerous cells ended in a long series of disappointments. The sharp end of this initial unfruitful era of tumor immunology is marked by dramatic observations reported in 1956 by Prehn and Main following an earlier report by Folley (1953): They induced in mice of the C57BL strain tumors by methylcholanthrene. They then transplanted pieces of a given tumor to syngeneic recipients resulting in progressive growth. Yet, if these primary growing grafts were either surgically removed or made to shrink following ligation of blood supply, a secondary graft of the same tumor was immunologically rejected. The rejection was tumor specific since primary grafts of normal tissues from the original tumor-bearing organism did not prime syngeneic animals to reject the tumor graft. Thus, for the first time, tumor specific transplantation antigen (TSTA) was demonstrated. Each tumor had a specific individual TSTA. Transplants of a given tumor did not immunize syngeneic animals against grafts of different methylcholanthrene-induced tumors from mice of the same strain (Prehn and Main, 1956). In fact, TSTA seemed to have elicited an immune response even in autochtunous animals (Klein *et al.*, 1960). To test whether the TSTA is tumor individual specific or whether the speci-

ficity reflects minor genetic differences between mice of the same strain, my student, A. Globerson, and I induced a few subcutaneous sarcomas in each mouse with benzpyrene. We demonstrated that each tumor within the same mouse had a different TSTA (Globerson and Feldman, 1964). Prehn's observations thus signified the initiation of a new era of tumor immunology characterized by a renewed conviction that immunogenic tumor-specific antigens may exist, although at that time the evidence was confined to tumors induced by polycyclic hydrocarbons.

IV. Actual Realities: T Cell Recognition of Cell Surface Epitopes

Attempts to purify and characterize TSTA have been, until recently, progressing very slowly. Since rejection of chemically induced tumors seemed mediated by T lymphocytes, the analysis of tumor antigens gained support by one of the most exciting discoveries of cellular immunology: the MHC restriction of immune recognition of cell surface epitopes by T cells. It appeared that cell surface antigens are not recognized via their native conformation, but in fact, protein antigens undergo intracellularly partial proteolysis, yielding peptides that bind to MHC molecules. The peptide–MHC complexes are translocated to the cell surface where the MHC molecules function as peptide presenters to T lymphocytes. MHC class II present peptides to helper T cells (Harding *et al.*, 1990), whereas MHC class I present peptides to precursors and mature cytotoxic T cells (CTLs). In humans, class I molecules include HLA-A, -B, and -C; in mice, they are represented by the H-2K, -D, and -L molecules. The MHC class I molecules consist of a 43–47 kDa heavy chain that includes three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$), a transmembrane domain, and a short cytoplasmic region. This heavy chain is noncovalently associated with a 12-kDa molecule, the $\beta 2$-microglobulin. X-ray crystallography analysis of HLA-A2 (Bjorkman *et al.*, 1987a,b) and of HLA-AW 68 (Garrett *et al.*, 1989) showed that the $\alpha 1$ and $\alpha 2$ domains are supported by the $\alpha 3$–$\beta 2$ microglobulin domain. The peptide-binding site is shaped like a groove, containing six pockets, that bind peptides of 8–20 amino acids (Bjorkman *et al.*, 1987a; Garret *et al.*, 1989). Such peptides derive from intracellularly synthesized antigenic proteins, such as viral proteins, or proteins in tumor cells mutated following the transforming processes. The peptides, following translocation to the endoplasmic reticulum, are assembled there with newly synthesized MHC class I and β-2 microglobulin molecules. The MHC–peptide complexes are then transported via the Golgi to the cell surface. In fact, the assembly of the peptides with the MHC class I stabilizes the latter (Ljunggren

et al., 1990). It appeared, therefore, that CTLs could be activated and expanded if tumor cells present to them tumor-specific peptides via MHC class 1. The CTLs thus generated could kill tumor cells provided the tumor cells express MHC class I and tumor-specific peptides. Such CTLs could then have therapeutic implications.

On the basis of these principles, I find interest in the experiments of Srivastava which were aimed at the identification and purification of TSTA (reviewed in Srivastava, 1993). Proteins were isolated by Srivastava from MCA-induced sarcomas capable of inducing specific transplantation immunity against each of the tumors. Such proteins seemed, therefore, to represent the TSTA of these tumors. Yet the purification of these proteins led to an unexpected paradox: they were found to belong to a family of proteins known as heat-shock proteins (HSP). These proteins, however, are among the most conserved proteins of the living systems. How could that be reconciled with the individual specificity of antigens of tumors induced by polycyclic hydrocarbons?

Srivastava and his group identified at first a heat-shock protein of 96 kDa (gp96), whereas Ulrich (1986) isolated from tumor cells an HSP of 84 and 86 kDa. It appeared that they belonged to the same family of HSP; an immunogenic gp96 was also subsequently isolated from tumor cells of uv-induced fibrosarcomas, which are also known to manifest individual tumor-specific TSTA, and in the Meth-A sarcoma an hsp70 was identified as an additional TSTA. Since sequence analysis failed to discover polymorphisms among HSP genes of different tumors, it seemed that the specific immunogenicity could not be attributed to the HSP per se. Rather, it appeared that the gp96 was a carrier of tumor-specific peptides, and Srivastava suggested that such peptides, derived from tumor proteins, are transported to the class I MHC molecules in the endoplasmic reticulum (ER) via the gp96 proteins. The gp96 thus functions as a peptide acceptor ("chaperone") in the ER lumen. In fact, tumor peptides not only seem to bind to HSP and generate immunogenic complexes capable of inducing tumor-specific class I-restricted CTL, but they also confer protection against a tumor graft. Preparations of gp96 obtained from influenza virus or SV40-infected cells induced in animals antigen-specific MHC class I-restricted CTL against the viral-infected cells. The concept of HSP chaperoning antigenic peptides is an attractive concept. It does, however, require further studies in which elution and characterization of antigen-specific peptides which form gp96 and hsp70 will be carried out, followed by reconstitutions by the peptides of empty gp96 and hsp70, and testing such reconstituted molecules for their capacity to induce specific CTL against tumors on viral-infected cells.

Be the function of HSPs in controlling cellular immune response as it may, our principle is beyond doubt: cytotoxic immune reactivity is mediated by MHC class I molecules presenting specific peptides to precursors of CTLs.

If host immune reactivity mediates not only rejection of tumor grafts but also participates in controlling tumor progression, then tumors which have lost cell surface expression of MHC class I could gain growth properties. Conversely, reexpression of MHC class I genes should confer immunogenic properties resulting in decreased tumorigenicity. The first study that used H-2 gene transfer to manipulate tumorigenicity was that of Hui *et al.* (1984) who experimented with K36.16 line of Gross leukemia virus transformed AKR cells, originated in an H-2^{K} mouse. The tumor cells are H-$2K^{K}$ nonexpressors, and transfection with H-$2K^{K}$ gene converted them to nontumorigenic cells. The abolishment of tumorigenicity was a function of their acquisition of immunogenic properties: the H-$2K^{K}$-restricted viral epitopes elicited specific CTL which prevented tumor growth. When tested in T-cell depleted mice, the transfectants did grow progressively.

V. MHC Class I Genes Control the Metastatic Phenotype of Tumor Cells

We aimed at studying solid metastatic tumors in mice as models for human malignant tumors. It was previously demonstrated that the cell population of a metastatic tumor is heterogeneous with regard to the capacity of its individual cells to generate metastasis (Fidler and Kripke, 1977). The first question we raised was whether there is a causal relationship between the imbalanced expression of MHC class I and the metastatic phenotypes within a given tumor. With Shraga Segal and our student Shulamit Katzav we initially studied the T10 sarcoma induced by methyleholanthrene in an (H-2^{K} × H-2^{b}) F1 mouse. By cloning its cells we observed that neither the metastatic nor the nonmetastatic clones of this tumor express the H-2K alleles. Yet there were clear differences in MHC class I expression between the metastatic and the nonmetastatic clones: metastatic clones, such as the IE7, expressed both the H-$2D^{K}$ and the H-$2D^{b}$, whereas nonmetastatic clones expressed the H-$2D^{b}$ only (DeBaetselier *et al.* 1980; Katzav *et al.*, 1981, 1982, 1983a,b). Immunoselection *in vivo* indicated that when the IE7 clone was made to lose the cell surface expression of H-$2D^{b}$, the retained H-$2D^{K}$ was associated not only with the retention, but in fact with an increase of its metastatic competence (Katzav *et al.*, 1983c, 1984).

To test whether induced expression after transfection with H-2K

genes would affect the metastatic properties, we collaborated with Dr. G. Hammerling of the DKFZ (The Center for Cancer Research) in Heidelberg for the transfection of cells of the IE7 clone (H-2D^{b+}, H-2D^{k+}) with the H-2K^{b}, H-2K^{k} genes or with H-2K^{b} + H-2K^{k} genes. The expression of either of the H-2K genes resulted in the abolishment of the metastatic phenotype, i.e., in the conversion of a malignant to a benign phenotype. This was associated with the acquisition of immunogenic properties: the H-2K transfectants elicited in syngeneic animals H-2K-restricted CTL. This was the first demonstration that transfer of syngeneic MHC class I genes into cells of a metastatic phenotype converted malignant to benign phenotypes (Wallich *et al.*, 1985).

To further analyze the MHC class I control of the metastatic phenotype of tumors, we turned to a tumor that has originated *spontaneously* in a C57BL(H-2^{b}) mouse: the 3LL Lewis lung carcinoma. Our studies on the lung carcinoma and on murine melanomas were carried out with Dr. Lea Eisenbach, who joined my laboratory in 1982 and devoted her excellent talents to the study of cancer metastasis designing experiments and leading the graduate students of our group. Cloning the cell population of the 3LL carcinoma, she first tested whether the differences between metastatic phenotypes and nonmetastatic phenotypes within the same tumor derive from differences in proteolytic enzymes which were claimed to function in cell dissemination. Previous studies of Liotta at the NIH demonstrated that cells of malignant tumors but not of benign tumors secrete collagenate type IV, capable of degrating collagen type IV, which is an important component of basement membranes of blood capillaries, thus enabling cell penetration through blood vessels (Garbisa *et al.*, 1980). Yet studies of metastatic and nonmetastatic clones of the same metastatic tumors indicated that both phenotypes secrete collagenate type IV (Eisenbach *et al.*, 1985a). Plasminogen activator (Wang *et al.*, 1950) also did not seem to act as a rate-limiting factor in controlling the metastatic properties of clones generated from malignant tumors (Eisenbach *et al.*, 1985b). It seemed, therefore, that metastatic competence of the different phenotypes within the murine malignant tumors that we studied is expressed at stages subsequent to the initial stage of cell dissemination. Hence, the interaction of the tumor cells, following intravasation, with the host immune system seemed a possible stage at which differential expression of the metastatic competence could take place. Since MHC class I molecules control host immune responses to cell surface antigens, Lea Eisenbach generated and tested a large number of clones of the Lewis lung carcinoma to discover that nonmetastatic clones expressed, like normal cells, both the H-2K^{b} and the H-2D^{b}, whereas in the metastatic clones H-2K^{b} expression was dramatically re-

duced. In fact, it appeared that the lower the cell surface H-2K/H-2D ratio, the higher was the metastatic competence of the clone (Eisenbach *et al.*, 1983, 1984a,b). Transfection of metastatic clones with H-2K^b genes, performed by our student Dani Plaksin, leading to cell surface expression of H-2K, resulted in the conversion of malignant clones to low or nonmetastatic phenotypes (Plaksin *et al.*, 1988). Since such transfectants did generate metastasis when transplanted to immune suppressed animals, the abolishment of the metastatic phenotype was attributed to *H-2K*-restricted immunogenic competence acquired by the transfectants. The H-2K molecules were thus assumed to present tumor-specific peptides to the host T cells. This was confirmed in experiments showing that the transfectants elicited in syngenesis animals H-2K^b restricted antitumor CTLs. Since the highly metastatic clones were not completely H-2K nonexpressors, we tested whether the low density of H-2K, which was not sufficient to confer immunogenicity on the tumor, was sufficient to make the cells susceptible to CTLs elicited by the H-2K^b transfectants. *In vitro* positive results led to the first treatment of tumor-bearing animals with gene-manipulated tumor cells as cellular vaccines: animals grafted with cells of a highly metastatic clone were subjected for 9 days following transplantation, when the local tumor was well established, to weekly vaccinations with inactivated (X-irradiated and mitomycine treated) H-2K^b-transfected cells. The immune response elicited by the transfectants caused significant suppression or complete prevention of tumor metastasis. It appeared that the H-2K^b of the tumor cells presenting to CTLs tumor-specific peptides induced a response that prevented metastatic growth (Plaksin *et al.*, 1988). We obtained similar results of suppressed metastatic competence, yet here also of reduced tumorigenicity, following transfection of B-16 melanoma with H-2K^b gene (Porgador *et al.*, 1990).

Could H-2K of other haplotypes present 3LL tumor-specific peptides to CTL, thus converting, following transfection, metastatic to nonmetastatic phenotypes? Our graduate student Cohava Gelber performed these experiments, transfecting cells of the metastatic clone D122 with H-2K^k, H-2K^d, or H-2K^{bm1} genes, then measuring the tumorigenic and metastatic properties of the transfectants in H-2K- and H-2D-matched recombinant congenic recipients. H-2K transfectants with each of the three H-2K genes grew locally in H-2-matched mice, but the generation of metastasis was abolished by each of the "foreign" H-2K alleles (Gelber *et al.*, 1989). These results suggested that putative tumor-associated antigens (3LL-TAAs) produced different immunogenic peptides, each presented by a different H-2K restriction molecule.

Furthermore, Hammerling *et al.* demonstrated that transfection of

3LL cells with human class I *CW3* genes followed by their transplantation into C57BL/6-CW3 transgenic mice also resulted in abrogation of the metastatic phenotype. It appeared that human HLA molecules presented a murine tumor peptide to the mouse T lymphocyte (Hammerling *et al.*, 1989).

All our *in vivo* studies on gene-modified 3LL tumor cells were made in syngeneic homozygous recipients. To get closer to the human situation, we tested the metastatic properties of our H-2K gene transfectants in compatible heterozygous recipients. These studies were performed by our then M.Sc. student, Ofer Mandelboim. Thus, tumor cells transfected with H-2K^b or H-2K^k genes were tested in (H-2^b × H-2^k) F_1 mice and cells transfected with H-2K^b or H-2K^d were tested in (H-2^b × H-2^d) F_1 mice. These single transfectants, which following transplantation into syngeneic homozygous animals, manifested abrogation of the metastatic competence, when tested in compatible heterozygous animals retained their metastatic phenotypes. We therefore subsequently doubly transfected the tumor cells with the two parental H-2K genes of each of the heterozygous recipient mice. Such double H-2K transfectants, when tested in compatible heterozygous mice, showed complete loss of their metastatic phenotype. In fact, even the growth of the local grafts of the double transfectants was either immunologically rejected or very much growth arrested (Mandelboim *et al.*, 1992). Such double transfectants were then tested as cellular vaccines in tumor-bearing animals. They were found to arrest metastatic growth when injected to animals bearing well-established tumor. The dramatic difference between the growth properties of the single H-2K transfectants and those of the double transfectants was unexpected. Measuring the levels of induced precursors of CTL between the two types of recipients provided a basis for the empirical observations on the difference in metastasis formation between the heterozygous and homozygous recipients, yet did not actually explain it. It did, however, raise fundamental questions regarding differences between the repertoire of T cells in heterozygous, as distinct from homozygous, animals.

Be the theory underlying the experimental results we obtained as it may, since human organisms are heterozygous the implication of our experiments for the generation of antimetastatic cellular vaccines by MHC class I gene insertion is that one would have to modify human tumor cells by insertion of HLA genes of the two parental haplotypes of each patient. We therefore searched for ways to bypass the necessity of generating vaccines tailor made according to the HLA genotype of each cancer patient.

We observed, like many others, that human malignant tumors mani-

fest significant levels of suppression of HLA class I gene expression (reviewed in Elliot *et al.*, 1989). Furthermore, in studies we carried out at the Memorial Sloan Kettering with Carlos Cordon Cardo and Zvi Fuchs, we observed that the frequency of HLA expressor tumor cells among the metastatic cell population was significantly lower than that among the populations of the primary tumor of the same patients. What was particularly significant was the observation that the disseminating intravascular tumor cells were practically HLA nonexpressors, suggesting that HLA nonexpressors had a selective advantage for metastatic dissemination (Cordon-Cardo *et al.*, 1991). It therefore appears that gene manipulation of human tumor cells which would result in high expression of HLA molecules could abolish their metastatic phenotype and make such cells candidates for cellular vaccines. Hence, bypassing the necessity for modifying tumor cells by HLA gene transfection according to the genotype of each individual patient by inserting genes that would upregulate the suppressed MHC genes of tumor cells seemed worth studying.

VI. Upregulating the Expression of Endogenous H-2K Genes

To test whether the H-2K gene of a highly metastatic low H-2K expressor is structurally intact, Dani Plaksin cloned the gene from a D122 genomic library and observed that no gross mutations, deletions, or rearrangements occurred in the K^b genomic clones. It thus appeared that the endogenous H-2K genes of the low expressor tumor cells are structurally intact, and that transacting factors suppressed its expression in the highly metastatic phenotypes (Plaksin *et al.*, 1993). Following transfection with the H-2K genes, the suppressor factors are diluted by excess of promoter sequences. Hence, Lea Eisenbach undertook the study of the transcriptional mechanism controlling the differential expression of MHC class I genes in high compared to low metastatic clones. Studies in several laboratories revealed that transcription factors involved in regulating MHC gene expression manifest binding to enhancer-like sequences of the murine MHC. The mouse MHC promoters contain two enhancer-like sequences: enhancer A, -203-158; enhancer B, -120-61; and an interferon responsive sequence at -163-19 (Kimura *et al.*, 1986). The NF_kB nuclear factor is a heterodimer of p50 and p65, whereas the KBF1 is a homodimer of the p50 kDa DNA-binding subunit of the NF_kB. The NF_kB and the KBF1 bind to the same sequences (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). Since high levels of H-2K transcripts were observed in low metastatic phenotypes, whereas

they were hardly detectable in high metastatic cells, we tested whether this is correlated with different transcription factors expressed in cells of high compared to low metastatic phenotypes. Plaksin conducted the experiments and observed that KBF1, the p50 homodimer, appears at higher levels in extracts of D122, a high metastatic clone, than in A9, a low metastatic clone. The p50 homodimer in the high metastatic cells was in excess of NF_kB (the p50–p65 homodimer). In contrast, in the A9, the low metastatic clone, the NF_kB was in excess of KBF1. This suggested that the p50 is associated with H-2K suppression, whereas the p65 is associated with H-2K activation. Indeed, transfection of A9 cells with p50 expression vector resulted in a significant decrease in both K^b-specific mRNA and in cell surface H-$2K^b$ molecules. In contrast, transfection of D122 cells with p65 resulted in almost complete elimination of KBF1 from the nuclear extract, in upregulation of K^b transcripts, and in elevated expression of cell surface K^b molecules (Plaksin *et al.*, 1993). It appeared, therefore, that changing the balance between NF_kB and KBF1, i.e., between p50–p65 heterodimers and p50–p50 homodimers, changed transcriptional activity of the K^b promoter. Elevated KBF1 is correlated with suppression of the gene, whereas elevated NF_kB is correlated with activation of the gene. K^b activated D122–p65 cells, when tested upon transplantation, showed decreased metastasis formation and increased survival. Yet the levels of these biological effects were lower than those obtained following transfection with the H-$2K^b$ gene.

A parallel study of the regulation of MHC class I expression derived from our earlier observation that low metastatic high H-2K expressor clones showed higher levels of c-*fos* mRNA and protein than high metastatic low H-2K expressors. The expression of c-*fos* seemed causally related to the activation of H-2K, since transfection of D122 cells with c-*fos* resulted in induction of transcription and of cell surface appearance of H-2K but not of some other gene products and in a concomitant reduction in the metastatic competence (Kushtai *et al.*, 1988, 1990). In human leukemic cell lines (U937, HL60) induction of differentiation by TPA to macrophage-like cells was associated with a transient induction of c-*fos* and increased expression of HLA. On the other hand, induction of differentiation in HL60 cells by DMSO, that does not involve signaling through c-*fos* resulted in decreased levels of HLA. In murine erythroleukemia cells, that express constitutively c-*fos*, differentiation by DMSO caused decreased expression of c-*fos* and in accordance abolishment of H-2 expression (Barzilay *et al.*, 1987).

Transcriptional regulation of c-*fos* is mediated via heterodimers of c-*fos* and members of the Jun family of protooncogenes (c-*jun*, jun B, and jun D). Indeed, we observed that transcripts of c-*fos*, c-*jun*, and jun-

B appear in much higher levels in the low metastatic H-2K expressors clone A9 than in the high metastatic, low H-2K expressor D122 of the 3LL Lewis lung carcinoma, and a similar correlation was observed among clones of the K1735 melanoma. Although transfection of our tumor cells showed that the c-*fos* and c-*jun* are reciprocally inducible, we found that double transfectants with fused c-*fos* plus c-*jun* constructs, ensuring the integration of the same copy number of c-*fos* and c-*jun* genes, resulted in the highest immunogenic effect: Not only was metastasis formation completely abolished, but even growth of the local tumor was, in most mice, immunologically prevented by a T cell response. Such double transfectants of c-*fos* plus c-*jun* could thus serve as an efficient cellular vaccine (Yamit-Hezi, M. Sci. Thesis, 1993).

However, the application of protooncogenes for gene therapy of human cancer may elicit significant reservations. Since δ-interferon (δ-IFN) is a known inducer of MHC class I expression, we initiated a long series of studies on the function of inserted δ-interferon genes in upregulating the expression the endogenously suppressed H-2K genes and thereby on the generation of antimetastatic cellular vaccines. These will be discussed under Section IX, A.

VII. The Tumor-Specific Immunogenic Epitope of a Murine Lung Carcinoma Is an Octapeptide Mutein of Connexin 37

We demonstrated that transfection of H-2K^b gene converted a highly metastatic to a nonmetastatic highly immunogenic phenotype capable of eliciting H-2K^b-restricted antitumor CTLs. We subsequently aimed at isolating and characterizing the H-2K^b-bound tumor-specific immunogenic peptides of the Lewis lung carcinoma and Ofer Mandelboim carried out this study. Crude peptide fractions (M_r>5000) from H-2K-transfected tumor cells were separated by reverse-phase HPLC, and individual fractions were loaded on RMA-S cells, of H-2^b origin, which are defective in their capacity to process proteins and hence express "empty" H-2K molecules. Such cells were acted upon by CTLs generated against H-2K^b-transfected 3LL cells. The most active fractions conferring susceptibility to the antitumor CTL were rechromatographed, and an active peak was sequenced, manifesting a major peptide, Phe–Glu–Gln–Asn–Thr–Ala–Gln–Pro (or Ala). An NBRF data bank search revealed homology with a peptide from the mouse gap-junction protein, connexin 37. Synthetic octapeptides were prepared with Pro (MUT1) or Ala (MUT2) at position 8, and both were found to stabilize H-2K^b molecules on the RMA-S cells. RMA-S cells thus loaded by either of the

synthetic peptides, then injected to syngeneic mice, induced antitumor CTL, which manifested specificities identical to CTLs generated against H-2K 3LL transfectants. It is important to note that the CTLs produced against the octapeptides also lysed cells of another H-2^b-originated lung carcinoma, the CMT-64, but did not lyse cells of other H-2^b-originated tumors. Thus, the octapeptides may represent a general lung carcinoma tumor-associated antigen (Mandelboim *et al.*, 1994).

RT-PCR tests on RNAs isolated from D122, H-$2K^b$-transfectant 3LL, and from normal H-2^b lung tissue indicated that both the normal connexin 37 protein and the Gln-mutated forms are expressed in the tumor cells. Mutations in connexin 37 may be causally related to the malignancy of lung carcinomas by impairing contact inhibition which in normal lung cells might be mediated by the normal gap-junction protein, the connexin 37. The cross-reactivity we observed with the nonrelated CMT-64 lung carcinoma suggests that common lung carcinoma immunogenic peptides may exist (Mandelboim *et al.*, 1994). Since recent studies of ours indicated that peptide vaccination of tumor-bearing animals elicited an antimetastatic immune response, immunotherapy by means of peptide epitopes might represent an effective antimetastatic modality.

VIII. The New Era of Molecularly Characterized Tumor-Associated Antigens

Do the observations we made in our studies of a murine lung carcinoma reflect a general reality of murine tumors? Are they relevant to human cancer? In fact, very extensive investigations were carried out during the past decade in which mouse and human tumors were subjected to analytical studies indicating the existence of immunogenic tumor-associated antigens. These constitute a new era of exciting observations of tumor-specific epitopes and of their coding genes. Although it is impossible to deduce at this point in time the probability of their application to clinical modalities of tumor immunotherapy, the basic observations made recently are of great interest. Here, I shall refer to just a few systems, which seem to me to be particularly significant, and will start with the unique series of observations made by Terry Boon and his associates studying both mouse and human tumors.

A. Murine Tumors

With regard to mouse tumors, Boon started with a rather artificial system in which experimental induction of antigens in an existing tumor was achieved: he used a line of a DBA/2 originated mastocytoma named

P815. Mutagen treatment of the tumor resulted in immunogenic mutants that lost tumorigenicity in syngeneic mice (tum$^-$ variants) because they acquired the capacity to elicit specific CTLs against the induced tum$^-$ transplantation antigens (P35B) (Szikora *et al.*, 1990; Van den Eynde *et al.*, 1991). Clones of these CTLs were produced, and when tested against transfectants with DNA from tum$^-$ cells could identify recipient cells expressing the tum molecule (i.e., antigen P35B). The gene P35B that was thus found to code for the tumor-rejection antigen is 6 kb long containing 11 exons, differing from its normal allele by a point mutation. A mutated peptide (replacing a Ser by an Asn residue) was thus presented to CTLs via MHC class I molecules. Another tum antigen discovered following mutagen treatment is the P91A, coded by a mutation of another gene on its exon 4, etc., each specifying a different immunogenic peptide presented by MHC class I to precursors of syngeneic CTLs (Sibille *et al.*, 1990; Chomez *et al.*, 1992). Thus, tum comprises a class of genes which in P814, under the influence of mutagens, expressed new epitopes recognized by CTL (Dyson *et al.*, 1992). Cells carrying the mutant alleles have impaired tumorigenicity. Antigens thus detected following mutagen treatment might represent the individual antigenic specificity manifested among chemically induced tumors in the classical studies of Prehn and Main and in the uv-induced tumors. Yet parallel studies by Terry Boon's group indicated that mouse tumors may also express cell surface tumor-rejection antigens resulting from the expression of genes that exist in normal cells but in an unexpressed form (Boon *et al.*, 1992). The P815A and P815B antigens coded by the P1A gene of the P815 mastocytoma represent the category of normal genes that were made to express themselves following transformation. Whether in spontaneous tumors of mice more tumor antigens result from gene mutations than from the activation of gene expression remains an open question. The latter may constitute a category of antigens that are shared by tumors of the same histological type (Boon *et al.*, 1992). They may then be more approachable targets for immunotherapy. The question then arises whether human tumors of the same histological type share tumor antigens, or whether a tumor of each individual expresses the antigens derived from different mutational events.

B. Human Tumors

Searching for endogenous peptides presented to T lymphocytes on human cancer cells, melanoma seemed the first attractive candidates for their exploration. They seemed attractive because (a) antigens in human melanomas recognized by HLA class I-restricted T cells were identified

and characterized (Wolfel *et al.*, 1989; Van den Eynde *et al.*, 1989); (b) TIL cells infiltrating human melanomas manifested following *in vitro* growth in the presence of IL-2, cytotoxic activity against melanoma target cells (Lotz, 1990; Itok *et al.*, 1988; Aebersold *et al.*, 1991; Rosenberg *et al.*, 1988); (c) following their injection into cancer patients they caused regression of established melanoma lesions (Aebersold *et al.*, 1991; Rosenberg *et al.*, 1988); (d) antimelanoma cytotoxic cells are $CD8^+$ T lymphocytes, recognizing cell surface epitopes in an HLA class I-restricted way (Hom *et al.*, 1991; Kawakami *et al.*, 1992; Muul *et al.*, 1987; Topalian *et al.*, 1989).

Terry Boon's studies on human tumors were focused on melanomas. Peripheral blood lymphocytes from a melanoma patient were stimulated to generate CTLs against a line of autologous human melanoma, and these CTLs recognized an antigen MZ2-E expressed on the autologous tumor cells. In fact, it is also expressed on melanomas of other patients and on some tumors of other histological types, but it is not expressed on normal cells (Degiovanni *et al.*, 1990; Van der Bruggen *et al.*, 1991). The tumor antigen—a nonapeptide—is presented to CTLs via HLA-1 and is coded by a third exon of the gene MAGE-1, which is expressed in 40% of melanoma samples (Marchand *et al.*, 1993). HLA-A2 was also observed to stimulate autologous CTLs, yet here the immunogenic peptide derived from a cDNA that corresponds to transcripts of the tyrosinase gene. This gene is active in all human melanomas tested (Traversari *et al.*, 1992). Although, unlike MAGE-1, it is also expressed on normal melanocytes, it could furnish a target for specific immunotherapy of human melanoma, even if effects of destruction of normal melanocytes will have to be expected (Wolfel *et al.*, 1993). HLA-A2 appears to represent the most common class I-restricting element of the antimelanoma CTLs. Hence, the question of the nature of the peptides presented via the $HLA\text{-}A2^+$ molecules constituted an obvious challenge. Lotz *et al.* (1993) eluted peptides from cell lines of $HLA\text{-}A2^+$ melanomas. They were demonstrated to contain TIL-reactive epitopes. Six epitopes have been identified (P1–P6), three of which (P1, P2, and P4) may represent the three clonal melanoma determinants discovered previously (Knwuth *et al.*, 1989; Wolfel *et al.*, 1989). Yet the sequence of these peptides was not determined. This was recently achieved by C. L. Slingluff *et al.* (1994), demonstrating that of several thousand peptides presented by the HLA-A2.1, at least 9 are recognized by melanoma-specific CTLs. One of these peptides, a nine-residue entity, was sequenced and shown to represent an epitope for CTL lines established from each of five different melanoma patients. This peptide may then be a candidate for a synthetic experimental antimelanoma vaccine.

A completely different set of human tumor-associated antigens was studied by Olivera Finn, focusing on glycosylated mucin molecules expressed on pancreatic, breast, and ovarian tumors. CTLs from cancer patients manifested an HLA nonrestricted cytotoxicity, yet the T cell receptor is an alpha–beta heterodimer typically found on MHC-restricted T cells (Barnd *et al.*, 1989). The epitope SM3 is part of multiple tandem repeats of a 20 amino acid sequence of the protein core of the mucin, and it therefore explains the lack of MHC restriction (Jerome *et al.*, 1991). The specificity of the CTL reactivity was elegantly demonstrated by testing CTL infiltrated into ovarian, pancreatic, or breast tumors. Several lines were generated from such CTLs which recognized in an MHC-nonrestricted pattern mucin-1 expressor carcinoma cells but not nonexpressor cells. Such CTL did lyse EBV-transformed cells that were made with polymorphic epithelial mucin complementary DNA following transfection to express mucin core–peptide. These CTLs did not lyse targets that were transfected with an expression construct containing a murine frameshift mutant cDNA. Yet, although the different lines of EBV and Burkitt lymphoma cells were transfected with the identical murine construct, they expressed different subsets of tumor-associated epitopes. This indicates that the specificity of these epitopes for tumors is not due to genetic alterations of the murine gene in tumors, but rather from post-translational modifications of normal gene products. Incomplete glycosylation of mucin might determine the appearance of different epitopes (Jerome *et al.*, 1992; Ioannides *et al.*, 1993).

IX. Tumor Cells Transduced by Cytokine Genes

I have reviewed in some detail the recent progress made with regard to the studies of Terry Boon's groups and of Olivera Finn since both of them are excellent examples of a new molecular approach to the old question of whether or not spontaneous tumors express tumor-associated antigens which can represent molecular targets for immunotherapy of cancer. Yet for immunotherapy as a clinical modality the expression of cell surface antigens on tumor cells presented by MHC molecules (i.e., of tumor-specific peptides) is necessary but may not be sufficient. Immunotherapy depends on the capacity to elicit within the cancer patient high enough levels of immune effector cells that could either eliminate tumor cells or at least prevent tumor progression. The immunogenic effects of antigens were demonstrated to be, among other factors, a function of a group of proteins collectively defined as cytokines or lymphokines synthesized and secreted by a variety of cells and each manifesting a large spectrum of cellular effects. They act locally as autocrine or paracrine signals, and most of them reveal regulatory

functions on cells of the immune system. Because of their local, paracrine function it appeared that the introduction of cytokine genes into tumor cells might amplify host antitumor responses significantly better than systemic administration of the cytokines. Cytokine gene therapy, based on the introduction of tumor cells modified via gene transfer to secrete IL-2 (Fearon *et al.*, 1990; Bubenik *et al.*, 1990; Gansbacher *et al.*, 1990), IL-4 (Tepper *et al.*, 1989; Blankenstein *et al.*, 1990; Golumtek *et al.*, 1990), IL-7 (Hock *et al.*, 1991), δ-IFN (Watanabe *et al.*, 1989; Restifo *et al.*, 1992), TNF-α (Blankenstein *et al.*, 1991), or GM-CSF (Colombo *et al.*, 1991; Dranoff *et al.*, 1991) was tested in a large number of murine tumors. Such cytokine gene-transduced tumors, in most cases, lost their tumorigenic properties while acquiring increased immunogenic potency, manifested in the capacity to (a) induce CTLs and additional immune effector cells, (b) immunize against a subsequent graft of parental tumor cells, (c) prevent growth of coinjected parental tumor cells, or (d) elicit an immune response effective in rejecting 1–3 day old tumors. In most tested cases $CD8^+$ and $CD4^+$ cells seemed to participate in the protective effects. Yet, in the first demonstration of the effects of an inserted cytokine gene (Tepper *et al.*, 1989), tumor cells transduced with IL-4 genes seemed to be infiltrated during their rejection by eosinophils, suggesting that the latter mediated the immunogenic effect. These studies established basic principles of the introduction of cytokine genes for immunotherapy of tumors, yet in most of these studies immunogenic animal tumors were used as distinct from metastatic nonimmunogenic models. Furthermore, in most studies immunization of healthy animals, followed by challenge of a subsequent parental tumor, rather than immunization of tumor-bearing metastatic cells, was investigated. None of the studies tested the effects of cytokine gene-transduced tumor cells on widely spread spontaneous metastasis, following surgical removal of the local tumor, which should represent models of immunotherapy via gene therapy of human tumors. We carried out studies on the properties of metastatic tumor cells transduced with cytokine genes, in particular on their function as cellular vaccines in animals bearing metastatic tumors. All these studies in our laboratory were performed by Angel Progador (reviewed in Porgador *et al.*, 1993a, 1994).

A. Tumor Cells Transduced by γ-Interferon Genes

We have known that γ-IFN is a potent inducer of MHC class I gene expression. More recently it was shown that genes involved in processing

and transport of peptides for MHC class I binding are also upregulated by γ-IFN. Hence, it seemed probable that the insertion of γ-IFN gene into tumor cells might increase their immunogenic properties. In fact, in earlier studies γ-IFN genes were introduced into two murine tumors: a murine neuroblastoma (Watanabe *et al.*, 1989) and a methylcholanthrene-induced fibrosarcoma (Gansbacher *et al.*, 1990). In both models γ-IFN-secreting tumor cells manifested reduction or abolishment of their tumorigenic properties. The γ-IFN-producing cells showed high elevation of MHC class I expression and of the capacity to induce CTLs. When injected into mice they induced protection against a subsequent graft of parental tumor cells.

Aiming at the induction of immunity against tumor metastasis we used retroviral vectors to introduce γ-IFN genes into cells of the high metastatic clone D122 (Porgador *et al.*, 1993b). As a function of the vector used, two types of IFN infectants were isolated: γ-IFN high secretors (128–256 IU/ml) and γ-IFN very low or nonsecretors (<21 IU/ml). In both infectants the expression of endogenous MHC class I was significantly upregulated, the secretors manifesting somewhat higher levels of MHC class I expression. Testing their biological properties we observed the following: both types, when inactivated by irradiation and then injected to normal mice, elicited similar levels of CTLs, and both protected mice to a similar extent against a subsequent graft of parental tumor cells. Thus, high levels of expression of MHC class I determined the induction of CTLs, and the latter seemed to have determined the state of protection against parental grafts. However, when mice carrying an established tumor of the metastatic clone were vaccinated, clear differences were observed between the IFN secretors and the nonsecretors. γ-IFN secretors were significantly more effective than nonsecretors in inducing host response against tumor metastasis. This suggested that effector cells additional to CTLs are induced by the secretors, and these are involved in preventing growth of tumor metastasis. Indeed, we found that the irradiated secretors induced, in addition to CTL, the generation of natural killer (NK) cells which seemed functional in arresting metastatic growth in tumor-bearing animals. Furthermore, to get closer to a model of immunotherapy of human cancer, we vaccinated animals from which the local tumor was surgically removed at a stage in which lung micrometastasis had already formed. Only the IFN secretors elicited immunity which nearly cured tumor metastasis completely. These observations suggest that γ-IFN-transduced tumor cells should be considered as candidates for a therapeutic modality in postoperative situations of human cancer.

B. Tumor Cells Transduced by IL-6 Genes

When tumor cells were transfected with IL-6 genes, we observed a correlation between the levels of IL-6 production by the transfected cells and *in vitro* growth retardation. The growth arrest was not a function of an autocrine effect of the secreted IL-6, since neutralizing antibodies to IL-6 did not prevent the growth arrest, neither did exogenous IL-6 inhibit the *in vitro* growth of D122 cells. The IL-6 transfectants significantly reduced their metastatic competence, most probably by inducing $CD8^+$ immune responses. When used as inactivated cellular vaccines, highly positive IL-6 transfectants protected the mice against metastatic growth of a subsequent graft of parental cells. Furthermore, when mice carrying a local parental tumor were similarly vaccinated, growth of lung metastasis was significantly reduced. Yet, a much smaller effect was obtained in postsurgical vaccination of established metastasis. Similar results were obtained when IL-6-transduced B16-F10.9 melanoma cells were tested.

C. Tumor Cells Transduced by IL-2 Genes

Interleukin-2, secreted primarily by activated CD4 lymphocytes, is a potent activator of CTL proliferation, of helper T cells, of NK cells, and of macrophages. An optimal therapeutic effect of IL-2 might be achieved by a local rather than a systemic application of IL-2, thus decreasing IL-2 toxicity and enabling optimal local levels of the cytokine at the tumor site. Gene insertion into murine tumor cells showed that secretors of IL-2 manifested reduced tumorigenicity and induced protective immunity against subsequent grafts of parental tumor cells, while eliciting antitumor CTLs. To explore the effects on metastatic cells, IL-2 gene was inserted via retroviral vectors into cells of the D122 clone, and both high and low secretors showed abolishment of tumorigenicity following transplantation (Porgador *et al.*, 1993c). In nude mice the low secretors retained their tumorigenic properties, and only the high secretors manifested reduction in tumorigenicity. Similar differences with regard to experimental metastasis were observed following intravenous injection of high and low IL-2 secretors. These results suggested that high IL-2 secretors seem to induce, in addition to T cell effectors, the generation of non-T cells. CTL again seemed sufficient for protective immunization against a subsequent graft of parental tumor cells, and here too vaccination with inactivated IL-2 infectants of mice carrying a small established tumor of parental cells prevented the generation of metastasis by the parental tumor. Yet, curing established metastasis following postsurgical vaccination was significantly less effective. Successful

treatment of established micrometastasis in a postsurgical protocol was achieved with cellular vaccines generated by double transfectants with H-2K + IL-2 genes, but not with H-2K + IL-6 or IL-2 + IL-6 genes. Cellular vaccines with combinations of genes should therefore be tested for the rejection of overt metastasis.

X. Target Organ Specificity of Metastasis

The ability of disseminating tumor cells to grow in a certain target organ constitutes the final limiting step of the metastatic cascade. Since different tumors tend to generate metastasis in different organs, it appeared that target organ specificity of metastasis might be based on specific paracrine growth signals generated in the different organs acting on the invading tumor cells. This concept, coined as the "seed" and "soil" hypothesis, was formulated by Paget in 1889, yet the experimental data were mostly limited to the demonstrations that organ conditioned media induced growth of tumor cells in culture which generate metastasis in these organs. Indeed, working with murine tumors which generated metastasis in the lungs, we observed that cells of clones of a high metastatic phenotype, but not of clones of a low metastatic phenotype, were growth induced in culture by lung-conditioned media. Assuming that the lung conditioned media contained growth factors capable of stimulating the metastatic cells, we explored putative receptors on such tumor cells. Having found that membrane vesicles from high metastatic clones showed a higher tyrosine kinase activity than membrane vesicles from low metastatic clones, we screened our tumor clones with probes of various tyrosine kinase domains of oncogenes and of growth factor receptors. We discovered that the high metastatic clones differentially expressed an mRNA homologous to v-*fms*$_x$ oncogene. In these studies performed by our students, Myoung Sol do and Cheryl Fitzer-Attas, a cDNA library was prepared from cells of the high metastatic clone, and applying the *fms* probe we isolated and sequenced a 6.5-kb cDNA encoding the murine PDGF$_\alpha$ receptor. To test whether expression of this gene would confer metastatic properties, we transfected cells of low metastatic clones, and observed that the transfectants acquired metastatic capacity exclusively to the lungs. We further found that lung conditioned media, but not kidney or liver, contain high levels of PDGF-like growth factors. It is therefore tempting to assume that the lung cell-secreted PDGF stimulate paracrinicity tumor cells expressing PDGF$_\alpha$ receptors thus determining the lung as the target organ for 3LL metastasis (Do *et al.*, 1992; Fitzer-Attas, 1993).

Will downregulation of PDFF$_\alpha$R expression downregulate metastasis?

We prepared a mammalian expression vector containing a c-terminal truncated form of $PDFF_{\alpha}R$ by site-directed mutagenesis. Since formation of heterodimers between a normal receptor molecule and a truncated one was shown to abolish phosphorization and signal transduction in a $PDGF_{\beta}R$ system, we transfected the $PDFF_{\alpha}R$ truncated gene into cells of one highly metastatic clone. The transfectants showed a significantly reduced metastatic competence, compared to that of parental cells or to mock transfectants, indicating that $PDFF_{\alpha}R$ expression controls the completion of the metastatic process.

This is the first system that clearly demonstrates a role of a growth factor receptor in proliferation in a specific target organ without any effect on local tumor growth. Specific blocking of such receptors might constitute another treatment modality for tumor metastasis. The use of anti-receptor antibodies or soluble receptors and of tyrosine kinase inhibitors (tyrophostins) on growth of murine lung metastasis is being investigated.

The approach aimed at controlling the growth of tumor metastasis by blocking growth factor receptors operating in metastatic growth recently gained significant support from the exciting observations of Dr. Mendelsohn's group, who are studying the function of monoclonal antibodies to EGF receptors in substantially enhancing the effects of doxorubicin and *cis*-diamminedichloroplatinum (*cis*-DDP) on established xenografts of cells of human tumor lines. Two anti-EGF receptor antibodies mAbs (528, isotype Ig62e and 225, isotype IgG1) were used in combination with either doxorubicin or *cis*-DDP in nude mice carrying established xenografts of two human cell lines: the A431 squamous cell carcinoma and the MDA-468 breast carcinoma, which express high levels of EGF receptors. The combination of both agents had a dramatic tumoricidal effect *in vivo,* manifested either in eradication of well-established tumors or in growth inhibition significantly greater than that produced by doxorubicin or DDP alone (Baselga *et al.,* 1993; Fan *et al.,* 1993). This constitutes the first demonstration that treatment of well-established tumors with a combination of anti-receptor antibodies and a chemotherapeutic agent resulted in tumor-free animals at 100 days follow-up. Based on these observations, clinical trials of combined therapy with human chimerized mAb 225 plus *cis*-DDP or doxorubicin will be initiated.

XI. Conclusion

It seems conceivable that tumor progression is based on a series of genetic changes in which mutations or deletions of suppressor genes,

modification of protooncogenes, or of genes which control growth signals and their receptors convert benign to malignant phenotypes. Yet, in many situations, therapeutic modalities have to be applied when a state of malignancy was already fully achieved. In such cases, the local tumor may still be heterogeneous with regard to the metastatic phenotype of its cell population. This heterogeneity, in our murine models, did not seem to be based on differential amplification of oncogenes or of modification of suppressor genes (p53, Rb, NM23). Furthermore, transfection of cells from our low metastatic clones with transforming oncogenes (V-Ha-*ras*, v-*fos*) did not result in the appearance of high metastatic phenotypes (Eisenbach *et al.*, 1986). In fact, many protooncogenes are similarly expressed in low and high metastatic clones of the same tumors (Eisenbach *et al.*, 1988). We therefore studied differences relevant to the category of interaction of tumors with host immune systems, focusing on tumor cell interaction with T lymphocytes.

One of the primary functions of T lymphocytes is identification and elimination of cells expressing abnormal proteins within the vertebrate organism. This is primarily a function of cytotoxic T lymphocytes which recognize, on the organism cell surface, peptides derived from intracellular proteins and transported in association with MHC class I molecules to the cell surface. CTLs recognizing abnormal peptides, derived from either exogenous viral proteins or from endogenous mutated tumor proteins, appear to function as the potent defense effectors against viral infection or cancerous cells. Yet autonomous host resistance to tumors by MHC-restricted CTLs is hardly functional against the local tumors. Rather, it seems to be operating primarily against the disseminating nonmetastatic phenotypes within the heterogeneous tumor cell population. This is deduced from our observation that (a) the metastatic phenotypes of murine and human tumors are characterized by impaired expression of MHC class I, in particular of the H-2K gene; and (b) restoration of H-2K expression following H-2K gene transfection converted metastatic to benign phenotypes, most probably by restoring the presentation to CTL of tumor-specific peptides. H-2K transfectants could be used, following inactivation, as cellular vaccines in tumor-bearing animals. The upregulation of the endogeneously suppressed H-2K was achieved by insertion of δ-interferon gene. δ-Interferon gene-transduced cells manifesting, in addition to MHC class I expression, local secretion of the cytokine, were found to be potent cellular vaccines when injected at advanced states of the disease, i.e., following surgical removal of the local tumor when lung micrometastasis is already formed. The requirement for effective antimetastatic vaccination of both class I expression and cytokine secretion was demonstrated in experiments in

which cellular vaccines were generated by transfection with both H-2K and IL-2 genes. We have identified, sequenced, and synthesized the tumor-specific octapeptides presented via the H-2K^b molecule to CTLs, which appears to be a mutein of connexin 37. The immunogenic effects of the octapeptides in tumor-bearing animals eliciting a cellular response which eradicated metastasis makes the isolation of this peptide a model for the generation of antimetastatic vaccines composed of synthetic peptides. Vaccination of patients with progressing human tumors with preparation of a single peptide may result in selection of antigen nonexpressors. Since class I MHC of heterozygous organisms is expected to present a number of tumor-specific peptides, one should aim at a cocktail of different peptides, thus reducing the probability of selection of antigen nonexpressor variants. Even then it is impossible to predict to what extent immunotherapy, either via gene therapy or by vaccination with the peptides themselves, might turn out to be an effective clinical modality.

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TUMOR SUPPRESSOR *p53* MUTATIONS AND BREAST CANCER: A CRITICAL ANALYSIS

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ADVANCES IN CANCER RESEARCH, VOL. 66

I. Introduction

Cancer of the breast is the most common neoplasm and the leading cause of cancer-related deaths among women in most developed and many developing countries (Parkin *et al.*, 1984; Boring *et al.*, 1992). For over half a century, the incidence of breast cancer has risen steadily, reaching as high as 4% per year in the mid-1980s (Harris *et al.*, 1992). The recent increase is thought to be due to earlier detection (Marshall, 1993). Nevertheless, the mortality rate has remained relatively constant for decades at ~27 per 100,000 women (Harris *et al.*, 1992; Marshall, 1993).

Risk factors associated with the disease include both personal and environmental sources which comprise family history of cancer, higher socioeconomic class, first childbirth after age 25, early age of menarche, late age of menopause, and high dietary fat intake (reviewed in Adami *et al.*, 1990; Mettlin, 1992). Although the probability of breast cancer increases with age (Kessler, 1992), premenopausal women constitute at least 15% of breast cancer deaths (Muir *et al.*, 1987). The statistics portray a dismal outcome; nevertheless, early diagnosis and treatment of breast cancer may result in a survival rate approaching 100% (American Cancer Society, 1993). Clearly, it is of public health importance to develop approaches for early diagnosis, treatment, and prevention of breast cancer.

II. Somatic Changes in Human Breast Cancer

A number of genetic alterations are frequently associated with cancer of the breast. These include the activation of protooncogenes, such as c-*MYC, INT2,* and *HER2* (c-*erb*B2/*neu*) (reviewed in Groner and Hynes, 1990; Callahan *et al.*, 1992a,b). Loss of heterozygosity (LOH) appears to account for the majority of mutations in primary breast cancer. Such genetic losses are believed to hallmark tumor suppressor genes. Putative

tumor suppressor genes which appear to be involved in breast cancer are the retinoblastoma susceptibility gene (*RB1*) (reviewed by Groner and Hynes, 1990), *BRCA1* (reviewed by Easton *et al.*, 1993), *NM23-H1* (Steeg *et al.*, 1993), *DCC* (Fearon et al., 1990), and *TP53* (*p53*).

Of the numerous genetic alterations associated with cancer of the breast, changes in *p53* are the most frequently identified. In breast tumors and tumor cell lines, the highest detectable LOH is at the *p53* locus on chromosome 17p (Mackay *et al.*, 1988; Cropp et al., 1990; Groner and Hynes, 1990; Sato *et al.*, 1990, 1991; Thompson *et al.*, 1990; Chen *et al.*, 1991a; Varley *et al.*, 1991; Callahan *et al.*, 1992b; Knyazev *et al.*, 1993). The same is true for most, if not all, other types of tumors (reviewed by Levine *et al.*, 1991; Caron de Fromentel and Soussi, 1992; Stratton, 1992; Donehower and Bradley, 1993). Furthermore, the finding that both alleles of *p53* are deleted and/or mutated in a majority of murine and human tumors suggests that mutation of *p53* is central to carcinogenesis (Levine *et al.*, 1991). To place in perspective the role *p53* mutations in the genesis of breast cancer, an overview of what is currently understood regarding the function of p53 is in order.

III. History of *p53*

A. Oncogene

p53 was discovered in the late 1970s as a cellular protein which was bound to the large tumor antigen (T-ag) in simian virus 40 (SV40)-infected or -transformed cells (DeLeo *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). Early studies showed that p53 quantities were elevated 5- to 100-fold in many transformed and tumor cell lines (DeLeo *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Reich *et al.*, 1983); however, the protein was virtually undetectable in normal cells (DeLeo *et al.*, 1979; Reich and Levine, 1984; Rogel *et al.*, 1985). The high levels of p53 in transformed cells were shown to be due to increased stability of the protein (Oren *et al.*, 1981; Reich *et al.*, 1983).

In 1983 Zakut-Houri *et al.* predicted the complete amino acid sequence of murine p53. A number of complete murine genomic and cDNA *p53* clones were subsequently isolated and sequenced (Bienz *et al.*, 1984; Jenkins *et al.*, 1984a; Parada *et al.*, 1984; Pennica *et al.*, 1984; Pinhasi and Oren, 1984). Expression analyses showed that p53 was capable of immortalizing low-passage rat cells in culture (Jenkins *et al.*, 1984a) and cooperating with an activated *ras* oncogene to transform primary cultures of rat embryo fibroblasts (Eliyahu *et al.*, 1984; Parada *et al.*, 1984). Reconstitution of *p53* expression in an Abelson murine

leukemia virus-transformed cell line resulted in lethal tumors when the cells were injected *in vivo* (Wolf *et al.*, 1984), and overexpression of *p53* in established rat cells caused tumorigenicity in nude mice (Eliyahu *et al.*, 1985). Consequently, *p53* was categorized as a cellular oncogene.

B. Tumor Suppressor Gene

The transforming properties of p53 were questioned when a murine *p53* cDNA clone, p11-4 (Pennica *et al.*, 1984), failed to cooperate with the *ras* oncogene in transformation assays (Hinds *et al.*, 1987; Finlay *et al.*, 1988). Hinds *et al.* (1989) demonstrated that p11-4 contained the bona fide wild-type (WT) *p53* cDNA and that mutation was required to render the p53 protein oncogenic. Thereafter, it was shown that the normal, WT *p53* gene product could suppress the growth of transformed cells *in vitro* (Baker *et al.*, 1990a; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990), the transformation of cells in culture by other oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989), and the tumorigenic potential of cells in animals (Chen *et al.*, 1990; Shaulsky *et al.*, 1991a). These observations, along with evidence of deletions and/or mutations of *p53* alleles in many human and animal tumors (Mowat *et al.*, 1985; Baker *et al.*, 1989; Nigro *et al.*, 1989), indicated that WT *p53* should be reclassified as a tumor suppressor gene.

Due to the unrecognized use of mutant (MT) *p53* in many studies published prior to 1990, much of those data have required reinterpretation. Other points of complication regarding the phenotypes of MT p53 are noteworthy: first, there is a spectrum of phenotypes conferred by various MTs of *p53*, ranging from simple loss of growth suppressor activity to gain of oncogenic potential (Zambetti and Levine, 1993). These characteristics seem to depend upon the specific *p53* mutation. Second, it is apparent that mutation of *p53* is very common in many established cell lines (Harvey and Levine, 1991; Kress *et al.*, 1992; Rittling and Denhardt, 1992; Ozbun *et al.*, 1993b). Moreover, the *p53* genotype may be subject to further mutation and evolution as cells are cultured *in vitro* (Ozbun *et al.*, 1993b). These observations make interpretation of *p53* functional studies carried out *in vitro* especially challenging.

C. Special p53 Reagents

Several reagents have been instrumental in the study of p53. The monoclonal antibodies PAb240, PAb246, and PAb1620 recognize epitopes present on different conformations of the murine and human p53

proteins. PAb240 (Greaves, 1988) and PAb1620 (Milner *et al.*,1987) recognize both murine and human p53, whereas PAb246 (Yewdell *et al.*, 1986) is murine specific. PAb246 and PAb1620 react with topologically related epitopes which are sensitive to denaturation. PAb240 detects a conserved amino acid motif which is hidden in the genotypically WT tumor suppressor protein but is commonly displayed on p53 molecules which have sustained mutations (Stephen and Lane, 1992). Thus, reactivities with PAb246/PAb1620 or PAb240 seem to be mutually exclusive on a given p53 molecule. Moreover, p53 proteins which are reactive with PAb246/PAb1620 (PAb246$^+$/PAb1620$^+$ and PAb240°) are typically classified as WT, whereas proteins recognized by PAb240 (PAb240$^+$ and PAb246°/PAb1620°) are categorized as MT. However, these simple classifications may not accurately reflect the genotype of *p53* in every situation, and consequently the wording "WT conformation" or "MT form" will be used herein to identify the folding of the p53 protein and not to imply a specific genotype.

The murine p53 MT which has an alanine to valine substitution at codon 135 (A135V) was shown to be conformationally and functionally temperature sensitive (ts) (Milner and Medcalf, 1990). The polypeptide is WT (PAb246$^+$) at the permissive temperature of 32.5°C but is MT (PAb240$^+$) at the nonpermissive temperature of 39°C. Furthermore, at 32°C the protein acts as a growth suppressor, whereas at 39°C the protein functions as a dominant oncogene (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). Other phenotypes associated with temperature sensitivity will be discussed as appropriate below.

IV. The *p53* Gene

A. Structure

The human *p53* gene has been cloned (Matlashewski *et al.*, 1984; Zakut-Houri *et al.*, 1985; Lamb and Crawford, 1986) and localized to the short arm of chromosome 17 (17p13.1) (McBride *et al.*, 1986; Miller *et al.*, 1986). The mouse genome contains both a functional murine *p53* gene that maps to chromosome 11 (Czosnek *et al.*, 1984; Rotter *et al.*, 1984) and a processed, inactive pseudogene that resides on chromosome 14 (Zakut-Houri *et al.*, 1983; Czosnek *et al.*, 1984). *p53* sequences have been determined from other vertebrates, including monkey (Rigaudy and Eckhart, 1989), chicken (Soussi *et al.*, 1988a), rat (Soussi *et al.*, 1988b), hamster (Legros *et al.*, 1992), trout (Caron de Fromentel *et al.*, 1992), and *Xenopus laevis* (Soussi *et al.*, 1987). No *p53* homologue has been found in species such as *Drosophila,* sea urchin, or yeast (Soussi *et al.*, 1990). A

comparison of the organization of the *p53* gene between human, mouse, rat and *X. laevis* revealed 11 similar exons (reviewed by Soussi *et al.*, 1990; Hulla and Schneider, 1993). Although the *p53* introns vary in length among the species, the splice sites occur at essentially homologous positions, and the first exon is noncoding in all cases (Soussi *et al.*, 1990).

B. Regulation

Several regulatory elements have been mapped within the *p53* gene (Fig. 1). Contrary to most class II eukaryotic promoters, *p53* contains no consensus TATA or CAAT boxes (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985; Lamb and Crawford, 1986; Soussi *et al.*, 1990).

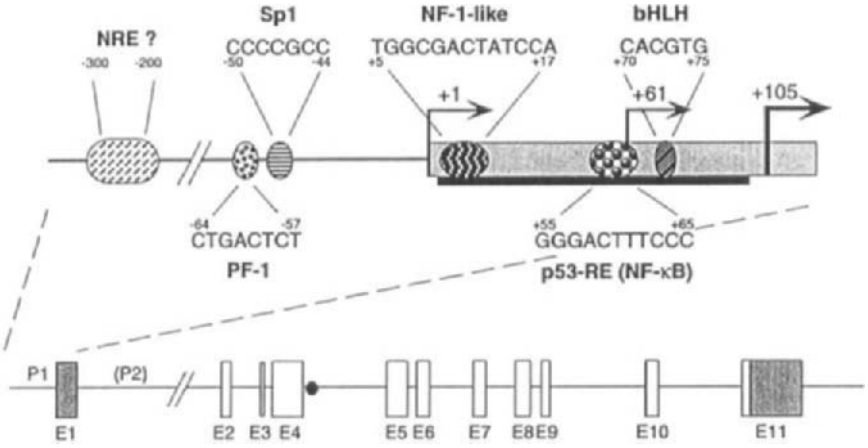

Fig. 1. The structure of the murine *p53* gene spanning 12 kilobases (modified from Donehower and Bradley, 1993). The 11 exons (E1–E11) are indicated by boxes at the bottom, with noncoding regions shaded (Bienz *et al.*, 1984). Two major promoters (P1, P2) have been described in the human *p53* gene (Reisman *et al.*, 1988). P1 is enlarged to indicate the known *cis*-acting elements: a controversial negative regulatory element (NRE) (Bienz-Tadmor *et al.*, 1985; Lamb and Crawford, 1986; Reisman *et al.*, 1988; Reisman and Rotter, 1989; Tuck and Crawford, 1989), p53 factor-1 (PF-1) (Ginsberg *et al.*, 1990), Sp1 motif (Reisman *et al.*, 1993), NF-1-like binding site (Ginsberg *et al.*, 1990), p53-responsive element (p53-RE) including the NF-κB site (Deffie *et al.*, 1993), and basic helix–loop–helix (bHLH) motif (Ronen *et al.*, 1991). The three transcriptional start sites are indicated by numbered arrows (Bienz *et al.*, 1984; Tuck and Crawford, 1989) and are reconciled to the first base of murine *p53* exon 1 [+1 was denoted as −216 by Bienz *et al.* (1984)]. The human major transcriptional start site at +105 is indicated by a bold arrow (Tuck and Crawford, 1989). A heavy line indicates the predicted hairpin structure at the 5′ end of murine exon 1 (Bienz *et al.*, 1984). The positive regulatory element contained within intron 4 is shown by a closed circle (Beenken *et al.*, 1991; Lozano and Levine, 1991).

C. Expression

The size of messenger RNA (mRNA) transcripts from the *p53* gene ranges from 1.8 to 3.0 kb depending upon the species; the disparity results from the variable length of the 3′ untranslated region (Soussi *et al.*, 1987). Differential splicing has been described for *p53* mRNA, although the biological relevance of such transcripts has not been determined (Arai *et al.*, 1986; Bendori *et al.*, 1987; Matlashewski *et al.*, 1987; Han and Kulesz-Martin, 1992).

p53 has been shown to be overexpressed in a variety of human and animal tumors but the cause is generally attributed to an accumulation of the protein rather than the message (reviewed by Levine *et al.*, 1991; Montenarh, 1992; Donehower and Bradley, 1993). Nonetheless, *p53* mRNA was reported to be present in higher amounts in several murine tumors (Rogel *et al.*, 1985).

Expression of *p53* mRNA seems to correlate with the proliferative state of cells. In murine fetal development, expression of *p53* mRNA peaked during organogenesis (Days 9–11), whereas the mRNA levels were considerably reduced from Day 11 and beyond (Rogel *et al.*, 1985). An analogous observation was made during chicken embryogenesis (Louis *et al.*, 1988). Furthermore, the amount of *p53* mRNA decreased in cells induced to differentiate (Oren *et al.*, 1982; Reich *et al.*, 1983; Dony *et al.*, 1985; Khochbin *et al.*, 1988; Khochbin and Lawrence, 1989). In a synchronous 3T3 cell population, an increase in the synthesis and steady-state levels of *p53* mRNA (and protein) was detected prior to DNA synthesis late in the G_1 phase of the cell cycle (Reich and Levine, 1984). In the mouse mammary gland, *p53* mRNA levels were relatively high during pregnancy, downregulated during lactation (differentiation), and then rose again after weaning when involution (programmed cell death or "apoptosis") of the gland began (Strange *et al.*, 1992).

V. The p53 Protein

A. Structure

The murine and human p53 proteins are 390 and 393 amino acids in length, respectively (Zakut-Houri *et al.*, 1983, 1985; Pennica *et al.*, 1984). The nucleic acid sequence homology is 81% between the two but is unequally distributed over the molecule (Zakut-Houri *et al.*, 1985). A comparison of predicted p53 amino acid sequences from human, murine, monkey, rat, chicken, trout, and frog reveals five clusters of amino acids (designated I, II, III, IV, V) which are highly conserved (see Fig. 2) (reviewed by Soussi *et al.*, 1987, 1990; Montenarh, 1992).

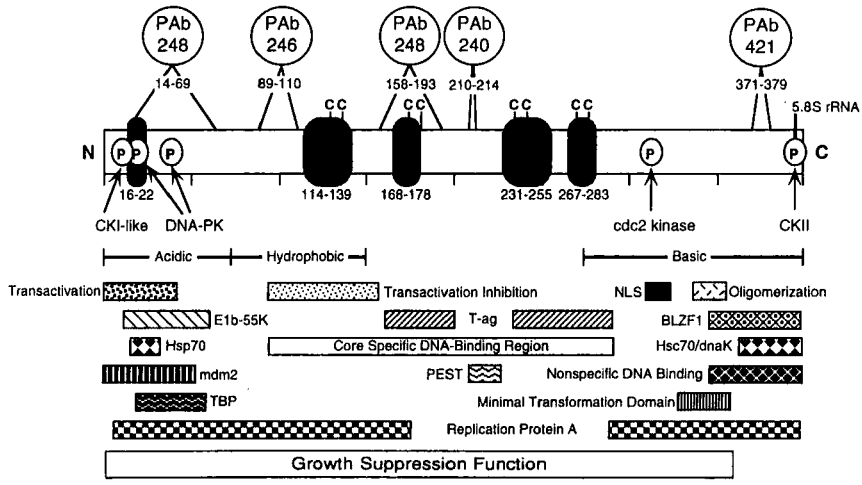

FIG. 2. Functional domains of murine p53. An open bar designates the 390 amino acid (aa) murine p53 polypeptide; all sequences were reconciled to the numbering of Pennica *et al.* (1984). Evolutionarily conserved domains are illustrated by shading on the protein (Soussi *et al.*, 1990); shown below are the corresponding aa residues. p53-specific monoclonal antibody epitopes are indicated above the protein: PAb248 (Wade-Evans and Jenkins, 1985; Greaves, 1988); PAb246 (Wade-Evans and Jenkins, 1985); PAb240 (Stephen and Lane, 1992); and PAb421 (Wade-Evans and Jenkins, 1985). Serine phosphorylation sites are denoted by a circled P with an arrow indicating the putative kinase: 9 (and possibly 7 and 12) by a casein kinase I-like protein (Milne *et al.*, 1992a); 15 and 37 by DNA-activated protein kinase (Lees-Miller *et al.*, 1992); 312 by cdc2 kinase (Bischoff *et al.*, 1990b; Stürzbecher *et al.*, 1990); and 389 by casein kinase II (Meek *et al.*, 1990). The acidic (aa 1–75), hydrophobic (aa 75–150), and basic (aa 265–390) regions of p53 are shown by heavy lines (Pennica *et al.*, 1984). Conserved cysteinyl residues (C) with homology to non-zinc-finger zinc-binding protein domains are indicated (Hainaut and Milner, 1993b). A small RNA (5.8S rRNA) is covalently linked to serine 389 (Samad and Carroll, 1991; Fontoura *et al.*, 1992). The transactivation domain (aa 1–42) (Unger *et al.*, 1992), transactivation repressing region (aa 90–157) (Liu *et al.*, 1993), major nuclear localization signal (aa 310–319) (Addison *et al.*, 1990; Shaulsky *et al.*, 1990b), the core region responsible for specific DNA binding (aa 99–289) (Pavletich *et al.*, 1994), PEST sequences (aa 211–230) (Rogers *et al.*, 1986), nonspecific DNA-binding motif (aa 343–390) (Shohat-Foord *et al.*, 1991), and the minimal transformation domain (Shaulian *et al.*, 1992; Reed *et al.*, 1993; Unger *et al.*, 1993) are designated. The smallest region of the protein required for growth suppression is shown (Shaulian *et al.*, 1992; Reed *et al.*, 1993; Unger *et al.*, 1993). Regions of p53 required for protein–protein interactions are indicated: p53 self-oligomerization (aa 337–352) (Stürzbecher *et al.*, 1992); adenovirus 5 E1b-55-kDa protein (aa 14–66) (Braithwaite *et al.*, 1991); SV40 large T antigen (aa 168–202 and 233–289) (Jenkins *et al.*, 1988); EBV BLZF1 (aa 343–390) (Zhang *et al.*, 1994); hsp70 (aa 16–35) (Lam and Calderwood, 1992); hsc70/dnaK (aa 363–390) (Hupp *et al.*, 1992); mdm2 (aa 1–52) (Chen *et al.*, 1993a); TBP, the TATA-binding protein (aa 20–57) (Liu *et al.*, 1993; Truant *et al.*, 1993); and replication protein A (Dutta *et al.*, 1993).

B. Phosphorylation

The most-studied modification of the p53 protein is phosphorylation (see Fig. 2). Phosphorylation of p53 was found to be regulated during the cell cycle, as it was underphosphorylated in G_0/G_1 compared with S phase (Bischoff *et al.*, 1990b). However, Segawa *et al.* (1993a) showed a correlation between a higher phosphorylation state for p53 *in vitro* and an increase in p53 activity in the presence of the G_1 cyclin, cyclin E, and its associated kinase, cdk2.

Murine WT p53 was phosphorylated at serine 389 (human 392) by casein kinase II (CKII) (Meek *et al.*, 1990), a reaction that appears to be required for the ability of p53 to bind to DNA with sequence specificity (see below) (Hupp *et al.*, 1992). In addition, an S389A MT p53 was unable to act as a growth suppressor *in vitro* (Milne *et al.*, 1992b). These data suggest that the antiproliferative activity of p53 is dependent upon sequence-specific DNA binding, which in turn may be regulated in part by phosphorylation at (murine) serine 389.

Purified human p53 was phosphorylated *in vitro* by cdc2 (Stürzbecher *et al.*, 1990); the primary site was serine 315 (analogous to mouse serine 312) (Bischoff *et al.*, 1990b; Stürzbecher *et al.*, 1990). The cdc2-mediated phosphorylation of p53 seems to be constant throughout much of the cell cycle, except for being lower in G_1 (Bischoff *et al.*, 1990b). However, alteration of human serine 315 or mouse serine 312 to a non-phosphorylatable residue had no detectable effect on phenotype (Meek and Eckhart, 1990; Nigro *et al.*, 1992).

p53 was phosphorylated at multiple sites *in vitro* by double-stranded DNA-activated protein kinase (DNA-PK) (Lees-Miller *et al.*, 1990): serine residues 7, 9, and possibly 18 and 37 were phosphorylated on synthetic murine p53 (Wang and Eckhart, 1992), and peptides from human p53 were phosphorylated on residues 15 and 37 (Lees-Miller *et al.*, 1992). Whereas changing amino acid 37 (S37A) of human p53 had no detectable effect, MT S15A reduced the ability to block the cell cycle ~50%. This suggests that phosphorylation of serine 15 may activate the growth-suppressive activities of p53 (Fiscella *et al.*, 1993). Interestingly, residue 15 (human) is the most highly evolutionarily conserved serine in the N-terminus (Soussi *et al.*, 1990).

Casein kinase I (CKI) was able to phosphorylate WT murine p53 expressed in *Escherichia coli* (Milne *et al.*, 1992a). The major serine residue targeted by CKI was 9; serines 7 and 12 were minor phosphorylation sites (Milne *et al.*, 1992a). p53 was shown to be an *in vivo* and *in vitro* substrate for protein kinase C (PKC); the phosphorylation site was localized to the C-terminus by V8 protease mapping (Baudier *et al.*, 1992).

Takahashi and Suzuki (1993) found evidence that growth stimulation of human breast cancer MCF7 cells by insulin-like growth factor I caused tyrosine phosphorylation of the endogenous WT p53; however, the phosphorylation sites were not mapped.

C. RNA Linkage

Phosphopeptide mapping of mouse p53 indicated a covalently linked RNA at serine 389 (Samad *et al.*, 1986). This was identified as the 157-nucleotide 5.8S rRNA, an essential component of the ribosome (Fontoura *et al.*, 1992). The functional significance of the linkage is currently unclear.

D. Subcellular Distribution

The major nuclear localization signal (NLS) of p53 is highly conserved among human, monkey, mouse, and rat but poorly homologous in chicken, frog, and trout (Soussi *et al.*, 1990). The NLS was sufficient to target proteins to the nucleus (Addison *et al.*, 1990); however, other factors apparently influence the localization of p53, as many p53 proteins with an intact NLS reside in the cytoplasm (Rotter *et al.*, 1983; Hinds *et al.*, 1987; Gannon and Lane, 1991; Ginsberg *et al.*, 1991b; Martinez *et al.*, 1991; Slingerland *et al.*, 1993).

The cellular distribution of p53 varies throughout the cell cycle (Shaulsky *et al.*, 1990a). Because the NLS is structurally linked to a cdc2 site (see Fig. 2), phosphorylation may have a role in regulating localization (Addison *et al.*, 1990). Newly synthesized p53 accumulated in the cytoplasm during G_1 growth-stimulated 3T3 cells (Shaulsky *et al.*, 1990a). The protein then localized in the nucleus for ~3 hr beginning at S phase and, following the initial round of DNA synthesis, accumulated again in the cytoplasm (Shaulsky *et al.*, 1990a). Takahashi and Suzuki (1994) showed cytoplasmic accumulation of p53 during maximum DNA synthesis of a number of normal human cell types. In herpesvirus-infected cells, p53 relocated to nuclear sites were DNA replication proteins were found, suggesting a role in the replication of DNA in normal cells (Wilcock and Lane, 1991). DNA-damaging agents caused the accumulation of nuclear p53 in fibroblasts and epithelial cells of murine, simian, and human origin (Fritsche *et al.*, 1993) and in murine thymocytes (Clarke *et al.*, 1993; Lowe *et al.*, 1993). These data indicate that nuclear WT p53 may participate in the cellular response to DNA damage and apoptosis (reviewed by Lane, 1992, 1993), whereas during DNA

synthesis under normal circumstances, WT p53 may be inactivated by nuclear exclusion.

E. Expression and Stability

In nontransformed cells, the p53 protein typically has a half-life of 20–40 min (Oren *et al.*, 1981; Reich *et al.*, 1983; Reich and Levine, 1984; Calabretta *et al.*, 1986). p53 levels in both mouse and human cells rise during progression into the cell cycle and peak in late G_1, just before S phase (Reich and Levine, 1984; Steinmeyer *et al.*, 1990). For the most part, protein levels of p53 mirror the decrease in mRNA levels during late embryogenesis and cellular differentiation (Chandrasekaran *et al.*, 1982; Oren *et al.*, 1982; Reich *et al.*, 1983; Shen *et al.*, 1983; Dony *et al.*, 1985; Rogel *et al.*, 1985; Khochbin *et al.*, 1988; Louis *et al.*, 1988; Khochbin and Lawrence, 1989).

The conformation of p53 may dictate how rapidly the protein is turned over. PEST sequences (regions rich in proline, glutamic acid, serine, and threonine) localized between amino acids 211 and 230 (murine p53) were proposed to confer instability to the protein (Rogers *et al.*, 1986) but many MT p53 proteins which maintain the PEST sequences manifest extended half-lives (Stürzbecher *et al.*, 1987a; Hinds *et al.*, 1990; Dittmer *et al.*, 1993). Interestingly, these residues encompass the PAb240 (MT) epitope (see Fig. 2) (Stephen and Lane, 1992). Huibregtse *et al.* (1991, 1993) suggested that the cellular E6-associated protein (E6-AP) is responsible for the normal, ubiquitination-dependent degradation of p53 (Gronostajski *et al.*, 1984; Ciechanover *et al.*, 1991). E6-AP is necessary to facilitate binding of the human papillomavirus (HPV) types 16 and 18 E6 oncoproteins to p53, thus promoting rapid degradation of p53 (Huibregtse *et al.*, 1991). Only p53 proteins in the WT (PAb1620$^+$) conformation were effectively targeted for rapid degradation by E6 and E6-AP although the epitope itself was not the basis for recognition (Crook and Vousden, 1992; Medcalf and Milner, 1993). Thus, rapid turnover of p53 correlates with the PEST sequences being folded such that they are not exposed on the surface of the p53 protein (i.e., WT p53 is PAb240°, and this unexposed epitope in WT p53 includes a portion of the PEST region).

WT p53 can be detected transiently in some cell types following various stimuli which induce differentiation (Kastan *et al.*, 1991b), apoptosis (Lowe and Ruley, 1993), and DNA damage (Hall *et al.*, 1993). A variety of DNA-damaging agents promote the nuclear accumulation of p53 (Maltzman and Czyzyk, 1984; Fritsche *et al.*, 1993; Hall *et al.*, 1993; Zhan

et al., 1993), which seems to begin at various points in the cell cycle (Fritsche *et al.*, 1993). An increase in the levels of p53 concomitant with inhibition of replicative DNA synthesis in response to DNA damage was reported (Kastan *et al.*, 1991a). The stockpiling of p53 in the nucleus was due to increased protein stability or increased translation, as no changes in p53 mRNA levels were observed (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991a, 1992; Kuerbitz *et al.*, 1992; Tishler *et al.*, 1993).

VI. Quaternary Structure of the p53 Protein

The p53 gene product interacts with a number of proteins, both cellular and viral in origin (Tables I and II).

A. Oligomerization

High-molecular-weight complexes of p53 from primate and rodent cells have been detected (McCormick *et al.*, 1981; Kraiss *et al.*, 1988; Schmieg and Simmons, 1988; Milner *et al.*, 1991). Oligomerization of p53 appears to be rapid and stable (Kraiss *et al.*, 1988), forming predominantly homotetrameric complexes (Stenger *et al.*, 1992; Stürzbecher *et al.*, 1992; Friedman *et al.*, 1993). An α-helical plus basic residue motif is apparently the major structural determinant of p53 oligomerization, whereby the α-helical region is responsible for dimerization, and then

TABLE I
Association of Tumor Suppressor Proteins with Gene Products of DNA Tumor Viruses

Virus	Viral proteins	Tumor suppressor proteins	Reference
Simian virus 40	Large T antigen	pRb p53	Reviewed by Ludlow (1993
Adenovirus 2, 5	Ela Elb (55 kDa)	pRb p53	Reviewed by Moran (1993)
Human papillomavirus 16, 18	E7 E6	pRb p53	Reviewed by Vousden (199
Epstein–Barr virus	EBNA-5	pRb p53	Reviewed by Klein (1993)
	BZLF1	p53	Zhang *et al.* (1994)
Hepatitis B virus	X protein	p53	Wang *et al.* (1994a)

TABLE II
INTERACTION OF p53 WITH CELLULAR GENE PRODUCTS

Protein	Functions	Reference
p53	Required for p53 activity	El-Deiry *et al.* (1992); Shaulian *et al.* (1992, 1993); Zauberman *et al.* (1993)
hsc70	Protein folding and trafficking	Pinhasi-Kimhi *et al.* (1986); Hinds *et al.* (1987); Stürzbecher *et al.* (1987a)
E6-AP	Promotes p53 degradation	Huibregtse *et al.* (1991, 1993)
cdc2, CKII	Protein kinases	Milner *et al.* (1989, 1990); Kraiss *et al.* (1990); Herrmann *et al.* (1991)
RPA	DNA replication factor	Dutta *et al.* (1993); He *et al.* (1993); Li and Botchan (1993)
S100b	Cell cycle and differentiation	Baudier *et al.* (1992)
Sp1	Transcription factor (housekeeping genes)	Borellini and Glazer (1993)
WT1	Transcription factor (tissue specific)	Maheswaran *et al.* (1993)
TBP	Transcription factor (general)	Seto *et al.* (1992); Liu *et al.* (1993); Martin *et al.* (1993); Ragimov *et al.* (1993)
CBF	Transcription factor (CAAT box)	Agoff *et al.* (1993)
mdm2	Possible transcription factor; inhibits p53 activity	Hinds *et al.* (1990); Momand *et al.* (1992); Wu *et al.* (1993)
ERCC3	Basic transcription factor (repair)	Wang *et al.* (1994a)

trimer and tetramer formation is facilitated by the basic amino acids located toward the C-terminus (Stürzbecher *et al.*, 1992). The minimal domain required for dimerization was mapped between murine amino acids 331 and 353 (Fig. 2) (Hupp *et al.*, 1992; Shaulian *et al.*, 1992, 1993). There is some disagreement concerning the role of tetramerization in p53 function (Hainaut *et al.*, 1994). Although tetramerization seems to be important in the nonspecific nucleic acid binding of p53 (Friedman *et al.*, 1993), there is no evidence that it is required for sequence-specific DNA binding (Wang *et al.*, 1994b). Most data suggest that simple dimerization is sufficient to contribute to WT p53 function (El-Deiry *et al.*, 1992; Shaulian *et al.*, 1992, 1993; Slingerland *et al.*, 1993; Tarunina and Jenkins, 1993; Zauberman *et al.*, 1993; Pietenpol *et al.*, 1994). It has been proposed that p53 binds to DNA as a dimer mediated by (murine)

residues 341–355 and that further oligomerization of dimers formed via the extreme C-terminus would inactivate p53 function (Hupp *et al.*, 1992; Tarunina and Jenkins, 1993). Thus, tetramerization may function to attenuate the growth-suppressive activities of WT p53 dimers specifically binding to and transactivating DNA sequences.

B. Complex Formation with Viral Oncoproteins

Since its discovery 15 years ago, the functional significance of p53 interaction with viral gene products (see Table I) has been extensively pursued (see reviews by Levine, 1990a; Howley, 1991; Ludlow, 1993; Moran, 1993; Vousden, 1993). The common result of such associations is to inactivate the WT suppressor functions of p53.

Although at least five different DNA viruses encode proteins which target p53, no consensus binding site has been defined in the viral gene products. Different p53 binding motifs are employed in each case (Fig. 2; for review see Prives and Manfredi, 1993). SV40 T-ag and HPV E6 proteins were shown to bind only WT forms of p53 (Lin and Simmons, 1990; Werness *et al.*, 1990; Huibregtse *et al.*, 1991; Cook and Vousden, 1992; Medcalf and Milner, 1993). In addition, SV40 T-ag, adenovirus (Ad) E1b-55K, HPV E6, Epstein–Barr virus BLZF1, and hepatitis B virus X (HBX) proteins inhibit the transactivation activity of WT p53 (Farmer *et al.*, 1992; Lechner *et al.*, 1992; Mietz *et al.*, 1992; Yew and Berk, 1992; Jiang *et al.*, 1993; Segawa *et al.*, 1993b; Wang *et al.*, 1994a; Zhang *et al.*, 1994). The ability of T-ag and HBX to block transactivation of p53 was mediated through an inhibition of sequence-specific DNA binding activity by p53 (Bargonetti *et al.*, 1992; Wang *et al.*, 1994a). That these viral gene products inhibit DNA binding and transcriptional activation functions of p53 strongly supports the hypothesis that these properties are central to the ability of p53 to mediate control of cell growth. Furthermore, the abrogation of p53 functions may be crucial to the replication and/or transforming abilities of these DNA viruses.

C. Cellular Protein Associations

The p53 polypeptide also interacts with a number of cellular proteins (Table II). As p53 has been shown to act as a transcriptional modulator, it is of interest that p53 interacts with several cellular transcription factors, including the CCAAT-binding factor (CBF) (Agoff *et al.*, 1993), the TATA-binding protein (TBP) (Seto *et al.*, 1992); Sp1 (Borellini and Glazer, 1993), ERCC3 (Wang *et al.*, 1994a), and Wilms' tumor suppressor

gene product (WT1) (Maheswaran *et al.*, 1993), and mdm2 (Hinds *et al.*, 1990; Momand *et al.*, 1992).

VII. Functional Properties of WT p53

A. Growth Suppression

In addition to p53 inhibition of oncogene-mediated transformation, the expression of WT *p53* may result in negative growth effects in various cells *in vitro*. The exact effect of p53 on growth seems to be cell-type specific. Consistent with the idea that p53 acts as a growth suppressor protein, many investigators have reported difficulty in obtaining cell lines which stably express a transfected WT *p53* gene (Eliyahu *et al.*, 1989; Baker *et al.*, 1990a; Chen *et al.*, 1990; Diller *et al.*, 1990; Casey *et al.*, 1991; Isaacs *et al.*, 1991; Johnson *et al.*, 1991; Kuerbitz *et al.*, 1992). Additionally, WT *p53* cotransfected with a selectable marker reduces the colony-forming efficiency of cells upon selection (Baker *et al.*, 1990a; Diller *et al.*, 1990; Mercer *et al.*, 1990; Isaacs *et al.*, 1991; Johnson *et al.*, 1991; Feinstein *et al.*, 1992). Few studies have investigated the effects of WT p53 replacement on cells *in vivo*. Reduced tumorigenicity in nude mice was described following introduction of WT *p53* into cell lines established from human tumors, including osteosarcoma (Chen *et al.*, 1990), neuroepithelioma (Chen *et al.*, 1991b), leukemia (Cheng *et al.*, 1992), colon carcinoma (Shaw *et al.*, 1992), and breast carcinoma (Wang *et al.*, 1993). Shaulsky *et al.* (1991a) demonstrated that WT *p53* was able to restrain tumor development in a murine pre-B cell line lacking endogenous *p53* expression when the cells were injected into syngeneic animals.

Deletion analyses showed that the majority of the p53 polypetide domains are required for growth suppression function, as assayed by the ability of p53 to suppress oncogene-mediated focus formation (Shaulian *et al.*, 1992; Reed *et al.*, 1993; Unger *et al.*, 1993). An exception is the report of Barak and Oren (1992) in which removal of the C-terminal 30 amino acids of murine p53 had no measurable effect on growth suppressor activity. Deletion of this region also activates the latent specific DNA-binding activity of p53, suggesting that the C-terminus may serve to attenuate the growth-suppressive function conferred by DNA binding (Hainaut and Milner, 1992; Hupp *et al.*, 1992). These data illustrate that transactivation and nucleic acid binding are important for growth suppressor function. Surprisingly, deletion of the p53 transactivation domain reduced transformation suppressive effects by only ~40% (Unger *et al.*, 1993). The acidic domain of herpesvirus protein VP16 was able

to substitute for the transactivation domain of p53; that the fusion protein retained full growth suppression functions implies the transactivation activity required for growth suppression of p53 is nonspecific (Reed *et al.*, 1993). In addition, Pietenpol *et al.* (1994) found that the yeast GCN4 dimerization domain could functionally replace the oligomerization region of p53, demonstrating that the core DNA-binding sequence is all that is essential for p53-specific growth suppression.

The regulated expression of *p53* in certain tissues suggests a role in differentiation. Consistent with this, differentiation was induced by the introduction of WT *p53* into two lymphoid cell lines, both of which failed to express endogenous *p53* (Shaulsky *et al.*, 1991a; Feinstein *et al.*, 1992). The finding that WT p53 can transactivate the enhancer region of the muscle-specific creatine kinase (MCK) gene implies a function in myogenesis (Weintraub *et al.*, 1991).

p53 seems to have a role in apoptosis, a type of programmed cell death, in numerous cell types. During involution of the murine mammary gland, *p53* mRNA levels peaked concomitant with apoptotic cell death and tissue remodeling (Strange *et al.*, 1992). Apoptosis was demonstrated in a human colon tumor cell line both *in vitro* and *in vivo* (tumor regression in a nude mouse) upon conditional expression of WT *p53* (Shaw *et al.*, 1992). Introduction of human WT *p53* was followed by growth suppression and induction of apoptosis in three-dimensional spheroids from human non-small-cell lung carcinoma lines in culture (Fujiwara *et al.*, 1993).

The ts A135V MT of mouse *p53* has been used to demonstrate apoptosis following a shift to the permissive temperature (WT) in Burkitt lymphoma cells (Ramqvist *et al.*, 1993), murine myeloid leukemia cells (Yonish-Rouach *et al.*, 1991), and murine erythroleukemia cells (Ryan *et al.*, 1993). Yonish-Rouach *et al.* (1991) reported that apoptosis was abated following induced differentiation. Ryan *et al.* (1993) utilized synchronized cell cultures to show that WT p53-induced cell death occurred predominantly at the G_1 phase where the cells had growth arrested. However, G_1 arrest was not sufficient to induce cell death, suggesting the apoptosis in those cells was a distinct feature of p53 activity. Cellular expression of the oncoprotein E1a induced high levels of WT p53 followed by apoptosis (Debbas and White, 1993; Lowe and Ruley, 1993).

Three laboratories established that dual pathways lead to apoptosis in murine thymocytes (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). Utilizing gene-targeted disruption of the *p53* gene in the germline of mice as first described by Donehower *et al.* (1992), a *p53* gene-dosage effect on the initiation of apoptosis brought on by agents causing DNA damage *in vivo* and *in vitro* was observed (Clarke *et al.*,

1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). However, thymocytes from the *p53* null mice were unchanged in their response to other apoptosis-inducing agents, such as glucocorticoids, calcium, and *in vitro* aging (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). In addition, hematopoietic cells from transgenic mice harboring MT *p53* transgenes or from *p53* null mice were more tolerant of ionizing radiation (i.e., apoptosis was not induced) than were cells from mice with WT *p53* (Lee and Bernstein, 1993; Lotem and Sachs, 1993). It was concluded that WT *p53* is necessary for radiation-induced apoptosis in the thymus but is not required for all apoptotic pathways (Clarke *et al.*, 1993; Lane, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993).

Recent studies suggest a role for p53, along with pRb, in the senescence (cellular aging) of human fibroblasts. Interaction with both pRb and p53 was necessary for the ability of viral oncoproteins to extend the life span of cells before the "crisis" which precedes senescence (Shay *et al.*, 1993). Unlike an antisense oligomer specific for *Rb*, an antisense *p53* oligomer alone was unable to extend the life span of normal human fibroblasts (Hara *et al.*, 1991). However, a cooperative effect in potentiating the life span of the cells was observed using a combination of the antisense *p53* and *Rb* oligomers (Hara *et al.*, 1991). These results indicate that there may be separate pathways to senescence in fibroblasts and that Rb and p53 may act cooperatively. Bischoff *et al.* (1990a, 1991) showed that nontumor fibroblasts from persons with germline *p53* mutations (e.g., Li-Fraumeni syndrome) had increased life spans and escaped senescence *in vitro*. However, Shay *et al.* (1993) found that human mammary epithelial cells had different immortalization requirements than did human fibroblasts or keratinocytes. Both E6 and E7 proteins of HPV 16 were necessary to produce rare immortalized fibroblasts and keratinocytes. In mammary epithelial cells, the E6 protein alone was sufficient, suggesting that only p53, and not Rb, was affected (reviewed by Shay *et al.*, 1993).

Expression of the *CIP1* (*SDI1* or *WAF1*) gene was shown to be upregulated in cells induced to synthesize increased levels of WT p53 (El-Deiry *et al.*, 1993); transcription of the same gene was found to be upregulated 10- to 20-fold in senescent diploid human fibroblasts (Noda *et al.*, 1994). These observations suggest a role for WT p53 in the onset of senescence in normal fibroblasts.

Early studies suggested that p53 has a positive role in the control of cell cycling and cellular proliferation. Cellular growth and DNA synthesis were blocked by either microinjection of monoclonal antibodies to p53 or expression of antisense *p53* RNAs (Mercer *et al.*, 1982, 1984; Shohat *et al.*, 1987; Deppert *et al.*, 1990). The interpretation of p53 as a

positive regulator appears to conflict with the modern dogma of p53 as a growth suppressor protein; however, it does agree with other studies indicating that p53 may behave as a growth promoter in some instances, an activity which is conformation dependent (discussed below and reviewed by Milner, 1991).

The growth suppression induced by the expression of WT *p53* has been demonstrated to result from failure of cells to progress through the cell cycle, and the p53-induced blockage appears to be at the G_0/G_1–S boundary (Mercer *et al.*, 1990; Martinez *et al.*, 1991; Nigro *et al.*, 1992). p53 appears to function in G_1 arrest in response to at least three types of stimuli. First, overexpression of exogenous p53 results in a currently undefined proliferative block of cells (Baker *et al.*, 1990a; Diller *et al.*, 1990; Mercer *et al.*, 1990; Martinez *et al.*, 1991; Feinstein *et al.*, 1992). Second, agents which induce DNA strand breaks (e.g., uv and γ-irradiation and several drugs) cause nuclear accumulation of p53 which is temporally in phase with arrest at G_1 (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991a, 1992; Kuerbitz *et al.*, 1992; Hall *et al.*, 1993). Third, gene amplification was drug [N-(phosphonacetyl)-L-aspartate [PALA]] induced only in cells which lacked WT *p53*, whereas cells with WT *p53* were growth arrested at G_1 (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Therefore, it appears that WT p53 is a G_1–S checkpoint which delays DNA synthesis to allow damage repair, thus preventing the inheritance of genomic mutations and amplifications in daughter cells. At this time it is unclear whether the same molecular mechanisms are utilized in each instance. If the DNA damage is irreparable (e.g., high doses of radiation), the proportionately high expression of p53 may activate the cell death pathway; however, the response of cells to increased levels of p53 (apoptosis or G_1 arrest) seems to be cell-type specific, as is the consequence of p53 loss.

The effects of WT p53 in growth arrest do not appear to be all encompassing, as WT p53 is not necessary for all instances of G_1 arrest. Cells containing MT p53 were appropriately blocked in G_1 in response to agents such as mimosine and methotrexate (Yin *et al.*, 1992), and cells expressing either WT or MT forms of p53 were equally affected by the alkylating agent ethyl methanesulfonate (Lee and Bernstein, 1993). Some tumor cell lines underwent PALA-induced gene amplification in the presence of the WT *p53* gene, indicating the presence of alternate pathways for the regulation of gene amplification (Livingstone *et al.*, 1992). Furthermore, there was no direct impact of *p53* status or G_1 checkpoint status on the radiosensitivity of some colorectal carcinoma cells (Slichenmyer *et al.*, 1993).

One way in which p53 might block cells from DNA replication is by

functionally inhibiting the DNA replication protein, replication protein A (RPA). In herpesvirus-infected cells, p53 colocalized with DNA replication proteins, including RPA (Wilcock and Lane, 1991), and both physical and functional p53–RPA interactions have been demonstrated *in vitro* (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993). RPA is a tripartite protein complex (13, 34, and 70 kDa) which appears to be the earliest factor recruited into the DNA replication initiation complex. The binding of RPA to single-stranded DNA (ssDNA) is necessary for the unwinding of the origin sequence, the first step in replication of DNA, and it has also been implicated in the process of DNA repair, recombination, and possibly transcription (He *et al.*, 1993; Li and Botchan, 1993; Pietenpol and Vogelstein, 1993; and references therein). The functional significance of RPA association with p53 is not clear, although it was suggested that the specific binding of RPA to p53 could interfere with DNA replication (Li and Botchan, 1993). He *et al.* (1993) proposed that RPA might stimulate the unwinding of DNA by helicases, such as T-ag, and that replication would be initiated when DNA polymerase-α was recruited into the RPA–helicase complex. Whereas p53 competes with DNA polymerase-α for binding to T-ag and subsequently blocks T-ag replication functions *in vitro* and *in vivo* (Braithwaite *et al.*, 1987; Gannon and Lane, 1987; Stürzbecher *et al.*, 1988b; Wang *et al.*, 1989; Friedman *et al.*, 1990), it follows that the ability of p53 to inhibit RPA from binding ssDNA and possibly to associate with DNA polymerase-α could be two means by which p53 might interfere with the replication of SV40 and cellular DNA. It should be interesting to determine whether p53 is able to prevent RPA from associating with DNA polymerase-α.

A second means whereby p53 may act globally to block cell cycle progression is by transactivation of genes such as *CIP1* (*WAF1/SDI1*) (El-Deiry *et al.*, 1993, 1994; Harper *et al.*, 1993). p21-Cip1 is a potent protein kinase inhibitor specific for cyclin-dependent kinases (Harper *et al.*, 1993). Expression of Cip1 suppressed the growth of tumor cells responsive to WT p53 (El-Deiry *et al.*, 1993) and inhibited DNA synthesis in young, proliferating cells (Noda *et al.*, 1994). Thus, expression of WT p53 may be necessary for the transactivation of other tumor suppressor genes with a subset of activities of WT p53 growth suppression. The synthesis of these genes could well be cell-type specific, which would help explain the various outcomes of WT p53 expression (i.e., differentiation, senescence, apoptosis, transient G_1–S blockage).

The underphosphorylated form of p53 found in G_0/G_1 is believed to exert negative control of the cell cycle (Bischoff *et al.*, 1990b). However, preventing the phosphorylation of human p53 at serine 15 reduced the

block at the G_1 to S transition (Fiscella *et al.*, 1993). Interaction with the S100b protein, implicated in cell cycle control, inhibited phosphorylation of p53 by PKC and also prompted disassembly of p53 oligomers, required for p53 activity (Baudier *et al.*, 1992). Thus, the regulation of p53 activity by phosphorylation is likely to be very complex throughout the cell cycle.

B. DNA Binding

The p53 protein has both nonspecific and sequence-specific DNA-binding activity. Lane and Gannon (1983) first demonstrated the binding of p53 to double-stranded DNA (dsDNA). Other reports confirmed that murine and human p53 bound nonspecifically to dsDNA (Steinmeyer and Deppert, 1988; Kern *et al.*, 1991a; Shohat-Foord *et al.*, 1991); murine p53 also bound ssDNA (Steinmeyer and Deppert, 1988). The 47 amino acids at the C-terminus apparently contain the nonspecific DNA-binding region (Shohat-Foord *et al.*, 1991). However, structural analyses of this DNA-binding region revealed none of the motifs characteristic of DNA-binding proteins, suggesting that p53 contains a novel DNA-binding site (Soussi *et al.*, 1990; Shohat-Foord *et al.*, 1991).

Purified synthetic human WT p53, in tetrameric complexes, bound to ssRNA and ssDNA with a higher affinity than to a p53-specific, dsDNA-binding site (from SV40, see Table III) (Oberosler *et al.*, 1993). The binding activity inhibited the helicase function of T-ag and *E. coli* DNA helicases I and II and was able to catalyze the renaturation of complementary RNA and DNA molecules (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994). The annealing activity was blocked by both PAb421 and PAb1620 monoclonal antibodies (see Fig. 2) (Oberosler *et al.*, 1993). These findings indicate that the C-terminal region of p53 might be responsible for binding to ss nucleic acids; further, the results implicate a more direct activity of p53 in the inhibition of DNA synthesis.

The finding that p53 could act as a transcriptional activator suggested that p53 might interact with DNA in a sequence-specific manner. In addition, purified recombinant WT p53 was shown to bind to Sp1 boxes close to the SV40 replication origin (Table III) (Bargonetti *et al.*, 1991). A consensus of DNA sequences was defined which were specifically associated with WT p53 by immunoprecipitation (using p53 monoclonal antibodies PAb421 and/or PAb1801) (Kern *et al.*, 1991b). These DNA fragments included repeats of the TGCCT motif (Table III) which has been observed in putative replication origins (Kern *et al.*, 1991b and references within); the consensus contained two copies of the 10-bp motif 5′-RRRC(A/T)(T/A)GYYY-3′ separated by 0–13 bp (El-Deiry *et*

TABLE III
DNA ELEMENTS TO WHICH p53 BINDS

Sequence[a]	Comments	Reference
TC**GAG CT**AAC TCCGC CCAGT TCCCC ATTCT CCGCC CCATG GC	Spl boxes/SV40 genome dimer	Bargonetti *et al.* (1991)
TGCCT TGCCT GGACT **TGCCT** GGCCT **TGCCT** TTT	Human *RGC* (ribosomal gene cluster)	Kern *et al.* (1991b); Farm *et al.* (1992)
AGACA TGCCT GTGCT CCTCT TT**TGC CT**TCT GCCAT	Human THE-1 (extra-chromosomal circles)	Kern *et al.* (1991b)
RRRC(A/T) (T/A)GYYY	Human consensus ("PG")/dimer	El-Deiry *et al.* (1992)
GCCC TGACG **TGTCC** CC	HTLV I enhancer[b]	Aoyama *et al.* (1992)
GGACA TGCCC GGGCA TGTCC	Human "CON" (palindrome)[b]	Funk *et al.* (1992)
GAACA TGTCT AAGCA TGCTG	Human *GADD45* (intron 3)	Kastan *et al.* (1992)
TG GCAAG CCTAT GACAT GGCCG GGGCC **TGCCT** CTCTC **TGCCT** CTGAC CCT	Murine *MCK* promoter	Zambetti *et al.* (1992)
GACA CTGGT CACAC TTGGC **TGCTT** AGGAA T	Murine (promoter activity)	Foord *et al.* (1993)
CCA **GGACA TGCCC GGGCA AGCCC** ATCG	Murine GLN LTR	Zauberman *et al.* (1993)
AGACA **TGCCC AGACA** TGTGT	Murine	Zauberman *et al.* (1993)
G **GGACT** TTCCC CTCCC ACGT	Murine *p53* promoter[b]	Deffie *et al.* (1993)
GGTCA AGTTG **GGACA** CGTCC GGCGT CGGCT GTCGG AG**GAG CTAAG TCC**TG ACA**TG TCT**	Murine *mdm2* (1st intron)	Wu *et al.* (1993)
AGC CGCCC **GGGCT** GGGG	c-*erb*A-α promoter	Shiio *et al.* (1993)
GAACA TGTCC CAACA TGTTG	Human *CIP1* promoter	El-Deiry *et al.* (1993)

[a] Bold, p53 consensus (PG) sequence (El-Deiry *et al.*, 1992). Single underlining, GC_3 motif which enhanced the ability of p53 to direct transactivation through the PG sequence (Shiio *et al.*, 1993). Double underlining, NF-κB element (Deffie *et al.*, 1993).

[b] No direct p53–DNA interaction observed.

al., 1992). Although this consensus is highly homologous to other p53 binding sites, p53 bound 5 to 15 times more tightly to this consensus than to the RGC and SV40 sequences, respectively (see Table III) (El-Deiry *et al.*, 1992); this consensus was designated "PG" (for poly-grip) (El-Deiry *et al.*, 1992; Kern *et al.*, 1992). The 10-bp dimer structure contains a mirror-image "half-site" which, arranged in any combination of directions, was sufficient for p53 binding (El-Deiry *et al.*, 1992). This type of DNA symmetry implied that p53 must be bound as a tetramer, in agreement with reports of p53 forming homotetramers both *in vitro* and *in vivo* (McCormick *et al.*, 1981; Kraiss *et al.*, 1988; Schmieg and Simmons, 1988; Stenger *et al.*, 1992; Stürzbecher *et al.*, 1992; Friedman *et al.*, 1993). However, it is also plausible that conformation, rather than tetramerization, is important for DNA binding (Halazonetis *et al.*, 1993; Wang *et al.*, 1994b).

Recently, the region necessary for specific DNA binding was mapped to the conformational "core" of the protein (human residues 102–292; mouse 99–289) (Srinivasan *et al.*, 1993; Bargonetti *et al.*, 1994; Pavletich *et al.*, 1994; Wang *et al.*, 1994b). This core region encompasses conserved regions II–V, the area where most mutations have been documented (Fig. 2). p53 reportedly is sensitive to the effects of sulfhydryl modifying agents (i.e., oxidants), and its specific DNA-binding ability is dependent upon the presence of reactive (i.e., reduced) sulfhydryl groups (Hainaut and Milner, 1993a; Hupp *et al.*, 1993). Conserved cysteinyl residues in this region are believed to be important in zinc binding (Fig. 2); chelation of zinc renders p53 unable to bind to DNA with sequence specificity (Hainaut and Milner, 1993a; Srinivasan *et al.*, 1993). Further, antibody PAb421 (specific to the C-terminus, see Fig. 2) stimulated the DNA binding of p53 by ≥90% to the p53 sequence (Hupp *et al.*, 1992). Other factors involving the C-terminus which enabled p53 to bind to PG DNA sequences included deletion or proteolysis of the 30 amino acids at the C-terminus, interaction with the *E. coli* hsc70 homologue dnaK, and phosphorylation by CKII (see Fig.2) (Hupp *et al.*, 1992).

Specific p53–DNA-binding sequences confer responsiveness to transactivation of a test gene by p53 (Farmer *et al.*, 1992; Funk *et al.*, 1992; Schärer and Iggo, 1992; Foord *et al.*, 1993). Shiio *et al.* (1992, 1993) identified an additional DNA motif (GC_3) which enhanced the ability of p53 to direct transactivation through the PG sequence. This GC_3 element (Table III, underlined) exists in other sequences found to contain p53-responsive elements (p53-RE) (Aoyama *et al.*, 1992; Funk *et al.*, 1992; Zambetti *et al.*, 1992; Zauberman *et al.*, 1993). It was noted that an additional factor(s) was required for p53 binding to these sequences (Aoyama *et al.*, 1992; Funk *et al.*, 1992). Thus, it is interesting that the

GC_3 element was shown to bind to a nuclear factor in cells lacking endogenous *p53* (Shiio *et al.*, 1993); this nuclear factor could be a p53-interactive protein.

Sequence-specific DNA binding by p53 was shown to be biologically relevant using elements from genes known to be transactivated by p53 (Table IV). Dissection of the MCK enhancer promoter, which was shown to be strongly transactivated by WT p53 (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992), revealed two types of p53–DNA-binding elements: two TGCCT repeats spaced by 12 bp (Kern *et al.*, 1991b) were adjacent to an Sp1-like motif (Bargonetti *et al.*, 1991; Zambetti *et al.*, 1992). A PG element was identified within the third intron of the human and hamster *GADD45* gene, the induction of which is dependent upon synthesis of p53 in response to DNA damage (Kastan *et al.*, 1992). The *GADD45* PG element bound p53 only in cells treated with DNA-damaging agents (Zhan *et al.*, 1993), indicating that p53 DNA-binding activity can be induced by such agents. In addition, WT p53 was able to transactivate the cellular retrovirus-like GLN element (Zauberman *et al.*, 1993), the *p53* gene (Deffie *et al.*, 1993), and the *CIP1/WAF1* gene (El-Deiry *et al.*, 1993, 1994) within their natural contexts. *CIP1/WAF1* was also induced in WT p53-containing cells in response to agents causing DNA damage, G_1 arrest, and apoptosis (El-Deiry *et al.*, 1994).

In total, the reports regarding DNA binding show that p53 is able to bind ss and ds nucleic acids with differing affinities. Furthermore, p53 has a strong affinity for the consensus sequence (PG), whereas p53 affinity decreased with an increasing number of mismatches. This implies that the array of DNA elements with which p53 can interact may vary depending upon the intracellular conditions (e.g., lower affinity sites may only be utilized when the concentrations of p53 reach a certain threshold, such as following DNA damage). Alternatively, modification of the p53 protein or interactions with other cell-type-specific proteins may modulate p53 affinity for various DNA motifs.

C. Transcriptional Activation and Repression

The first 42 amino acids in the acidic N-terminal region of p53 act as a potent transcriptional activator (Fig. 2) (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990; Unger *et al.*, 1992). In addition, overexpression of the WT p53 protein has been demonstrated to either transactivate or repress a number of cellular and viral promoters linked to reporter genes (Table IV).

The ability of p53 to bind to DNA with sequence specificity correlates with the ability to transactivate transcription in yeast (Kern *et al.*, 1992;

TABLE IV
TRANSCRIPTIONAL MODULATION BY WT p53

Cellular/ viral	Element[a]	Effect[b]	Reference
Cellular	*MCK* (E–P)	A	Weintraub *et al.* (1991)
	GLN LTR	A	Zauberman *et al.* (1993)
	p53 (P)	A, R	Ginsberg *et al.* (1991a); Deffie *et al.* (1993)
	c-*erb*A-α (P)	A	Shiio *et al.* (1993)
	CIP1	A	El-Deiry *et al.* (1993)
	EGF R	Ø	Aoyama *et al.* (1992); Chin *et al.* (1992)
	(α)1 collagen (P)	Ø	Deffie *et al.* (1993)
	Human H*ras*1 (P)	Ø	Aoyama *et al.* (1992); Chin *et al.* (1992)
	MHC class I (P)	Ø	Ginsberg *et al.* (1991a)
	Rb	R, Ø	Shiio *et al.* (1992); Deffie *et al.* (1993)
	PCNA (P)	R, Ø	Subler *et al.* (1992); Mack *et al.* (1993)
	MDR1/pgp1	R	Chin *et al.* (1992); Zastawny *et al.* (1993)
	IL-6 (P)	R	Santhanam *et al.* (1991); Margulies and Sehgal (1993)
	c-*jun* (P)	R	Ginsberg *et al.* (1991a)
	c-*fos* (P)	R	Ginsberg *et al.* (1991a); Santhanam *et al.* (1991); Aoyama *et al.* (1992)
	c-*erb*B2	R	Aoyama *et al.* (1992)
	hsc70 (P)	R	Ginsberg *et al.* (1991a)
	hsp70 (P)	R	Agoff *et al.* (1993)
	MHC class I	R	Santhanam *et al.* (1991)
	GADD45	R	Zhan *et al.* (1993)
	β-actin (P)	R	Ginsberg *et al.* (1991a); Santhanam *et al.* (1991); Aoyama *et al.* (1992); Chin *et al.* (1992); Zhan *et al.* (1993)
Viral	RSV LTR	R	Jackson *et al.* (1993); Aoyama *et al.* (1992); Chin *et al.* (1992); Subler *et al.* (1992)
	SV40 (early P)	R, Ø	Jackson *et al.* (1993); Aoyama *et al.* (1992); Chin *et al.* (1992); Shiio *et al.* (1992); Subler *et al.* (1992); Subler *et al.* (1992); Zhan *et al.* (1993)
	HIV LTR	R	Subler *et al.* (1992); Zhan *et al.* (1993)

(*continued*)

TABLE IV *(Continued)*

Cellular/ viral	Element[a]	Effect[b]	Reference
	HSV UL9 (P)	R	Subler *et al.* (1992)
	CMV (early P–E)	R	Jackson *et al.* (1993); Subler *et al.* (1992)
	HTLV I	A, R	Aoyama *et al.* (1992); Deb *et al.* (1992); Subler *et al.* (1992)
	Ad major late (P)	Ø	Deffie *et al.* (1993)

[a] E, enhancer; P, promoter.
[b] A, activation; R, repression; Ø, no effect.

Schärer and Iggo, 1992) and mammalian cells (Funk *et al.*, 1992; Kern *et al.*, 1992; Zambetti *et al.*, 1992). However, some data suggests that DNA binding is not an absolute requirement for transactivation or tumor suppression. Although dimerization-defective p53 molecules (i.e., monomers) retained the activities of suppressing oncogene-mediated focus formation (Shaulian *et al.*, 1992; Slingerland *et al.*, 1993) and transactivation (Shaulian *et al.*, 1993; Tarunina and Jenkins, 1993), they were not able to bind detectably to certain specific DNA sequences *in vitro* (Shaulian *et al.*, 1993; Tarunina and Jenkins, 1993; Zauberman *et al.*, 1993). It is not clear whether monomeric p53 mediates transactivation via other proteins interacting with a p53-RE, but other factors do seem to be required for p53 interactions with some DNA elements (see Table III) (Aoyama *et al.*, 1992; Funk *et al.*, 1992).

The reported ability of WT p53 to modulate transcription of a certain promoter has been variable (Table IV). For example, results using the SV40 early promoter ranged from a 50-fold reduction to no effect (Aoyama *et al.*, 1992; Chin *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992; Jackson *et al.*, 1993). It is noteworthy that an array of cell lines (e.g., HeLa, NIH 3T3, CV-1) was used in the experiment but HeLa cells were utilized in reports of 50-fold (Jackson *et al.*, 1993) and 16-fold reductions (Subler *et al.*, 1992) and no effect on the SV40 early promoter (Shiio *et al.*, 1992). The differences in p53-mediated repression when certain promoters were directly compared in different cell lines were attributed to cell-type specificity (Weintraub *et al.*, 1991; Subler *et al.*, 1992; Jackson *et al.*, 1993). The level of p53 overexpression probably influenced the results, as carefully controlled experiments showed that repression was dependent upon the dose of WT p53-expressing plasmids

(Chin *et al.*, 1992; Lechner *et al.*, 1992; Jackson *et al.*, 1993). Finally, the species (human or murine) of p53 may have influenced the results. The observations indicate the only limited comparisons should be made between these types of experiments; Table IV serves merely to summarize available reports concerning the transactivational activity of p53.

Different mechanisms appear to govern the ability of p53 to activate some promoters and to repress others. None of the promoters repressed by WT p53 (Table IV) has been shown to contain a p53-RE. However, promoters activated by p53 (e.g., SV40, *MCK*, c-*erb*A-α, HTLV I, GLN LTR, *p53*, *CIP1/WAF1*) contain p53-RE; *GADD45* and *mdm2* have a p53-RE within an intron (see Table III). Seto *et al.* (1992) defined a mechanism by which p53 could negatively regulate certain genes: WT p53 associates with the TATA-binding protein (TBP) and may interfere with the ability of TBP to initiate transcription from a minimal promoter.

TBP forms a central portion of the transcription factor IID (TFIID) multiprotein transcriptional preinitiation complex; the association of these factors is an ordered process in which TBP, along with TFIIA, binds to the promoter (Martin *et al.*, 1993; Ragimov *et al.*, 1993 and references therein). TBP is common to both TATA and initiator (pyrimidine-rich sequences) activities but distinct combinations of TBP-associated factors (TAFs) are utilized in each case (Mack *et al.*, 1993 and references therein). p53 was shown to interact with TBP contained within the holo–TFIID complex (Liu *et al.*, 1993). The p53–TBP complex may bind to the TATA box element (Martin *et al.*, 1993), although a conflicting study reported that WT, but not MT, p53 prevented the stable association of TBP and TFIIA with the TATA element (Ragimov *et al.*, 1993). The TPB-binding domain was localized to the acidic transactivational region between p53 amino acids 20 and 57 (Fig. 2) (Jackson *et al.*, 1993; Liu *et al.*, 1993). The segment of p53 between amino acids 93 and 160 was shown to decrease the transcriptional activation potential of the acidic domain by 100-fold, suggesting that these residues function to attenuate the strong transactivational domain at the N-terminus of p53 (Liu *et al.*, 1993).

Overexpression of WT p53 specifically repressed transcription initiation from promoters dependent upon the TATA element but had no effect upon promoters which direct transcription via an initiator motif (Mack *et al.*, 1993). It was suggested that as p53 complexes with TBP it excludes a TAF required for TATA-mediated, but not for initiator-mediated, transcription (Seto *et al.*, 1992; Mack *et al.*, 1993). The fact that overexpression of p53 was needed to produce repression of specific genes is physiologically consistent with the accumulation of p53 in cells

exposed to DNA-damaging agents and the transient (p53-mediated) G_1 block that follows (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991a; Fritsche *et al.*, 1993; Hall *et al.*, 1993; Kessis *et al.*, 1993; Tishler *et al.*, 1993).

Preliminary studies indicate that p53 can repress some promoters by mechanisms other than through the TBP. p53 was shown to complex with another transcription factor, the CBF, and repress CBF-directed transcription from the promoter of human *HSP70* (Agoff *et al.*, 1993). Downregulation of the interleukin-6 (IL-6) promoter by WT p53 was facilitated through a C/EBPβ-binding site (Santhanam *et al.*, 1991; Margulies and Sehgal, 1993); however, there was no evidence that p53 could bind the C/EBPβ transcription factor (Margulies and Sehgal, 1993).

D. Hypothesis for p53 Biological Functions in Growth Control

A relatively straightforward model for p53 function in growth suppression and arrest of cells in G_1 includes three possible roles for p53; these roles need not be mutually exclusive (Fig. 3). The first is by transcriptional repression of TATA-mediated gene expression (Seto *et al.*, 1992; Mack *et al.*, 1993). A second may be through p53-RE-induced expression of genes which prevent passage from G_1 to S (e.g., transactivation of *GADD45* and *CIP1/WAF1*) (Hartwell, 1992; Kastan *et al.*, 1992; El-Deiry *et al.*, 1994). The third activity of p53 could be direct control of DNA replication by blocking the replication machinery (e.g., helicase, RPA, and ERCC) (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993; Oberosler *et al.*, 1993; Wang *et al.*, 1994a).

It is also plausible that high-affinity p53 binding to DNA elements has additional effects on control of DNA replication. This hypothesis is based upon a number of observations. First, the p53 consensus motif TGCCT is associated with DNA replication origins (Bargonetti *et al.*, 1991; Kern *et al.*, 1991b). Second, the affinity of p53 for ss nucleic acids is higher than that for the dsDNA consensus motif (Oberosler *et al.*, 1993). It is possible that p53 binds to the consensus motif in a ssDNA form. Finally, p53 is able to reanneal ss nucleic acids (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994). Together, these findings suggest a model for the binding of p53 to replication origins and subsequent prevention of unscheduled (virally induced) or damaged DNA replication (Fig. 3). These activities might help explain the more global suppressive effects of the expression of WT p53 in many cell types.

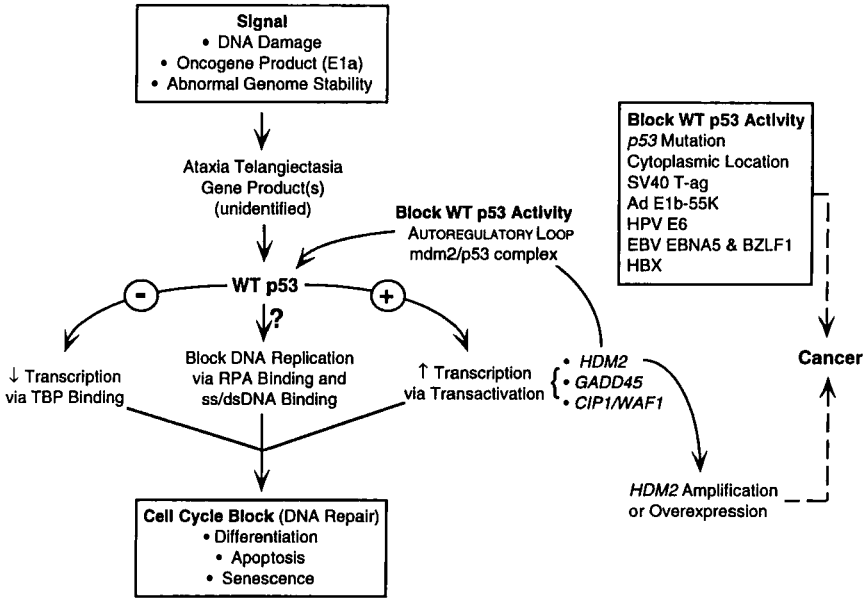

FIG. 3 Putative signal pathway for the activity of p53 (modified from Zambetti and Levine, 1993). Signals such as DNA damage (Kastan *et al.*, 1991a, 1992; Kuerbitz *et al.*, 1993) or the oncogene Ad E1a protein (Lowe and Ruley, 1993) cause an increase of cellular p53 protein and ultimately result in a G_1 block. In contrast, no such increase in p53 is found in cells from patients with ataxia–telangiectasia, which lack the normal delay in DNA synthesis following exposure to ionizing irradiation (Kastan *et al.*, 1992). Negative control by WT p53 may be mediated through interaction with TBP which inhibits the expression of genes with TATA-dependent promoters (Seto *et al.*, 1992; Liu *et al.*, 1993; Martin *et al.*, 1993; Ragimov *et al.*, 1993). Conversely, WT p53 may transactivate genes which contain p53-RE. Such genes include the *mdm2/HDM2* oncogene which is involved in the autoregulatory loop of p53 activity (Wu *et al.*, 1993); the growth arrest and DNA damage gene *GADD45* (Hartwell, 1992; Kastan *et al.*, 1992); and *CIP/WAF1* (El-Deiry *et al.*, 1993), which is upregulated in senescent cells (Noda *et al.*, 1994) and whose product inhibits cyclin-dependent kinases (Harper *et al.*, 1993). A third possible way in which p53 may cause cell cycle block is by directly inhibiting DNA replication. This could occur by functionally inactivating the DNA replication protein RPA (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993) and/or by directly binding to DNA replication origins to prevent unwinding. Perturbation of WT p53 activity by mutation of the gene, cytoplasmic sequestering of the protein, or inactivation of the protein by DNA tumor virus oncoproteins or overexpressed mdm2/HDM2 protein can lead to uncontrolled cell proliferation, and ultimately to tumorigenesis (reviewed by Zambetti and Levine, 1993).

VIII. Properties and Phenotypes of *p53* Mutants

Donehower *et al.* (1992) showed that p53 was not essential for cell survival or for development and differentiation *in vivo* by engineering a

mouse in which both *p53* alleles were deleted. These mice, however, were susceptible to tumors at early ages, indicating that WT *p53* is required for long-term normalcy of cells (Donehower *et al.*, 1992). This system is an important tool with which to study normal p53 functions. However, the relevance of the *p53* null mouse to human tumorigenesis is limited by the fact that human tumors rarely lose p53 expression completely but rather synthesize MT p53 (reviewed by Caamano *et al.*, 1992; Caron de Fromentel and Soussi, 1992). In this respect, heterozygote mice, carrying one WT allele and one null allele of *p53*, may be a better model.

A variety of mutations have been shown to exert common effects on the structure and function of p53, resulting in a protein that is phenotypically distinct from the WT gene product (see Table V). Not only do many p53 MTs lose the ability to act as a growth suppressor (i.e., a loss of function), they also behave as dominant oncogenes (i.e., a gain of function). In general, gain-of-function MT forms of p53 are capable of immortalizing primary rodent cells in culture (Jenkins *et al.*, 1984a; Rovinski and Benchimol, 1988) and of cooperating with an activated *ras* oncogene to transform such cells (Eliyahu *et al.*, 1984, 1988; Parada *et al.*, 1984; Finlay *et al.*, 1988; Hinds *et al.*, 1990). MT *p53* was also found to enhance transformation by acting synergistically with other oncogene pairs, such as Ad Ela plus *ras*, HPV E6 plus *ras*, and *myc* plus *ras* (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Peacock *et al.*, 1990; Crook *et al.*, 1991a). Expression of certain p53 MTs adds to the growth advantage of cells in culture by increasing the plating efficiency of G418-resistant colonies (Finlay *et al.*, 1989; Gerwin *et al.*, 1992; Dittmer *et al.*, 1993), stimulating

TABLE V
GENERALIZED PHENOTYPES OF WT VERSUS MT p53[a]

Phenotype	WT	MT
Half-life	20–30 min	≥2 hr
PAb246/1620 reactivity	+	−
PAb240 reactivity	−	+
Hsc70 binding	−	+
Cellular immortalization	−	+
Ras cooperation	−	+
Increase in growth advantage *in vitro*	−	+
Growth suppression	+	−
Specific DNA binding	+	−
TATA-mediated transcriptional repression	+	−

[a] See text for specific references.

oncogene-mediated focus formation (Eliyahu *et al.*, 1989), augmenting saturation density and colony formation in soft agar (Chen *et al.*, 1990; Dittmer *et al.*, 1993), and increasing resistance to the negative growth factor, transforming growth factor-β_1 (Gerwin *et al.*, 1992). MT p53 was found to increase the life span of normal human epithelial cells prior to senescence but the MT was unable to immortalize the human cells (Wyllie *et al.*, 1993). Further, there appears to be a strong selection for mutation of *p53* during the establishment of murine cell lines *in vitro* (Harvey and Levine, 1991; Kress *et al.*, 1992; Rittling and Denhardt, 1992; Jerry *et al.*, 1993; Ozbun *et al.*, 1993b).

Few studies have examined the growth advantage that MT p53 may provide *in vivo*, aside from approaches using nude mice. In one instance, expression of a mutated *p53* gene in murine leukemia cells lacking endogenous *p53* resulted in cells which were more malignant when injected into syngeneic animals (Wolf *et al.*, 1984; Shaulsky *et al.*, 1991a). A second study showed that transfection of a MT *p53* increased the metastatic capacity of bladder carcinoma cells inoculated intravenously into syngeneic mice (Pohl *et al.*, 1988). Analysis of two MT *p53* alleles from a case of human acute myelogenous leukemia revealed each *p53* allele to have a different missense mutation, suggesting a recessive activity *in vivo*. However, when overexpressed *in vitro*, each MT was capable of acting as a dominant oncogene by cooperating with an activated *ras* oncogene (Slingerland and Benchimol, 1991). Employing a syngeneic *in vitro–in vivo* mouse mammary epithelial cell (MMEC) system (see below), we have found that the cellular environment influences the ability of a given MT p53 to provide a cell with a growth advantage (Ozbun *et al.*, 1993b). Analysis of the FSK3 MMEC line showed a population mixed with respect to *p53* genotypes. One subpopulation of MT (233-S-234) *p53*-expressing cells was selected in a preneoplastic mammary outgrowth *in vivo* (TM3), whereas another minor population of MT (A135P) *p53*-expressing cells was selected during passage of FSK3 cells in culture (Ozbun *et al.*, 1993b). When FSK3 cells were subdivided and cultured in parallel *in vitro*, *p53* genotypes continued to evolve and diverge, suggesting that selective pressures exerted on MMEC populations *in vivo* are different from pressures present *in vitro* (Ozbun *et al.*, 1993b). Together, these findings indicate the caution should be exercised when attempting to correlate the effects of *p53* mutations assayed in cell culture systems with the *in situ* phenotypes of MT *p53* in tumorigenesis.

The general lack of *in vivo* functional data on p53 has made it difficult to correlate the phenotypes MT *p53* may possess in an artificial culture system with phenotypes observed in the *milieu* of a whole animal. Recent-

ly, substantial effort has been directed at determining how a *p53* MT gains the ability to act as a dominant oncogene.

A. Conformation and Stability

MT forms of p53 are typically PAb240$^+$ (Gannon *et al.*, 1990; Stephen and Lane, 1992) and PAb246°/PAb1620° (Milner and Cook, 1986; Yewdell *et al.*, 1986; Milner *et al.*, 1987; Finlay *et al.*, 1988; Stürzbecher *et al.*, 1988a; Hinds *et al.*, 1989; Cook and Milner, 1990), whereas the WT p53 conformation is PAb246$^+$/PAb1620$^+$ and PAb240° (Cook and Milner, 1990; Gannon *et al.*, 1990). However, a number of exceptions have been noted: (i) a variable fraction of the p53 molecules in cells expressing a MT *p53* gene can be PAb246$^+$, a phenomenon termed "pseudo-wild type" (Bártek *et al.*, 1990b; Gannon *et al.*, 1990); (ii) WT p53 briefly loses PAb246 reactivity (PAb246°) during cell division (Milner, 1984; Milner and Watson, 1990; Mosner and Deppert, 1992; Ullrich *et al.*, 1992; Zhang *et al.*, 1992); (iii) when translated *in vitro*, genotypically WT *p53* can fold into either the WT or MT conformation (Cook and Milner, 1990), and cotranslation of WT and MT *p53* results in the formation of mixed oligomers, both in the MT (PAb240$^+$) conformation (Milner and Medcalf, 1991). The PAb246$^+$ and PAb246° conformations have been proposed to represent functionally distinct forms of the p53 protein: PAb246° acts as a growth promoter, and PAb246$^+$ is a growth suppressor (Cook and Milner, 1990; Milner, 1991).

The mechanisms involved in the conformational regulation of p53 have been extensively studied by Milner and co-workers, primarily using lymphocyte cultures (Milner, 1984; Milner and Cook, 1986; Gamble and Milner, 1988) and *in vitro* transcription/translation (Hainaut and Milner, 1993a,b). The folding of p53 translated *in vitro* was shown to be dependent upon metal ions, specifically zinc; the WT conformation of genotypically WT *p53* is increasingly lost in the presence of zinc chelators (Hainaut and Milner, 1993b). The zinc requirement is mediated by cysteinyl residues in the protein, and two motifs with homology to known "non-zinc-finger" zinc-binding protein domains are located within p53 in conserved regions II, III, IV, and V (Fig. 2) (Hainaut and Milner, 1993b). In addition, sulfhydryl oxidizing agents perturb the WT conformation and subsequently inhibit the sequence-specific DNA-binding ability of p53 (Hainaut and Milner, 1993a; Hupp *et al.*, 1993). The conformational change from WT to MT (i.e., growth suppressor to growth promoter) is fully reversible, although the entropic equilibrium appears to favor the MT growth-promoter form. The addition of zinc or sulfhydryl reducing agents reverses the folding and DNA-binding ability,

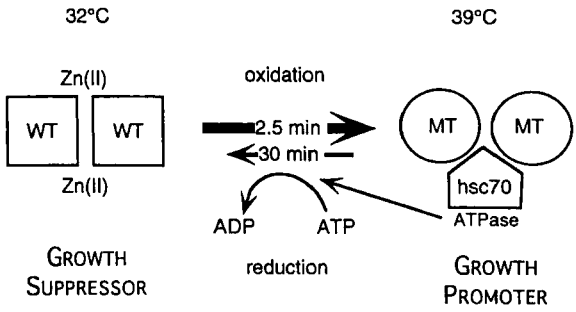

FIG. 4. A hypothetical model depicting the factors that may influence the folding and consequently the phenotype, of the p53 protein, shown as a dimer. The ts MT of p53 (A135V) is WT at the permissive temperature (32°C) and MT at the nonpermissive temperature (39°C) (Milner and Medcalf, 1990). Oxidation, chelation of zinc, and/or mutation results in the conversion from WT to MT p53 (Hainaut and Milner, 1993a,b; Hupp *et al.*, 1993). Zinc, reducing agents, or ATP is required to facilitate the change of p53 from MT to WT (Hainaut and Milner, 1992, 1993a,b; Hupp *et al.*, 1993). The ATPase activity of hsc70, bound only to MT p53, may contribute to the ATP-dependent switch from MT to WT (Clarke *et al.*, 1988).

suggesting that reduction of thiols favors the WT conformation and suppressor activity (Hainaut and Milner, 1993a; Hupp *et al.*, 1993). Although ATP is not required for the rapid shift from WT to MT p53, it is necessary for the full, and more lengthy, conversion of MT to WT form of p53 upon temperature shift (Fig. 4) (Hainaut and Milner, 1992). Redox modulation apparently is important in the function of other transcriptional factors; indeed, this may provide a biochemical mechanism whereby p53 exerts both positive and negative growth activities in response to diverse physiological stimuli (Hainaut and Milner, 1993a; Hupp *et al.*, 1993 and references therein). It was proposed that, because the two zinc-binding loops flank the Pab240 epitope (see Fig. 2), a diversity of mutations in the conserved domains could disrupt this conformationally critical region and, subsequently, the WT conformation and activities (Hainaut and Milner, 1993a,b).

Additional observations support that conformation is an important regulator of p53 function. Only p53 in the WT form is targeted by DNA tumor virus oncoproteins (Lin and Simmons, 1990; Medcalf and Milner, 1993), and only WT p53 is rapidly degraded by HPV E6 and E6-AP (Crook and Vousden, 1992; Medcalf and Milner, 1993). Only "MT" p53 interacts with the heat-shock cognate 70 protein, hsc70 (see below). In addition, the murine A135V MT p53 switches conformation coincident with phenotypic characteristics upon temperature change (Milner and Medcalf, 1990).

The conformation and stability of p53 appear to be intrinsically linked. Increased stability of the protein is one of the most frequent phenotypes associated with *p53* mutation (Finlay *et al.*, 1988; Halevy *et al.*, 1989; Hinds *et al.*, 1990; Iggo *et al.*, 1990; Kraiss *et al.*, 1991; Dittmer *et al.*, 1993). Although the biochemical basis for MT p53 stabilization is unclear, both the specific mutation and cell-type-specific factors appear to influence the stability of p53 (Halevy *et al.*, 1989; Zambetti and Levine, 1993). However, a murine p53 miniprotein including amino acids 302–390 was capable of complexing to WT p53 and extending its half-life to 3–4 hr (Shaulian *et al.*, 1992). This miniprotein abrogated WT p53 sequence-specific DNA binding and exhibited other dominant oncogenic activities (discussed below; Shaulian *et al.*, 1992). Thus, an increase in the half-life of p53 directly correlates with the transformed phenotype but inversely correlates with functions which are attributed to the WT tumor/growth suppressor p53 protein (Kraiss *et al.*, 1991). It follows that the loss of tumor suppressor activity must somehow disengage p53 from its normal pathway of rapid turnover, resulting in cellular accumulation.

B. Subcellular Distribution

Cytoplasmic localization is a common feature of many, but not all, MT forms of p53 (Hinds *et al.*, 1987; Gannon and Lane, 1991; Ginsberg *et al.*, 1991b; Martinez *et al.*, 1991; Slingerland *et al.*, 1993). The ts MT appears to act dominantly by sequestering WT p53 in the cytoplasm at critical times during the cell cycle (Martinez *et al.*, 1991). A labile cellular protein was shown to be necessary for the retention of ts MT p53 in the cytoplasm (Gannon and Lane, 1991). Thus, it would appear that MT p53 proteins are inhibited to variable extents in their normal subcellular transport such that some MT molecules spend more time in the cytoplasm than in the nucleus. However, nuclear localization is apparently necessary for the activity of p53, as both WT suppressor and MT oncogenic activities of different p53 proteins were completely abolished upon mutation of the nuclear localization signal (Shaulsky *et al.*, 1991b). In some cases nuclear localization contributed to, rather than hindered, the transforming activity of MT p53 (Shaulsky *et al.*, 1990b, 1991b).

C. hsc70 Binding

Heat-shock proteins (hsp) are members of a family of proteins generally defined as molecular chaperones, functionally involved in the transport, folding, and assembly of numerous proteins (Hainaut and Milner,

1992 and references therein). Hsps are induced to different degrees by certain types of stress, including heat, DNA damage, ethanol, or developmental signals (Clarke *et al.*, 1988 and cited references).

Transforming MT forms of p53 were shown to stably interact with the 70-kDa heat-shock protein cognate, hsc70, whereas WT p53 did not (Pinhasi-Kimhi *et al.*, 1986; Hinds *et al.*, 1987; Stürzbecher *et al.*, 1987a, 1988a; Finlay *et al.*, 1988; Ehrhart *et al.*, 1989; Hainaut and Milner, 1992; Lam and Calderwood, 1992). It appears that sequences at both the N- and C-termini of p53, as well as the folding of p53, are involved in the interaction with hsc70 (see Fig. 2) (Stürzbecher *et al.*, 1988a; Hainaut and Milner, 1992; Hupp *et al.*, 1992, 1993; Lam and Calderwood, 1992). The ability of p53 to complex with hsc70 correlates with both stabilization of p53 (Gronostajski *et al.*, 1984; Pinhasi-Kimhi *et al.*, 1986; Finlay *et al.*, 1988; Stürzbecher *et al.*, 1988a) and MT conformation (Hinds *et al.*, 1987; Stürzbecher *et al.*, 1987a, 1988a; Finlay *et al.*, 1988; Hainaut and Milner, 1992, 1993b). However, a cause–effect relationship between stability and hsc70 binding has not been established.

p53–hsc70 complexes have been localized to both the cytoplasm and the nucleus of cells (Stürzbecher *et al.*, 1987a). The ratio of hsc70 to p53 appears to be 1:1 (Pinhasi-Kimhi *et al.*, 1986; Hainaut and Milner, 1992), although a significant proportion of cellular hsc70 seems to be monomeric (Pinhasi-Kimhi *et al.*, 1986; Clarke *et al.*, 1988). p53 expressed *E. coli* interacted with a bacterial hsp, dnaK, indicating conservation of function (Clarke *et al.*, 1988). Physiological levels of ATP dissociated purified complexes of p53–hsc70 or p53–dnaK *in vitro*, suggesting that the intrinsic ATPase activity of the heat-shock protein regulates the stability of the complex (Clarke *et al.*, 1988). Hsp70 was shown to associate with ts MT p53 just subsequent to the rapid conformational change of p53 from WT (30°C) to MT (37°C), and the addition of a nonhydrolyzable ATP analog substantially stabilized the hsp70–MT p53 complexes (Hainaut and Milner, 1992). However, other evidence indicates that additional ATP-mediated actions, aside from the hsc70 ATPase, are necessary for the refolding of p53 into the WT conformation (Hainaut and Milner, 1992). These data suggest that the interaction of hsc70 with MT p53 may be a reaction to the MT conformation and that the hsc70 ATPase activity may be involved in the ATP-dependent conformational change from MT to WT p53 protein (see Fig. 4). Furthermore, the fact that direct association with dnaK activated the cryptic sequence-specific DNA binding of p53 suggests another means whereby hsc70 may regulate p53 function (Hupp *et al.*, 1992; Lane *et al.*, 1993). Interestingly, overexpression of rat *hsc70* suppressed oncogene-mediated transforma-

tion (A135V *p53* plus *ras* or *myc* plus *ras*), although the mechanism of this suppression is unknown (Yehiely and Oren, 1992).

D. Phosphorylation State

Whether the phosphorylation state of p53 influences its transformation ability is not known. p53 was more highly phosphorylated in SV40-transformed cells than in parental nontransformed cell lines (Stürzbecher *et al.*, 1987b; Meek and Eckhart, 1988; Scheidtmann and Haber, 1990), suggesting an involvement with cellular transformation. However, no qualitative differences in phosphorylation sites were observed among primary, immortalized, established, SV40-transformed, or chemically transformed mouse cells (Patschinsky and Deppert, 1990). A limited amount of data indicate the MT *versus* WT p53 proteins may be phosphorylated differently; phosphorylation of human serine 15 (murine 12) appeared to be dependent upon the ability of p53 to fold into the WT conformation (Ullrich *et al.*, 1993). No other major differences in the phosphorylation of MT p53 have been described (Picksley *et al.*, 1992; Slingerland *et al.*, 1993; Ullrich *et al.*, 1993). The fact that no naturally occurring *p53* genes have been isolated with mutation(s) at the conserved serine residues thought to be phosphorylation sites suggests that the ability to regulate its phosphorylation state is also important for oncogenic forms of p53.

E. DNA Binding and Transactivation Activities

The domains of p53 required for the activities of DNA binding and transactivation are undoubtedly important for its tumor suppressor functions. Further, that mutations have rarely been reported in the N-terminal transactivation region or in the C-terminal oligomerization domain suggests that such p53 MTs may not be functional and implies further that these sequences may be necessary for the acquired activities of MT p53. MT p53 proteins that fail to suppress growth generally are impaired for activities of nonspecific nucleic acid affinity (Steinmeyer and Deppert, 1988; Kern *et al.*, 1991a; Shohat-Foord *et al.*, 1991; Friedman *et al.*, 1993; Oberosler *et al.*, 1993), sequence-specific DNA binding (Bargonetti *et al.*, 1991, 1992; Kern *et al.*, 1991b; El-Deiry *et al.*, 1992; Foord *et al.*, 1993; Zhang *et al.*, 1993), and transactivation (Fields and Jang, 1990; Raycroft *et al.*, 1990, 1991; Aoyama *et al.*, 1992; Farmer *et al.*, 1992; Kern *et al.*, 1992; Schärer and Iggo, 1992; Zambetti, *et al.*, 1992; Deffie *et al.*, 1993; Zhang *et al.*, 1993).

Most MT–WT p53 heterocomplexes appear to be unable to bind to a p53-RE (Bargonetti *et al.*, 1992; Kern *et al.*, 1992). However, a MT from a Li-Fraumeni family (R248W) complexed to WT p53 could bind to the RCG motif at levels close to that of pure WT p53 complexes but homo-oligomeric R248W (MT–MT) p53 complexes were unable to bind to the sequence (Bargonetti *et al.*, 1992). These data indicate that some p53 MTs may have a dominant negative effect on WT p53 by abrogating sequence-specific DNA-binding activity, whereas other MTs may have a WT activity in the presence of genuine WT p53. Cotransfection of WT with some MT forms of p53 resulted in transactivation via a p53-RE which was decreased proportionally to the ratio of MT to WT to WT p53 (Weintraub *et al.*, 1991; Farmer *et al.*, 1992; Kern *et al.*, 1992; Schärer and Iggo, 1992). Further, there was variability between different MTs and their ability to abrogate WT p53-mediated transactivation (Chen *et al.*, 1993b). Inhibition from the PG element was due to a failure of the mixed (WT–MT) p53 oligomers to bind to the DNA sequences (Kern *et al.*, 1992). Thus, different MT proteins exhibit varying abilities to bind to distinct p53-RE (i.e., various MT p53 proteins bound to certain DNA elements but failed to bind to others) (Bargonetti *et al.*, 1991, 1992; Kern *et al.*, 1991b; El-Deiry *et al.*, 1992; Zhang *et al.*, 1993). These reports demonstrate the heterogeneity in the transactivational activities of various p53 MTs is dependent upon the protein's ability to bind to the specific p53-RE. Therefore, the possibility exists that some MTs retain the ability to activate or repress a subset of p53-regulated genes, which would help to explain the difference in the transforming potentials of various MTs. This may also have implications for the preponderance of certain p53 MTs (i.e., mutational "hot-spots") in certain tumor types.

The gain-of-function activity reported for many p53 MTs may be explained, in part, by the finding that promoters normally repressed by WT p53 were *activated* by MT forms of p53 (Table VI). This was shown to be physiologically relevant in at least two cases. First, the multidrug resistance gene (*MDR1*) is overexpressed in a number of human cancers; WT p53 repressed transcription from the *MDR1* promoter, whereas MT p53 specifically upregulated the expression of *MDR1* (Chin *et al.*, 1992). These results were confirmed in cells lacking endogenous *p53* in which five different missense MT p53 proteins activated the *MDR1* promoter to varying levels (Dittmer *et al.*, 1993). Second, IL-6 expression is often increased in neoplastic cells, and in contrast to WT p53, MT p53 stimulated expression from the IL-6 promoter (Margulies and Sehgal, 1993).

TABLE VI
PROMOTERS TRANSACTIVATED BY MT p53 PROTEINS

Promoter[a]	Reference
IL-6	Margulies and Sehgal (1993)
MDR1	Chin *et al.* (1992); Dittmer *et al.* (1993)
pgp1 (hamster mdr)	Zastawny *et al.* (1993)
RB1	Shiio *et al.* (1992)
PCNA	Deb *et al.* (1992)
CMV IE	Deb *et al.* (1992)
RSV LTR	Deb *et al.* (1992)
HTLV I LTR	Deb *et al.* (1992)

[a] PCNA, proliferating cell nuclear antigen; CMV IE, cytomegalovirus immediate early; RSV LTR, rous sarcoma virus long terminal repeat; HTLV LTR, human T cell leukemia virus LTR.

F. TRANSFORMATION ABILITY

In one model, the mechanism by which p53 acts oncogenically is through a dominant negative interaction with WT p53 (Baker *et al.*, 1989; Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). It is assumed that the MT is capable of abrogating or attenuating the suppressive effects of the WT protein (Herskowitz, 1987). Evidence in support of this hypothesis comes from studies showing that MT p53 can oligomerize with WT (presumably in the same manner that WT p53 forms dimers) (Eliyahu *et al.*, 1988; Rovinski and Benchimol, 1988; Finlay *et al.*, 1989; Milner and Medcalf, 1991). In addition, the ability to form dimers appears to be critical for the transformation ability of various p53 MTs (Slingerland *et al.*, 1993). The smallest segment of p53 required for *in vitro* transformation of cells with endogenous p53 includes murine p53 residues 320–360 (Fig. 2) (Shaulian *et al.*, 1992; Reed *et al.*, 1993; Unger *et al.*, 1993). The fact that only 29 residues (321–349 in mouse) are highly conserved among species suggests that this region, common to the oligomerization domain (Fig. 2), is most important for transformation (Reed *et al.*, 1993). No synergism was observed using a miniprotein (amino acids 302–360) and the A135V p53 MT, suggesting that the two mutants transform cells through the same mechanism (Shaulian *et al.*, 1992). These data suggest that oligomerization with endogenous WT p53 could be a major factor in the transforming potential of a given MT p53. However, this is probably not the only mechanism, as certain MTs that demonstrate equivalent interactions with WT p53 transform with different efficiencies (Halevy

et al., 1990). Few mutations have been mapped to the C-terminal region; perhaps such mutations would interfere with the oligomerizing/transforming domain, and the MT monomers would be unable to provide any selective growth advantage to cell. However, whether a miniprotein which contains the oligomerization region can promote transformation *in vivo* or enhance transformation by MT p53 in cells which express no WT p53 remains to be tested.

Other data, generated from both *in vitro* and *in vivo* experiments, argue against the dominant negative theory as the sole mechanistic explanation for MT p53 activity. First, a substantial excess of MT over WT p53 is typically required to stimulate transforming capabilities *in vitro* (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Chen *et al.*, 1990; Johnson *et al.*, 1991; Michalovitz *et al.*, 1991; Weintraub *et al.*, 1991). Second, only rarely are WT *p53* alleles found together with MT *p53* alleles in tumors *in vivo* (Chiba *et al.*, 1990; Davidoff *et al.*, 1991c; Halevy *et al.*, 1991). Rather, it is most common for one allele to contain a missense mutation accompanied by the deletion of the other allele (Nigro *et al.*, 1989; Baker *et al.*, 1990b; Slingerland *et al.*, 1991), suggesting that MT p53 acts in a recessive, not a dominant negative fashion *in vivo*. Third, the introduction of many MT forms of p53 into cells which lack endogenous *p53* expression enhances the transformed phenotype *in vitro* and tumorigenicity *in vivo*, suggesting a gain of function beyond the simple ability to block the activity of WT p53 (Wolf *et al.*, 1984; Chen *et al.*, 1990; Shaulsky *et al.*, 1991a; Dittmer *et al.*, 1993). Fourth, approximately 90% of cervical carcinomas express HPV oncoproteins E6 and E7 and, consequently, have undetectable levels of WT p53 protein (reviewed by Howley, 1991; Vousden, 1993). Metastases arising from HPV-positive tumors often have acquired mutations in *p53* (Crook and Vousden, 1992). Among the remaining tumors which lack HPV, many have *p53* genes with missense mutations (Crook *et al.*, 1991b,c, 1992), and these carcinomas are typically more aggressive and invasive than those with HPV (Riou *et al.*, 1990). These data suggest that cells with MT *p53* are more malignant than those which simply lack WT p53.

IX. *p53* Mutations and Breast Cancer

A. Types of *p53* Alterations

Mutations in *p53* are generally found to be somatic in breast tumors, although inherited mutations have been demonstrated in patients from cancer-prone families in which females tend to have a high incidence of breast cancer (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Prosser *et al.*, 1992).

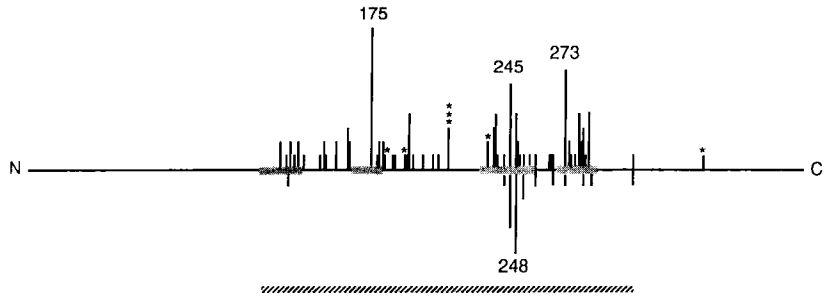

FIG. 5. The distribution and frequency of documented *p53* point mutations in sporadic and familial human breast cancer. Above the axis are sporadic *p53* mutations (Nigro *et al.*, 1989; Bártek *et al.*, 1990b; Malkin *et al.*, 1990; Prosser *et al.*, 1990; Børresen *et al.*, 1991, 1992; Chen *et al.*, 1991a; Davidoff *et al.*, 1991b,c; Devilee *et al.*, 1991; Kovach et al., 1991; Osborne *et al.*, 1991; Runnebaum *et al.*, 1991; Varley *et al.*, 1991; Coles *et al.*, 1992; Sommer *et al.*, 1992; Thompson *et al.*, 1992; Thor *et al.*, 1992; Carrere *et al.*, 1993). Asterisks denote nonsense mutations. Below the axis are mutations from families with inherited cancer syndromes (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Law *et al.*, 1991; Metzger *et al.*, 1991; Santibàñez-Koref *et al.*, 1991; Caron de Fromentel and Soussi, 1992; Prosser *et al.*, 1992; Sameshima *et al.*, 1992; Sidransky *et al.*, 1992). The shaded bars indicate the evolutionarily conserved domains (II, III, IV, V; aa 117–142, aa 171–181, aa 234–258, aa 270–286, respectively) of the human p53 protein from Soussi *et al.* (1990). The hatched line at the bottom includes exons 5–8, the regions commonly sequenced.

It is important to recognize limitations common to many of the studies of *p53* mutations in human breast cancers. The two techniques most commonly used to study p53 are nucleic acid sequencing and immunohistochemical staining for protein accumulation. Early studies reporting *p53* mutations in tumors, tumor cell lines, and xenografts found that most MTs were concentrated in the four highly conserved domains which are encompassed by exons 5–8 (regions II, III, IV, V) of the protein (Baker *et al.*, 1989; Nigro *et al.*, 1989). The majority of subsequent investigations analyzed the *p53* gene or cDNA only across these exons; however, *p53* mutations have been detected outside of this region (Fig. 5, Table VII). Thus, the number of *p53* mutations in human cancer may be underestimated. Furthermore, some reports concluded that the *p53* gene was WT based upon sequence analysis of only exons 5–8 (Børresen *et al.*, 1991; Moll *et al.*, 1992; Sommer *et al.*, 1992).

Detection of p53 by immunohistochemistry (IHC) is generally interpreted to indicate mutation in the coding sequences, because the WT protein is normally present at undetectable levels in cells (Rodrigues *et al.*, 1990; Purdie *et al.*, 1991). As summarized above, a number of missense mutations within the coding sequences of p53 have been

TABLE VII
SUMMARY OF NONMISSENSE p53 MUTATIONS IN HUMAN BREAST CANCER

Codon(s) affected	Result	Reference
33–126[a]	Exon 4 deleted	Osborne *et al.* (1991)
140	Frmeshift	Coles *et al.* (1992)
144	1 Amino acid deleted	Sommer *et al.* (1992)
167	1 bp deleted (frameshift)	Varley *et al.* (1991)
172	2 bp deleted (stop codon)	Børrensen *et al.* (1991)
175–180	In-frame deletion	Davidoff *et al.* (1991b)
187 splice site	Exon 6 deleted (stop codon)	Jolly *et al.* (1994)
201	1 bp deleted (frameshift)	Prosser *et al.* (1990)
233	Frameshift	Sommer *et al.* (1992)
235–239	14 bp deleted (frameshift	Kovach *et al.* (1991)
252–254	3 Amino acids deleted	Sommer *et al.* (1992)
255	1 Amino acid deleted	Coles *et al.* (1992)
329[a]	1 bp deleted (frameshift)	Kovach *et al.* (1991)
Intron 9[a]	Splice junction mutation	Sommer *et al.* (1992)
364–393[a]	Exon 11 deleted	Runnebaum *et al.* (1991)

[a] Outside of regions normally sequenced (see Fig. 5).

demonstrated to increase the protein's stability. However, in most breast cancer studies employing IHC, no verification of mutations in *p53* was made, and lack of detectable mutations across exons 5–8 was interpreted to indicate that WT p53 was stabilized (Børresen *et al.*, 1991; Moll *et al.*, 1992; Sommer *et al.* 1992; Allred *et al.*, 1993). The recent finding that the *mdm2* oncogene product can bind to, stabilize, and inactivate the normal functions of WT p53 may partially explain the significance of accumulation of WT p53 in tumorigenesis (Momand *et al.*, 1992; Finlay, 1993; Wu *et al.*, 1993). Therefore, it may be accurate to interpret accumulation of p53 as an alteration which inactivates the tumor suppressor activity, but it is likely improvident to simply assume that increased stability indicates a *p53* genetic alteration (Wynford-Thomas, 1992; Vojtések and Lane, 1993). Additionally, not all *p53* mutations which inactivate the tumor suppressor function are predicted to result in protein accumulation (e.g., nonsense, frameshifts, deletions). It is important not to overinterpret results based on incomplete sequence analysis or IHC.

There are six possible types of mutations in *p53*, all of which could potentially disrupt the tumor suppressor activity of p53: (i) LOH (reduction in gene dosage); (ii) complete deletion (loss of expression); (iii) partial deletions and frameshifts (fusions or truncations); (iv) splicing errors (domain deletions, truncations, or frameshifts); (v) nonsense

(truncations); and (vi) missense (amino acid substitutions). All of these mutations have been observed in one or another human tumor type; however, missense mutations leading to amino acid changes are the most common *p53* alterations in human breast cancer, as in other tumors (reviewed by Lane and Benchimol, 1990; Levine, 1990b; Hollstein *et al.*, 1991; Michalovitz *et al.*, 1991; Stratton, 1992; Donehower and Bradley, 1993). Complete loss of p53 expression has not been reported for human breast tumors, although it has been noted in a number of other tumor types, e.g., osteosarcoma (Masuda *et al.*, 1987; Romano *et al.*, 1989; Miller *et al.*, 1990; Hovig *et al.*, 1992), erythroleukemia (Mowat *et al.*, 1985; Wolf and Rotter, 1985; Ben-David *et al.*, 1988, 1990), and lung (Sameshima *et al.*, 1990). Furthermore, mice deficient in endogenous *p53* develop a diverse array of tumor types but very few cases of mammary carcinoma (Donehower *et al.*, 1992). This suggests a requirement for a putative oncogenic contribution conferred by many *p53* mutations in mammary tumorigenesis.

Some breast cancer studies report greater than 60% LOH on chromosome 17p (Mackay *et al.*, 1988; Davidoff *et al.*, 1991b). Early studies showed that allelic loss on chromosome 17p was accompanied by a mutation in the other *p53* allele (Nigro *et al.*, 1989; Prosser *et al.*, 1990). However, a proportion of breast tumors with point mutations in *p53* also expressed a WT allele (Davidoff *et al.*, 1991b; Osborne *et al.*, 1991) or had no evidence of LOH at 17p (Deng *et al.*, 1994), suggesting that LOH may be a later event. This is consistent with the idea that MT *p53* initially provides a cell with a growth advantage and that a further advantage is obtained upon loss of the remaining WT *p53* allele (Nigro *et al.*, 1989). Conversely, whether simple reduction of *p53* gene dosage contributes to tumorigenesis *in vivo* has not been demonstrated directly. Supportive of this possible mechanism is the finding that a number of breast tumors with LOH near the *p53* gene had no detectable mutation in the remaining allele (although only exons 5–8 were sequenced), and there was no p53 accumulation in the tumors (Davidoff *et al.*, 1991b; Osborne *et al.*, 1991). Alternatively, this may indicate there is the involvement of a gene on 17p other than *p53* in a subset of breast tumors. It appears that neither LOH nor mutation of p53 are prerequisite for the other event to occur in breast tumorigenesis.

Most studies report no detection of the p53 protein using IHC during any state of normal mammary gland differentiation (Bártek *et al.*, 1990a; Davidoff *et al.*, 1991b; Varley *et al.*, 1991; Marchetti *et al.*, 1993). However, Moll *et al.* (1992) observed cytoplasmic expression of p53 in a normal lactating breast tissue sample but found no mutations in exons 4–10 of the *p53* cDNA. Some cytoplasmic p53 was detected in normal

asynchronous human breast epithelial cells (Takahashe and Suzuki, 1994). They found predominantly cytoplasmic distribution of p53 during maximal DNA synthesis when the cells were epidermal growth factor stimulated following growth arrest. Cytoplasmic p53 was also found in other normal, exponentially growing human cell types (Takahashi and Suzuki, 1994), indicating that nuclear exclusion might be a mechanism of inactivating the growth-suppressive effects of p53 during active DNA synthesis.

Nuclear accumulation of the *p53* gene product has been documented in as many as 53% of breast tumors analyzed by IHC (Cattoretti *et al.*, 1988; Bártek *et al.*, 1990a,b, 1991; Davidoff *et al.*, 1991a,b; Horak *et al.*, 1991; Iwaya *et al.*, 1991; Ostrowski *et al.*, 1991; Varley *et al.*, 1991; Walker *et al.*, 1991; Heyderman and Dagg, 1992; Sommer *et al.*, 1992; Thor *et al.*, 1992; Allred *et al.*, 1993; Silvestrini *et al.*, 1993; Singh *et al.*, 1993). That protein stabilization results from mutation of *p53* has been shown directly in many cases of breast cancer (Bártek *et al.*, 1990b; Børresen *et al.*, 1991; Davidoff *et al.*, 1991b; Varley *et al.*, 1991; Sommer *et al.*, 1992; Thor *et al.*, 1992; Marchetti *et al.*, 1993; Singh *et al.*, 1993). However, in a few cases, no mutations were detected across the conserved regions, suggesting accumulation of WT p53 (Børresen *et al.*, 1991).

Detection of p53 protein in the cytoplasm of breast cancer cells also has been reported (Cattoretti *et al.*, 1988; Bártek *et al.*, 1990a; Chang *et al.*, 1991; Doglioni *et al.*, 1991; Horak *et al.*, 1991; Iwaya *et al.*, 1991; Varley *et al.*, 1991; Heyderman and Dagg, 1992; Sommer *et al.*, 1992; Domagala *et al.*, 1993; Stenmark-Askmalm *et al.*, 1994). The histological type of breast cancer may also influence the localization of p53 accumulation, as Domagala *et al.* (1993) found cytoplasmic staining of p53 primarily in lobular carcinomas. In some instances the *p53* nucleotide sequence of these cytoplasmic proteins was determined across exons 5–8 but no mutations were detected (Moll *et al.*, 1992; Sommer *et al.*, 1992). Accumulation of cytoplasmic p53 was detected in the MCF7 breast cancer cell line, which has genotypically WT *p53* across exons 4–9. Growth-arrested MCF7 cells were found to have cytoplasmic p53 coincident with DNA replication upon both serum stimulation (Suzuki *et al.*, 1992; Takahashi *et al.*, 1993) and insulin-like growth factor I stimulation (Takahashi and Suzuki, 1993). Thus, inactivation of WT p53 functions by nuclear exclusion also may have a role in mammary tumorigenesis. As the cytoplasmic p53 was tyrosine phosphorylated in insulin-like growth factor I-stimulated MCF7 cells, it was proposed that phosphorylation might function to inactivate p53 at the onset of cellular DNA replication (Takahashi and Suzuki, 1993).

B. mdm2 Interaction

A 90-kDa protein, identified as the product of the murine double minute 2 (*mdm2*) gene, was demonstrated to coimmunoprecipitate with p53 from cell lines transformed with MT *p53* and activated *ras* oncogenes (Hinds *et al.*, 1990; Momand *et al.*, 1992). *mdm2* was first identified as an amplified gene associated with double minutes in a spontaneously transformed Balb/c 3T3 derivative (3T3DM) (Cahilly-Snyder *et al.*, 1987); additionally, overexpression of *mdm2* in rodent cells resulted in their tumorigenicity in nude mice (Fakharzadeh *et al.*, 1991). The human homologue of *mdm2* (*HDM2*) maps to chromosome 12q13–14 (Oliner *et al.*, 1992). This chromosomal region is reportedly altered in a number of human malignancies, including soft tissue sarcomas (Turc-Carel *et al.*, 1986; Mandahl, 1989; Meltzer *et al.*, 1991; Oliner *et al.*, 1992), osteosarcomas (Oliner *et al.*, 1992; Ladanyi *et al.*, 1993), gliomas (Reifenberger *et al.*, 1993), and breast carcinomas (Sheikh *et al.*, 1993).

Although no specific function has been assigned to mdm2, the amino acid sequence suggests an involvement in transcriptional regulation (Fakharzadeh *et al.*, 1991). mdm2 apparently controls the activity of p53 in G_1, and p53 and mdm2 seem to be subject to autoregulatory feedback control (see Fig. 3) (Wu *et al.*, 1993). In 3T3DM cells, both mdm2 and p53 had half-lives of 20 min and both were localized in the nucleus (Olson *et al.*, 1993). mdm2 and mdm2–p53 complex levels increased as the cells reached late G_1 phase in the cell cycle following serum stimulation (Olson *et al.*, 1993). Although mdm2 binds both MT and WT p53, enhanced mdm2 binding is observed in cells which express higher levels of WT p53 (Barak and Oren, 1992). The rise in detection of p53–mdm2 complexes in these cells was attributed to an increase in mdm2 protein resulting from elevation of *mdm2* mRNA levels in response to WT p53 protein (Barak *et al.*, 1993; Wu *et al.*, 1993). Moreover, WT p53 activated expression from a *p53* DNA-binding sequence (see Table III) located in the first intron of the *mdm2* gene (Wu *et al.*, 1993).

Recent studies indicate that *mdm2* overexpression may be another mechanism by which cancer cells overcome p53-regulated growth control without selecting for *p53* mutations per se. In a number of the tumors in which *mdm2* was amplified, the *p53* gene was determined to be WT (Oliner *et al.*, 1992; Reifenberger *et al.*, 1993). Additionally, transfection of WT p53 into cells which express two MT *p53* alleles (Eliyahu *et al.*, 1988) resulted in the overexpression of *mdm2* (Otto and Deppert, 1993). Finlay (1993) showed that *mdm2* behaved much like a MT *p53* gene; overexpression of *mdm2* immortalized primary REFs and cooperated

with an activated *ras* oncogene to transform such cells. Also, *mdm2* was able to abrogate the growth-suppressive activities of WT *p53* in transformation assays *in vitro* (Finlay, 1993) and to inhibit the WT p53 activities of sequence-specific DNA binding (Zauberman *et al.*, 1993) and transactivation (Momand *et al.*, 1992; Oliner *et al.*, 1993). Consistent with this, mdm2 appears to bind p53 in the N-terminal transactivation domain (see Fig. 2) (Chen *et al.*, 1993a; Oliner *et al.*, 1993; Olson *et al.*, 1993). These findings support the idea that mdm2 binds to and functionally inactivates p53 in a manner similar to that of the DNA tumor virus oncoproteins. In summary, p53 upregulates expression of mdm2 which, in turn, negatively regulates the activity of p53 (see Fig. 3). It is currently unclear whether mdm2 is able to simply inactivate the tumor suppressor function of p53 or whether the interaction has an added oncogenic function.

Overexpression of *HDM2* mRNA has been correlated with estrogen receptor (ER) expression in human breast cancer cell lines; however, *HDM2* gene amplification was not detected (Sheikh *et al.*, 1993). Further, estrogen stimulation enhanced the levels of *HDM2* mRNA in cells transfected with the ER gene (Sheikh *et al.*, 1993). It is interesting that ER-negative breast tumors generally have a high frequency of *p53* mutation, whereas ER-positive tumors have lower incidences of MT *p53* but overexpression of HDM2 (Allred *et al.*, 1993; Sheikh *et al.*, 1993). Thus, it was proposed that p53 accumulation in breast tumors with no detected *p53* mutations may arise from an HDM2–p53 interaction (Allred *et al.*, 1993; Sheikh *et al.*, 1993).

C. Summary of *p53* Mutations

Up to 40% of primary breast carcinomas have been shown to express MT *p53* genes (Osborne *et al.*, 1991). A feature of *p53* mutations in all tumors is that their distribution does not appear to be random. The majority are clustered between amino acids 130 and 290, with most occurring within four evolutionarily conserved regions of the protein (see Figs. 2 and 5) (reviewed by Hollstein *et al.*, 1991; Levine *et al.*, 1991; Caron de Fromentel and Soussi, 1992; Donehower and Bradley, 1993). However, this may reflect in part the bias in a majority of studies to analyze only those regions and not the extreme 5′ and 3′ ends of the *p53* coding region (summarized above).

Germline *p53* mutations have been detected in many families with the Li-Fraumeni syndrome (LFS) cancer predisposition (see Fig. 5). Although p53 protein levels in the LFS cells were reportedly no higher than those in normal cells (Malkin *et al.*, 1990), biochemical and genetic

analyses of nontumor cells from two members of a cancer family indicated accumulation of p53 despite finding the gene was WT (Barnes *et al.*, 1992). These findings indicate that p53 in families predisposed to cancer may be inactivated by a number of mechanisms, just as occurs in sporadic tumors.

Several types of observations suggest that the germline mutation in *p53* is important in the process of tumorigenesis in persons with LFS. A comparison of tumors from five members of a LFS family indicated that each of the tumors had maintained the germline *p53* MT allele, whereas the WT allele was lost, suggesting that loss of WT p53 contributes to tumor formation (Srivastava *et al.*, 1992). A functional assessment of certain MT p53 proteins common to familial cancers showed that the majority of the MTs had lost the WT ability to suppress the growth of tumor cells lacking p53 (Frebourg *et al.*, 1992). A thorough investigation of the *in vitro* properties of more than six LFS-associated mutations found that the proteins demonstrated a spectrum of both WT and MT p53 characteristics (e.g., listed in Table V), depending upon the specific mutation (Milner and Medcalf, 1991; Hao *et al.*, 1993; Mukhopadhyay and Roth, 1993; Srivastava *et al.*, 1993). Furthermore, unlike normal human fibroblasts which have never been observed to undergo spontaneous immortalization in culture, nontumor fibroblasts from LFS cancer patients escaped senescence and had extended life spans (Bischoff *et al.*, 1990a). Together, these findings suggest that LFS *p53* MTs may not have a fully transforming phenotype in the presence of WT *p53*. This would help to explain how cells might tolerate the expression of a MT and a WT *p53* allele but become transformed upon loss of the single WT allele. However, similar to the variable functionality of p53 MTs in spontaneous tumors, the spectrum of phenotypes in LFS is probably dependent upon the specific *p53* mutation.

Compared with other human tumor types, somatic *p53* MT in breast cancer appears to be more dispersed over the regions between exons 5 and 9 (Levine *et al.*, 1991; Caron de Fromentel and Soussi, 1992; Donehower and Bradley, 1993). However, codons 175, 245, and 273 are frequently targeted for mutation in breast tumors, accounting for 23% of 105 documented *p53* mutations (see legend to Fig. 5 for references). Codons 175 and 273 are also highly targeted for mutations in many other tumor types (Caron de Fromentel and Soussi, 1992; Donehower and Bradley, 1993).

An analysis of the *p53* mutations in sporadic breast tumors indicated a high proportion of GC–TA transversions, a strong implication for environmental carcinogens (Børresen *et al.*, 1991; Coles *et al.*, 1992). However, the highly mutated codons 175 and 273 were shown to be

methylated *in vivo* (Rideout *et al.*, 1990), consistent with CG–TA transitions at CpG dinucleotides, a change attributed to cytosine deamination (i.e., 5-methylcytosine → thymidine) and common in spontaneous mutations (Coles *et al.*, 1992). The overall pattern of *p53* mutations in breast tumors is distinct from that in other human tumors in which the predominant *p53* mutations occur at CpG dinucleotides (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992). The germline *p53* mutations observed in familial cancers also tend to be CG–TA transitions at CpG dinucleotides (Caron de Fromentel and Soussi, 1992). These comparisons indicate that multiple factors probably are involved in breast tumorigenesis. Exposure and susceptibility of specific tissues to environmental carcinogens helps to explain the differences in frequency and distribution of *p53* mutations among various tumor types (Caron de Fromentel and Soussi, 1992). Another possibility, that has not been addressed experimentally, is that tissue-specific *p53* mutations (hot spots) may be selected based upon their ability to promote growth by interacting with tissue-specific factors. Of interest is a recent report of a correlation between histological types of breast tumors and the distribution of *p53* mutations (Marchetti *et al.*, 1993). Of mutations in exon 5, 63% were in medullary carcinomas, whereas the mutations in lobular and ductal types were more dispersed in exons 6, 7, and 8 (Marchetti *et al.*, 1993). The significance of this correlation is not currently clear, although the immune system may have some involvement (see below).

D. Antibody Response

Cancer patients may develop an antibody response to p53 which is dependent upon the specific mutation (Winter *et al.*, 1991; Davidoff *et al.*, 1992) and is correlated with poor prognosis for breast cancer (Schlichtholz *et al.*, 1992). Approximately 10–15% of breast cancer patients have circulating p53 antibodies (Crawford *et al.*, 1982; Schlichtholz *et al.*, 1992; Lubin *et al.*, 1994), most of which were found to be of the IgG class (Lubin *et al.*, 1994). Patients with tumors that expressed MT p53 forms capable of forming a complex with hsc70 had mounted an immune response to p53 (typically more transforming MTs); conversely, those who synthesized MT p53 proteins that failed to bind to hsc70 had no detectable p53 antibodies (Davidoff *et al.*, 1992). A significant association was demonstrated between hsc70 binding and *p53* mutation in exons 5 and 6, whereas the proteins unable to complex hsc70 were mutated in exons 7 and 8 (Davidoff *et al.*, 1992). The exceptions were mutations at cysteine residues in exons 7 and 8 which enabled the

MT p53 proteins to bind to hsc70 and elicit an immune response (Davidoff *et al.*, 1992).

The biochemical basis for the p53-induced immune response is unknown; however, several lines of evidence suggest that it is dependent upon a self-immunization process resulting from the accumulation of the protein rather than a mutational alteration causing a novel epitope. First, a significant relationship was found between tumor levels of p53 protein and the patient's p53 antibody response (Lubin *et al.*, 1994). Second, the sera appeared to recognize both WT and MT forms of p53 (Schlichtholz *et al.*, 1992; Labrecque *et al.*, 1993). Third, the immunodominant regions of p53 were localized at both N- and C-terminal regions of the protein (Schlichtholz *et al.*, 1992; Lubin *et al.*, 1994), which are hydrophilic and include areas rarely found to contain mutations. Fourth, the patient responses were identical to those of animals immunized with high levels of p53 (Schlichtholz *et al.*, 1992; Lubin *et al.*, 1994). Additionally, SV40 tumor-bearing animals make antibodies to the p53 protein (Linzer and Levine, 1979), which is known to be WT and accumulates to high levels via interaction with T-ag (Lin and Simmons, 1990). The mechanism of immune presentation is unclear as there is no evidence for p53 protein in human serum samples (Winter *et al.*, 1991; Hassapoglidou *et al.*, 1993).

E. Prognostic Significance

Although accumulation of p53 has been noted in benign breast disease (Heyderman and Dagg, 1992), most investigations suggest that detection of p53 expression may be a valuable prognostic indicator for breast tumors (Davidoff *et al.*, 1991a; Horak *et al.*, 1991; Iwaya *et al.*, 1991; Walker *et al.*, 1991; Callahan, 1992; Porter *et al.*, 1992; Thor *et al.*, 1992; Allred *et al.*, 1993; Thor and Yandell, 1993). The factors having a statistically significant correlation with p53 detection and poor prognosis varied among studies and included the following: low estrogen receptor expression (ER negative) and high expression of epidermal growth factor receptor (EGFR positive) (Cattoretti *et al.*, 1988; Walker *et al.*, 1991), only ER negativity (Iwaya *et al.*, 1991; Thompson *et al.*, 1992), only EGFR positivity (Horak *et al.*, 1991), and only low progesterone receptor expression (Davidoff *et al.*, 1991a). Whether overexpression of *HER2* (c-*erb*B2) correlates with p53 accumulation is controversial (Chang *et al.*, 1991; Horak *et al.*, 1991; Iwaya *et al.*, 1991; Walker *et al.*, 1991). Thor *et al.* (1992) and Friedrichs *et al.* (1994) concluded that high p53 expression was an independent prognostic marker, whereas Ostrowski *et*

al. (1991) surmised that detection of p53 expression was likely to be of no clinical value. Stenmark-Askmalm *et al.* (1994) found the former to be true when both cytoplasmic and nuclear accumulation of p53 were considered. The best consensus on the value of p53 accumulation as a prognostic indicator was that detection of p53 expression by IHC was an independent predictor of early disease recurrence in patients whose lymph nodes were not involved (i.e., node-negative tumors) (Isola *et al.*, 1992; Thor *et al.*, 1992; Allred *et al.*, 1993; Knyazev *et al.*, 1993; Silvestrini *et al.*, 1993). This same later conclusion was reached in a pilot study employing single-stranded conformation polymorphism analysis to screen for mutations across *p53* exons 5–9 (Ellege *et al.*, 1993). It was stressed in the IHC studies that an absence of guidelines for performing IHC (e.g., antibodies and tissue fixatives used and length of patient follow-up time) aimed at relating p53 expression to known prognostic factors makes comparisons of the studies difficult, and thus limits the clinical usefulness of the IHC assays at present (Callahan, 1992; Thor and Yandell, 1993).

Most breast tumors analyzed for p53 alterations are invasive ductal carcinomas which make up 65–80% of malignant breast neoplasms (Marchetti *et al.*, 1993). In studies including various histological types of breast tumors, those considered more prognostically favorable (e.g., medullar, mucinous, papillary, and lobular) had a lower incidence of *p53* mutation (Davidoff *et al.*, 1991a; Marchetti *et al.*, 1993). No *p53* mutations were found in the mucinous and papillary types, which are associated with a high degree of differentiation and a more favorable prognosis. Surprisingly, medullary carcinomas had the highest proliferative index (i.e., growth rate) and the highest frequency of *p53* mutations (Davidoff *et al.*, 1991a; Marchetti *et al.*, 1993). Domagala *et al.* (1993) made essentially the same observations using IHC to analyze nuclear p53 accumulation. These studies indicate the lack of a simple correlation between alteration of p53 and prognostic significance; however, the specific *p53* mutation and subsequent antibody response may partially help to explain this apparent conundrum.

Patient antibody response to p53 was tightly associated with the presence of high histologically graded breast tumors, indicative of poor prognosis (Schlichtholz *et al.*, 1992). This is especially interesting in light of the finding that only the more transforming p53 MTs (by *in vitro* analyses) were shown to elicit an immune response (Davidoff *et al.*, 1992). This is further support in favor of the hypothesis that certain p53 MTs may have an added function *in vivo*. The fact that no increase in the presence of p53 antibodies was found in patients with relapse or metastases suggests that induction of the antibodies is an early event, indepen-

dent of disease progression (Schlichtholz *et al.*, 1992). The presence of p53 antibodies may, therefore, be an early marker of disease risk.

Accumulation of p53 was also seen in breast carcinomas *in situ* (a relatively early stage tumor), albeit at much lower frequencies (13–20%) (Bártek *et al.*, 1990a; Davidoff *et al.*, 1991c; Thor *et al.*, 1992). This indicates that mutation of *p53* may be an early event in malignant transformation. From patients in whom the early tumors expressed MT *p53* genes, the same MTs were found in more advanced tumors (secondary and metastatic) (Davidoff *et al.*, 1991c). This suggests a role for the MT *p53* in breast cancer progression. Additionally, compared with sporadic breast cancers, increased incidences of p53 protein accumulation were observed in cancers from patients with Li-Fraumeni syndrome, familial breast cancer, and the familial breast/ovarian cancer syndrome (Thor *et al.*, 1992). Thus, *p53* mutation may either have an added role or act at an earlier step in the familial cancer disorders.

If mutation of the *p53* gene is an early event in breast tumorigenesis, this might help explain the array of mutations and genomic losses associated with the disease, considering that p53 is believed to function in preserving the integrity of the genome by regulating DNA replication (reviewed by Hartwell, 1992; Lane, 1992). This is also consistent with the correlation between LOH and 17p and DNA aneuploidy in primary breast tumors (Chen *et al.*, 1991a).

F. Evidence for Tumor Suppressor Activity

Although the growth suppressor activity of *p53* has been demonstrated in many types of tumor cells and cell lines, relatively few studies have focused on breast tumor cells. WT *p53* has been introduced into three established breast cancer cell lines and a murine mammary cell line known to express only MT *p53* genes. Casey *et al.* (1991) transfected human cell lines (MDA-MB468 and T47D) with plasmids containing *p53* (WT or MT), expressed from the CMV promoter, plus a neomycin-resistance gene (neo^R). MT *p53* had no deleterious effects on these cell lines but the cells did not tolerate expression of WT *p53*. Only 10% of the neo^R cell colonies contained intact exogenous WT *p53*, and ~1% ($\frac{1}{91}$) of the neo^R colonies expressed WT *p53* mRNA; sequence determination of the exogenous WT *p53* in this single clone revealed that a mutation had occurred following transfection (Casey *et al.*, 1991). Thus, high expression of WT *p53* was incompatible with the *in vitro* growth of two human breast cancer cell lines. Retroviral-mediated introduction of WT *p53*, expressed from the Mo-MuLV LTR, into human breast cancer cell lines (MDA-MB468 and BT549) resulted in a reduction in both

colony-forming ability in agar and tumorigenicity in nude mice (Wang *et al.*, 1993). However, the growth rates of the cell lines *in vitro* were unaffected by WT *p53*. Wang *et al.* (1993) reasoned that their ability to propagate breast tumor cell line colonies expressing WT *p53*, compared with the inability demonstrated by Casey *et al.* (1991), might be due to a gene dosage effect (i.e., retroviral transfer results in a single integration, whereas transfection generally results in high copy numbers). Thus, the WT *p53* gene was likely to be much more highly expressed in the former study compared with the latter report.

In contrast to the cell lines with endogenous MT *p53*, the human breast tumor cell line MCF7 expresses *p53* which is WT across exons 4–9, and the cells were unaffected both *in vitro* and *in vivo* by exogenous WT *p53* expressed at high levels (Casey *et al.*, 1991). It was concluded that some breast cancers arise without inactivation of *p53*. However, it seems equally possible that an alteration has occurred either in another pathway or downstream of *p53* (e.g., overexpression of *mdm2*); this change might override the requirement for *p53* mutation and would presumably render the cells refractory to the effects of WT *p53*.

In no instances did introduction of exogenous MT *p53* (A143V) increase the growth potential (plating efficiency, growth rate, or saturation density) of human breast cancer cell lines which expressed endogenous MT *p53* (and no WT) (Casey *et al.*, 1991), suggesting that an additional MT *p53* gene has no added effect in the absence of WT *p53*.

Merlo *et al.* (1994) infected HC11, a murine mammary cell line, with a retrovirus expressing WT *p53* from the inducible metallothionine promoter. Clones containing the input WT *p53* DNA were dramatically growth inhibited when expression was induced with cadmium, and WT *p53*-expressing cells were more anchorage dependent than the parental, untransfected cells (Merlo *et al.*, 1994). In no cases was DNA fragmentation observed in the WT *p53*- expressing cells, suggesting that activation of apoptosis was not responsible for the inhibition of growth (Merlo *et al.*, 1994).

X. Systems for Studying Genetic Changes Involved in Breast Cancer

A. Limitations of the Human System

For the most part, studies of breast cancer in the human system have been limited to analysis of tumor cells and several established breast tumor cell lines. Technical difficulties associated with analysis of small early lesions, as well as the impracticability of studying human pre-

neoplasia *in vivo,* have precluded efforts to determine at what stages in mammary tumorigenesis genetic changes occur.

B. *In Vivo* Murine Models

Mouse model systems offer a number of advantages and have provided much of the information regarding the alterations involved in mammary cancer (reviewed by Medina, 1982). Inbred mouse strains are a great asset as they allow *in vivo* analyses by virtue of easy transplantation and manipulation. In addition to the occurrence of spontaneous tumors, mammary neoplasias can be easily induced at variable incidences and with reproducible results using a variety of agents (Medina, 1982 and references therein).

Two minimal changes in growth control required for mammary tumorigenesis include cellular immortalization and liberation from environmental constraints (reviewed by Medina, 1982, 1988). The acquisition of immortality is an early, preneoplastic step, and this preneoplastic intermediate in tumorigenesis appears to be common to both human and mouse mammary systems (Medina, 1988; Page and Dupont, 1990). Hyperplastic alveolar nodules (HAN) are the best-characterized early lesions. Morphologically, HAN are similar to the differentiated alveolar cells typically present in pregnant mammary gland. In addition, preneoplastic cells resemble their normal homologues in that they are dependent upon local environmental factors responsible for regulation, thus their growth is limited to the mammary fat pad (Medina, 1982, 1988). However, unlike their normal alveolar counterparts, the preneoplastic HAN cells are transformed and have an increased probability of tumor formation (Medina, 1982, 1988).

Murine infection with the retrovirus mouse mammary tumor virus (MMTV) is associated with clonal outgrowth of HAN and malignant mammary tumors (Varmus, 1982). Insertional mutagenesis has afforded the identification of five murine genes which are activated in mammary tumors induced by MMTV; *wnt1, int2, int3, hst,* and *wnt3* (reviewed by Peters, 1990; Callahan *et al.,* 1992a). However, there is no evidence for viral etiology in human breast cancer, and only the *INT2* and *HST* genes have been implicated in the genesis of human breast tumors (Callahan *et al.,* 1992a).

Several *in vivo* models using transgenic mice carrying oncogenes have been developed for breast cancer. Nevertheless, such transgenes alone are not sufficient to induce mammary cancer (Callahan *et al.,* 1992a,b and references therein). These studies support the multistep model for

tumorigenesis of the breast (Newbold, 1985; Barrett, 1987; Vogelstein and Kinzler, 1993).

C. *In Vitro* Systems

Many efforts to understand tumorigenesis *in vivo* have utilized immortalized and transformed cells propagated *in vitro*. Cell culture systems have established the stage-wise progression of neoplastic transformation (for review see Barrett and Fletcher, 1987). The studies of Newbold *et al.* (1982) provided initial evidence that immortalization may be essential for tumorigenesis. Added support came from the finding that normal cells could be immortalized by the introduction of certain oncogenes (reviewed by Hunter, 1991). Although cell culture systems provide the advantage of being able to quantitatively analyze defined cellular populations, there are major environmental differences between *in vivo* and *in vitro* systems which must be considered (Barrett and Fletcher, 1987).

Studies in the murine mammary system have greatly benefited from the ability to manipulate and analyze lesions *in vivo*. However, until recently, there were few cell lines which could serve as *in vitro* models for the *in vivo* stages of tumorigenesis. This was due primarily to the difficulty of reproducibly establishing murine mammary epithelial cell lines (MMECL) which maintained the *in vivo* morphogenic abilities of mammary epithelial cells. Although preneoplastic lines induced by a variety of agents exist, most have been serially transplanted *in vivo* for 1 or 2 decades (Medina, 1988). Consequently, it is questionable whether the genetic changes present in these lines are directly relevant to the preneoplasia.

D. MMECL *in Vitro–in Vivo* System

Recently, Kittrell *et al.* (1992) described a protocol for reproducibly establishing MMECL *in vitro*. These cells maintain their morphogenic ability when transplanted at low passages into the cleared mammary fat pad of syngeneic animals. In some instances the cells fill the fat pads as normal ductal outgrowths, whereas other lines give rise to preneoplastic HAN lesions (Kittrell *et al.*, 1992). The *in vivo* outgrowths can be reestablished *in vitro* or can be serially transplanted to other animals, allowing analysis of the genetic alterations involved in the early stages of mammary tumorigenesis (Kittrell *et al.*, 1992).

The parental (FSK) cell lines have similar growth requirements of insulin and epidermal growth factor (EGF) in a low serum medium

(Kittrell *et al.*, 1992). In general, the FSK cell lines at passages (P) 1–4 gave rise to normal ductal outgrowths, at P6–12 ductal or alveolar hyperplasias were occasionally produced, and no outgrowths were yielded after P12. It was concluded that some factor necessary for successful morphogenesis *in vivo* was lost or diluted during passage of the cell lines *in vitro* (Kittrell *et al.*, 1992).

The four ductal mammary epithelial outgrowth (EL) lines resemble normal ductal outgrowths except that the EL lines are immortal *in vivo* (Medina and Kittrell, 1993). The EL lines demonstrate a normal ductal morphology, are dependent upon ovarian hormones, and are nontumorigenic, indicating that immortalization is not tightly linked to preneoplasia (Medina and Kittrell, 1993).

The six TM preneoplastic outgrowth lines share a number of characteristics reported for other transplantable HAN lines and fit the criteria for mouse mammary preneoplasias (Medina *et al.*, 1993a and references therein). In general, the outgrowth lines are morphologically characterized as alveolar hyperplasias (Medina *et al.*, 1993a). They demonstrate moderate dependence upon ovarian hormones for growth and alveolar differentiation and display various extents of constitutive expression of casein, a milk protein (Medina *et al.*, 1993a). The TM lines give rise to tumors at higher incidences than do normal mammary cells, producing mainly type B adenocarcinomas (Medina *et al.*, 1993a). Finally, the TM outgrowth lines are immortal (Medina *et al.*, 1993a). The exception to these characteristics is the TM3 outgrowth. Although TM3 is immortal, it never has produced a tumor and is completely ovarian dependent for growth and alveolar differentiation, like normal mammary cells (Medina *et al.*, 1993a).

A comparison of the TM cell lines established from the preneoplastic *in vivo* outgrowths with their respective FSK cell lines showed that the TM cell lines had a decreased dependence upon EGF and serum for their growth *in vitro* (Medina *et al.*, 1993b). The TM3 cell line was an exception in that it was highly EGF dependent (Medina *et al.*, 1993b). Considering the growth requirements for the preneoplastic lines, there is a striking association between ovarian independence and EGF independence in the tumorigenic TM2, 4, 6, and 9 lines. Conversely, EGF dependence correlated with ovarian dependence in TM3 which has produced no tumors (Medina *et al.*, 1993a,b). EGF is an important growth factor in normal mouse mammary development, and loss of EGF responsiveness is common in the transformation of rodent mammary tumors (Medina *et al.*, 1993b and references therein). Further, the EGF receptor-related protein c-*neu* (*HER2*) is overexpressed in human breast tumors (Groner and Hynes, 1990; Callahan *et al.*, 1992b).

Expression of certain genes implicated in mammary cancer was also analyzed in normal mammary cells and compared to those in TM cell lines, outgrowths, and tumors; there was no alteration of the c-*neu*, c-H-*ras*, c-*myc*, or *Rb* genes in any of the cells (Medina *et al.*, 1993b). However, there was an increase in the synthesis of the endogenous MMTV-LTR mRNA levels, consistent with observations made in other preneoplasias (Medina *et al.*, 1993b). Also in accord with data from human breast tumors, TM tumors contained virtually no transcript from gelsolin, a gene product involved in polymerization of actin filaments RNA (Medina *et al.*, 1993b).

Expression of p53 was investigated in the outgrowths, their cell lines, and tumors (Jerry *et al.*, 1993; Ozbun *et al.*, 1993a). An excellent correlation was seen between the alteration of *p53* and the development of preneoplasia *in vivo* (Ozbun *et al.*, 1993a). IHC analysis of the p53 protein in the EL cells showed that the protein was not overexpressed (Medina and Kittrell, 1993). This suggested that none of the EL lines contained MT *p53* and indicated that mutation of *p53* was not necessary for the *in vivo* immortalization of MMEC. Conversely, all five HAN outgrowths demonstrated alteration of p53; furthermore, the mutations were maintained in the tumors arising from the preneoplasias (Table VIII) (Jerry *et al.*, 1993).

TABLE VIII
p53 MUTATIONS IN OUTGROWTH CELL LINES AND TUMORS

	p53 Status: amino acid changes (IHC expression)[a]	
Outgrowth	Cell line	Tumor
TM2H	Single allele 112 stop (none)	Single allele 112 stop (none)
TM3	Single allele 233–234 serine insertion (nucleus)	N/A[b]
TM4	123–129 deletion C138T (nucleus)	123–129 deletion C138T (nucleus)
TM9	WT V170M (cytoplasm)[c]	WT (nucleus)
TM10	N/A[d]	ND[e] (nucleus)

[a] Adapted from Jerry *et al.* (1993) and Ozbun *et al.* (1993a).
[b] No tumors have arisen from TM3.
[c] IHC was performed only on the TM9 outgrowth *in vivo*, not on the cell line.
[d] No cell line was established from TM10.
[e] The TM10 tumor was not sequenced as there was no cell line with which to make a comparison.

The expression of p53 detectable by IHC was apparently inconsistent between the outgrowths and tumors *in vivo* (Table VIII). For example, only a fraction of the TM4 and TM3 HAN outgrowth cells *in situ* stained positively in the nuclei; however, virtually all nuclei were positive in the TM4 and TM3 cultured cell lines and the TM4 tumors (Jerry *et al.*, 1993). Cytoplasmic localization of WT p53 was found for the TM9 outgrowths, whereas in the TM9 tumors the WT p53 was only present in the nuclei (Jerry *et al.*, 1993). As with studies of human breast cancer, these results indicate that the growth environment *in vivo* may modulate the expression and localization of p53 and that other factors besides actual mutation may interfere with the normal function of p53.

Observations from the MMEC outgrowths and outgrowth cell lines indicate that at least two, and probably three, separate steps are necessary for evolution to preneoplasia (illustrated in Fig. 6). This is compatible with the theory that multiple stages are required for tumorigenesis (Newbold, 1985; Barrett, 1987; Vogelstein and Kinzler, 1993). Overall, data from this murine *in vitro–in vivo* system have provided evidence and examples for multiple, defined stages between normal mammary cells and preneoplasia prior to tumor formation. Alterations in *p53* appear to be an early event in this system (Fig. 6) and are reminiscent of many studies of p53 in human breast cancer. For example, *p53* is frequently mutated and is stabilized in breast tumors (Bártek *et al.*, 1990b; Børresen *et al.*, 1991; Davidoff *et al.*, 1991b; Varley *et al.*, 1991; Sommer *et al.*, 1992; Thor *et al.*, 1992; Marchetti *et al.*, 1993; Singh *et al.*, 1993). Also, in

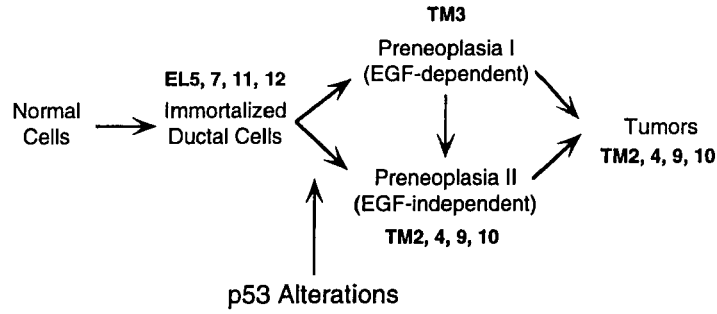

FIG. 6. Model for progression to mammary neoplasia in the murine system. The EL outgrowth lines represent a minimal alteration; the cells behave normally *in vivo* but are immortal (Medina and Kittrell, 1993). TM3 outgrowth cells appear to be an intermediate lesion between immortal (EL) mammary cells and fully hyperplastic (other TM) cells (Medina *et al.*, 1993b). This suggests that at least two forms of preneoplasia can arise and that the properties of immortality and hyperplasia are separable from the tumorigenic properties which characterize mammary tumorigenesis. p53 alterations are important in the formation of the preneoplastic stage (Jerry *et al.*, 1993; Ozbun *et al.*, 1993a).

many cases accumulation of p53 was not a result of mutation and was localized both in the cytoplasm (Moll *et al.*, 1992; Sommer *et al.*, 1992; Suzuki *et al.*, 1992; Takahashi *et al.*, 1993; Takahashi and Suzuki, 1993) and in the nucleus (Børresen *et al.*, 1991).

XI. Summary

Alterations in the tumor suppressor gene *p53* are the most commonly identified changes in cancer, including neoplasia of the breast. The activity of p53 is regulated post-translationally. Phosphorylation state, subcellular localization, and interaction with any of a number of cellular proteins are likely to influence the function of p53. The exact effect of p53-mediated growth suppression seems to be cell-type specific but appears to be directly related to the ability of p53 to act as a specific transcriptional activator. The role that transcriptional repression plays in the function of WT p53 is less clear. It is also possible that p53 has a more direct activity in DNA replication and repair.

Most documented *p53* mutations result in single amino acid substitutions which may confer one or more of a spectrum of transforming abilities on the protein. Mutation may lead to nuclear accumulation of p53 protein; however, inactivation of p53 by nuclear exclusion and interaction with the mdm2 protein also appear to be important in tumorigenesis. Used in conjunction with other established factors, accumulation of cellular p53 may be a useful prognostic indicator in breast cancer.

A syngeneic mouse model system yielded evidence that *p53* mutations are important in the early, preneoplastic stages of mammary tumorigenesis. This murine system may provide the ability to investigate the functions of p53 in the early stages of breast cancer which are technically difficult to examine in the human system.

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p53: A CELL CYCLE REGULATOR ACTIVATED BY DNA DAMAGE

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I. Introduction

Mutations in the p53 tumor suppressor gene occur at high frequency in a large variety of human tumors (Hollstein *et al.*, 1991; Harris, 1993). These mutations include deletions of the entire gene, leading to complete loss of p53 expression, and missense point mutations that give rise to mutant p53 proteins lacking wild-type p53 activity. The fact that the great majority of p53 mutations are missense mutations indicates a positive selection for mutant p53 rather than complete p53 loss during tumor development. In this respect, p53 differs from other tumor suppressor genes, e.g., the retinoblastoma (*RB*) gene, which is usually inactivated by nonsense mutations. All in all, p53 mutations occur in around

50% of human tumors and represent the most common genetic change in cancer cells.

The high frequency of p53 alterations in human cancer suggests that this gene plays a critical role in normal control of cell growth. A number of biological and biochemical functions have now been ascribed to wild-type p53. Importantly, mutant p53 proteins derived from human tumors are defective in some of these functions, suggesting that such functions are relevant for p53-mediated growth control and tumor suppression. Further studies of p53 may open possibilities for clinical therapy of cancer in the future, for instance, the design of drugs that restore wild-type p53 function in tumor cells. This article will review some of the recent progress in the exciting p53 field. On the basis of this information, we propose a model for activation of p53 in response to DNA damage.

II. Biological Activities of p53

A. Induction of G1 Arrest and Suppression of Tumor Cell Growth

Expression of exogenous wild-type p53 in various transformed cells carrying mutant p53 or completely lacking p53 inhibits cell proliferation (Baker *et al.*, 1990; Casey *et al.*, 1991; Diller *et al.*, 1990; Johnson *et al.*, 1991; Martinez *et al.*, 1991; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990). More specifically, using constitutively expressed wild-type p53 (Diller *et al.*, 1990), wild-type p53 driven by the dexamethasone-inducible MMTV promoter (Mercer *et al.*, 1991), or a temperature-sensitive mouse mutant p53 gene (Martinez *et al.*, 1991), wild-type p53 was shown to block cell cycle progression in the G1 phase.

The generation of p53 gene knockout mice (Donehower *et al.*, 1992) has allowed a direct examination of the role of p53 in development and tumorigenesis. Mice homozygous for an inactivated p53 allele appeared normal at birth but were highly susceptible to tumors. By 6 months of age, 74% of the animals had tumors, and all of the animals were dead within 10 months. Mice carrying one wild-type and one inactivated p53 allele were also susceptible to spontaneous tumors, although these tumors developed with a much longer latency period. At least some of the tumors that arose in the heterozygous animals had lost the remaining wild-type p53 allele. These experiments thus confirm that p53 plays an important role as tumor suppressor *in vivo*. Moreover, the normal appearance of the p53 knockout mice at birth suggests that the presence of

wild-type p53 is not an absolute requirement for cell division, differentiation, and embryogenesis.

Studies of the *in vitro* growth properties of cells derived from p53-deficient mice lend further support to the notion that p53 acts as a cell cycle checkpoint control in G1. p53-deficient (−/−) mouse embryo fibroblasts divide more rapidly under normal and low density conditions and contain fewer cells in G1 compared to wild-type (+/+) and heterozygous (+/−) cells (Harvey *et al.,* 1993). In addition, the p53-negative fibroblasts do not reach senescence. An enhanced proliferative potential *in vitro* was also observed in epithelial cells of the lens, mammary gland, and seminal vesicles, and in neural precursor cells, but not in hepatocytes and cardiac muscle cells from p53-deficient mice (Tsukada *et al.,* 1993).

B. Involvement of p53 in Differentiation

Wild-type p53 has also been implicated in B cell differentiation. Shaulsky *et al.* (1991) showed that constitutive expression of exogenous wild-type p53 in a p53-negative pre-B cell line resulted in a more differentiated phenotype. Furthermore, treatment of pre-B and B cells with the differentiation inducer lipopolysaccharide caused an increased expression of *p53* mRNA. Wild-type p53 was shown to transactivate the promoter control sequences of the kappa light-chain gene (Aloni-Grinstein *et al.,* 1993). Exogenous wild-type p53 induced expression of the differentiation marker hemoglobin in human erythroid chronic myelogenous leukemia cells (Feinstein *et al.,* 1992) and in Friend virus-transformed mouse erythroleukemia cells (Johnson *et al.,* 1993). Hence, loss or mutation of p53 may prevent differentiation and thereby block cells in a less mature proliferating state.

Recent data also indicate that p53 is involved in control of meiosis during spermatogenesis in mice (Almon *et al.,* 1993). Increased p53 expression was observed in tetraploid primary spermatocytes of the meiotic pachytene phase in the spermatogenic pathway (Schwartz *et al.,* 1993).

These observations are in apparent conflict with the results obtained with the p53 knockout mice, whose normal phenotype at birth indicates that p53 is not required for normal differentiation during embryogenesis. It remains possible, however, that the p53 null mice show more subtle defects in, for instance, B cell development and spermatogenesis, that have so far been undetected. Alternatively, other cellular proteins may substitute for the differentiation-inducing but not for the tumor suppressor activity of p53 in the p53-deficient mice.

C. Induction of Apoptosis

Using a temperature-sensitive mutant p53 construct, expressed as mutant p53 at 37°C and wild-type p53 at 32°C, Yonish-Rouach *et al.* (1991) found that wild-type p53 can trigger cell suicide, or apoptosis, in p53-negative mouse myeloid cells. Cells grown at 32°C showed characteristic morphological changes, and DNA from these cells gave rise to a ladder on agarose gel electrophoresis, a hallmark of apoptotic death. Expression of wild-type p53 has been shown to elicit apoptosis in other cell types as well, including a human colon tumor-derived cell line (Shaw *et al.*, 1992), mouse erythroleukemia cells (Ryan *et al.*, 1993; Johnson *et al.*, 1993), a Burkitt lymphoma line carrying mutant p53 (Ramqvist *et al.*, 1993), and a p53-negative v-*myc* retrovirus-induced mouse T lymphoma line (Wang *et al.*, 1993a). Based on the latter two studies, performed in our laboratory, we proposed that cell suicide by apoptosis is a consequence of contradictory growth signaling: stimulation of proliferation by constitutively expressed *myc* and induction of growth arrest by wild-type p53. Apoptosis may be viewed as a default option in a situation in which G1 arrest is prevented by deregulated expression of growth stimulatory genes (Oren, 1994).

The idea that apoptosis could result from mutually incompatible signals is further supported by studies of apoptosis induced by the adenovirus E1A oncoprotein. Like *myc*, E1A has growth-promoting capacity. E1A causes accumulation of p53, and subsequently apoptosis (Debbas and White, 1993; Lowe and Ruley, 1993). It is significant, however, that induction of apoptosis by E1A occurs only in cells with intact wild-type p53 function. Likewise, coexpression of wild-type p53 and E2F-1, a transcription factor that activates c-*myc* and other growth-promoting genes and that is liberated from a complex with the RB protein by E1A, was shown to induce apoptosis in mouse embryo fibroblasts (Wu and Levine, 1994).

E1A-induced apoptosis can be suppressed by mutant forms of p53, and by the adenovirus E1B 55K and 19K oncogene products. The E1B 55K protein binds p53 and blocks its functions, whereas the E1B 19K protein averts apoptosis in a more indirect way. Our own experiments showed that wild-type p53-triggered apoptosis is blocked by *bcl-2*, a gene known to inhibit some apoptotic pathways (Wang *et al.*, 1993b). Similarly, Chiou *et al.* (1994) demonstrated that *bcl-2* prevents p53-induced apoptosis but not growth arrest. Thus, *bcl-2* can serve as a modulator of p53 function. A possible mechanism by which E1B 19K and *bcl-2* prevent p53-induced apoptosis is inhibition of p53-mediated transcriptional repression, as suggested by Shen and Shenk (1994).

The molecular mechanisms that mediate p53-induced apoptosis remain unknown, but a clue comes from the demonstration that wild-type p53 upregulates expression of the *bax* gene and simultaneously downregulates *bcl-2* expression (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994). Overexpression of *bax* can accelerate apoptosis and interfere with the apoptosis-inhibiting function of *bcl-2* by forming heterodimers with *bcl-2*. The ratio between *bax* and *bcl-2* probably determines whether a cell will enter apoptosis or survive after receiving an apoptotic signal (Oltvai *et al.*, 1993).

D. p53 AS GUARDIAN OF THE GENOME

Several studies have suggested that one important physiological function of wild-type p53 is to curb cell cycle progression or induce apoptosis following certain types of DNA damage. Ionizing irradiation causes a rapid accumulation of p53 protein (Kastan *et al.*, 1991). The observed accumulation appears to be a result of increased p53 protein stability and depends on ongoing translation (Kuerbitz *et al.*, 1992). Fritsche *et al.* (1993) reported that p53 accumulates in the nucleus following treatment of cells with DNA-damaging agents such as cisplatin, mitomycin C, etoposid and other drugs, as well as energy-rich irradiation. The accumulation of p53 can be detected a few hours after treatment, depending on the agent used, and may remain detectable in surviving cells for at least 20 days.

Introduction of DNA restriction enzyme or nuclease in the nuclei of cultured cells, and even the process of DNA transfection itself, can induce p53 accumulation (Lu and Lane, 1993; Nelson and Kastan, 1994). An increase in p53 protein levels has also been demonstrated *in vivo* after uv irradiation of human skin (Hall *et al.*, 1993). DNA strand breaks were shown to be both necessary and sufficient to trigger wild-type p53 accumulation in cells exposed to DNA-damaging agents (Nelson and Kastan, 1994).

A cause and effect relationship between expression of wild-type p53 and the G1 arrest that occurs after irradiation was established by the demonstration that ionizing irradiation induces G1 arrest in p53-negative cells after transfection of wild-type p53, but not in cells with endogenous wild-type p53 following transfection of mutant p53 (Kuerbitz *et al.*, 1992). Burkitt lymphoma cells expressing mutant p53 failed to arrest in G1 following irradiation (O'Connor *et al.*, 1993). The ability of p53 to induce G1 arrest correlated with the radiosensitivity of the Burkitt lymphoma cells. Moreover, experiments using bone marrow cells

derived from mutant p53-transgenic mice demonstrated that expression of mutant p53 conferred increased resistance to ionizing irradiation (Lee and Bernstein, 1993).

Studies of thymocytes from p53 knockout mice have confirmed that p53 is required for irradiation-induced apoptotis (Clarke *et al.*, 1993; Lowe *et al.*, 1993). These cells entered apoptotis after treatment with glucocorticoids, but were extraordinarily resistant to the induction of apoptosis by irradiation, both *in vivo* and *in vitro*. Analysis of spontaneous and irradiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice revealed that the loss of p53 renders the epitelial cells essentially radioresistant *in vivo* (Merrit *et al.*, 1994). This result is consistent with earlier studies by Kastan *et al.* (1992), showing that primary p53-negative fibroblasts derived from p53 null mice do not arrest in G1 following ionizing irradiation. Loss of *p53* function alone, with no other genetic abnormalities, is thus sufficient for the loss of irradiation-induced G1 arrest. Kastan *et al.* (1992) also demonstrated that expression of the *GADD45* gene, known to be induced by DNA damage, was dependent on wild-type *p53* function. In addition, cells from patients with the radiosensitive cancer-prone syndrome ataxia telangiectasia (AT) showed a defective p53 response to ionizing irradiation (Kastan *et al.*, 1992; Khanna and Lavin, 1993). *p53* accumulated normally in these cells after uv treatment, however (Khanna and Lavin, 1993), suggesting that there is more than one pathway responsible for induction of p53. Lu and Lane (1993), on the other hand, did not find any defects in the p53 response in AT cells, but found that cells from at least some patients with Bloom's chromosome instability syndrome failed to accumulate p53 after uv treatment. These findings implicate a gene or genes that are defective in AT and Bloom's syndrome cells upstream of p53 in a signal transduction pathway that controls cell cycle progression following DNA damage.

The fact that cells defective in wild-type p53 function are unable to undergo the G1 arrest necessary to repair damaged DNA implies that cells carrying mutant p53 or no p53 should sustain a larger number of genetic lesions than cells expressing functional wild-type p53. In agreement with this notion, p53-negative mouse embryo fibroblasts were significantly more prone to gene amplification, measured as frequency of *CAD* gene amplification using a PALA selection assay, than fibroblasts carrying wild-type p53 (Livingstone *et al.*, 1992). A large fraction of the p53-negative fibroblasts showed aneuploidy already at passage 3 in culture. A higher incidence of cells with abnormal chromosome numbers was evident also among mouse embryo fibroblasts heterozygous for an inactivated p53 allele, but not until passage 25 (Harvey *et al.*, 1993).

Similarly, human fibroblasts expressing the human papilloma virus (HPV) *E6* gene, whose product binds to and causes the degradation of p53, showed altered cell cycle regulation and increased ability to amplify the *CAD* gene (White *et al.*, 1994).

Taken together, these data suggest that p53 protects cells from genomic injury. If DNA is damaged, the p53 protein accumulates and blocks entry into S phase. The transient delay at the G1 checkpoint permits repair of damaged DNA prior to DNA replication. If the repair fails, p53 may trigger apoptosis, and thus prevent propagation of cells that have sustained mutation (Lane, 1992).

III. Biochemical Functions of p53

A. Regulation of DNA Replication

Experiments using replication of viral DNA as a model system have indicated that p53 may fulfill a role in control of DNA replication. Mouse p53 can inhibit SV40 replication both *in vivo* and *in vitro* (Stürzbecher *et al.*, 1988; Braithwaite *et al.*, 1987). Mutant p53 proteins did not inhibit SV40 replication, although some of them were able to form complexes with the SV40 large T antigen. Detailed examination of the mechanisms underlying inhibition of SV40 replication *in vivo* revealed that both mouse and human wild-type p53 can interfere with the initiation event of DNA replication. Binding to the large T antigen is probably not necessary for inhibition, since at least one p53 mutant that is unable to interact with large T still inhibits SV40 DNA replication (Stürzbecher *et al.*, 1992). p53 reduced binding of large T to the SV40 origin of replication and abolished its helicase activity (Stürzbecher *et al.*, 1988; Wang *et al.*, 1989; Friedman *et al.*, 1990). Additionally, p53 can compete with DNA polymerase-α for binding to the large T antigen (Gannon and Lane, 1987). Immunopurified wild-type but not mutant p53 protein was shown to bind specifically to DNA sequences adjacent to the SV40 origin of replication (Bargonetti *et al.*, 1991).

More recently, Oberosler *et al.* (1993) showed that p53 can inhibit the activity of various helicases *in vitro,* including DNA helicase I and II of *Escherichia coli* and human p68 RNA helicase, probably without complexing with these proteins. Since the unwinding of DNA is a basic step in the initiation of DNA replication *in vivo,* p53 may directly inhibit DNA replication by blocking this process.

Studies of herpes simplex virus type 1 (HSV-1)-infected cells provide additional evidence that p53 participates in control of DNA replication. Immunostaining showed that p53 localized at sites of viral DNA

synthesis in herpes virus-infected cells, along with ICP8, the major DNA-binding protein of HSV-1, and cellular proteins including RPA, PCNA, and the RB protein (Wilcock and Lane, 1991). Furthermore, work by Kanda *et al.* (1994) implicates p53 in positive regulation of polyoma virus DNA replication through a p53 DNA binding site. The involvement of p53 in control of DNA replication is also supported by the observation that p53 can complex with the cellular DNA replication factor RPA, alternatively designated SSB, single-stranded DNA-binding protein (Dutta *et al.*, 1993; Li and Botchan, 1993; He *et al.*, 1993) (see Section IV.B).

Work of Waga and colleagues (1994) has demonstrated yet another mechanism by which p53 may regulate DNA replication. The p21 protein, which is the product of one of the genes induced by p53 (see Section III.D), was shown to inhibit the ability of PCNA to activate DNA polymerase δ by complexing with PCNA and thus directly block DNA replication *in vitro*.

B. Sequence-Specific DNA Binding

The wild-type p53 protein binds DNA in a sequence-specific manner. As shown in Table I, several specific DNA-binding sites for p53 have been identified by various techniques. Using a modified DNA-binding immunoassay followed by PCR amplification of the selected human genomic DNA fragments, El-Deiry *et al.* (1992) defined a consensus p53-binding site consisting of two copies of the 10 base pair element 5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′, separated by up to 13 base pairs. Comparisons with previously identified p53-binding sites reveal reasonable similarity to the RGC- and MCK-binding sites, with a few mismatches (Table I), whereas the SV40 sequence, previously shown to bind immunopurified p53 protein *in vitro*, is less related (Bargonetti *et al.*, 1991).

The specific DNA-binding activity of p53 has been mapped to the central portion of the protein, roughly corresponding to amino acid residues 100 to 300 (Fig. 1) (Bargonetti *et al.*, 1993; Halazonetis and Kandil, 1993; Pavletich *et al.*, 1993; Wage *et al.*, 1993c). A great majority of the point mutations found in human tumors are located in this region (Harris, 1993). Tumor-derived mutant forms of p53 have reduced specific DNA-binding activity, as a rule (for a review, see Zambetti and Levine, 1993), strongly suggesting that the specific DNA-binding activity of p53 is important for its tumor suppressor function.

The p53 protein forms stable homooligomers (Stenger *et al.*, 1992; Stürzbecher *et al.*, 1992; Friedman *et al.*, 1993). Oligomerization is medi-

TABLE I

p53 DNA-BINDING ELEMENTS

Consensus site	5′-PuPuPu C (A/T) (A/T) G PyPyPy-3′	El-Deiry *et al.* (1992)
p53 CON	GGA CATG CCC GGG CATG TCC	Funk *et al.* (1992)
BC	GGG CATG TCC GGG CATG TCC	Halazonetis *et al.* (1992)
syncon	AGCTTAGA CATG CCT AGA CATG CCTA	Hupp *et al.* (1992)
PG	TTGCCTGGA CTTG CCTGG ×16	Kern *et al.* (1991)
RGC	GAT tGc CTTG CCT GGA CTTG CCT GGC CTTG CCTTTT	Kern *et al.* (1991)
SV40	GCCATGGGGCGCAGAATGGGGAACTGGGCGGAGTTAGCTCGA	Bargonetti *et al.* (1992)
MCK	TCGAG tGG CAAG CCT A tGA CATG gCC GGG CcTG CCTCTCTCTGC	Zambetti *et al.* (1992)
GLN LTR	CCAGGA CATG CCC GGG CAAG CCC CATG	Zauberman *et al.* (1993)
GADD45	TGGTACAGAA CATG TCT AAG CATG CTg GGGACT	Kastan *et al.* (1992)
MDM2	GGt CAAG TTg GGA CAcG TTC AGc tAAG TCC tGA CATG TCT	Barak *et al.* (1993); Wu *et al.* (1993)
WAF1	GAA CATG TCC cAA CATG TTg	El-Deiry *et al.* (1993)
p53	G GGA CTTt CCC TCCCACTGT	Deffie *et al.* (1993)
MgBH6	GACACTGGTC AcA CTTG gCT GCTTAGGAAT	Foord *et al.* (1993)
cycG	AGACcTGCCC GGG CAAG CCT	Okamoto and Beach (1994)

Note. The underlined regions represent homology to the consensus site with mismatches identified by small lettering. Note that all genes known to be transactivated by p53 contain imperfect binding site (one to three mismatches per double 10-mer) without spacer nucleotides between the 10-mers.

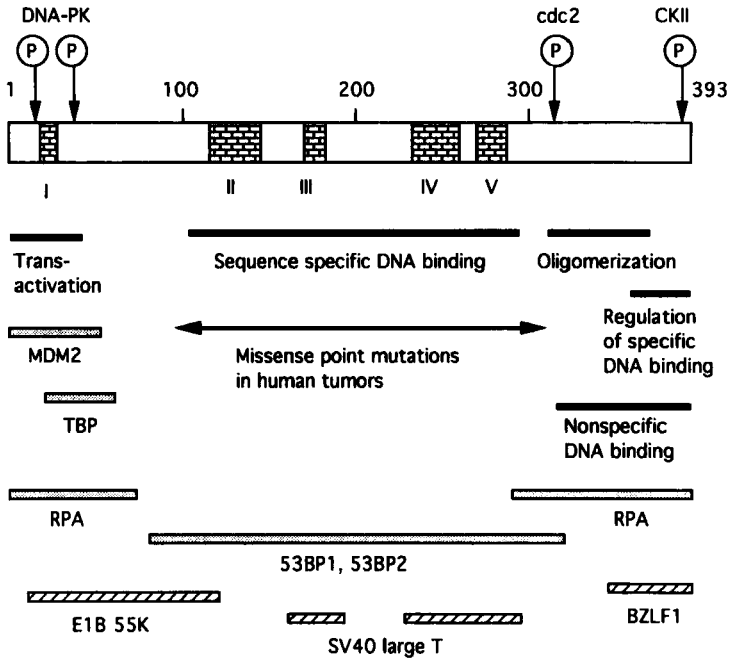

FIG. 1. Functional domains of the human p53 protein and regions involved in complexing with cellular and viral proteins. The functional domains include the N-terminal transactivation domain, the zinc-containing sequence-specific DNA-binding domain, and the C-terminal region, containing the oligomerization domain. The C-terminus also possesses nonspecific DNA-binding activity. Phosphorylation sites for dsDNA-PK (Ser-15 and Ser-37), p34-cdc2 (Ser-315), and CKII (Ser-392) are indicated. The TBP and MDM2 interaction domains map to the N-terminal region, whereas RPA binds to both the N-terminal and the C-terminal part of the p53 protein. The newly discovered 53BP1 and 56BP2 proteins bind to the central core domain responsible for specific DNA binding. The adenovirus E1B 55K protein complexes with p53 through an N-terminal region that includes the transcriptional activation domain, while the SV40 large T antigen binds to the central core domain. The EBV-encoded BZLF1 protein interacts with a C-terminal region of p53. Five conserved regions are indicated (I–V).

ated by a region in the C-terminal domain (Shaulian *et al.*, 1992; Stürzbecher *et al.*, 1992; Pavletich *et al.*, 1993) (Fig. 1). An α-helix, spanning amino acid residues 334–356, was shown to be important for dimerization, whereas a basic region comprising residues 363–386 was implicated in formation of higher oligomeric complexes (tetramers) (Stürzbecher *et al.*, 1992). In contrast, Pavletich *et al.* (1993) found that a region corresponding to amino acid residues 311–367 is sufficient for tetramerization, while Wang *et al.* (1994b) identified a strong tetrameriz-

ation domain between amino acid residues 323 and 355 and a weaker oligomerization domain in the central core domain of p53. Analysis of the three-dimensional structure of the C-terminal region of p53 (amino acid residues 319–360) using multidimensional heteronuclear magnetic resonance (NMR) spectroscopy has supported the involvement of this domain in mediating tetramerization (Clore *et al.*, 1994).

The fact that p53 can form oligomers and that the consensus DNA-binding motif contains four copies of a pentamer sequence suggests that p53 binds DNA as a tetramer. Band shift assays using wild-type and dimeric and monomeric deletion mutant p53 proteins have demonstrated that in fact both p53 tetramers and dimers but not monomers bind specifically to DNA (Halazonetis and Kandil, 1993; Tarunina and Jenkins, 1993). On the other hand, Friedman *et al.* (1993) used Southwestern analysis and found that p53 monomers can also bind DNA.

While the binding of *in vitro* translated p53 to the p53 CON sequence (Table I) requires complexing with other factors provided by the nuclear extract (Funk *et al.*, 1992), p53 can efficiently bind the RGC and BC sequences in the absence of nuclear extract (El-Deiry *et al.*, 1992; Halazonetis *et al.*, 1993). p53 from cellular extracts seems to have the highest affinity for the p53 CON sequence, since oligonucleotides containing the RGC, MCK, or SV40 sequences could not compete out oligonucleotides containing the p53 CON sequence from the complex with p53 in a band shift assay (Zhang *et al.*, 1993a). These results indicate that the recognition of a given DNA sequence by p53 is modulated by the interaction with cellular proteins or factors.

Some mutant *p53* alleles clearly act as oncogenes, as demonstrated by their ability to transform cells in culture (Eliyahu *et al.*, 1984; Parada *et al.*, 1984; Jenkins *et al.*, 1984; Zambetti *et al.*, 1992b). In these cells, the mutant p53 protein interferes with the function of the endogenous wild-type p53 protein in a dominant negative fashion, by forming hetero-oligomers with the wild-type protein and forcing the latter into a mutant conformation, unable to bind DNA (Milner and Medcalf, 1991; Milner *et al.*, 1991; Kern *et al.*, 1992; Shaulian *et al.*, 1992). Oligomerization-defective p53 mutants cannot transform rat embryo fibroblasts together with mutant *ras* (Slingerland *et al.*, 1993). Furthermore, C-terminal miniproteins of p53, containing amino acid residues 302–390 or 302–360, were found to oligomerize with wild-type p53, abrogate sequence-specific DNA binding by coexpressed wild-type p53, and transform rat embryo fibroblasts (Shaulian *et al.*, 1992).

A complex containing the DNA-binding core domain of p53 (amino acid residues 102 to 292) bound to a specific target DNA sequence has been crystallized and its structure determined (Cho *et al.*, 1994). Analysis

of this structure reveals that the core domain consists of a β sandwich that functions as a scaffold for a loop–sheet–helix structure and two loops. Residues within the loop–sheet–helix motif interact in the major groove of DNA, whereas one of the two loops interacts in the minor groove through the arginine at position 248. The second loop stabilizes the loop that interacts directly with DNA. A tetrahedrally coordinated zinc atom helps to hold the two loops together. Importantly, the structural analysis of the core domain reveals that amino acid residues frequently mutated in human tumors are located in the regions of the core domain that interact with DNA, i.e., the loop–sheet–helix and the two loops. These motifs correspond well to the four conserved regions of the core domain (Fig. 1).

C. Transcriptional Activation

The N-terminal domain of p53 (amino acid residues 1–42; Fig. 1) is a strong transcriptional activator, as first shown in yeast using a GAL4–p53 fusion protein (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990). Due to its sequence-specific DNA binding activity, located in the central core domain, and N-terminal transcriptional activator domain, the wild-type p53 protein can transactivate promoters that contain a specific p53-binding motif. Naturally occurring mutant p53 proteins usually have reduced or no transactivating activity (Farmer *et al.*, 1992; Kern *et al.*, 1992; Unger *et al.*, 1992; Chen *et al.*, 1993b).

At least some mutant p53 proteins retain the ability to stimulate transcription through certain target DNA sequences, however. In cotransfection experiments, the mutant p53 proteins Trp-248, His-175, and Glu-281 enhanced p53 CON-mediated transactivation of a reporter gene by wild-type p53, but decreased RGC-mediated transactivation (Zhang *et al.*, 1993b). All three mutants failed to activate both RGC- and p53 CON-mediated transcription in the absence of wild-type p53. Some mutant p53 proteins (Val-143 and His-273) can bind p53 CON but not RGC *in vitro,* and can transactivate reporter gene expression from promoters containing a p53 CON motif, but not from those containing an RGC motif (Zhang *et al.*, 1993a; Pietenpol *et al.*, 1994; Chen *et al.*, 1993b). The Ile-246 and Leu-248 mutants are likewise able to activate transcription through the p53 CON element but not through the RGC element (Chen *et al.*, 1993b). Nevertheless, when the ability of mutant p53 proteins to activate transcription via imperfect p53-binding sites from human genomic DNA was tested, all mutant proteins failed to transactivate (Pietenpol *et al.*, 1994). The inability of these p53 mutants to transactivate correlated with an inability to suppress tumor cell

growth. Thus, the difference between wild-type and mutant p53 proteins is that the mutant proteins are unable to transactivate promoters containing target sequences with mismatches relative to the p53 consensus sequence, such as those found in the natural promoters induced by wild-type p53.

It is conceivable that some mutant p53 proteins have an altered binding specificity for DNA. As a result, they may illegitimately transactivate a new set of genes, different from the natural targets of p53. This idea provides an explanation for the observation that certain p53 mutations not only inactivate its wild-type function, but lead to a gain of function as well. Dittmer *et al.* (1993) found that introduction of mutant p53 in p53 null cells caused an increased plating efficiency *in vitro* or an enhanced tumorigenic potential *in vivo*. In keeping with this notion, common mutant forms of p53 can transactivate the promoter of the human multidrug resistance gene (Chin *et al.*, 1992; Dittmer *et al.*, 1993).

Monomeric p53 deletion mutant proteins lacking the C-terminal oligomerization domain have been shown to transactivate a p53-responsive promoter and also suppress oncogene-mediated transformation, although they did not show detectable specific DNA binding *in vitro* (Shaulian *et al.*, 1993; Tarunina and Jenkins, 1993). These results could indicate that monomeric p53 is functionally active and can bind to a p53 target DNA sequence *in vivo*, although the band shift assay employed did not allow detection of a weak interaction between the p53 monomers and DNA. If so, the formation of dimers and tetramers may not be essential for specific DNA binding and transactivation, but could be important for stabilizing the p53:DNA complex. Alternatively, the p53 deletion mutants that lack the C-terminal oligomerization domain may still form oligomers *in vivo* and thus bind DNA, as suggested by the observation that the central core domain of p53 has weak oligomerization activity (Wang *et al.*, 1994b).

D. Genes Activated by Wild-Type p53

The muscle creatine kinase gene promoter harbors a consensus p53-binding site and is transactivated by wild-type p53 (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992a). Another p53-binding site, similar to the p53 CON element, resides within the LTR of an endogenous retrovirus-like element, GLN LTR (Zauberman *et al.*, 1993). The expression of exogenous GLN elements was significantly induced upon activation of wild-type p53 in cells carrying a temperature-sensitive p53 mutant (Zauberman *et al.*, 1993) So far, the relevance of MCK or GLN LTR transactivation for the known biological activities of p53 is unclear. p53

has also been shown to upregulate the RB and the epidermal growth-factor receptor gene promoters (osifchin *et al.*, 1994; Deb *et al.*, 1994), and induce *bax* mRNA and protein levels (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994). In addition, Deffie *et al.* (1993) have shown that wild-type p53 can transactivate its own promoter through a sequence that is identical to the NF-κB-binding site and that also matches the El-Deiry consensus p53-binding site at 9 of 10 positions (Table I), although electrophoretic mobility shift assays failed to show significant binding of p53 to this site. Okamoto and Beach (1994) recently identified the mouse cyclin G gene as a transcriptional target of p53. This raises the possibility that cyclin G is involved in p53-mediated tumor suppression.

As discussed under Section II.D, Kastan and colleagues (1992) have identified the *GADD45* gene as a cellular target of p53. This gene is among a series of genes whose expression is stimulated when cells suffer DNA damage (Fornace *et al.*, 1989). *GADD45* has growth-suppressing properties on its own (Zhang *et al.*, 1994b). A p53-responsive element that matches the consensus binding site at 19 of 20 nucleotides and binds purified p53 protein *in vitro* was identified in the third intron of the *GADD45* gene (Table I; Kastan *et al.*, 1992).

Wild-type p53 induces expression of *MDM2*, a previously identified oncogene (Fakharzadeh *et al.*, 1991; Barak *et al.*, 1993; Wu *et al.*, 1993). A p53 DNA-binding site containing a few mismatches in comparison with the El-Deiry consensus motif is located within the first intron of *MDM2* (Wu *et al.*, 1993; Table I). The observations that the *MDM2* gene product, p90, can form a complex with p53 (Barak and Oren, 1992; Momand *et al.*, 1992) (see Section IV.B) and that overexpression of *MDM2* results in inhibition of p53-mediated transactivation (Momand *et al.*, 1992) have led to the assumption that *MDM2* serves as a feedback regulator of p53 function (Wu *et al.*, 1993). Overexpression of *MDM2* may therefore promote oncogenesis by blocking the activity of wild-type p53 (Finlay, 1993).

The amount of *MDM2* mRNA increases upon DNA damage in cells carrying wild-type p53, but not in cells expressing mutant p53 or no p53 protein at all (Perry *et al.*, 1993; Chen *et al.*, 1994). Moreover, Price and Park (1994) found a defective induction of *MDM2* mRNA levels in AT cells, where the accumulation of p53 protein in response to DNA damage is delayed and/or reduced.

The p53-responsive element in the first intron of the *MDM2* gene mediates transactivation of *MDM2* from an internal promoter (Juven *et al.*, 1993). This indicates that p53 can influence the relative abundance of different *MDM2* transcripts within the cell, in addition to upregulat-

ing *MDM2* expression. The identification of multiple MDM2 proteins and MDM2–p53 protein complexes substantiates this notion (Olson *et al.*, 1993; Haines *et al.*, 1994). Among four MDM2 proteins studied, three were found to associate with the p53 protein and inhibit its transactivation function. In contrast, one of the proteins, lacking the first 49 amino acids, was unable to complex with p53 and block p53-mediated transactivation (Haines *et al.*, 1994). Although the full-length *MDM2* mRNA and the *MDM2* mRNA initiated from the p53-dependent promoter in intron 2 are expected to have the same coding potential (exon 1 and 2 in mouse *MDM2* are noncoding), the full-length mRNA is primarily translated into truncated MDM2 proteins lacking the N-terminal p53-binding domain, whereas the *p53*-induced mRNA yields both truncated and full-length MDM2 proteins, at least *in vitro* (Barak *et al.*, 1994). In conclusion, these data suggest that p53 can induce the expression of MDM2 proteins that are capable of binding to and inhibiting the function of p53, while an MDM2 protein species that is unable to bind p53 is expressed largely independently of p53.

Using a subtractive hybridization approach, El-Deiry and co-workers (1993) identified a gene designated *WAF1*, whose expression is directly induced by wild-type p53 and that is most likely an important downstream effector of p53 function. The *WAF1* gene is localized to human chromosome 6p21.2 and its sequence, structure, and activation by p53 are conserved in rodents. Introduction of *WAF1* cDNA suppresses the growth of various human tumor cells in culture. A 20-bp sequence with two mismatches in comparison with the El-Deiry consensus p53-binding site was identified 2.4-kb upstream of the *WAF1* coding sequence (see Table I). A *WAF1* promoter fragment containing this p53-binding site conferred p53-mediated transactivation upon a reporter gene. *WAF1* expression is induced in cells undergoing wild-type p53-associated G1 arrest or apoptosis (El-Deiry *et al.*, 1994). More recently, Michieli *et al.* (1994) showed that *WAF1* can also be induced in a p53-independent manner.

The same gene was also identified using entirely different strategies. Studies of the subunit composition of cyclin/cyclin-dependent kinase (cdk) complexes showed frequent loss of a p21 component in transformed cells (Xiong *et al.*, 1993b). *In vitro* reconstitution of quaternary cyclin/cdk complexes revealed that p21 inhibits the activity of each member of the cyclin/cdk family. The novel gene, designated *p21* (Xiong *et al.*, 1993a) or *CIP1* (cdk-interacting protein 1; Harper *et al.*, 1993), was found to be identical to *WAF1*. This gene was also cloned as a cDNA differentially expressed in senescent cells (Noda *et al.*, 1994).

The combined data on *WAF1/p21/CIP1* suggest the following model for the function of wild-type p53 (Fig. 2): DNA damage triggers accumulation of p53 protein, leading to transactivation of *WAF1/p21/CIP1*. If DNA damage has occurred prior to S phase, the WAF1 protein can prevent phosphorylation of cyclin-dependent kinase substrates through inhibition of cyclin-dependent kinase activity, and subsequently block cell cycle progression. However, if DNA is damaged during S phase, p21 can block DNA replication by binding to PCNA (as discussed under Section III.A). In tumor cells lacking wild-type p53 function, this pathway would be defective, permitting cell division in the presence of damaged DNA. This would either lead to mitotic failure or the accumulation of mutations and chromosomal aberrations.

E. Transcriptional Repression

In addition to its ability to positively regulate specific promoters containing a p53 binding motif, p53 can repress transcription from a variety of promoters, including the c-*fos*, c-*jun*, c-*myc*, *IL-6*, and *RB* promoters (Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Lecher *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992; Ragimov *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Osifchin *et al.*, 1994). In contrast, the activity of an MHC class I gene promoter was not affected (Ginsberg *et al.*, 1991; Ragimov *et al.*, 1993). Available evidence suggests that p53 inhibits transcription through a direct interaction with TBP, the TATA-binding protein (Seto *et al.*, 1992; Truant *et al.*, 1993; Liu *et al.*, 1993). Mack *et al.* (1993) found that p53 specifically represses TATA-mediated but not initiator-mediated transcription, although transcription from both types of promoters requires TBP. Overexpression of p53 reduced TATA-mediated transcription initiation from a plasmid containing a tandem TATA and initiator element downstream of the SV40 21-bp repeats, while initiator-mediated transcription was unaffected. This suggests that p53-mediated repression of transcription is not simply a result of squelching TBP (Mack *et al.*, 1993). Significantly, both wild-type and mutant p53 can bind TBP but only wild-type p53 interferes with the binding of TBP and TFIIA to the TATA motif (Ragimov *et al.*, 1993). An inhibitory effect of p53 on TBP binding to the TATA motif in the absence of a p53-binding site has also been reported by Chen *et al.* (1993a).

In addition, p53 has been shown to repress transcription by interaction with the transcription factor CBF (CCAAT binding factor). CBF in complex with E1A activates transcription from the hsp70 promoter, whereas CBF in complex with p53 has the opposite effect. It is conceiv-

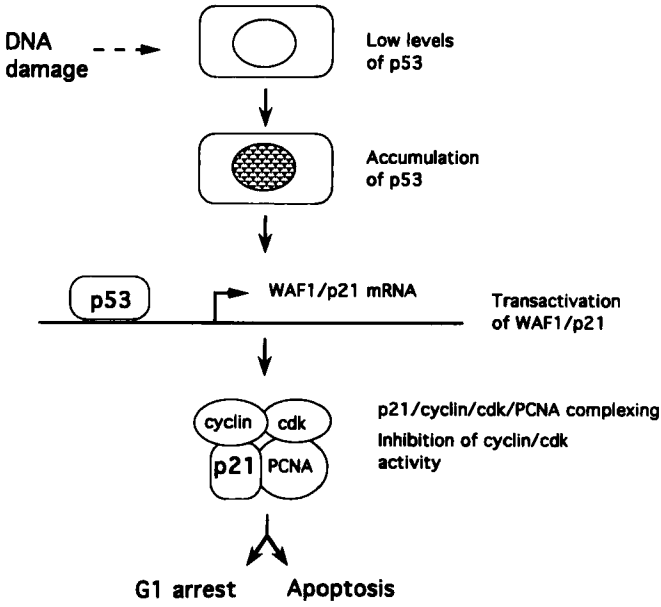

FIG. 2. p53-mediated cell cycle division arrest or apoptosis in response to DNA damage. Cellular p53 protein levels rise upon DNA damage. p53 binds to a specific target DNA sequence in the WAF1 promoter and stimulates WAF1 transcription, resulting in increased levels of the WAF1 protein, p21. This protein binds and blocks the activity of cyclin/cdk complexes. As a result, phosphorylation of cyclin/cdk substrates is inhibited, leading to cell cycle arrest in the G1 phase and/or cell suicide by apoptosis. Complexes containing p21, cyclin, and cdk also contain PCNA (proliferating cell nuclear antigen). The interaction of p21 with PCNA may interfere with DNA replication.

able that complexing with p53 changes the ability of CBF to interact properly with the hsp70 promoter (Agoff *et al.*, 1993).

Recent work has demonstrated that expression of the *bcl-2* gene is downregulated by p53 (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994). This effect appears to be mediated by a negative control element located in the 5′ untranslated region of *bcl-2* (Miyashita *et al.*, 1994b).

While the low levels of p53 protein present in normal cells are probably insufficient to shut off transcription through interaction with TBP and other factors required for efficient transcription, the accumulation of p53 protein in response to DNA damage is likely to result in significant p53-mediated repression of various promoters, including those of c-*myc* and other growth-promoting genes.

F. Promotion of DNA Renaturation and Strand Transfer

Oberosler *et al.* (1993), Bakalkin *et al.* (1994), and Brain and Jenkins (1994) have independently demonstrated that wild-type p53 can catalyze renaturation of complementary single-stranded (ss) DNA oligonucleotides. p53 was also shown to promote strand exchange between double-stranded (ds) DNA and a complementary ssDNA strand (Bakalkin *et al.*, 1994), and to promote RNA reannealing and strand exchange (Oberosler *et al.*, 1993).

Some proteins or compounds promote spontaneous strand transfer, a process that can be blocked by a single mismatch in the region of branch migration (Panyutin and Hsieh, 1993). However, the p53-promoted strand exchange is due to an enzymatic activity of p53, since p53 catalyzed strand transfer of duplex substrates containing one or four mismatches relative to the acceptor DNA strand as efficiently as strand transfer of perfectly matched oligonucleotides (Bakalkin *et al.*, 1994). The three mutant p53 proteins, Glu-213, Ile-237, and Tyr-238, failed to catalyze DNA renaturation and strand transfer (Bakalkin *et al.*, 1994).

The observation that p53 is able to reassociate nucleic acids suggests that p53 can counteract DNA and RNA helicases by promoting reannealing in processes like DNA replication, recombination, and repair, and RNA splicing. This activity may at least in part depend on the interaction of p53 with the DNA replication factor RPA (see Section IV.B).

Wild-type p53 has significantly higher affinity for short (36–76 nucleotides) than for longer (>462 nucleotides) ssDNA oligonucleotides, indicating that short ssDNA fragments and/or DNA ends are the prime targets for p53 (Bakalkin *et al.*, 1994). Indeed, our electron microscopy analysis of p53–ssDNA complexes revealed preferential binding of p53 to ssDNA ends (in 90% of complexes observed) (Bakalkin *et al.*, 1994). These findings raise the possibility that *p53* serves as a sensor of DNA strand breaks *in vivo*. This would be consistent with the observation that DNA strand breaks are necessary and sufficient to trigger *p53* accumulation (Nelson and Kastan, 1994). It is possible that p53 may participate directly in DNA repair by promoting the renaturation of protruding ssDNA ends in cells that have suffered DNA damage. p53 could also promote the proper joining of incorrectly renatured DNA through its ability to catalyze strand transfer. If this p53 function is important *in vivo*, DNA strand breaks will not be efficiently repaired in cells lacking p53 or expressing mutant p53, resulting in genomic instability. Thus,

p53-mediated DNA reannealing and strand transfer could play a role in tumor suppression.

IV. Interactions with Viral and Cellular Proteins

A. Complexing with Viral Oncoproteins

Transforming proteins encoded by three different DNA tumor viruses, the SV40 large T antigen, the adenovirus E1B 55-kDa protein, and the human papilloma virus 16/18 E6 protein, can form complexes with p53 (Linzer and Levine, 1979; Lane and Crawford, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990; Scheffner *et al.*, 1990). The same viruses also encode proteins that bind the RB protein, the product of another tumor suppressor gene (for a review, see Wiman, 1993). The common association between the viral oncoproteins and p53 represents a remarkable case of convergent evolution and provides strong evidence that p53 is a critical cell cycle regulator. Impairment of p53 function may serve the viral strategy by relieving the G1 block imposed by p53, allowing the infected cell to enter S phase and hence replicate viral DNA.

Whereas complexing with the SV40 large T antigen or the E1B 55-kDa protein leads to stabilization of p53 (Oren *et al.*, 1981; Reich *et al.*, 1983), binding to the HPV 16/18 E6 protein results in the degradation of p53 via the ubiquitin-dependent proteolytic system (Scheffner *et al.*, 1990; Werness *et al.*, 1990). As a result, cells producing HPV 16/18 E6 protein express little or no p53. Complexing between E6 and p53 requires an additional cellular protein, E6-AP, that induces E6- and ubiquitin-dependent degradation of p53 (Huibregtse *et al.*, 1993). The SV40 large T antigen interacts with the central DNA-binding domain of p53 (Fig. 1) and inhibits specific DNA binding (Bargonetti *et al.*, 1992) as well as p53-mediated transactivation of a reporter gene (Mietz *et al.*, 1992; Jiang *et al.*, 1993; Segawa *et al.*, 1993). Unlike large T, the adenovirus E1B 55-kDa protein binds to the N-terminal transactivating domain of p53 (Fig. 1; Kao *et al.*, 1990), suggesting that the E1B 55-kDa protein does not directly interfere with specific DNA binding. Instead, work by Yew *et al.* (1994) shows that the E1B 55-kDa protein is a general transcriptional repressor that inhibits transcription of p53 responsive genes by binding to p53. Thus, although the viral transforming proteins use different mechanisms to abrogate p53 function, the common denominator is loss of p53-mediated transactivation of specific target genes.

More recent studies have shown that additional transforming DNA viruses encode proteins that interact with p53. The Epstein–Barr virus

(EBV)-encoded EBNA-5 protein, required for EBV-mediated B cell transformation, can complex with p53 and RB as well (Szekely *et al.*, 1993). The transforming protein X of hepatitis B virus and the immediate-early protein BZLF1 of EBV have been shown to bind p53 both *in vitro* and *in vivo* and to inhibit its sequence-specific DNA-binding and transactivation activity (Fietelson *et al.*, 1993; Wang *et al.*, 1994a; Zhang *et al.*, 1994a). Moreover, Speir *et al.* (1994) found that the IE84 protein of human cytomegalovirus (HCMV) interacts with p53 in coronary restenosis. p53 protein levels were induced upon HCMV infection of smooth muscle cells, and the viral IE84 protein, which is thought to participate in activating cellular DNA replication, was shown to physically bind p53 and abrogate p53-mediated transactivation of a *CAT* reporter gene.

B. p53-Associated Cellular Proteins

p53 interacts with several cellular proteins that may play a role in regulating p53 function. The MDM2 gene product, p90, can form oligomeric complexes with wild-type and mutant p53 and block p53-mediated transactivation of a p53-responsive promoter (Momand *et al.*, 1992; Hoppe-Seyler and Butz, 1993; Brown *et al.*, 1993). Since MDM2 expression is induced by p53, MDM2 may act as a p53 antagonist that modulates the p53 response to DNA damage (Chen *et al.*, 1994).

The region of p53 required for MDM2 binding (residues 1–52) corresponds almost exactly to the acidic transactivation domain of p53 (Oliner *et al.*, 1993; Chen *et al.*, 1993c) and overlaps with the binding domains for the adenovirus E1B 55-kDa protein and the cellular TBP and RPA proteins (Fig. 1). The hydrophobic amino acid residues Leu-22 and Trp-23, shown to be critical for the transactivating function of p53 (Lin *et al.* 1994), are important for binding of both the MDM2 and the adenovirus E1B 55-kDa protein.

Overexpression of MDM2 increases the tumorigenic potential of NIH3T3 cells (Fakharzadeh *et al.*, 1991) and immortalizes primary rat embryo fibroblasts and transforms rat embryo fibroblasts together with an activated *ras* gene (Finlay, 1993). In addition, MDM2 can overcome wild-type p53-mediated suppression of transformed cell growth (Finlay, 1993). Thus, MDM2 has the properties of an oncogene. *MDM2* gene amplification has been observed in several types of human sarcomas carrying wild-type p53 (Oliner *et al.*, 1992, Ladanyi *et al.*, 1993), suggesting that p53 mutation and *MDM2* overexpression represent two alternative tumorigenic pathways, both involving loss of p53 function. However, *MDM2* overexpression, due to gene amplification or other genetic

lesions, has also been observed in tumors that overexpress p53 (p53 overexpression is usually associated with missense mutation). Cordon-Cardo *et al.* (1994) found that around 11% of soft tissue sarcomas overexpressed both *MDM2* and p53. This group of patients had the shortest survival times. Therefore, the combination of *MDM2* overexpression and p53 mutation appears to endow cells with a selective advantage during tumorigenesis, perhaps indicating that *MDM2* overexpression in cells carrying wild-type p53 only partially inhibits wild-type p53 function and/or that mutant p53–MDM2 protein complexes may perform new functions that contribute to the malignant phenotype.

The p53-binding domain of the MDM2 protein is located within amino acid residues 19 to 102, a region which is highly conserved (93% identity) between human and mouse, and is distant from the putative transactivation domain of MDM2. As discussed above, one form of the MDM2 protein lacks the p53-binding domain, indicating that this MDM2 protein can function independently of p53 (Haines *et al.*, 1994).

p53 also complexes with the TATA box-binding protein, TBP, now widely recognized as a universal eukaryotic transcription factor. TBP is part of the initiation complexes formed on TATA-containing and TATA-less RNA polymerase I, II, and III promoters (for a review, see Hernandes, 1993). The wild-type p53 protein was shown to repress transcription *in vitro* from a minimal promoter by direct interaction with human TBP (Seto *et al.*, 1992; Truant *et al.*, 1993). p53 cooperates with either recombinant TBP or partially purified TFIID in binding to a DNA element containing both a p53-specific binding motif (RGC) and a TATA box (Chen *et al.*, 1993a). These findings are consistent with the proposed mechanism of action of acidic transactivation domains and suggest that p53 activates transcription through the formation of a more stable p53–TFIID-promoter complex. Cotransfection of both p53 and TBP in *Drosophila* Schneider cells resulted in higher expression of a *CAT* reporter gene than the sum of the *CAT* expression seen when the plasmids were transfected separately, indicating that p53 and TBP can act synergistically to activate transcription *in vivo* (Chen *et al.*, 1993a).

The TBP-binding domain of p53 was mapped to the acidic transactivation domain between residues 20 and 57 (Fig. 1), whereas p53 binds to the conserved region of TBP, including residues 220–271 (Liu *et al.*, 1993). p53 can interact with TBP when it is associated with multiple TAFs, since p53 binds to holo-TFIID at least as well as to isolated TBP (Liu *et al.*, 1993).

Removal of the 60 or 75 C-terminal amino acids, but not N-terminal residues, resulted in truncated p53 proteins incapable of repressing transcription from promoters lacking a p53-binding motif (Sang *et al.*,

1994; Kanda *et al.*, 1994). In contrast, such proteins were able to transactivate p53-responsive promoters. A role of the C-terminal domain in p53-mediated tumor suppression is suggested by the identification of a C-terminal truncated mutant p53 allele in a Li-Fraumeni family (Plummer *et al.*, 1994). We and others have shown that the C-terminal domain is important for the DNA-reannealing activity of p53 (Brain and Jenkins, 1994; Bakalkin *et al.*, 1995). The requirement of the C-terminal domain for repression of transcription may indicate that DNA reannealing is involved in this process. Recent findings concerning the interaction of the acidic domain of p53 with the p62 component of the TFIIH transcription factor (Xiao *et al.*, 1994), which was shown to have helicase activity, are consistent with the idea that inhibition of helicase activity by p53 is important for its transrepression function. Wild-type and mutant p53 can also form complexes with ERCC3, another component of TFIIH. This transcription factor has helicase activity and is involved in transcription-coupled DNA repair (Schaeffer *et al.*, 1993; Wang *et al.*, 1994a). The interaction of p53 with TBP or other transcription factors at a promoter lacking a p53-binding motif may result in dissociation of the transcription initiation complex from the promoter, due to p53-mediated inhibition of the unwinding of the DNA strands necessary for opening of the promoter and initiation of transcription.

Another putative cellular partner of p53 is the product of the Wilms tumor suppressor gene, WT1, a transcription factor that binds to the early growth response gene 1 consensus sequence and mediates transcriptional repression (for a review, see Haber and Housman, 1992). Transfection of WT1 into Saos-2 cells, lacking endogenous p53, resulted in a 13.6-fold activation of transcription from a promoter containing an EGR1 site. However, WT1 caused a 10-fold suppression of transcription from the same promoter in A1.5 cells, stably transfected with a temperature-sensitive p53 mutant, under conditions favoring the wild-type p53 conformation, and a 3-fold activation in the presence of mutant p53. These data suggest that transcriptional repression by WT1 is due to its interaction with wild-type p53. In contrast, cotransfection of WT1 with wild-type p53 consistently enhanced expression of a *CAT* reporter gene driven by a p53-responsive promoter in a cooperative manner (Maheswaran *et al.* 1993).

DNA-binding heterocomplexes induced by GM-CSF in human erythroleukemia cells were shown to contain both p53 and the transcription factor Sp1 (Borellini and Glazer, 1993). The latter complexes probably contained mutant p53, as indicated by the fact that Sp1 was most efficiently coimmunoprecipitated by the mutant p53-specific antibody PAb240.

Thus, it appears that p53 can interact with a whole range of transcription factors and modulate their activity. As a consequence, p53 may regulate the expression not only of these genes that harbor a p53 consensus binding site, but a large number of other genes as well. Moreover, the activity and DNA-binding specificity of p53 itself may be regulated through these interactions, perhaps allowing a more fine-tuned control of p53 function depending on the particular transcription factor environment of a given cell. This notion is supported by the observation that the ability of p53 to bind specific target DNA is influenced by the presence of a nuclear extract (see Section III.B).

The finding that p53 binds replication protein A (RPA) (Dutta *et al.*, 1993; Li and Bothchan, 1993; He *et al.*, 1993) provides further evidence for the involvement of p53 in regulation of DNA replication. RPA consists of three polypeptides, p70, p34, and p13, and is conserved from *Saccharomyces cerevisiae* to man (Fairman and Stillman, 1988; Brill and Stillman, 1989). This single-stranded DNA-binding factor is a component of the initiation complex and is essential for the first step of DNA replication, i.e., DNA unwinding at the origin of replication (Stillman, 1989, Borowiec *et al.*, 1990). RPA itself has unwinding activity (Georgaki *et al.*, 1992). The p53 protein can form complexes with purified RPA *in vitro* and also *in vivo*, since RPA was coimmunoprecipitated from cell lysates by p53-specific antibodies (Dutta *et al.*, 1993). This finding is consistent with the previous colocalization of p53 and RPA in herpes virus-infected cells (Wilcock and Lane, 1991). The major p53-binding domain in the RPA holocomplex is located in the N-terminal part of p70. As shown in Fig. 1, both the acidic transactivation domain (amino acid residues 1–73) and the C-terminal region of p53 (residues 289–393) can bind RPA (Dutta *et al.*, 1993; Li and Botchan, 1993; He *et al.*, 1993).

Complexing with the full-length p53 protein inhibited binding of RPA to ssDNA *in vitro* (Dutta *et al.*, 1993). This raises the possibility that the increase in p53 protein levels in response to DNA damage inhibits DNA replication by blocking the action of RPA. The interaction of the p53–RPA complex with helicases of the DNA replication machinery may result in inhibition of DNA unwinding due to p53-mediated reannealing of ssDNA. That, in turn, may lead to the dissociation of the replication–initiation complex and consequently to arrest of DNA replication. Also, p53 may sequester RPA at DNA strand breaks through its affinity for ssDNA ends, thus facilitating the assembly of repair complexes. However, the fact that common mutant forms of p53 derived from human tumors (His-175 and His-273) can also bind RPA and interfere with its single-stranded DNA-binding activity indicates that there is no strong selection against p53–RPA complexing during tumor development.

Using the two-hybrid system, Iwabuchi *et al.* (1994) identified two novel p53-interacting proteins, designated 53BP1 and 53BP2. Both proteins bind to the central DNA-binding domain of p53 and appear to block specific DNA binding. Two mutant p53 proteins, His-175 and His-273, did not complex with 53BP1 and 53BP2. It will be important to determine whether naturally occurring mutant forms of p53 consistently fail to bind 53BP1 and 53BP2. If so, these interactions are likely to be important for p53-mediated tumor suppression. A reasonable hypothesis is that 53BP1 and 53BP2 in some way regulate the specific DNA binding of p53.

V. Regulation of p53 Function

A. Regulation of p53 Protein Levels

p53 is normally an unstable protein with a half-life of 15–30 min (Gannon *et al.*, 1990; Oren *et al.*, 1981; Reihsaus *et al.*, 1990). As a result, p53 levels in normal cells are almost undetectable and probably insufficient to suppress growth. The observations that p53 is degraded via the ubiquitin-dependent proteolytic system *in vitro* through the interaction with the HPV 16/18 E6 and the cellular E6–AP proteins (Scheffner *et al.*, 1990; Werness *et al.*, 1990; Huibregtse *et al.*, 1993) and that p53 accumulates in certain cell lines defective in the ubiquitin pathway (Chowdary *et al.*, 1994) suggest that the p53 levels are normally regulated *in vivo* through the ubiquitin-mediated proteolytic pathway. The N-terminal domain of p53 contains a PEST sequence thought to be the target for enzymes of the ubiquitin-dependent proteolytic system. The possibility that this domain is relevant for regulation of p53 stability is supported by the observation that a truncated p53 protein that lacked the N-terminal region was expressed at 40-fold higher levels than full-length p53 (Unger *et al.*, 1993).

When cells suffer DNA damage, the p53 protein accumulates, resulting in G1 arrest or apoptosis. The accumulation of p53 is not due to increased transcription, but appears to occur by post-translational stabilization. The p53 protein that accumulates upon DNA damage retains a wild-type conformation, since it is not recognized by the mutant-specific monoclonal antibody PAb240 (Liu *et al.*, 1994). This stabilization of the p53 protein clearly represents an important regulatory mechanism that allows the cell to switch on p53 only when it is needed, i.e., in the case of genomic injury, and maintain a proliferative potential under normal conditions. The molecular events that trigger accumulation of p53 are unknown, but stabilization may involve adoption of a more stable pro-

tein conformation, perhaps induced by phosphorylation (see below). In addition, cellular proteins or factors that bind p53 may induce a conformational shift that is critical for recognition of p53 by enzymes of the ubiquitin-dependent proteolytic system. Changes in the expression or activity of these factors may thus modulate ubiquitin-mediated degradation of p53.

Many tumor cells express high levels of mutant p53 proteins, readily detected by immunohistochemistry (Bartek *et al.*, 1991). The prolonged half-life of mutant p53 proteins has been regarded as a direct consequence of conformational changes due to the missense point mutations. More recent evidence suggest that other factors determine the stability of p53 in tumor cells, however. Normal cells from individuals in certain cancer-prone families express high levels of wild-type p53 (Barnes *et al.* 1992). Furthermore, a mouse temperature-sensitive mutant p53 protein was stable when transfected in human breast carcinoma cells carrying endogenous mutant p53, but unstable and expressed at low levels when transfected in another human breast carcinoma cell line that produces endogenous wild-type p53 (Vojtesek and Lane, 1993). Studies of fibroblasts from Li-Fraumeni family individuals, carrying an inherited mutant p53 allele, have revealed that the mutant allele is expressed at low levels, like the wild-type allele. In contrast, tumor cells from these patients, in which the wild-type p53 allele is lost, express high levels of mutant p53 protein. These findings demonstrate that p53 mutation per se is not sufficient for stabilization of the protein. Rather, the tumor cell environment appears to be the crucial factor that leads to increased stability of p53. This environment may in some aspects resemble the environment in normal cells exposed to DNA-damaging agents, as suggested by Lane (1994).

B. Phosphorylation of p53

p53 is phosphorylated by several protein kinases, including casein kinases I and II, cdc2, and cdk2, and DNA-activated protein kinase (DNA-PK) (for a review, see Meek, 1994). Phosphorylation sites in the p53 molecules are shown in Fig. 1. The functional significance of p53 phosphorylation is poorly understood. Phosphorylation of human p53 at Ser-392 by casine kinase II has been shown to activate specific DNA binding *in vitro* (Hupp *et al.*, 1992). In mouse p53, Ser-386 is a target site for casein kinase II (Meek *et al.*, 1990). Substitution of Ser-386 for Ala in mouse p53 resulted in loss of wild-type p53-mediated growth suppression (Milne *et al.*, 1992), suggesting that Ser-386 phosphorylation is functionally important. In contrast, Pietenpol *et al.* (1994) did not find any

effect of substitution of Ser-392 in human p53 for Ala or Asp on transactivation of a reporter gene and suppression of transformed cell growth. This discrepancy may reflect a difference between mouse and human p53 with regard to the role of phosphorylation by casein kinase II, or may be due to differences in the experimental systems used.

DNA-PK is a DNA-dependent Ser/Thr kinase that phosphorylates a number of nuclear proteins, including p53, Sp1, SV40 large T antigen, Oct-1 and Oct-2, RNA polymerase II, and c-myc. Ser-15 and Ser-37 in human p53, and Ser-7 and Ser-18 in mouse p53 have been identified as target sites for DNA-PK (Lees-Miller *et al.*, 1992). At least Ser-15 in human p53 is phosphorylated *in vivo* (Ullrich *et al.*, 1993). While a p53 mutant in which Ser-37 had been substituted for Ala was able to block cell cycle progression as efficiently as wild-type p53, the Ser to Ala change at residue 15 decreased the ability of p53 to prevent cells from entering S phase (Fiscella *et al.*, 1993). Significantly, two out of three stable lines transfected with the Ser-15 to Ala mutant failed to produce detectable levels of p53 protein, although p53–Ala-15 mRNA was expressed. This may indicate that Ser-15 phosphorylation plays a role in stabilization of p53 (see below).

In addition, the N-terminus of p53 has a target site for phosphorylation by members of the mitogen-activated protein (MAP) kinase family. Serum stimulation of quiescent cells or exposure of cells to uv irradiation causes MAP kinase activation. These findings raise the possibility that the MAP kinase pathway is involved in regulation of p53 activity following uv-induced DNA damage (Devary *et al.*, 1992; Milne *et al.*, 1994; Meek, 1994).

C. Activation of p53 through Conformational Alteration

The specific DNA binding of p53 is activated *in vitro* by binding of the monoclonal antibody PAb421 to the C-terminal end of p53 (Hupp *et al.*, 1992). Phosphorylation at Ser-392 by casein kinase II, binding to the *E. coli* DnaK protein, and removal of the 30 C-terminal amino acids or partial proteolysis by trypsin has the same effect. These results imply that the C-terminal p53 domain negatively regulates specific DNA binding. The C-terminal modifications may trigger an allosteric shift in the p53 molecule, leading to exposure of the specific DNA-binding site in the central core domain. Multidimensional NMR studies have suggested that the C-terminal tail of p53 (residues 361 to 393) may interact with the central core domain and thereby modulate its specific DNA-binding activity (Clore *et al.*, 1994). Hence, p53 may normally exist in a latent

conformation (Hupp *et al.*, 1992) and may only become activated in the presence of the proper signals upon DNA damage. Interestingly, the specific DNA-binding activity of several mutant p53 proteins can be rescued *in vitro* by PAb421, DnaK, or C-terminal truncation (Hupp *et al.*, 1993; Halazonetis and Kandil, 1993).

An alternatively spliced mouse p53 mRNA (Arai *et al.*, 1986; Han and Kulesz-Martin, 1992) is translated into a protein in which the C-terminal 26 residues have been replaced by 17 unrelated residues and which is preferentially expressed during the G2 phase of the cell cycle (Kulesz-Martin *et al.*, 1994). Since the C-terminal domain appears critical for regulation of specific DNA binding, the product of the alternatively spliced p53 mRNA may show constitutive or altered DNA binding and as a consequence, altered biological function. Recent data confirms this idea (Wu *et al.*, 1994).

Wild-type p53 is structurally flexible and appears to reversibly adopt a "mutant" conformation under certain conditions *in vitro* (Milner and Medcalf, 1991; Hainaut and Milner, 1992) and *in vivo* during cell division (Milner, 1984; Milner and Watson, 1990; Ullrich *et al.*, 1992; Zhang *et al.*, 1992). On the basis of these and other results, Milner (1994) has proposed that p53 exists in three distinct conformational and functional states. The suppressor form is recognized by the human wild-type p53-specific monoclonal antibody PAb1620 but not by PAb421 ($1620^+/421^-$). This form suppresses cell proliferation and preserves quiescence. The promoter form, on the other hand, reacts with PAb421 but not with PAb1620 ($1620^-/421^+$). It is associated with mitogenic commitment and is thought to promote cell growth. The third form ($1620^+/421^+$) is present in dividing cells and acts as a sensor that can be converted to either of the other two forms of p53. Conversion to the suppressor form in G1-arrested cells is associated with loss of the 421 epitope and increased phosphorylation (Ullrich *et al.*, 1992). One possibility is that the phosphorylation at Ser-392 in human p53 required to activate specific DNA-binding results in the loss of the 421 epitope.

The sensor form is stabilized by metal ions, probably zinc (Hainaut and Milner, 1993a) and reducing conditions (Hainaut and Milner, 1993b). Thus, the folding of wild-type p53 is dependent on physiological parameters such as availability of metal ions (zinc) and reduction oxidation; chelating and oxidizing agents disrupt this structure and favor the promoter form. These data are consistent with the studies of the crystal structure of the central core domain of p53, showing that this domain contains a zinc atom that appears to stabilize the structural elements that are in direct contact with DNA (Cho *et al.*, 1994).

Only the 1620^+ form of p53 can bind DNA in a sequence-specific

manner (Hainaut and Milner, 1993b; Halazonetis *et al.*, 1993). It remains unclear whether specific DNA binding causes a conformational shift in the p53 molecule. Halazonetis *et al.* (1993) found that the binding of wild-type p53 to a specific DNA-binding motif is associated with loss of the 1620 epitope, indicating a "mutant" conformation. The mutant-specific antibody PAb240 did not recognize p53 bound to DNA, however. Hainaut and Milner (1993b), in contrast, reported that p53 bound to DNA is 1620^+.

The conformational flexibility of p53 is further demonstrated by the fact that the transactivating capacity of certain mutant human p53 proteins (Ala-143, Leu-173, and Ile-247) is temperature sensitive (Chen *et al.*, 1993b; Zhang *et al.*, 1994c). These mutants were able to transactivate a p53-responsive promoter at 30 or 32.5°C, but not at 37°C. The Ala-143 mutant showed stronger specific binding to the CON and RGC DNA motifs at the lower temperature and was recognized by PAb1620 at 32.5°C but not at 37°C.

A majority of the point mutations found in tumors change the conformation of the p53 protein, exposing an epitope recognized by the monoclonal antibody PAb240. The effect of different point mutations on the structure of p53 has been illuminated by the analyses of the crystal structure of the central core domain of p53 bound to DNA (Cho *et al.*, 1994). The p53 point mutations can be divided into two classes. The first class of mutations affect amino acid residues that directly contact DNA, e.g., Arg-248 and Arg-273. The structure of these mutant proteins is not significantly altered. They do not bind the heat-shock protein hsp70 and are not recognized by the mutant-specific PAb240 antibody. The second class of mutations, including mutations at for instance Val-143 and Arg-175, cause substantial denaturation of the p53 molecule, leading to loss of the wild-type specific PAb1620 epitope and exposure of the PAb240 epitope, which is not exposed on the native wild-type protein. These mutants bind hsp70 and are probably deficient in specific DNA binding due to extensive unfolding of the protein. Therefore, the crystal structure data suggest that the $PAb240^+$ mutant conformation is not a well-defined alternative conformation. Rather, the $1620^-/240^+$ mutants are at least partially denatured and unfolded.

D. DNA Strand Breaks: Direct Activator of p53?

Nelson and Kastan (1994) demonstrated that only DNA strand breaks, but not other DNA lesions, are capable of inducing p53 accumulation. This observation and our own finding that p53 binds to

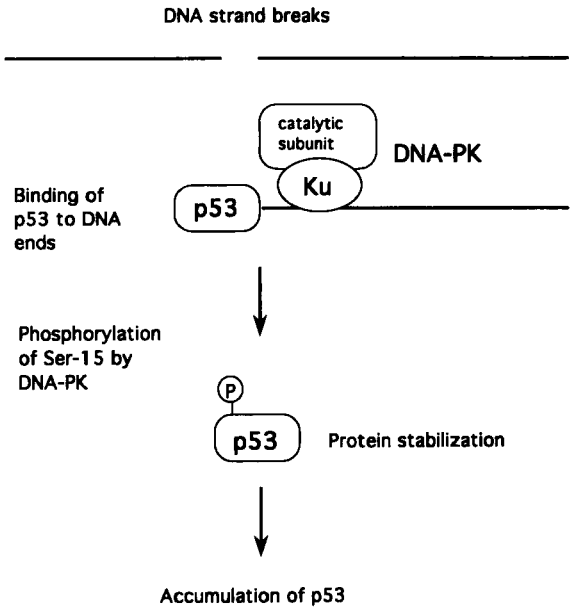

FIG. 3. Model for stabilization of p53 through phosphorylation by DNA-PK in response to DNA damage. p53 binds DNA ends generated by DNA-damaging agents. DNA-PK, which is composed of a catalytic subunit and the Ku subunit, has affinity for DNA ends and is also activated by DNA ends. A p53 molecule colocalized on the same DNA molecule is phosphorylated by DNA-PK at Ser-15 and Ser-37. This induces stabilization of p53 and thus accumulation of the protein.

ssDNA ends (Bakalkin *et al.*, 1994) have led us to suggest that p53 may serve as a cellular sensor of DNA strand breaks. The interaction of p53 with ssDNA ends in damaged DNA could trigger a conformational change of the protein, converting it to a more stable form, and hence, induce p53 accumulation. As shown in Fig. 3, this process may involve the DNA-dependent protein kinase, DNA-PK, that binds to and is activated *in vitro* by DNA ends (Gottlieb and Jackson, 1993). This kinase is composed of one catalytic 350-kDa subunit and a DNA-binding component, Ku, originally identified as an antigen detected by sera from patients with various autoimmune diseases (Mimori *et al.*, 1981). Given the fact that DNA-PK is activated by DNA ends and that it phosphorylates p53 (see above), it is tempting to speculate that DNA damage triggers phosphorylation of p53 at Ser-15 through activation of DNA-PK, leading to stabilization of p53 and subsequently growth arrest or apoptosis (Fig. 3). This idea is consistent with the data indicating that the N-terminus is important for stability and is also supported by the fact that p53

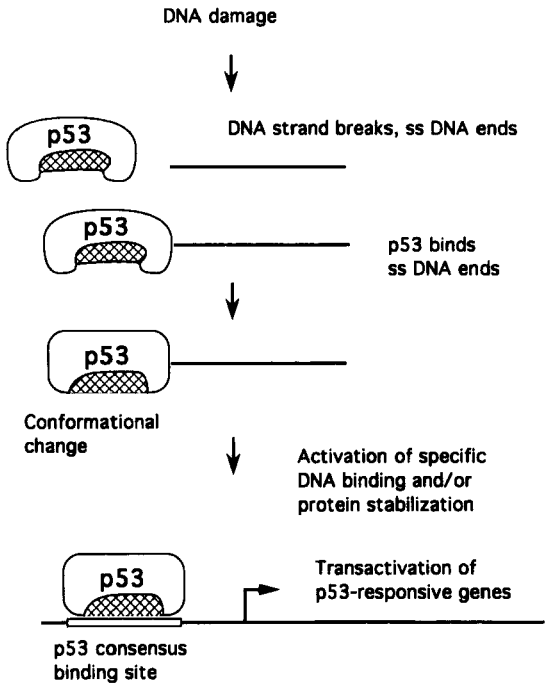

FIG. 4. Model for activation of p53 through a conformational shift induced by the interaction with ssDNA ends. p53 interacts with ssDNA ends that arise after DNA damage through a C-terminal ssDNA-binding site. This triggers a conformational change in the p53 protein, leading to the activation of specific DNA binding mediated by the central core domain. p53 will now bind to its specific DNA target sequences and transactivate p53-responsive genes.

and DNA-PK must be bound to DNA at nearby sites in order for DNA-PK to efficiently phosphorylate p53 (Lees-Miller *et al.*, 1992). Similarly, the transcription factor Sp1, another substrate for DNA-PK, is only phosphorylated efficiently by DNA-PK if Sp1 and DNA-PK are co-localized on the same DNA molecule (Gottlieb and Jackson, 1993).

We have mapped the ssDNA end-binding site to the C-terminal domain (residues 320–393) of p53 (Bakalkin *et al.*, 1995). As discussed above, various manipulations with the C-terminal p53 domain have been shown to activate specific DNA binding *in vitro*. These observations have led us to propose the model shown in Fig. 4. According to this model, the interaction of the C-terminal domain with ssDNA ends causes a conformational change in the p53 protein analogous to that induced by different modifications of the C-terminus. This conformational change may result in the activation of specific DNA binding by the

central core domain. Cellular factors (for instance, proteins encoded by putative AT genes), may recognize altered conformation of p53 and preserve it by binding and/or phosphorylation. The model also predicts that the conformational state that is activated for specific DNA binding has reduced affinity for ssDNA ends, leading to the release of p53 from ssDNA ends. Thus, the interaction of p53 with ssDNA ends generated by DNA damage would cause transcriptional activation of p53-responsive genes, including *WAF1* and other growth-suppressive genes, and subsequently cell cycle arrest and/or initiation of an apoptotic program.

When combined, the two models shown in Figs. 3 and 4 provide a molecular link between DNA damage and p53-mediated G1 arrest and apoptosis. A scenario could be envisioned in which phosphorylation by DNA-PK also plays a role in activating specific DNA binding of p53 and that the interaction with ssDNA ends stabilizes p53 through a conformational shift and thus triggers accumulation of the protein. In any case, the proposed models should be amenable to experimental verification.

ACKNOWLEDGMENTS

The authors are supported by the Swedish Cancer Society (Canderfonden), Åke Wibergs Stiftelse, Magn. Bergvalls Stiftelse, and Stiftelsen Wenner-Gren Center.

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CYCLINS AND CYCLIN-DEPENDENT KINASES: THEME AND VARIATIONS

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I. Introduction

This is a review on cyclins, so it will come as no surprise that we will be primarily concerned with the cell cycle. Cyclins are the activating partners of a family of protein kinases, the cyclin-dependent kinases (CDK), and are intimately concerned with regulating and coordinating DNA replication and cell division. But it is increasingly apparent that the cyclin-CDK motif is used to control processes quite separate from the cell cycle, the most recent example being the response to phosphate starvation in budding yeast. As more and more diverse cyclins and their partner CDKs are identified, more and more cellular processes are likely to be found to be regulated by these highly flexible protein kinase complexes. In this overview I will introduce the features of the cyclins and their partner CDKs and the ways in which cyclin–CDK complexes can be regulated. I will go on to outline what we know of the specific roles of

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individual cyclin–CDKs in the cell cycle and finish with the non-cell cycle functions that have recently been elucidated. But first, a quick introduction to the cell cycle.

II. Checkpoints and the Cell Cycle

The cell cycle describes the series of steps by which a cell coordinates the processes of DNA duplication and cell division, and is generally divided into four phases; cell division (M phase), the first gap phase (G1), DNA replication (S phase), and the second gap phase (G2) (for a review see Murray and Hunt, 1993). A cell can also exit the cell cycle into a state of quiescence (G0) or into the meiotic cell cycle. The cell cycle proceeds via a number of "controlpoints" (schematically shown in Fig. 1). These are points at which the cell monitors the correct completion of a process, such as DNA replication, and whether conditions are favorable to go on to the next stage (Hartwell and Weinert, 1989). For example, in the absence of growth factors, tissue culture cells will exit from G1 phase

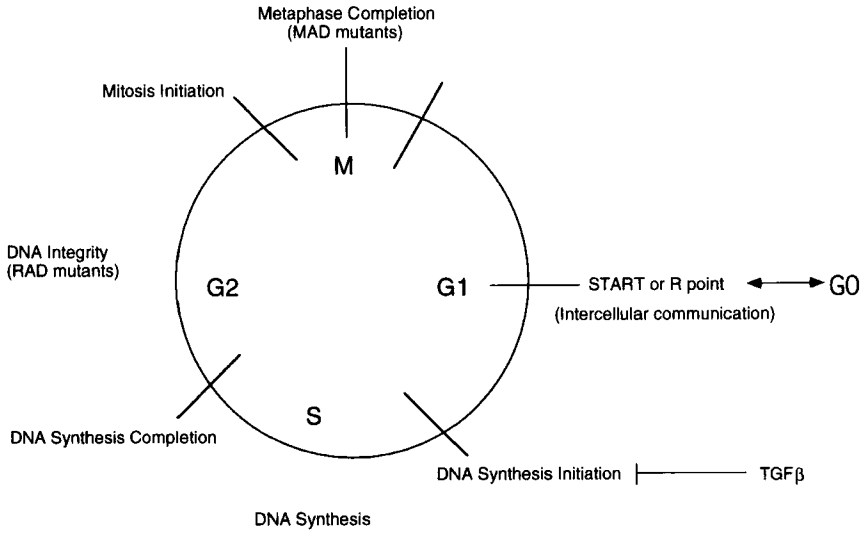

FIG. 1. Cell cycle controlpoints. Illustrated are some of the major control and "checkpoints" in the cell cycle. They include; START, at which the cell commits itself to another round of DNA replication; the initiation of DNA synthesis and of mitosis; the completion of DNA synthesis; and the detection of a correctly formed spindle and metaphase plate at the end of metaphase. Yeast mutants that are defective in detecting incorrect mitosis are MAD mutants. Between the completion of DNA replication and mitosis the integrity of the DNA is monitored, yeast mutants defective in this are RAD mutants.

into G0. This controlpoint (R) is roughly equivalent to START in yeast cells, at which the cell makes the decision whether to commit itself to another round of DNA replication, to sporulate under unfavorable conditions, or to mate if mating factors are present. Several controlpoints are regulated by a related family of protein serine/threonine kinases, which share the need to bind a cyclin to be active; hence their designation as CDKs (see Tables IA–IC).

III. Cyclins

Cyclins were originally identified (on July 23, 1983) by Tim Hunt as proteins that were strongly synthesized after fertilization of marine invertebrate eggs, and then rapidly and specifically destroyed at each mitosis (Evans *et al.*, 1983). After the first few cyclins were cloned and sequenced, it was noted that they all had in common an ~100 amino acid region of homology, which was called the cyclin box (Hunt, 1991). Subsequently, this part of the protein has been shown to be involved in binding to the CDK family of protein kinases (Kobayashi *et al.*, 1992; Lees and Harlow, 1993). On the basis of sequence homology to the cyclin box consensus, there are now more than 12 different cyclins in budding yeast, and a similar number (named from A to H) in mammalian cells (Tables IA–IC). Genetic analyses in budding yeast have defined two broad classes of cyclins, the G1 or START cyclins and the G2 or mitotic cyclins, according to where they act in the cell cycle.

IV. Cyclins Are Regulated by Proteolysis

The two classes of cyclins can be distinguished according to their degradation pattern through the cell cycle. Thus, the G1 cyclins are short-lived proteins that are rapidly turned over ($t_{\frac{1}{2}}$ ~30 min) throughout the cell cycle. This instability is conferred by PEST sequences that are C-terminal to the cyclin box (reviewed in Reed *et al.*, 1991). In contrast the mitotic cyclins are stable through most of interphase, but are rapidly degraded when cells enter mitosis (Evans *et al.*, 1983; Hunt *et al.*, 1992; Minshull *et al.*, 1989). The mitotic cyclins are degraded by proteasomes in an ubiquitin-dependent pathway (Friedman and Snyder, 1994; Glotzer *et al.*, 1991). The instability of the mitotic cyclins at cell division is conferred by a region of the protein N-terminal to the cyclin box called the "destruction box" (Glotzer *et al.*, 1991), although both cyclin A and cyclin B2 are only be degraded if they can bind to cdc2 (Stewart *et al.*, 1994; Van der Velden and Lohka, 1994). The destruction box consensus sequence differs between the subclasses of the mitotic cyclins, the A and

TABLE IA

BUDDING YEAST CYCLINS, CDKS, AND CDIS

Cyclin	Subfamily	CDK	CDI	Phase	Substrates	Features
Cln1	G1 cyclin	Cdc28	Farl?	START	SBF (activates)	
Cln2	G1 cyclin	Cdc28	Farl	START	SBF (activates)	Forms a complex with Swi4
Cln3	G1 cyclin	Cdc28	?	START	SBF (activates)	Couples cell size to the cell cycle?
Clb5	B-type	Cdc28	Sic1	S phase	?	Necessary for efficent DNA replication
Clb6	B-type	Cdc28	Sic1?	S phase	?	Necessary for efficent DNA replication
Clb3	B-type	Cdc28	?	G2 phase	?	
Clb4	B-type	Cdc28	?	G2 phase	?	
Clb1	B-type	Cdc28	?	M phase	SBF (inhibits)	
Clb2	B-type	Cdc28	?	M phase	SBF (inhibits)	Required for mitosis. Nondestructible mutant blocks in anaphase
Hcs26	G1	Cdc28?	?	START?		
OrfD	G1	Cdc28?	?	START?		
Pho80	Hcs26/OrfD	Pho85	Pho81	None	Pho4	Regulates phosphate metabolism
Ccl1		Kin28	?	?	?	

TABLE IB
FISSION YEAST CYCLINS, CDKS, AND CDIS

Cyclin	Subfamily	CDK	CDI	Phase	Substrates	Features
cig1	B-type	cdc2	rum1?	G1/S?		
cig2	B-type	cdc2	rum1?	G1/S?	DSC-1?	
cdc13	B-type	cdc2	rum1?	G2-M		Primary mitotic cyclin
puc1	B-type	cdc2	?	Meiosis		

B type cyclins, and may account for the different characteristics of their destruction. It is possible that the different destruction box sequences are recognized by different ubiquitin ligases, which might account for the differences in the timing of their destruction. However, this cannot be the whole story, because recent data show that the same ubiquitin conjugating enzyme in budding yeast (UBC9) is involved in the degradation of an S phase and an M phase cyclin (Clb5 and Clb2, respectively; Seufert *et al.,* 1994). Once cells enter mitosis the A-type cyclins are destroyed first, during metaphase. The B-type cyclins are degraded later, and this correlates with the transition from metaphase to anaphase (Hunt *et al.,* 1992; Minshull *et al.,* 1990). In addition, the B-type cyclins are able to activate the destruction of both A- and B-type cyclins in *in vitro* extracts, whereas the A-type cyclins cannot (Félix *et al.,* 1990; Luca *et al.,* 1991). Moreover, there is evidence to suggest that cyclin B-cdc2 kinase activity activates a cyclin-specific ubiquitin ligase (Hershko *et al.,* 1994).

A variety of proteins are degraded in sequence during mitosis, and this is important for the proper regulation of mitosis (Brown *et al.,* 1994). However, it is not clear whether it is different proteases or different ubiquitin ligases that are activated in sequence, or alternatively, whether different substrates only become available to the ligases at different points in mitosis. Whatever the mechanism, the destruction of the different cyclins responds to distinct checkpoints. Thus, if the mitotic spindle is prevented from forming correctly, for example by disrupting microtubules with colchicine, then cyclin A is still degraded, but cyclin B remains stable and accumulates to supraphysiological levels (Hunt *et al.,* 1992). This led to the idea that cyclin B destruction was the signal for the cell to enter anaphase. However, recent data suggest that this is not the case. If cyclin B destruction is inhibited in *Xenopus* extracts using a vast excess of the N-terminus of cyclin B as a competitive inhibitor, the sister chromatids still separate even though cyclin B–cdc2 kinase activity remains high (Holloway *et al.,* 1993; van der Velden and Lohka, 1993).

TABLE IC
MAMMALIAN CYCLINS

Cyclin	Subfamily	CDK	CDI	Phase	Substrates	Features
A	Mitotic	cdc2, CDK2	p21?	S, G2, M	RF-A? E2F-1, centrosomes	Interacts with p107, p130, E2F
B1	Mitotic	cdc2	p21?p24?	Mitosis	Lamins, caldesmon, vimentin	Degraded at metaphase–anaphase Nondestuctible mutant blocks cells in mitosis
B2	Mitotic	cdc2	p21?p24?	Mitosis	Golgi/endoplasmic reticulum?	Degraded at metaphase–anaphase
B3	Mitotic	cdc2	p21?p24?	Mitosis	?	Nuclear B type only in chicken cells so far
C	G1	?	?	?		
D1	G1	CDK2, 4, 5, 6	p16, p21, p27	START	Retinoblastoma?	*PRAD1* and *Bcl1* protooncogene
D2	G1	CDK2, 4, 5, 6	p16, p21, p27	START	Retinoblastoma?	*vin-1* protooncogene
D3	G1	CDK2, 4, 5, 6	p16, p21, p27	START	Retinoblastoma?	
D3	G1	CDK2, 4, 5, 6	p16, p21, p27	START	Retinoblastoma?	
E	G1	CDK2	p21, p27	G1/S	Retinoblastoma? RF-A?	Interacts with p107, p130, E2F
F						~80 kDa, largest cyclin known
G	Cig1-like					
H		$p40^{MO15}$			T-loop threonine	

High cyclin B–cdc2 kinase activity apparently causes a block in late anaphase (Holloway *et al.*, 1993; Surana *et al.*, 1991). Therefore, cyclin B destruction is normally coincident with the beginning of anaphase, but does not initiate anaphase, perhaps because the ubiquitin ligases or proteases responsible for allowing chromatids to separate are activated at the same time as the cyclin B-specific proteolysis pathway.

Recent evidence from budding yeast shows that the period of instability of the mitotic cyclins extends into early interphase until cells pass START and become committed to another round of DNA replication (see below) (Amon *et al.*, 1994). The mitotic cyclin-destruction system is inactivated by G1 cyclin–CDK activity, ensuring that in each new round of the cell cycle, G1 cyclins must accumulate before the mitotic cyclins (Amon *et al.*, 1994).

The specific degradation of the cyclins has profound implications for the control of the cell cycle because degrading a cyclin necessarily inactivates the partner kinase. Thus, one role of the cyclins is to enable the cell to turn on and off a specific set of protein kinases at particular points in the cell cycle. The other role of the cyclins is to target their partner kinase to particular parts of the cell. For example, the A-type cyclins are nuclear proteins, whereas the B-type cyclins remain in the cytoplasm until mitosis and subsequently associate with the mitotic apparatus (Gallant and Nigg, 1992; Ookata *et al.*, 1992; Pines and Hunter, 1991). The different locations of the cyclin–CDK complexes necessarily restrict the substrates available, which is likely to be important *in vivo*, because *in vitro* most cyclin–CDK complexes recognize the same basic consensus sequence; (K/R)-S/T-P-X-(K/R).

V. How CDKs Are Activated by Binding Cyclins

Clues to the mechanism by which the cyclins activate their partner CDK have been afforded by the resolution of the crystal structure of human CDK2 (De Bondt *et al.*, 1993). A comparison between the crystal structure of the inactive, monomeric CDK2 with the active cAMP-dependent protein kinase (PKA) structure (Knighton *et al.*, 1991) suggests that cyclin binding would need to alter the conformation of CDK2 in two ways to activate it. First, the N-terminal lobe of CDK2 would have to change so as to alter the conformation of a bound ATP molecule. The scissile bond between the γ and β phosphates needs to be brought into alignment with the hydoxyl group of the substrate. Second, a region in the middle of CDK2, called the T loop, needs to be moved away from its position where it obscures the substrate binding cleft of the enzyme. The T loop also includes a conserved threonine residue (T160 in CDK2)

which has to be phophorylated for full activity (Ducommun *et al.*, 1991; Gould *et al.*, 1991) (reviewed in Draetta, 1993). Phosphorylation of T160 in CDK2 would facilitate its interaction with basic residues on the C-terminal lobe of the protein if the T loop were to move into the conformation of the analogous region in PKA. Both of these changes, altering the alignment of ATP and of the T loop, could be achieved if cyclin binding were to cause an alpha-helical region (α12) of CDK2 to melt (De Bondt *et al.*, 1993, reviewed in Pines, 1993). Whether this is indeed the means by which cyclins activate their partner CDKs awaits the resolution of an active cyclin–CDK complex crystal structure.

In addition, it appears that binding different cyclins changes the specificity of any one CDK. In part this may be through targeting to a different part of the cell, but it also appears as if the affinity for particular substrates is also changed (Peeper *et al.*, 1993). This facet is especially important in the yeasts in which one CDK is bound by several different cyclins according to the stage of the cell cycle, and thus has a different role to perform and different substrates to phosphorylate. Recent studies modeling the sequence of cdc2 onto the crystal structures of CDK2 and PKA predict that mutations known to prevent cyclin binding map to the active site of the enzyme. This suggests that cyclin residues could have a direct effect on substrate binding (Endicott *et al.*, 1994).

VI. Modulation of the Cyclin–CDK Complexes: Phosphorylation and Inhibitor Proteins

The basic motif of a pool of inactive protein kinases which are activated by proteins whose synthesis and destruction are regulated has an inherent flexibility, allowing any one type of cyclin–CDK to respond to a particular change in events through a change in the level of the cyclin. But further modulation is possible because once formed, the cyclin–CDK complexes can be activated or inhibited by phosphorylation and by binding a number of different inhibitor proteins (CDIs) (Fig. 2).

A. Phosphorylation

To be activated fully, the CDK component of the cyclin–CDK complex needs to be phosphorylated on a conserved threonine in the T loop (T160 in CDK2). The analogous residue (threonine or tyrosine) is phosphorylated in most, if not all, other families of protein kinases. In PKA and the *src* family this is an autophosphorylation event, whereas in the MAP kinases two residues in this region are phosphorylated by the MEK protein kinases (see Marshall, 1994). The protein kinase responsible for

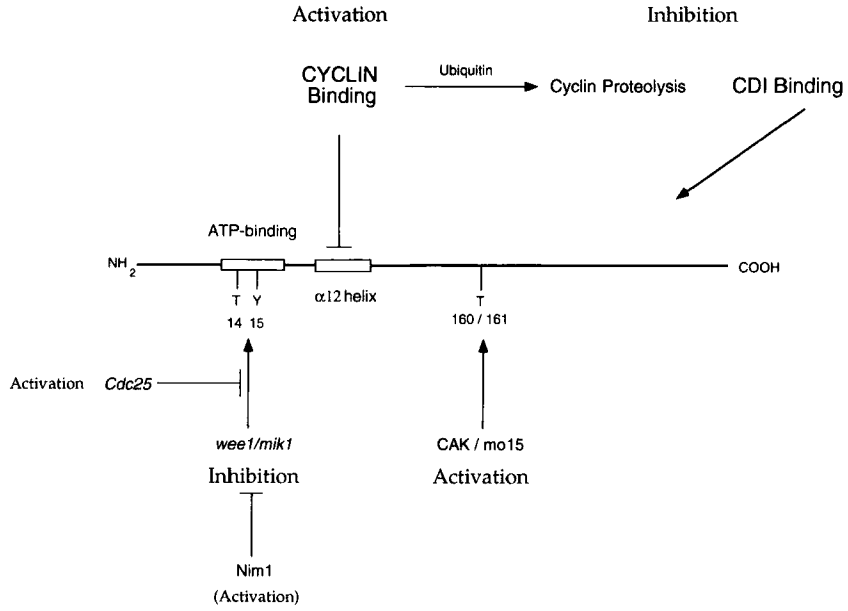

FIG. 2. Activation and inhibition of cyclin-dependent kinases. The activatory and inhibitory phosphorylation sites of a generic cyclin-dependent kinase are shown, with the kinases and phosphatases responsible for their modulation. The activatory binding of a cyclin is shown as disrupting the α12 helix (see text). The binding sites for the CDI proteins have not been mapped thus far.

phosphorylating the T loop threonine of cdc2, CDK2, and CDK4 has been identified as p40^{MO15} (Fesquet *et al.*, 1993; Kato *et al.*, 1994; Poon *et al.*, 1993; Solomon *et al.*, 1993), a protein that is ~40% identical to cdc2 in the kinase domain. This observation suggested that p40^{MO15} might itself need to be activated by a cyclin-like partner, and this has just been shown to be the case. Cyclin H is the apparent partner for p40^{MO15} (Fisher and Morgan, 1994; Makela *et al.*, 1994), but it is not yet clear to what influences, external or internal, cyclin H synthesis and degradation respond. Of course, this also raises the issue of whether p40^{MO15} itself needs to be activated by phosphorylation, and if so, is this by another cyclin–CDK? Also unresolved is the question of whether cyclin H-CDK7 activates all types of cyclin–CDKs, or whether there are different activating kinases for different CDKs. Cyclin H-CDK7 has been shown to be able to phosphorylate cdc2, CDK2, and CDK4, but CDK7 has also been found to be almost exclusively nuclear, which raises the question of whether there is any cytoplasmic cyclin H-CDK7 to phosphorylate the cyclin B-cdc2 complex (see below) (Tassan *et al.*, 1994).

Cyclin–CDKs can also be inhibited by phosphorylation. There are two inhibitory phosphorylation sites in mammalian cdc2 and CDK2, threonine 14 and tyrosine 15 (Krek and Nigg, 1991; Norbury *et al.*, 1991). Only the tyrosine is phosphorylated in yeast (Gould and Nurse, 1989). These sites lie within the ATP-binding region of the CDK and interfere with phosphate transfer to a bound substrate (Atherton *et al.*, 1993). The phosphorylation and dephosphorylation of tyrosine 15 have been most clearly defined, in particular for the mitotic cdc2–cyclin B complex in fission yeast. The protein kinases that phosphorylate Y15 are the products of the *wee1* and *mik1* genes (Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Russell *et al.*, 1989; Russell and Nurse, 1987), the antagonistic phosphatase is the product of the *cdc25* gene (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Girard *et al.*, 1992; Lee *et al.*, 1992; Millar *et al.*, 1991; Russell and Nurse, 1986; Sebastian *et al.*, 1993; Strausfeld *et al.*, 1991), and both kinases and phosphatases are themselves regulated by phosphorylation. Thus, during interphase *wee1* is active and *cdc25* is not, and the reverse is true in mitosis (Smythe and Newport, 1992).

The wee1 protein kinase is negatively regulated by the nim1 protein kinase (Coleman *et al.*, 1993; Tang *et al.*, 1993; Wu and Russell, 1993) and by a second mitotic kinase that has yet to be identified (Coleman *et al.*, 1993). Nim1 is activated at the end of G2 phase in order to inactivate wee1, thereby facilitating the dephosphorylation of cdc2. In budding yeast the wee1 homolog has been shown to be able to discriminate between G1 phase and G2 phase Cdc28 complexes (Booher *et al.*, 1993). (Cdc28 is the primary budding yeast CDK.) Only one cdc2–tyrosine 15 kinase activity has been identified in mammalian cells (McGowan and Russell, 1993; Parker and Piwnica, 1992), and this is more related to mik1 than wee1 (T. Hunter, personal communication). It is not known whether human mik1 is responsible for phosphorylating and inactivating all types of cyclin–CDK complexes; although this seems unlikely given that there are three types of cdc25 proteins in mammalian cells (see below). The protein kinase that phosphorylates threonine 14 has been partially purified. It is found associated with the membrane fraction of *Xenopus* eggs and is clearly different from the mik1 kinase (Kornbluth *et al.*, 1994).

At mitosis cdc25 is activated by phosphorylation (Hoffmann *et al.*, 1993; Kumagai and Dunphy, 1992). The kinase responsible for the initial phosphorylation of cdc25 has not been unambiguously identified, but the sites are good substrates for the cyclin–CDKs. As a consequence cdc25 and the mitotic CDK, cyclin B–cdc2, form a positive feedback loop; a little activated cdc25 will dephosphorylate a fraction of the accu-

mulated pool of cyclin B–cdc2, which will in turn activate more cdc25. Thus, the activation of cyclin B–cdc2 becomes irreversible.

Mammalian cells have a family of cdc25 proteins; cdc25A, B, and C (Galaktionov and Beach, 1991), which appear to interact with different cyclin–CDK complexes. Cdc25A is a substrate for the cyclin E-CDK2 complex at the entry into S phase (Hoffmann *et al.*, 1994), and has been shown to activate the cyclin A–CDK2 complexes in S phase (Jinno *et al.*, 1994), whereas cdc25C activates the cyclin B–cdc2 complex at mitosis (Hoffmann *et al.*, 1993; Kumagai and Dunphy, 1992). It has not yet been shown whether cdc25A and cyclin E-CDK2 or cyclin A–CDK2 also form a positive feedback loop.

B. CDK Inhibitors

Another means by which the protein kinase activity of the cyclin–CDK complexes can be modulated has recently come to light. There are a variety of inhibitor proteins (CDIs) that bind specifically to the CDKs and inhibit them in a stoichiometric fashion. Some of these proteins are important in signal transduction, and others in the proper coordination of cell cycle phases. In mammalian cells some of the proteins are potential tumor suppressors because they are lost when cells are transformed.

Budding yeast have at least two CDIs important in cell cycle regulation. The first of these is unusual in being much larger than the other known CDIs (which are all around 20 kDa). This is the 120-kDa Far1 protein, and it is important in arresting cells at START in response to mating factor (Chang and Herskowitz, 1990). Budding yeast mating factors bind to G protein-coupled serpentine receptors and activate a MAP kinase cascade. At the end of the cascade the MAP kinase homolog, FUS3, phosphorylates Far1, which then binds to and inhibits the G1 cyclin–CDK complex, Cln2–Cdc28 (Chang and Herskowitz, 1992; McKinney *et al.*, 1993; Tyers and Futcher, 1993; Valdivieso *et al.*, 1993; Peter and Herskowitz, 1994). This effectively blocks cells at START. The other budding yeast CDI, Sic1, is important in regulating the cell cycle between START and the beginning of DNA replication (Mendenhall, 1993; Nugroho and Mendenhall, 1994). Sic1 accumulates in G1 cells and binds to the S phase cyclin–CDK complex, Clb5–Cdc28. When the cell initiates S phase it degrades Sic1, again by a ubiquitin-dependent pathway, thus activating Clb5–Cdc28 (Schwob *et al.*, 1994). The Cdc34 protein encodes an ubiquitin ligase (Goebl *et al.*, 1994), and a cell mutant in Cdc34 blocks in late G1. This cell cycle block can be overcome by deleting sic1, suggesting that Cdc34 regulates sic1 destruction and that sic1 is the most important substrate of Cdc34 at the G1-S transition (Schwob *et al.*, 1994).

So far one CDI has been identified in fission yeast. This is the rum1 (Replication Uncoupled from Mitosis) protein (Moreno and Nurse, 1994), necessary to coordinate DNA replication and mitosis. Rum1-defective cells are unable to recognize whether they have replicated their DNA or not, and thus cells in G1 enter aberrantly into mitosis. If rum1 is overproduced in cells, they fail to recognize that they have replicated their DNA and thus continuously replicate (Moreno and Nurse, 1994). Similarly, when a yeast containing a temperature-sensitive cdc2 is raised to the restrictive temperature, cdc2 is degraded, and the cell will arrest in either late G2 or at START (Nurse and Bisset, 1981). On returning to the permissive temperature, cdc2 is resynthesized but it is always the G1 form of cdc2 (Broek *et al.*, 1991). Therefore, cells will replicate their DNA whether they were originally blocked in G1 or G2 (Broek *et al.*, 1991). The crucial differences between the G1 and G2 forms of cdc2 have not been defined, but it is likely that rum1 is important in distinguishing between them.

Seven potential CDIs have been identified in mammalian cells; p15, p15.5, p16, p18, p21, p24, and p27 (reviewed in Hunter, 1993; Nasmyth and Hunt, 1993; Pines, 1994). p16 is deleted in more than 50% of human tumor cell lines and binds specifically to the cyclin D–CDK4 complexes (see below). p24 was isolated as a protein that binds human cdc2 in a yeast 2 hybrid screen (Gyuris *et al.*, 1993; Hannon *et al.*, 1994). From its sequence, p24 is a member of the dual specificity protein phosphatase family, and thus potentially capable of dephosphorylating phosphoserine/threonine or phosphotyrosine. However, at present its substrates and physiological role are unknown. p15, p21, and p27 are implicated in arresting the cell cycle in response to negative regulators (see below).

Thus, the cyclin–CDK motif offers a remarkably versatile means of regulation and of integrating a variety of influences at any one control point. However, although the modulation of many of the different cyclin–CDK complexes has been elucidated, the exact roles of each cyclin–CDK complex in the cell cycle are much less clear. In the rest of this review I will attempt to assign the different cyclin–CDK complexes to particular cell cycle checkpoints, according to the weight of current data, with the caveat that this is undoubtedly an oversimplification.

VII. START

In this consideration we should start at the beginning by beginning at START, the major cell cycle controlpoint in most somatic cells. At START there is a clear interaction between the cyclin–CDK complexes

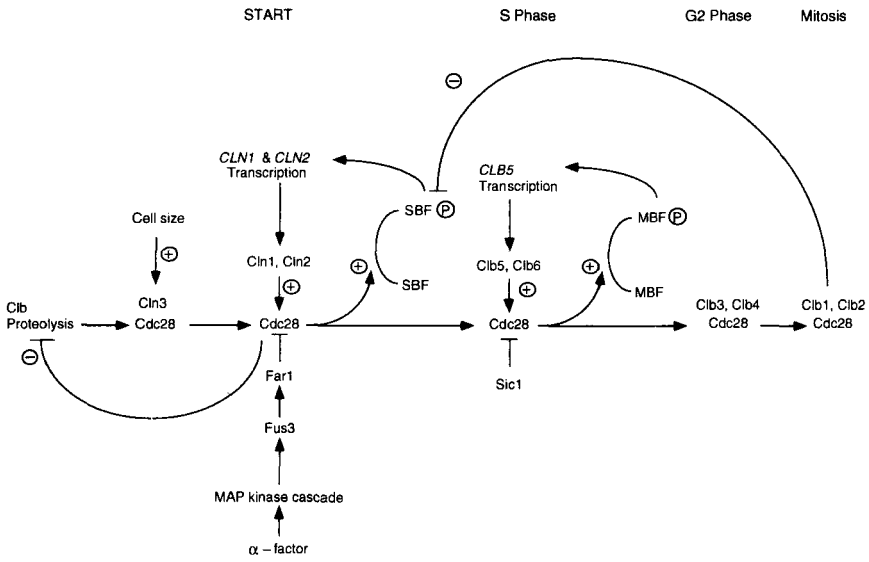

FIG. 3. Schematic view of the interactions between the cyclins, Cdc28, and the two known CDIs (Far1 and Sic1), in budding yeast. Interactions with the SBF and MBF transcription factors are also shown.

and certain transcription factors in both types of yeast (reviewed in Koch and Nasmyth, 1994 (Fig. 3) and there are indications that an analogous interaction may also occur in mammalian cells.

In budding yeast START is controlled by the Cdc28 protein kinase in complexes with three different G1 cyclins; Cln1, Cln2, and Cln3 (Richardson *et al.*, 1989). [Two other relatives of the Clns, OrfD and Hcs26 (Ogas *et al.*, 1991), have also been isolated but their role in the cell cycle, if any, is not clear.] START is made irreversible by a positive feedback loop between the G1 cyclin–CDK complexes and the SBF transcription factor complex (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991). SBF is composed of the Swi4 and Swi6 proteins (Primig *et al.*, 1992). Swi4 itself is thought to bind directly to DNA (Primig *et al.*, 1992; Sidorova and Breeden, 1993), whereas Swi6 has a regulatory role. SBF binds to the sequence CACGAAA, which is called the Swi4–Swi6-dependent cell cycle box (SCB), and is present in the promoters of several genes activated at START. The levels of Cln1 and Cln2 are determined by the rate of their transcription (Wittenberg *et al.*, 1990), which initiates at START, and the promoters of both Cln1 and Cln2 have the SCB sequence (Cross *et al.*, 1994). By contrast, Cln3 is present at low levels throughout the cell cycle (Tyers *et al.*, 1992). Thus,

between the end of the previous mitosis and START, there is only one cyclin present, Cln3. Current thinking has it that the Cln3–Cdc28 complex is involved in triggering START in a cell size-dependent fashion, through its phosphorylation and concommitant activation of SBF (Tyers *et al.*, 1993). Activated SBF will promote CLN2 transcription, so more Cln2 will bind and activate more Cdc28, setting up a positive feedback loop on Cln2 synthesis. However, there must be other (as yet unidentified) components involved in periodic CLN2 transcription because deleting the SCB sequences from the CLN2 promoter diminishes the level of transcription but does not abolish its cell cycle dependence (Cross *et al.* 1994; Stuart and Wittenberg, 1994). Similarly, CLN2 synthesis is still cell cycle dependent even in the absence of Swi4.

The Swi6 protein is also a component of the MBF transcription factor which regulates the DNA synthesis genes that are activated after START in late G1 phase (Koch *et al.*, 1993; Verma *et al.*, 1992). MBF recognizes the SCB-related sequence ACGCGTNA which contains the recognition site for the *Mlu*I restriction enzyme and, hence, is called the *Mlu*I cell cycle box (MCB). The other subunit of MBF is MBP1 (Koch *et al.*, 1993), which has structural homology to Swi4, and like Swi4, is the DNA-binding component. Cells deleted for MBP1 still transcribe DNA synthesis genes, but transcription of some of these genes, such as DNA polymerase, is no longer periodic in the cell cycle. The parallels between SBF and MBF, a common subunit and a second subunit from the same family of proteins, mean that it is very possible that MBF will interact with the cyclin–CDK complexes in late G1/early S phase. Indeed, the promoters of the S phase cyclins, Clb5 and Clb6, have MCB sites essential for their periodic transcription (McIntosh 1993).

Eventually, SBF-dependent genes are repressed in G2 phase when the Clb cyclins appear (Clb1-4), and the Swi4 protein is found associated with Clb2 in anti-Clb2 immunoprecipitates (Amon *et al.*, 1993). This suggests that the Clb2–Cdc28 complex may directly inhibit transcription of SBF-dependent genes, a phenomenon that is markedly analogous to the repression of E2F transcription by mammalian cyclin A (see below).

VIII. Fission Yeast START

A strikingly similar set of components regulate late G1 phase in the fission yeast (Fig. 4). The DNA synthesis genes in *S. pombe* are regulated by the DSC1 transcription factor which also binds to MCB-like sites (Reymond *et al.*, 1993). DSC1 is essential for the cell to enter S phase and is composed of the cdc10 protein (Aves *et al.*, 1985), which resembles Swi6, and the Res1/Sct1 protein (Caligiuri and Beach, 1993; Tanaka *et*

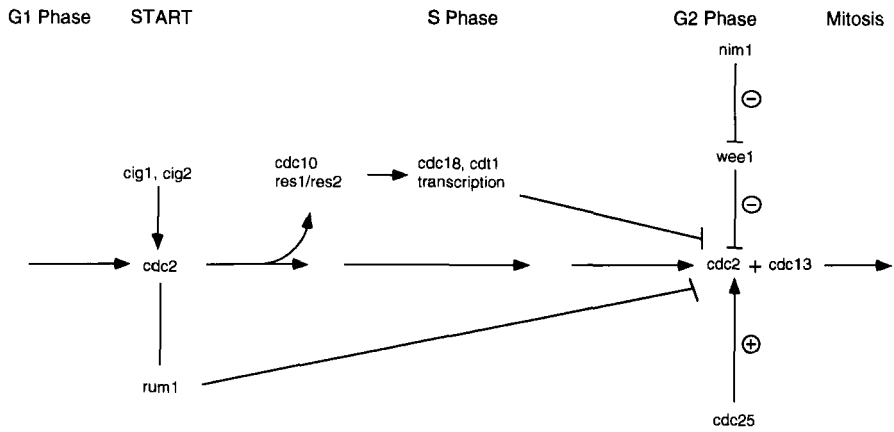

FIG. 4. Schematic view of the interactions between the cyclins, Cdc2, and rum1 in fission yeast. Interactions with the DSC-1 transcription factor (cdc10 + Res1 or Res2) and its target genes required for S phase, *cdc18* and *cdt1*, are also shown.

al., 1992), which is similar to Swi4. As in budding yeast, cdc10 also binds to a second Swi4-related protein, Res2 (Miyamoto *et al.*, 1994), to form a transcription factor complex which is less important in the mitotic cell cycle, but nevertheless overlaps in function with DSC1. The cdc10/Res2 complex has a much more important role in the meiotic cell cycle. A further parallel with budding yeast is that DSC1-binding activity is enhanced at START in a cdc2-dependent fashion (Reymond *et al.*, 1993).

At present the fission yeast cyclins which activate cdc2 at START are not clearly defined. The two most likely candidates are the *cig1* and *cig2* genes (Bueno and Russell, 1993; Connolly and Beach, 1994), which are B-type cyclins. Although *cig1* mRNA levels are constant, *cig2* mRNA is periodic in the cell cycle, peaking in late G1/early S phase. Neither *cig1* nor *cig2* is essential for cell viability, but cells lacking *cig2* do undergo a higher frequency of conjugation than wild-type cells (Connolly and Beach, 1994). The decision to conjugate is made in G1 phase, so this phenotype suggests that *cig2* may be important in regulating G1 phase. Moreover, cells which lack both *cig1* and *cig2* show a synthetic phenotype indicative of defects in coupling DNA replication to cytokinesis, and a high proportion of the cells seem to be delayed in G1 phase (Connolly and Beach, 1994). These results suggest that *cig1* and *cig2* may both be required for the cell to complete correctly the G1/S checkpoint. However, cells are still viable even without both *cig1* and *cig2*, whereas cdc2 is essential to traverse START (Nurse and Bisset, 1981), so there must be other activators of cdc2 in G1 phase.

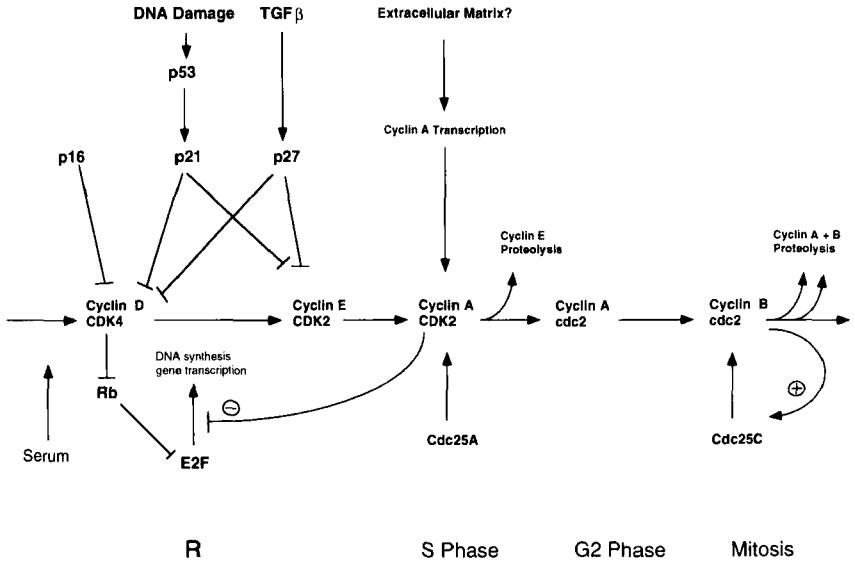

FIG. 5. Schematic view of the interactions between the cyclins, CDKs, and three of the known CDIs (p16, p21, and p27) in mammalian cells. Interaction with the E2F transcription factor and the retinoblastoma protein by the D-type cyclins, and the activation of the cyclin A and cyclin B complexes by specific types of cdc25, are shown.

IX. Mammalian START

In mammalian cells the approximate equivalent to START is the restriction point (R) (Fig. 5), and the D-type cyclins are most likely to be involved in its regulation. R is a rather nebulous stage in the cell cycle after which cells no longer require the presence of serum to commit themselves to initiating DNA replication.

D-type cyclins have a very short half-life (~30 min) and their synthesis is highly growth-factor dependent; when growth factors are withdrawn, cyclin D synthesis ceases immediately (Matsushime *et al.*, 1991). This has led to the idea that the D-type cyclins act as growth-factor sensors (Sherr, 1993) (see below). It is therefore not surprising that the D-type cyclins are the cell cycle components that have been most closely linked to oncogenesis (reviewed in Motokura and Arnold, 1993). The three types of D cyclin, D1, D2, and D3, are cell-type specific. Most cells express D3, and either D1 or D2 (but not all three). Cyclin D1 is the *CCND1* gene and maps to chromosome 11q13. It has been identified as the parathyroid adenoma (*PRAD1*) protooncogene and as the most likely candidate for

the *Bcl1* protooncogene (Motokura *et al.*, 1991; Withers *et al.*, 1991). Wild-type cyclin D1 is overexpressed as *PRAD1* because of a chromosomal inversion, inv (11)(p15;q13) such that it comes under the control of the parathyroid promoter (Motokura *et al.*, 1991). These are benign, noninvasive tumors which suggests that overexpression of cyclin D1 is a purely proliferative lesion. In centrocytic lymphomas a chromosomal translocation at the *Bcl1* breakpoint, t(11;14)(q13;q32), brings cyclin D1 under the influence of the immunoglobulin heavy-chain enhancer (Withers *et al.*, 1991). Cyclin D2, the *CCND2* gene (Xiong *et al.*, 1992a), maps to chromosome 12p13. In retroviral-induced mouse T cell leukaemias, the *CCND2* gene has been identified as the vin-1 site of integration of a murine provirus (Hanna *et al.*, 1993), which leads to cyclin D2 overexpression. Human cyclin D3, the *CCND3* gene (Motokura *et al.*, 1992; Xiong *et al.*, 1992a) maps to chromosome 6p21 but has not yet been identified as a protooncogene, although the 6p21 region is rearranged in several lymphoproliferative disorders. Recent work has shown that overexpressing D-type cyclins alone is not sufficient to transform a cell, which is not surprising given the need for cooperation between onocogenes in tumorogenesis (Hunter, 1991) (see below).

The D-type cyclins are able to bind to the retinoblastoma tumor suppressor protein (Rb) through an L–X–C–X–E motif in their N-terminus (Dowdy *et al.*, 1993; Kato *et al.*, 1993). In insect cells, baculovirus-produced human cyclins D2 and D3 bind to Rb much more tightly than does cyclin D1. D-type cyclins bind to several different CDKs; CDK2, CDK4, CDK5, and CDK6 (Bates *et al.*, 1994; Matsushime *et al.*, 1992; Xiong *et al.*, 1992b). Of these, the main and consistent partner appears to be CDK4 (Matsushime *et al.*, 1992); in many cell types CDK2, CDK5, and CDK6 are not associated with cyclin D (Bates *et al.*, 1994). CDK4 is unusual among the CDKs in that it associates with its partner cyclin for only a short period in the cell cycle, in late G1 and early S phase (Matsushime *et al.*, 1992), and its synthesis is subject to regulation by negative growth factors such as TGFβ (Ewen *et al.*, 1993). The cyclin D–CDK4 complex has a very limited substrate specificity; *in vitro* the best substrate found to date is Rb (Matsushime *et al.*, 1994).

X. D-Type Cyclins as Regulators of Rb

Because the D-type cyclins bind Rb *in vivo*, and Rb is their best substrate *in vitro*, it is likely that D-type cyclins regulate Rb in the cell cycle. Rb is underphosphorylated throughout G1 phase, phosphorylated at the G1/S transition, and remains phosphorylated until late mitosis (DeCaprio *et al.*, 1992, 1989). Only the hypophosphorylated form of Rb is

able to block cells in G1 phase, and it binds a large number of proteins. The most relevant to the cell cycle are the family of transcription factors collectively known as E2F. E2F has been implicated as responsible for transcribing a set of genes at the end of G1 phase that are required for DNA synthesis, such as thymidine kinase, ribonucleotide reductase, DHFR, and DNA polymerase α (reviewed in La Thangue, 1994; Nevins, 1992). E2F is a dimer composed of a member of the E2F family (Helin *et al.*, 1992; Kaelin *et al.*, 1992) (at least four different cDNAs have been isolated) and a member of the DP family (Bandara *et al.*, 1993; Girling *et al.*, 1993) (of which three cDNAs have been found so far). Thus, one role for the D-type cyclin kinase might be to phosphorylate Rb in order to release E2F, and so turn on genes required for S phase.

It is obviously tempting to draw parallels between the interaction between the D-type cyclins, Rb and E2F, and the control by yeast G1 cyclin–CDK complexes of the SBF, MBF, and DSC-1 transcription factors. This may well be a valid comparison, although none of the E2F or DP cDNAs sequences thus far closely resemble the Swi4 or Swi6 families.

Cyclin D1 and Rb seem to be especially interdependent. Most cells will arrest before S phase when anti-cyclin D1 antibodies are microinjected in early to mid G1 phase, adding support to the need for cyclin D1 to traverse G1 phase (Lukas *et al.*, 1994b; Müller *et al.*, 1994). However, microinjecting anti-cyclin D1 antibodies does not arrest cells that lack a functional Rb protein (Lukas *et al.*, 1994a). (Rb can either be disabled through its deletion or mutation, or through sequestration by DNA tumor virus products such as SV40 T Ag, adenovirus E1A, or the papilloma virus E7 protein.) It also appears that there is a significant downregulation in the amount of cyclin D1 in cells that effectively lack Rb. This has led to the proposal that hypophosphorylated Rb is involved in the stimulation of cyclin D1 transcription, and that the almost exclusive role of cyclin D1 is to inactivate Rb so that cells can replicate their DNA. Thus, cyclin D1 synthesis and Rb phosphorylation would form a negative feedback loop in late G1 phase (Müller *et al.*, 1994).

XI. D-Type Cyclins Are Involved in Differentiation

There is increasing evidence that the D-type cyclins and Rb play an important role in the switch between proliferation and differentiation. The 32D myeloid cell line normally expresses cyclins D2 and D3 in a growth factor-dependent manner and proliferates in culture until G-CSF is added, which induces the cells to differentiate (Kato and Sherr, 1993). When the cells are transfected with either cyclin D2 or D3 under a constitutive promoter, the cells are unable to differentiate in the pres-

ence of G-CSF; they continue to proliferate until they die. Constitutive expression of cyclin D1 has no effect on their differentiation, nor does expression of cyclin D2 and D3 mutants that are unable to bind Rb (Kato and Sherr, 1993). There are also some data to suggest that CDK4 needs to be downregulated in order to allow differentiation (Ewen *et al.,* 1993). Thus, one way to think of how D-type cyclins could act as protooncogenes is as follows. If D-type cyclin synthesis becomes constitutive and independent of the presence of growth factors, the cell cycle machinery would receive the signal that growth factors are constantly present, and so would proliferate rather than differentiate. This is one of the conditions necessary for transformation, so cyclin-D expression would therefore be expected to cooperate with other oncogenes in transformation, and recent data show cooperation with *myc* in transgenic mice (Bodrug *et al.,* 1994) and with *ras* and defective E1A protein in cultured cells (Hinds *et al.,* 1994).

Recently, p16, a protein with four ankyrin repeats, has been found to specifically bind and inhibit CDK4 (Serrano *et al.,* 1993). p16 was initially described as binding more cyclin D–CDK4 complexes in T Ag-transformed cells (Serrano *et al.,* 1993) and later found to be deleted in a large number of tumor cell lines (Kamb *et al.,* 1994; Nobori *et al.,* 1994), but not in primary tumors. The *p16* gene is very closely linked to a gene encoding an almost identical protein that is ~24 amino acids longer. The genes map to chromosome 9p12 and are rearranged, deleted, or mutated in a majority of later stage gliomas, leukemias, and melanomas (Kamb *et al.,* 1994). However, little is known of the physiological role of p16. In tandem with the gene encoding p16 is one encoding the closely related protein p15. In HaCaT cells the levels of p15 mRNA and protein are dramatically increased after TGFβ treatment, implicating p15 (and p27, see below) as the means by which TGFβ blocks the cell cycle (Hannon and Beach, 1994).

XII. The G1 to S Cyclins

Overexpressing D-type cyclins alone only moderately accelerates the cell's entry into S phase, although Rb is phosphorylated much earlier than usual (Quelle *et al.,* 1993; Resnitzky *et al.,* 1994). The E-type cyclins are thought to act after the D-type cyclins at the G1–S transition itself and be important in the initiation of DNA replication. However, like the D-type cyclins, overexpressing cyclin E only moderately advances entry into S phase (Ohtsubo and Roberts, 1993). However, there are suggestions that cyclin E regulates a different aspect of G1 phase compared with the D-type cyclins.

The E-type cyclins bind and activate CDK2 (Dulic *et al.*, 1992), and this complex is essential for the cell to begin DNA replication. The best evidence for this comes from studies on developing *Drosophila* embryos. In *Drosophila* embryogenesis, the disappearance of cyclin E transcripts after mitosis 16 causes cells to stop dividing and arrest in G1 (Knoblich *et al.*, 1994). Some cells go on to complete endoreplication (DNA synthesis without cell division) after cycle 16, and the presence of cyclin E transcripts correlates exactly with cells that are capable of endoreplication. Furthermore, cells of a *Drosophila* mutant in cyclin E are unable to enter S phase after the maternal store of cyclin E has been exhausted.

Ectopically expressed cyclin E is sufficient to cause postmitotic G1 cells to undergo another round of DNA replication and cell division. Ectopic cyclin E was also sufficient to stimulate the accumulation of cyclins A and B which are essential for mitosis. There was no increase in the mRNA levels of cyclins A or B in these experiments, which suggests that, like the G1 cyclins in budding yeast (Amon *et al.*, 1994), cyclin E could stabilize cyclin A and B by shutting off the proteolytic machinery (Knoblich *et al.*, 1994). It will be interesting to determine whether this is also the case in mammalian cells. Another point of interest is that cyclin E can trigger either another complete round of DNA replication and mitosis or of DNA rereplication. This is a developmentally controlled switch in the cell cycle which could obviously involve a protein similar to rum1 in fission yeast.

As yet the substrates of the cyclin E–CDK2 complex are unclear. One effect of cyclin E–CDK2 may be on the transcription of genes required for S phase because it is associated with the E2F transcription factor in a complex with the Rb-related proteins p107 (Lees *et al.*, 1992) and p130 (Hannon *et al.*, 1993). Thus, by analogy with MBF and DSC1, it is possible that cyclin E–CDK2 modulates E2F activity to promote the transcription of S phase. Interestingly, the E2F subunit (E2F-4) associated with cyclin E and p107 differs from those which associate with Rb (E2F1, E2F2, and E2F3), although DP1 is common to both complexes (La Thangue, 1994). Thus, the E2F complex regulated by Rb and the D-type cyclins may well have different properties from the E2F regulated by cyclin E–CDK2 and p107.

Because of its role in promoting S phase, the cyclin E–CDK2 complex is the target of a number of negative growth factors which arrest cells in G1 phase. Prominent among these is TGFβ. TGFβ activates/unmasks a CDK inhibitor, $p27^{KIP1}$, a heat-stable protein that is constitutively present in latent form, and is also unmasked when cells reach confluence (Koff *et al.*, 1993; Polyak *et al.*, 1994b). $p27^{KIP1}$ has a high affinity for

cyclin E–CDK2, but is also bound by CDK4 and by the D-type cyclins (Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994). This has led to the suggestion that $p27^{KIP1}$ may be involved in regulating the normal transition from G1 to S phase by binding initially to cyclin E–CDK2, and is then sequestered by accumulating cyclin D-CDK4 complexes (Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994). This model parallels the regulation of G1–S in budding yeast by $p40^{Sic1}$, with the difference that $p40^{Sic1}$ is inactivated by proteolysis rather than being sequestered by another cyclin–CDK.

$p27^{KIP1}$ is 42% identical in its N-terminus to another mammalian CDI, p21, which is very important in the p53-mediated arrest of the cell cycle in response to DNA damage (Duli'c *et al.*, 1994; El-Deiry *et al.*, 1994, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). The p21 promoter has a p53-binding site, and p21 transcription is activated by wild-type, but not mutant, p53 (El-Deiry *et al.*, 1993). p53 is thought to be activated when DNA is damaged in G1 phase, which would lead to increased p21 synthesis. p21 binds and inhibits a wide variety of cyclin–CDK complexes, including cyclin D–CDK4, cyclin E–CDK2, and cyclin A–CDK2. Thus, increased levels of p21 in G1 phase would prevent cells entering S phase. Conversely, in cells with mutant p53 the failure to induce p21 after DNA damage might lead to the replication of damaged DNA and could explain the increased incidence of chromosomal abnormalities and genetic instability in oncogenesis. p21 has also recently been shown to bind and inhibit components of the DNA replication machinery, in particular to PCNA, the processive subunit of DNA polymerase δ (Waga *et al.*, 1994). Thus, DNA damage in S phase would also block DNA replication directly through the mediation of p21. The one caveat to these observations is that all the data derive from an *in vitro* system and have yet to be confirmed by *in vivo* experiments. p21 levels are also increased 10- to 20-fold in senescent cells, which may partially explain why these cells are no longer able to progress through the cell cycle (Noda *et al.* 1994).

XIII. S-Phase Cyclins

Once cells have entered S phase there is another set of cyclin–CDKs that is necessary for continued DNA replication. In the budding yeast the Clb5 and Clb6 cyclins are important in the regulation of S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Both cyclins belong to the B-type cyclin family, and their deletion causes dramatic

delays in progress through S phase. Clb5 and Clb6 transcription begins in late G1 phase, but the Clb5–Cdc28 complex remains inactive because it is bound to the budding yeast CDI, $p40^{Sic1}$. Once cells are ready to enter S phase, $p40^{Sic1}$ is degraded by the ubiquitin pathway and the Clb5–Cdc28 complex is activated (Schwob *et al.*, 1994).

In mammalian cells, cyclin A in a complex with CDK2 appears to be required for efficient DNA replication. Cells microinjected with anti-cyclin A antibodies are able to initiate at most 10% of normal DNA replication (Tsai *et al.*, 1993). In some respects cyclin A replaces cyclin E, which is rapidly degraded in early S phase. Thus, cyclin A is also found to be associated with p107, p130, and E2F (Ewen *et al.*, 1992; Lees *et al.*, 1992). These interactions are likely to be important to the correct regulation of the cell cycle because the E1A-transforming protein of adenovirus disrupts this complex by sequestering cyclin A (Pines and Hunter, 1990) and p107.

There are now some indications to how cyclin A–CDK2 influences transcription. Cyclin A appears to bind directly to E2F1 (the subunit that interacts with pRb) and through this binding CDK2 is able to phosphorylate the associated DP-1 subunit (Krek *et al.*, 1994). This phosphorylation event inhibits E2F DNA-binding activity. Neither cyclin E nor the mitotic cyclin B1 are able to bind directly to E2F1 (Krek *et al.*, 1994). An interpretation of these results suggests that as cells enter S phase, cyclin A synthesis beings, and this downregulates the transcription of the genes transcribed by E2F that initiated in late G1 phase; a model reminiscent of the effect of the Clb–Cdc28 complexes on SBF transcription in budding yeast (Amon *et al.*, 1993).

An intriguing observation suggests that cyclin A synthesis may be influenced by the extracellular matrix. A stable cell line of NRK cells expressing ectopic cyclin A is able to grow in suspension, whereas the parental cell line is anchorage dependent (Guadagno *et al.*, 1993). This suggests that cyclin A transcription is stimulated by a signal from surface adhesion molecules as a late event in G1 phase.

There is one other instance of an alteration in cyclin A having a profound effect on cell behavior. The cyclin A gene was found to be the unique insertion site of a hepatitis B virus (HBV) in one clonal hepatoma (Wang *et al.*, 1990). Due to this integration the N-terminus of cyclin A, including the destruction box, was replaced by the preS protein of HBV (Wang *et al.*, 1992), and this chimeric protein was unable to be degraded in the normal fashion in mitosis. This event could have contributed to the transformation of the cell, but it is not known how the chimeric HBV–cyclin A protein perturbed the cell cycle. By analogy with E1A, it may have interfered with the p107/E2F interaction, or

alternatively a nondegradeable cyclin A may alter the control of START by its aberrant presence in G1 phase. However, changes in cyclin A are a rare event in most cancers.

As with cyclin E the substrates of cyclin A–CDK2 in S phase are mostly undefined. One likely substrate is RF-A (Dutta and Stillman, 1992), a helicase that is part of the DNA replication machinery. RF-A is phosphorylated in S phase cells, which enhances its helicase activity, and the sites of phosphorylation *in vivo* can be phosphorylated by cyclin A–CDK2 *in vitro* (Dutta and Stillman, 1992).

Cyclin A plays a further role in G2 phase cells, but its exact function is frustratingly unclear. In mammalian cells cyclin A forms a new complex with cdc2 in G2 phase (Pagano *et al.*, 1992), but the differences between this complex and the cyclin A–CDK2 complex are not known, although in early frog embryos cyclin A appears to be exclusively bound to cdc2. Cells of a *Drosophila* that is mutant in cyclin A arrest in G2 phase and are unable to enter mitosis (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1990). However, an observation using cycling *Xenopus* egg extract is directly at odds with the role of cyclin A in promoting M phase inferred from the *Drosophila* results. It was found that when cyclin A is ablated in frog extracts, the extract goes prematurely into M phase (Walker and Maller, 1991). These conflicting results from *Drosophila* and *Xenopus* have yet to be reconciled.

More satisfying are experiments showing that cyclin A-associated kinase activity has profound effects on the microtubule system (Verde *et al.*, 1992). In *Xenopus* extracts cyclin A–cdc2 kinase activity substantially enhances the nucleating ability of centrosomes, while the microtubules remain at their interphase length. In contrast, active cyclin B–cdc2 kinase effectively depresses centrosome nucleation and causes microtubules to shorten to their mitotic length. It is very tempting to draw parallels between these effects and the changes in microtubule behavior as the cell enters mitosis. Thus, at the beginning of mitosis, when cyclin A is most active, the centrosomes nucleate and begin to form the long spindle asters. Later, once the spindle forms, the centrosomes no longer nucleate fresh microtubules and the spindle microtubules shrink. At this point cyclin A is in the process of being degraded and the B-type cyclin–cdc2 complexes are most active.

XIV. Mitotic Cyclins

Thus, we come to the last part of the cell cycle, mitosis itself. The decision to enter mitosis is a very carefully regulated event (reviewed in Dunphy, 1994). It is at this checkpoint that the control of the phosphorylation

state of Y15 (and T14 in mammalian cells) in cdc2 appears to be most crucial. Fission yeast that have a mutant wee1 protein and which overproduce cdc25 are unable to prevent entry into mitosis in the presence of unreplicated DNA (Enoch and Nurse, 1990). Hence, there is stringent control on the activation of the T14/Y15 phosphatase, cdc25, as part of a feedback loop with the B-type cyclins and on the antagonistic protein kinases, wee1 and mik1 by the nim1 protein kinase. The one exception to this is Cdc28 in budding yeast. Cdc28 is phosphorylated on the Y15 equivalent, Y18, but mutating this to a nonphosphorylatable phenylalanine has no obvious phenotype (Amon *et al.*, 1992; Sorger and Murray, 1992). By comparison, in fission yeast the same mutation in cdc2 causes the cell to enter mitosis prematurely (Gould and Nurse, 1989). Thus, there must be some other means of regulating Cdc28 kinase activity at mitosis, perhaps by a CDI.

Part of the additional control may reside in the cyclin partners of Cdc28. In fission yeast, cdc2 only seems to interact with one B-type cyclin, cdc13 (Booher and Beach, 1988; Goebel and Byers, 1988; Solomon *et al.*, 1988), whereas in budding yeast Cdc28 interacts with at least four B-type cyclins (Fitch *et al.*, 1992; Richardson *et al.* 1992); Clb3 and Clb4 in late S phase/G2 phase, and later Clb1 and Clb2. Of these, only a deletion in Clb2 causes a pronounced mitotic defect. The various roles of the other Clb types in mitosis have not been defined. The likelihood is that at least one Clb will be involved in the reorganization of the microtubules at mitosis because this seems to be one of the primary functions of the B-type cyclins in mitosis. The evidence for this stems from the observations on the effects of cyclin–CDK kinases on microtubules in *Xenopus* extracts mentioned above (Verde *et al.*, 1992), and that B-type cyclins are associated with the centrosomes and spindle poles in all organisms studied thus far. In fission yeast, cdc13 is associated with the spindle poles during mitosis (Alfa *et al.*, 1990); in starfish oocytes (Ookata *et al.*, 1993) cyclin B associates with the spindle—apparently through MAP4—and human cyclin B1 (Pines and Hunter, 1991) and chicken cyclin B2 (Gallant and Nigg, 1992) bind to the centrosomes (Bailly *et al.*, 1992) and mitotic apparatus.

Starfish B, chicken B2, and human B1 cyclins also share another interesting property. These cyclins are cytoplasmic in interphase, but at the beginning of mitosis—before nuclear envelope breakdown—they rapidly translocate into the nucleus (Gallant and Nigg, 1991; Ookata *et al.*, 1992; Pines and Hunter, 1991). The reason for this is still unclear. One possibility is that this is another means to control the timing of cdc2 activation, that cdc2 is dephosphorylated only in the nucleus by cdc25c, which most groups report to be a nuclear protein. However, this attrac-

tive hypothesis has recently been thrown into question by another report that cdc25c is cytoplasmic and translocates into the nucleus at the same time as cyclin B–cdc2 (Heald *et al.*, 1993).

The cyclin B–cdc2 complexes are not exclusively concerned with rearranging microtubules in mitosis, although this is an important role. They have also been implicated in initiating several of the other major changes in the cell behavior and architecture at mitosis by phosphorylating key substrates (reviewed in Nigg, 1993). Cyclin B–cdc2 has been shown to phosphorylate components of the transcriptional apparatus in mitotic *Xenopus* extracts, which downregulate *Pol*III-mediated transcription (Gottesfeld *et al.*, 1994; Hartl *et al.*, 1993). Cyclin B–cdc2 appears to be directly responsible for phosphorylating and thus disassembling the nuclear lamina (Peter *et al.*, 1991, 1990) and intermediate filaments (Chou *et al.*, 1990) and caldesmon, which weakens its interaction with the actin cytoskeleton (Yamashiro and Matsumura, 1991; Yamashiro *et al.*, 1991). These are important events in disassembling the nucleus and in allowing the cell to round up and divide in mitosis. The vesicular compartment of the cell is also extensively modified at mitosis (reviewed in Warren, 1993). The Golgi and endoplasmic reticulum bud themselves into a multitude of vesicles because mitotic kinases inhibit vesicle fusion (Thomas *et al.*, 1992; Stuart *et al.*, 1993; Woodman *et al.*, 1993). Recent data suggest that the form of cdc2 responsible is probably cyclin B2–cdc2 because this complex associates almost exclusively with the vesicle compartment of human cells and remains associated throughout mitosis (M. Jackman, M. Firth, and J. Pines, manuscript submitted for publication).

All these changes will persist in the cell while the cyclin B–cdc2 kinases remain active until the metaphase–anaphase transition, when the B-type cyclins are degraded. The phosphatases which reverse the changes and reestablish the interphase state have been partially defined through genetic analysis in yeast. They belong to both the PP1 and the PP2A classes, but this is beyond the scope of this review.

XV. Nonmitotic Cell Cycle Cyclins

Finally, there are the recent exciting results showing that cyclins and CDKs are involved in regulating processes other than the cell cycle.

Perhaps it was to be expected that there would be cyclins specific to meiosis. Several examples are now known, including a second cyclin A in both *Xenopus* and in mouse meiosis (M. Carrington, personal communication), and the puc1 cyclin in fission yeast (Forsburg and Nurse, 1991). Fission yeast meiosis also involves the res2 transcription factor (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994).

The most extensive data on a non-cell cycle role for the cyclin–CDK motif are from the budding yeast phosphate response. When yeast cells are placed under conditions of low phosphate the *Pho5* gene is induced through the mediation of the Pho2 and Pho4 transcription factors. By contrast, in conditions of high phosphate, Pho4 is unable to bind DNA because it is phosphorylated by a protein kinase that is effectively a cyclin–CDK (Kaffman *et al.*, 1994). This is the Pho80–Pho85 complex. Pho85 is a close structural homologue to Cdc28 (Toh-e *et al.*, 1988) and is activated by binding the Pho80 protein, a close relative of the Hcs26 and OrfD cyclins (Kaffman *et al.*, 1994) (which are most similar to the Clns, but whose function, as mentioned above, is unclear). Furthermore, Pho81, a small protein with several ankyrin repeats, has recently been shown to bind and inhibit the Pho80–Pho85 complex, in striking parallel with other recently cloned CDIs (Hirst *et al.*, 1994; Scheider *et al.*, 1994).

In mammalian cells the normally G1 phase protein kinase, CDK5, is also used as a CDK in postmitotic neuronal cells. In association with a novel, neuronal-specific activator with little homology to cyclins, p35, CDK5 is the primary neurofilament kinase (Hellmich *et al.*, 1992; Lew *et al.*, 1992).

XVI. With Apologies to Elgar

Thus, it is clear that the versatile cyclin–CDK theme has been varied by the cell to orchestrate cellular processes as diverse as DNA replication and mitosis, phosphate metabolism, and neuronal structure. Indeed, cyclin–CDK complexes are more than simply the regulators of particular reactions; there is good evidence from elegant experiments in fission yeast that the cell defines which state of the cell cycle it is in by which cyclin–CDK complex is present (Broek *et al.*, 1991, Hayles *et al.*, 1994). With some exceptions, the exact roles and substrates of the kinase complexes remain mysterious, but with the current furious pace of research these variations should not remain enigmas for long!

Acknowledgments

My thanks to Anna Meddins for comments on the manuscript and to the Cancer Research Campaign for financial support.

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MOLECULAR GENETICS OF 11q23 CHROMOSOME TRANSLOCATIONS

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I. Introduction

A wide variety of nonrandom chromosome alterations have been identified in many different human tumors. These were first extensively studied in leukemias and lymphomas, and more recently the work has been extended to nearly every type of human neoplasm (Heim and Mitelman, 1987). The findings have had useful clinical applications in diagnosis and prognosis, and, most importantly, with the advent of modern molecular techniques, have provided valuable clues to the location of "cancer genes" that contribute to human tumorigenesis (Nowell, 1994). A large number of these genes, both known and previously unknown, have now been cloned and characterized, and these data are being rapidly translated into clinical applications, including the design of innovative specific therapies.

In most cases, the new cancer genes identified through this approach, particularly in the leukemias and lymphomas, have proved to be oncogenic only in a specific cell lineage and, in some cases, only at a particular stage of differentiation (Nowell, 1994). A remarkable exception is a gene recently identified at chromosome band 11q23 that appears to be involved in a spectrum of hematopoietic tumors with a variety of

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phenotypes. When alterations were first identified in this chromosome region, primarily translocations, they were reported not only in leukemias but also in some lymphomas and occasional solid tumors (e.g., Ewing's sarcoma) (Mitelman, 1991). Subsequently, as improved cytogenetic and molecular genetic techniques have been applied, it has become apparent that several different genes in this chromosome region are involved in neoplasia (Kobayashi *et al.*, 1993a). However, it is also now clear that one particular gene, variously designated *ALL-1, MLL, HRX,* or *HTRX,* has a critical role in multiple leukemic subgroups as well as occasional lymphomas, primarily by forming a "fusion gene" with many partners from different chromosomes (Cimino *et al.*, 1991; Ziemen Van Der Poel *et al.*, 1991; Gu *et al.*, 1992a; Tkachuk *et al.*, 1992; Djabali *et al.*, 1992), and occasionally through involvement in deletions and trisomies of chromosome 11.

This review will attempt to briefly summarize the cytogenetic and clinical findings that accompany alterations of the *ALL-1* gene, which are present in 5–10% of all human leukemias, and then consider in some detail the molecular characteristics of this locus and its multiple fusion partners. Since this is the only human cancer gene that interacts with such a wide variety of other loci, resulting in tumors with a spectrum of phenotypes, we will also offer some speculation concerning both the mechanisms and significance of these associations and the possible clinical utility to which these observations might be put.

II. Cytogenetics of 11q23 Abnormalities

Karyotypic alterations in band 11q23 were first reported in the late 1970s as a t(4;11)(q21;q23) translocation in acute lymphocytic leukemia in young children (Oshimura *et al.*, 1977; Van den Berghe *et al.*, 1979). As cytogenetic techniques have improved and been corroborated by other methods, no fewer than 20 different 11q23 translocations have now been identified in various childhood and adult acute leukemias (Hunger *et al.*, 1993; Thirman *et al.*, 1993). The t(4;11) rearrangement remains the most frequent, with acute lymhocytic leukemia also associated with t(1;11) and t(11;19) translocations as well as occasional deletions involving band 11q23 (Raimondi, 1993; Kobayashi *et al*, 1993b). A karyotype of leukemic cells with the t(4;11) abnormality is shown in Fig. 1. Translocations observed in acute nonlymphocytic leukemias of varying phenotypes have included predominantly a t(9;11), usually with a monoblastic phenotype (Fourth International Workshop, 1984), as well as t(1;11), t(2;11), t(6;11), t(10;11), t(11;17), t(11;19), and rarely others. Rearrangements in this region, as either translocations or deletions, have also been

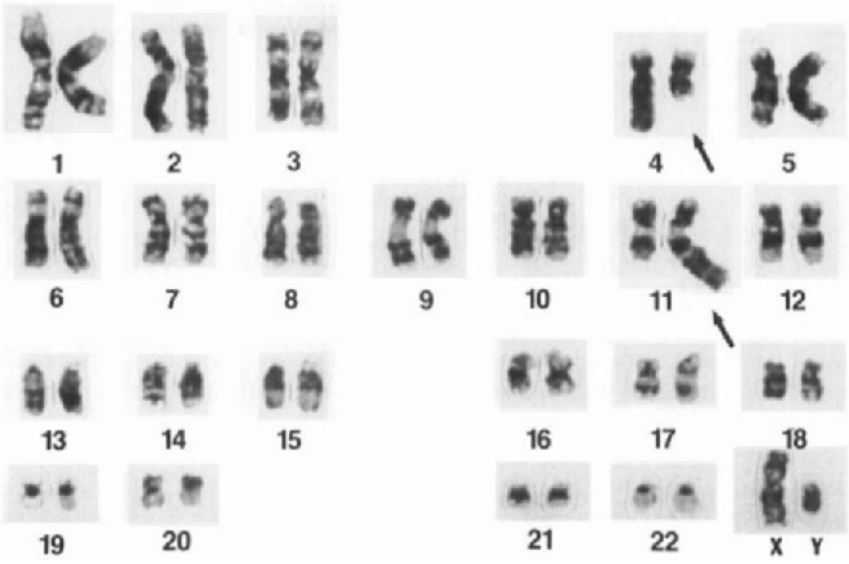

FIG. 1. Karyotype of leukemic cell from a 3-month-old infant with acute mixed-lineage leukemia, showing the characteristic t(4;11)(q21;q23) translocation. No other karyotypic alterations are observed.

reported in a few lymphomas, as well as in the blast crisis of chronic myelogenous leukemia and in myelodysplastic disorders (Thirman *et al.*, 1993; Hunger *et al.*, 1993; Mitelman, 1991).

An interesting subset of acute leukemias, particularly in children, that commonly have 11q23 rearrangements are acute myeloid leukemias secondary to previous chemotherapy with the topoisomerase inhibitor epipodophyllotoxin (Pui *et al.*, 1991).

It should be noted that "deletions" in band 11q23 in primary leukemias have recently been found, by molecular studies, to represent a heterogeneous phenomenon. Those with more proximal breakpoints are usually true deletions, involving either the *ALL-1* locus or, in some cases, a putative suppressor gene located proximal to *ALL-1* (Kobayashi *et al.*, 1993b). Some small distal deletions, however, have been shown by molecular dissection to represent submicroscopic translocations between the *ALL-1* gene and one of the various donor loci involved in such rearrangements [e.g., AF6 from chromosome band 6q27 (Prasad *et al.*, 1993)]. Interestingly, recent study of several cases of acute leukemia with an extra copy of chromosome 11 has demonstrated that rearrangement of *ALL-1* can also occur in this circumstance (Schichman *et al.*, 1994); so

it is now apparent that a variety of different mechanisms can result in significant structural and functional alteration of this important locus.

III. Clinical Considerations

The various acute leukemias, both lymphoid and myeloid, with cytogenetic and molecular evidence for alterations in *ALL-1*, uniformly carry a poor prognosis. The t(4;11) translocation is the most common genetic alteration in early childhood leukemia, and this, along with other rearrangements of the *ALL-1* gene, sometimes submicroscopic, apparently contributes to at least 70% of the acute leukemias occurring in children under the age of 12 months (Chen *et al.*, 1993a; Cimino *et al.*, 1993; Raimondi, 1993). These alterations are typically associated with a very high leukocyte count and often with involvement of the central nervous system. The disorder is often classified clinically as "B cell precursor ALL," although the cells typically demonstrate both lymphocytic and myeloid markers and are also CALLA negative. The ambiguous lineage has led to speculation as to whether this, and other leukemias associated with *ALL-1* rearrangements, arises in a multipotential stem cell.

The same is true of the adult leukemias with 11q23 rearrangements. All are present as acute leukemias, and the phenotype may be either lymphoid or myeloid, according to the particular donor gene involved in the translocation with *ALL-1*. Most common among those in the myeloid lineage is the t(9;11) translocation, usually with an acute monoblastic phenotype (M5a), and the others are often either monocytic or myelomonocytic (M5,M4) (Heim and Mitelman, 1987; Mitelman, 1991). It is not clear whether all of these subgroups arise from a multipotential stem cell or whether different *ALL-1* translocations have an oncogenic effect in different hematopoietic lineages at various stages of differentiation.

The secondary leukemias with 11q23 rearrangements typically present with a myeloid phenotype. As with the *de novo* leukemias, these neoplasms are clinically aggressive and respond poorly to therapy. In children who have received topoisomerase inhibitors for a previous neoplasm, 80–90% of the secondary leukemias have 11q23 alterations (Hunger *et al.*, 1993), and unlike therapy-induced leukemias following other types of genotoxic therapy in both children and adults, those with 11q23 abnormalities typically arise quite soon after previous therapy, without an intermediate myelodysplastic phase (Pedersen-Bjergraad *et al.*, 1990; Prieto *et al.*, 1990; Kearney *et al.*, 1992). This rapid expansion and progression of the secondary neoplasms provides an interesting

parallel to the rapid appearance of the *de novo* leukemias in infants that involve *ALL-1*. The latter has suggested to some investigators that the mutagenic event triggering these leukemias in infants may have occurred *in utero* and perhaps might reflect exposure of the mother to some mutagenic agent. Evidence supporting this notion has been recently presented (Ford *et al.*, 1993; Gill Super *et al.*, 1994).

IV. Cloning the Breakpoint Cluster Region

The association of band 11q23 with a variety of chromosome translocations prompted attempts to examine genes mapped to this region for their location with regard to the breakpoints and for possible rearrangements. Using somatic cell hybrids (Savage *et al.*, 1988; Yunis *et al.*, 1989; Wei *et al.*, 1990) and *in situ* fluorescent hybridization (FISH) (Rowley *et al.*, 1990) it was determined that several translocations, in particular t(4:11), split 11q23 between the *NCAM* and *CD3* genes and a group of genes which included *PBGD, CBL2, THY1*, and ETS1.

In parallel, by applying pulse-field electrophoresis, a long-range restriction map was constructed and positioned the *CD3, PBGD, CBL2*, and *THY1* genes within an area of 1.8 mb (Tunnacliffe and McGuire, 1990). Taken together with the previous information, it was possible to localize 11q23 breakpoints to an area of 750 kb between *CD3G* and *PBGD* (Tunnacliffe and McGuire, 1990). Subsequently, probes for these two genes detected in pulse-field electrophoresis analysis rearranged DNA fragments in leukemic cells with the t(4:11) abnormality (Das *et al.*, 1991; Chen *et al.*, 1991).

A novel approach to identify the DNA region which spans the breakpoints was devised by Rowley and her colleagues (Rowley *et al.*, 1990). A yeast artificial chromosome (YAC) containing the *CD3D* and *CD3G* genes was cloned from a human YAC library and was used as a probe in a FISH analysis on normal and leukemic metaphase cells. This probe hybridized to the normal chromosome 11 as well as to the der (11) and der (4) chromosomes of the RSV 4:11 cell line established from a patient with the t(4:11) aberration. This indicated that the breakpoint was within the 350 kb YAC insert. Similar results were obtained when the YAC probe was hybridized to leukemic cells with t(9:11), t(6:11), or t(11:19), implying that in these translocations as well the breakpoints were bracketed by the YAC insert. The same approach was subsequently extended with similar results to the t(5:11), t(10:11), and t(X:11) abnormalities (Kearney *et al.*, 1992).

Next, the same YAC alone was subjected to partial enzymatic digestion and the resulting fragments were shotgun cloned into a cosmid

vector (Cimino *et al.*, 1991). Repeat-free probes obtained from the cosmids were used to screen Southern blots of DNAs from tumors exhibiting translocations. One such probe, corresponding to sequences at the center of the YAC and positioned around 120 kb telomeric of the *CD3D* gene, detected rearrangements in DNAs from leukemic cells from ALL and AML patients with seven types of 11q23 abnormalities. The breaks clustered within a region of 6 kb (extended later to 8.5 kb) designated *ALL-1* (Cimino *et al.*, 1991, 1992). [A larger region was identified in parallel by pulse-field electrophoresis techniques, and the corresponding locus was termed *MLL* (Ziemin-Van der Poel *et al.*, 1991)]. Subsequent analysis with related probes (Hunger *et al.*, 1993; Thirman *et al.*, 1993) increased the number of 11q23 abnormalities in which the *ALL-1* locus is rearranged to 27 (Table I). The multitude of partners involved in recombination with the *ALL-1* locus on chromosome 11 is unprecedented and surpasses the number of genetic partners to the immunoglobulin and T cell receptor genes. This suggests either an unusual fragility of the breakpoint cluster region or nonstringent requirements from the partner genes. Also unusual in leukemias associated with *ALL-1* rearrangements is the spectrum of clinical settings which includes ALL, AML, non-Hodgkin's lymphomas, and acute phase of chronic myeloid leukemia (Corral *et al.*, 1993; Hunger *et al.*, 1993; Thirman *et al.*, 1993). An additional class of malignancies in which the *ALL-1* locus is rearranged are secondary leukemias, mostly AMLs, arising as a consequence of treatment with topo II inhibitors, particularly epipodophyllotoxin analogues. Eighteen of 20 such secondary leukemias with 11q23 abnormalities showed rearrangement of the *ALL-1* locus (Hunger *et al.*, 1993; Gill Super *et al.*, 1993).

The vast majority of 11q23 abnormalities involve the *ALL-1* locus. However, in isolated cases, other genes, designated *RCK* and *PLZF* located telomeric or centromeric, respectively, to *ALL-1* were found rearranged (Akao *et al.*, 1992; Lu and Yunis, 1992; Chen *et al.*, 1993b).

Anticipating that molecular diagnosis will be more sensitive than cytogenetic examination, investigators used the *ALL-1* probe in Southern analysis of DNAs from patients. In one such study shown in Table II (Cimino *et al.*, 1993), 15 infants with ALL were divided into patients who showed *ALL-1* rearrangements and patients who did not. The infants in the first group showed hyperleukocytosis and early treatment failure. This group included several patients whose leukemic cells showed a normal karyotype. Infants in the second group presented with lower WBC counts and had a good treatment outcome. Another study (Chen *et al.*, 1993a) in which 30 patients were examined reached similar results. It projected at 4 years 15 and 80% survival for patients with and without

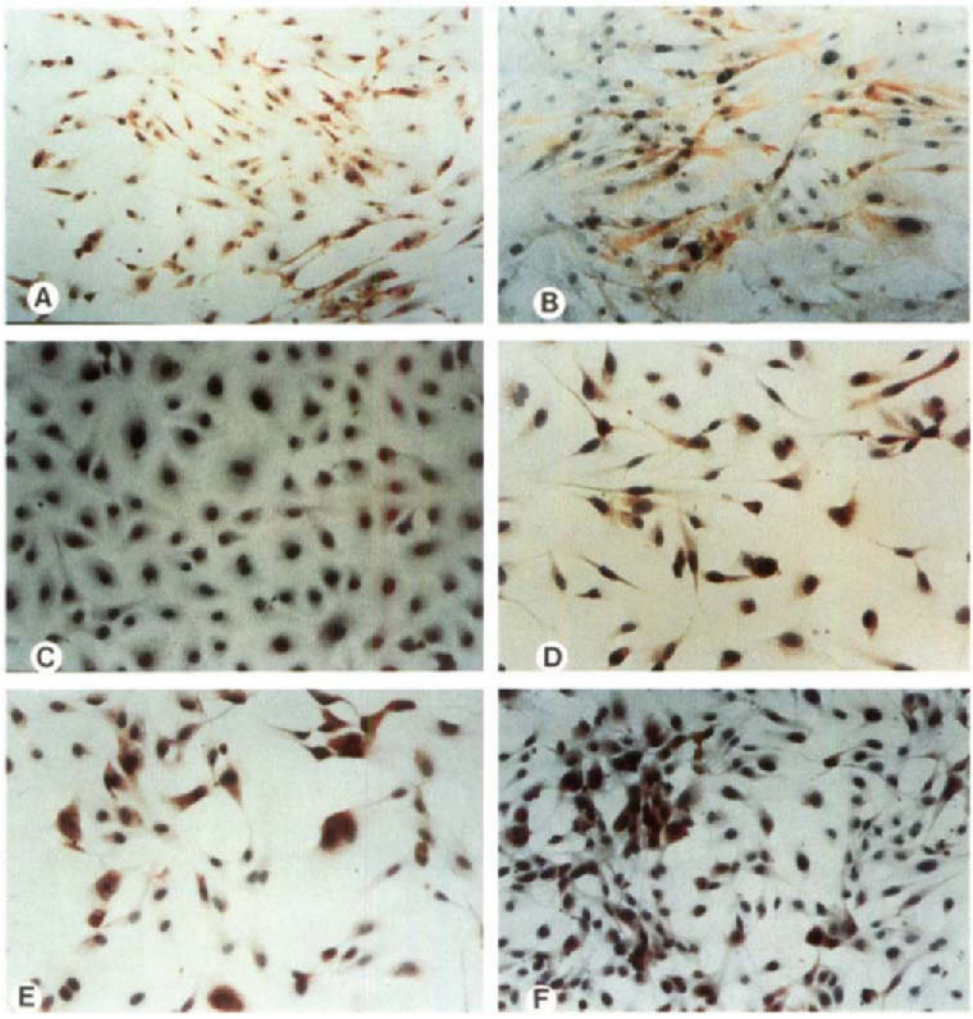

FIG. 1. Conversion of human endothelial cells to a KS tumor cell-like phenotype. Cell cultures were immunostained as described previously (Naidu *et al.*, in press). Positive cytoplasmic reactivity is indicated by red (alkaline phosphatase) staining. KS tumor cells grown in KSGM were stained for factor XIIIa (1A) and for VCAM-1 (1B). HUVECs were stained for factor XIIIa before (1C) and after (1D) a 24-hr incubation in KSGM. HUVECs exposed to 250 units/ml of purified native murine scatter factor (SF) (1E) or recombinant human SF (1F) for 24 hr were stained for factor XIIIa. Magnification, ×60.

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TABLE I
ALL-1 GENE REARRANGEMENTS IN LEUKEMIAS WITH 11q23 ABNORMALITIES

Cytogenetic abnormality	Type of leukemia
t(1:11)(p32:q23)	ALL
t(1:11)(q32:q23)	CML-bc
t(1:11)(q21:q23)	AML-M4
t(2:11)(p21:q23)	ALL
t(4:11)(q21:q23)	ALL
t(6:11)(q27:q23)	AML and ALL
t(6:11)(p12:q23)	NH lymphoma
t(7:11)(p15;q23)	AML-M7
t(9:11)(p22;q23)	AML and ALL
t(9:11)(p24:q23)	AML-M5[a]
t(9:11)(q21;q23)	AML-M5[a]
t(10:11)(p11:q23)	AML-M4
t(10:11)(q22;q23)	AML-M5
t(11:15)(q23;q15)	AML
t(11:16)(q23;p13)	ALL[a]
t(11:17)(q23:q21)	AML-M5
t(11:17)(q23;q25)	AML-M2
t(11:18)(q23;p21)	NH lymphoma
t(11:19)(q23:p13)[b]	ALL and AML
t(11:22)(q23;q12)	AML-M5
t(X:11)(q13;q23)	ALL
del (11(q23)	ALL and AML
inv(11)(q14;q23)	NH lymphoma
ins(10;11)(p11:q23;q24)	AML-M5
inv ins (10;11)(p11:q23;q13)	AML-M5
ins(10:11)(p13;q23;q24)	AML-M5

[a] Secondary leukemia.
[b] At least two separate sites are present on 19p13.

involvement of the *ALL-1* locus, respectively. These results strongly suggest that infants with acute leukemia should be tested for rearrangement of the *ALL-1* locus, and that therapy should be adjusted accordingly.

The appearance of infant acute leukemia in very young children raised the suspicion of a prenatal transformation event. This was further supported by high concordance rate of the disease in monozygotic twins (McMahon and Levey, 1964; Clarkson and Boyse, 1971). The identification of the *ALL-1* locus enabled direct examination of this idea. Using an *ALL-1* probe in Southern analysis of genomic DNAs from the leukemic cells of four pairs of identical twins, identical rearrangements were observed in each pair (Ford *et al.*, 1993; Gill Super *et al.*, 1994). This result

TABLE II

CLINICAL FEATURES AT PRESENTATION OF INFANTS WITH ACUTE LEUKEMIA AND TREATMENT OUTCOME

Patient no.	Age	WBC ($\times 10^9$/liter)	11q23 Abnormalities	ALL-1 rearrangement	Clinical outcome (months)
1	5 Months	500	+	+	Died + 16
2	20 Days	100	+	+	Died + 1
3	5 Days	104	+	+	Died + 15
4	4 Months	361	+	+	Died + 24
5	12 Months	12	NA	+	Died + 6
6	12 Months	40	+	+	Died + 1
7	13 Days	804	NA	+	Died + 9
8	3 Months	940	−(Normal karyotype)	+	Died + 2
9	8 Months	756	−(Normal karyotype)	+	Died + 12
10	7 Months	340	−(Normal karyotype)	+	Died + 1
11	2 Months	37	−(Normal karyotype)	+	Died + 7
12	9 Months	677	−(Normal karyotype)	+	CCR + 31
13	3 Months	92	−(Normal karyotype)	−	CCR + 40
14	6 Months	13	NA	−	CCR + 52
15	2 Months	91	−	−	CCR + 64

Note. CCR, continuous complete remission; NA, not available.

could best be explained by a clonal event *in utero* in one twin followed by transfer of the malignant clone through vascular anastomoses or through the maternal circulation to the second twin. Presumably, in most nontwinned infant leukemias as well, *ALL-1* is rearranged during pregnancy. As was pointed out (Ford *et al.*, 1993) the high concordance rate, the short latency, and the synchrony of clinical onset in identical twins suggest that rearrangement of *ALL-1* is either sufficient for leukemia development or renders the affected cells at a very high risk for necessary secondary mutations.

V. *ALL-1:* The Human Homologue of *Drosophila trithorax*

A. Cloning and Structural Analysis of *ALL-1*

Nonrepetitive probes isolated from DNA segments located centromeric or telomeric of the breakpoint cluster region detected in Northern blot analysis a major large transcript, later estimated to be of 15 kb (Ziemin-Van der Poel *et al.*, 1991; Cimino *et al.*, 1992). This transcript as well as one or two slightly smaller RNAs were identified in a variety of hematopoietic and nonhematopoietic cell lines. The same probes detected altered transcripts in cell lines with the t(4:11) abnormality, as well as in somatic cell hybrids containing the der 11 or der 4 chromosomes (Cimino *et al.*, 1992; Gu *et al.*, 1992b). This implied that the t(4:11) aberration cleaved a gene spanning the breakpoint into two segments each transcribed in the leukemic cells. Utilizing genomic repeat-free probes to screen cDNA libraries, and subsequently using end-probes for further cDNA cloning, contigs of 14–15 kb were established (Gu *et al.*, 1992b; Tkachuk *et al.*, 1992). These cDNAs originated from the major 15-kb RNA previously described. The gene transcribed into this RNA was designated *ALL-1, HRX, MLL,* or *HTRX1* (Gu *et al.*, 1992a; Tkachuk *et al.*, 1992; Ziemin-van der Poel *et al.*, 1991; Djabali *et al.*, 1992). Analysis of the hybridization pattern of cDNA fragments to cloned genomic DNA indicated that the *ALL-1* gene is composed of a minimum of 21 exons distributed over 100 kb. The first intron is the largest, spanning 35 kb. The breakpoint cluster region encompasses exons 5–11 delineated by two *Bam*H1 sites 8.4 kb apart. The RNA contains an open reading frame encoding a protein of 3968 amino acids. Three domains within the protein demonstrate homology to the *trithorax* gene of *Drosophila* (Mazo *et al.*, 1990). The first two, located around the center of the protein (aa 1427–1627, 1868–1976), correspond to a region of *trithorax* containing zinc-binding motifs. Cysteins and histidines

in this region are conserved in the two proteins and can be arranged into 6 or 10 zinc fingers. These two regions are conceivably involved in DNA or protein binding. The third region of homology constitutes the extreme C-terminus. In this domain the two proteins show 82% similarity and 61% identity. This last domain (aa 3754–3968) was recently identified in another *Drosophila* gene, *Enhancer of zeste,* which has a function related to that of *trithorax* (Jones and Gelbart, 1993). The similarity in size between proteins and transcripts of ALL-1 and *trithorax,* the sequence homology, and the colinearity of the homologous domains suggest that ALL-1 is the human homologue of *trithorax.*

ALL-1 contains motifs not found in *trithorax:* (1) three short regions at the N-terminus of the protein (aa 170–182, 217–228, 301–309) which match the consensus of AT hook motif. The latter, initially identified within the high mobility group (HMG) protein HMG-I(Y), mediates binding of these proteins to AT-rich sequences in the minor groove of target DNAs and is thought to promote the activity of transcription factors involved in transcriptional regulation of these DNAs (Du *et al.,* 1993). (2) Two motifs at the N-terminus (aa 171–174, 326–329) belonging to the "SPKK" consensus which also were shown to bind to the minor groove of AT-rich DNA (Churchill and Zuzuki, 1989). (3) A motif (aa 1186–1252) homologous to a region in mammalian DNA methyl transferase (MTase) (Ma *et al.,* 1993; Domer *et al.,* 1993), thought to be capable of distinguishing hemimethylated from unmethylated DNA. Thus, the state of methylation of a target gene may influence the ability of ALL-1 to regulate its expression. The various recognized elements in the ALL-1 protein are shown in Fig. 2.

The murine *ALL-1* gene was recently cloned and sequenced (Ma *et al.,* 1993). Comparison of the human and murine proteins (Fig. 2) shows four domains in which the homology exceeds 93%, separated by three shorter regions with 66–69% homology. The former domains presum-

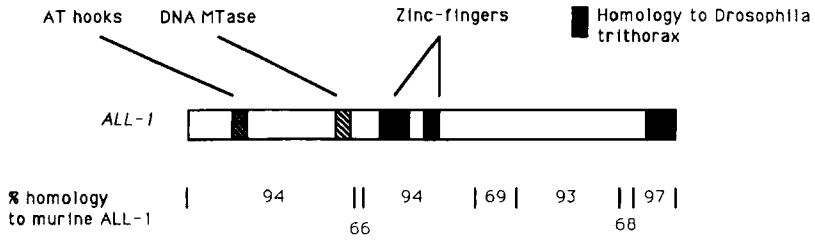

FIG. 2. Sequence motifs in the predicted ALL-1 protein (3969 amino acids) and division of the protein to regions with high or moderate homology to murine ALL-1. MTase, methyltransferase. Data are from references cited in the text.

ably contain the functional elements of the protein and mediate binding to DNA and proteins. Both murine and human genes give rise to alternatively spliced RNAs varying by a stretch of nine nucleotides located at the end of an exon within the zinc fingers domain (Ma *et al.*, 1993). A second alternative splice removes 2.5 kb (exon 3) of coding sequences including the AT hooks of *ALL-1* and gives rise to the minor RNA transcript of ~12.5 kb, apparent in most cell lines examined (Domer *et al.*, 1993). Splicing of exons 3–5 was detected in the B-1 cell line (Leshkowitz, unpublished observations).

B. *Drosophila trithorax*

In *Drosophila melanogaster* the activity of the homeotic genes of the *Antennapedia (ANT-C)* and *bithorax* complex *(BX-C)* determine the various body structures along the anterior–posterior axis (Lewis, 1978). Loss of expression of a homeotic gene at any time during development causes alteration in the segmental identity of cells normally expressing this gene (Lewis, 1963). Similarly, ectopic homeotic gene expression can result in transformation of the cells which normally do not express this gene. The striking phenotypes resulting from misexpression of homeotic genes enabled isolation of mutations in both *cis*-acting regulatory sequences as well as in transacting genes. The latter distribute into two groups—*trithorax (trx)* and *Polycomb (Pc)*—named after the best studied members of each group (reviewed in Kennison, 1993; Paro, 1993). The genes of the *trithorax* group positively regulate homeotic gene expression, while the *Polycomb* group genes maintain the repressed state of homeotic genes. Genes of both groups have multiple genetic targets, some of which, such as *ANT-C* and *BX-C,* are in common. By *in situ* hybridization to polytene chromosomes it was shown that *Pc* is associated with more than 100 loci in the genome, including the *ANT-C* and *BX-C* complexes (Zink and Paro, 1989). By the same technique the trx protein was found bound in a minimum of 16 sites on polytene chromosomes (Kuzin *et al.*, 1994).

The identification within the *Pc* protein of a motif, the chromodomain, shared with the hetrochromatin-associated protein HP1 encoded by the suppressor of position effect variegation Su(var)205 suggested that, by analogy to HP1, the mechanism by which *Pc* is silencing its target genes involves restructuring of chromatin (heterochromatization) into nonexpressed large domains (Paro and Hogness, 1990). It was further suggested that *Pc* is acting in a large multimeric complex composed of the *Pc* group proteins (Franke *et al.*, 1992). It is tempting to speculate that the *trx* group genes would act by a mechanism related to that of the

Pc group genes, namely by affecting chromatin structure so as to allow transcription of the target genes. Circumstantial evidence supporting this idea came from studies of the *brahma (brm)* gene, a member of the *trx* group genes, which encodes a protein related to the yeast transcriptional activator SNF2/SW12 (Tamkun *et al.*, 1992). The activity of the latter is made dispensable in yeast by mutations in genes (*SIN*) which affect chromatin structure (Peterson and Herskowitz, 1992). Thus, *brm* is related to the yeast SWI proteins which counteract proteins that repress transcription by influencing chromatin structure; the latters will be analogous to the *Pc* genes group in *Drosophila*. Recently, it was shown that the human homologue of *brm* is present in HeLa cells in a large protein complex of about 2×10^6 Da (Khavari *et al.*, 1993). This further extends the similarity between a gene of the *trx* genes group to *Pc*.

Very recent studies (Sedkov *et al.*, 1994) demonstrate complex pattern of *trx* RNAs in *Drosophila* embryos. At least five RNA species are made by a process of alternative splicing. Most important, it appears that transcription of the *BX-C* and *ANT-C* genes is regulated by different *trx* products. The identification of alternatively spliced *ALL-1* RNAs in human and mice (Domer *et al.*, 1993; Ma *et al.*, 1993) reemphasizes the similarity between the *Drosophila* and mammalian genes. Presumably, different products of *ALL-1* will regulate expression of different groups of target genes during embryo development and possibly during differentiation of adult tissues.

VI. Fusion of *ALL-1* to Partner Genes

Altered *ALL-1* RNAs detected in cell lines with the t(4:11) or t(11:19) abnormalities were further characterized by cDNA cloning and sequencing (Gu *et al.*, 1992a; Tkachak *et al.*, 1992). Two reciprocal fusion transcripts were identified. The first, originating from the der 11 chromosome, contained the 5′ *ALL-1* sequences fused to DNAs from chromosome 4 or 19. The second species was transcribed from the der 4 or der 19 chromosomes and contained RNAs from these chromosomes linked to 3′ *ALL-1* sequences. The fused RNAs encoded chimeric proteins. The genes from chromosomes 4 and 19 were designated *AF-4* and *ENL*, respectively. These findings suggested that 11q23 chromosome aberrations result in chimeric ALL-1 proteins. The breakpoints in ALL-1 occur in the region delineated by exons 5 and 11. Within this region, exons 7–11 begin in the first residue of a codon; this enables alternative splicing of exons of the partner genes to *ALL-1* exons located further away from the genomic breakpoint, without shifting of the open reading frame.

In the initial analysis of cell lines with the t(4:11) or the t(11:19)

abnormalities, both reciprocal fused transcripts were detected in all cell lines examined. In subsequent RT-PCR analysis of ALL patients with t(4:11) (Downing *et al.*, 1994), 23 of 23 patients showed the RNA originating from the der 11 chromosome, and 16 of 19 contained the reciprocal transcript. This suggested that expression of the *ALL-1/AF-4* RNA, but not of the *AF-4/ALL-1* transcript, is indispensable for leukemogenicity. In two other instances, leukemic cells from a patient with t(4:11) and a cell line with the (X:11) aberration contained RNAs transcribed from the der 11 chromosome but not from the other derivative (Morrissey *et al.*, 1993; McCabe *et al.*, 1994). Further evidence that the critical product of 11q23 translocations is present within the der 11 chromosome comes from cytogenetic studies of variant translocations made by several groups over the years. Analysis of these studies (Rowley, 1992) indicated that in each case the der 11 alone was cytogenetically identical to that seen in the standard two-way translocations, and therefore was the constant feature in these 11q23 abnormalities. Finally, FISH analysis has indicated the deletion of 3′ *ALL-1* sequences in some patients with *ALL-1* rearrangements (Cherif *et al.*, 1994). Based on all of the above, it appears likely that the chimeric protein encoded by the der 11 chromosome is essential for the leukemogenesis process. However, this needs to be confirmed in appropriate model systems.

The cloning and characterization of the genes which recombine with *ALL-1* (partner genes) was hampered by the nonavailability of cell lines for most of the abnormalities; this precluded the preparation of RNAs in amounts and quality sufficient for construction of cDNA libraries. One approach taken to circumvent this problem was to clone the genomic junction fragment from patient's DNA and utilize it to obtain repeat-free probes for screening of normal cDNA libraries. Following sequencing of the cDNA, primers were designed for an RT-PCR reaction on patient's RNA to confirm the presence of a chimeric *ALL-1* transcript. A second approach applied (for cloning of *AF-1P*) consisted of PCR amplification of the RNA junction by using *ALL-1* primers in conjunction with tagged random primers; subsequently, the PCR products were further amplified with nested primers.

To date, the partner genes from chromosomal regions 1p32, 4q21, 6q27, 9p22, 17q21, and 19p13 were cloned and designated *AF-1P, AF-4, AF-6, AF-9, AF-17,* and *ENL,* respectively (Bernard *et al.*, 1994; Nakamura *et al.*, 1993; Prasad *et al.*, 1993, 1994; Tkachuk *et al.*, 1992; Morrissey *et al.*, 1993). All of these genes are expressed in most or all cell types, but their function is not yet known. Only two of the partner genes, *AF-9* and *ENL,* are related by sequence homology. Schematic representation of the proteins encoded by the six genes is shown in Fig. 3. In this scheme, the chimeric proteins thought to be directly involved in leukemogenicity are

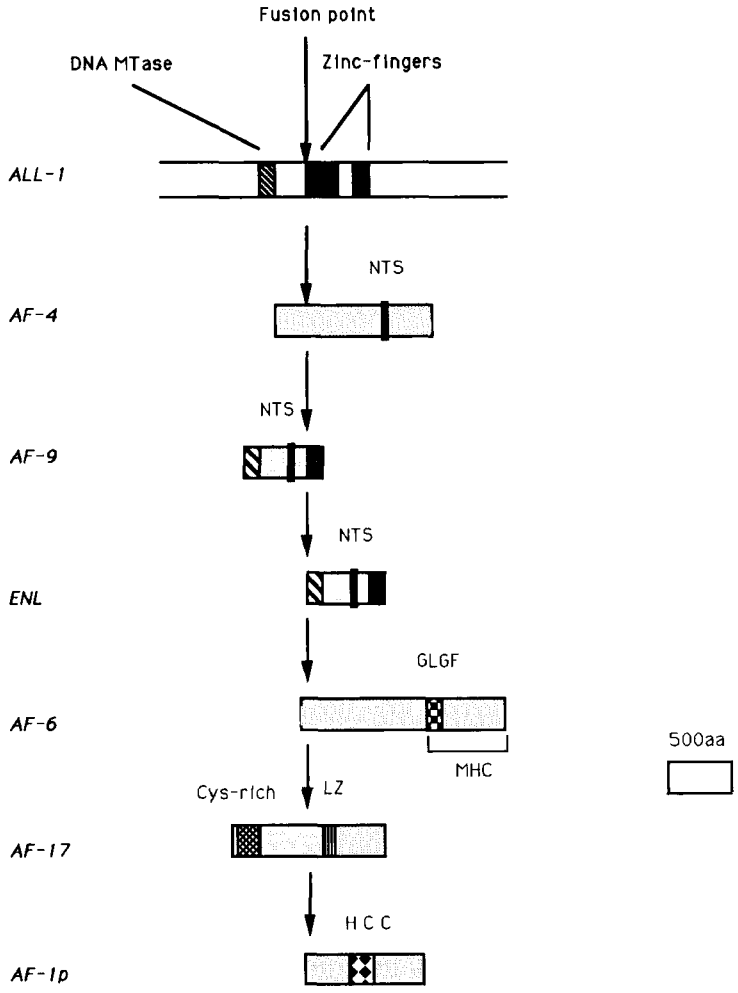

FIG. 3. Sequence motifs in proteins encoded by *ALL-1's* partner genes. Arrows indicate fusion points to the ALL-1 protein. NTS, nuclear targeting sequence; MHC, homology to the myosin heavy chain of *Dictyostelium discoidenum;* LZ, leucine zipper; HCC, helical coiled coil domain. Boxes at the C-terminus and N-terminus of AF-9 and ENL indicate regions of highest homology between the two proteins. Indicated domains are not drawn to scale. Data are from references cited in the text.

composed of the N-terminal ALL-1 polypeptide and the C-terminal (right side of arrows) segments of the partner proteins. In three of the proteins (ENL, AF-6, AF-1P) the fusion point is very close to the N-terminus, so that the polypeptide linked to the C-terminal part of ALL-1 is very

short and not likely to contribute a functional domain; this further supports the idea that the reciprocal product is the species essential for leukemogenicity.

AF-4, AF-9, and ENL contain nuclear targeting signals and are probably nuclear proteins. The three proteins are rich in serines and prolines. AF-9 and ENL show 56% identity and 68% similarity. The homology is the highest (~80% identity and ~90% similarity) at the N- and C-termini of the two proteins (Nakamura *et al.*, 1993). Only the C-terminus of AF-9 is included in the ALL-1/AF-9 protein; presumably, this domain contains the critical element provided to the chimeric protein. Very recently the yeast gene ANC1 was cloned and found to share extensive similarity with the N- and C-termini of AF-9 and ENL (Welch and Drubin, 1994). The product of this gene is a nuclear protein and is involved in organization of actin filaments (Welch and Drubin, 1994). Most importantly, Burton and his colleagues have now found that this protein is physically associated with yeast RNA polymerase II (Werel *et al.*, submitted for publication). This suggests that AF-9 and ENL are transcription factors.

The AF-6 protein shows high similarity but low identity over the C-terminal 600 amino acids to the myosin heavy chain from *Dictyostelium discoidenum*. This region in the latter protein is part of the tail domain which is thought to function in assembly of a myosin filament. Within this homology segment, AF-6 contains the GLGF motif (Prasad *et al.*, 1993) present within several proteins which are associated with the cytoskeleton and are involved in signal transduction or synaptic organization. AF-6 contains clusters of prolines, glutamines, and charged amino acids.

The most notable feature of the AF-17 partner polypeptide is the leucine zipper protein dimerization motif located 3′ of the fusion point (Prasad *et al.*, 1994). At the N-terminus of the protein there is a cystein-rich domain with homology to the nuclear protein Br140. Segments rich in alanines, glycines, glutamines, or prolines are present within AF-17 partner polypeptide.

The *AF-1P* gene is the human homologue of the murine *eps15* gene which encodes a cytoplasmic protein containing a domain predicted to have a coiled coil structure that has similarity to several proteins, including myosin heavy chains, and is involved through protein–protein interactions in filament formation (Bernard *et al.*, 1994). Domains with abundant glutamines or acidic amino acids distribute within the AF-1P protein.

The availability of probes and sequences of some partner genes enabled development of RT-PCR assays to diagnose and monitor patients (Biondi *et al.*, 1993; Hilden *et al.*, 1993; Downing *et al.*, 1993). In

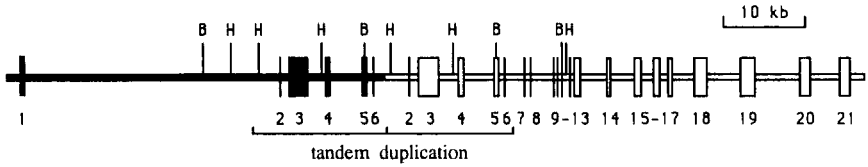

FIG. 4. Tandem duplication of *ALL-1* exons 2–6 in the leukemic cells of some patients with AML. B, *Bam*HI; H, *Hin*dIII. Genomic fusion point between exons 1–6 and exons 2–21 is at the junction of the black and white lines. Data are from Schichman *et al.* (1994).

addition, it demonstrated that some 11q23 abnormalities are "masked" translocations. Thus, a typical del (11q23) aberration was found by using an AF-6 probe to represent a (6:11)(q27;q23) chromosome translocation (Prasad *et al.*, 1993). By utilizing FISH technology, other investigators showed that several terminal deletions of 11q23 corresponded to (6:11) translocations (Kobayashi *et al.*, 1993c).

While most *ALL-1* rearrangements involve partner genes, some do not. In two AML patients with trisomy 11 but no 11q23 abnormality, and in one AML patient with a normal karyotype (Schichman *et al.*, 1994) the *ALL-1* gene has undergone tandem duplication of exons 2–6 (Fig. 4). Sequencing analysis indicated an in-frame fusion, predicting synthesis of a partially duplicated protein. In the altered protein, a truncated ALL-1 polypeptide encoded by ALL-1 exons 1–6 (heavy line in Fig. 4) is fused to a truncated protein encoded by exons 2–21. Tandem duplication of exons 2–8 was recently identified in some other AML patients (Schichman *et al.*, in press).

Obtaining some of the partner genes involved cloning of genomic breakpoints and this enabled accurate localization of the latter within the *ALL-1* gene. In all 14 patients and cell lines analyzed (Gu *et al.*, 1992, 1994; Negrini *et al.*, 1993), the breakpoints occurred between exons 6 and 9; 8 of the breaks occurred in the intron between exons 6 and 7 composed mainly of four Alu repeats. Four other Alu repeats are present between exons 8 and 10 (Gu *et al.*, 1994). Possibly, the high density of Alu sequences in the breakpoint cluster region makes the latter more prone to recombination events. Analysis of three breakpoints indicated extra nucleotides inserted between the joined chromosomes, in conjunction with adjacent heptamer-like or nonamer-like sequences (Gu *et al.*, 1992b; Negrini *et al.*, 1993). This suggested the possibility that in those cases the immunoglobulin VDJ recombinase was involved. In contrast, sequence analysis of several other breakpoints (Prasad *et al.*, 1993; Nakamura, unpublished) did not show the above features.

VII. Models for ALL-1 Leukemogenicity

When tumor-associated gene fusion results in synthesis of a chimeric protein it is not always obvious which of the two partner polypeptides carries the critical activity. In the case of 11q23 abnormalities, the multitude of partner polypeptides suggests that the alteration of ALL-1 function is the critical outcome. This contention is supported by the identification of several cases of AML in which *ALL-1* is rearranged with no involvement of a partner gene (Schichman *et al.*, 1994). Based on the identification of putative DNA binding domain(s) in *ALL-1* and in analogy with other gene fusions in which transcription factors were involved (Kamps *et al.*, 1990; Hunger *et al.*, 1992; de Thé *et al.*, 1991; Kakizuka *et al.*, 1991), Tkachuk *et al.* (1992) proposed that the ALL-1/ENL protein is a hybrid transcription factor in which the AT hook motifs would localize the hypothetical transactivation domain of ENL to regulatory sites or enhancer cores. This model, which emphasizes gain of function, predicts that the partner polypeptides would contain transactivation domains, and that the hybrid proteins will be directed to normal targets of ALL-1 (possibly also to additional sites).

In recent experiments with the ENL partner polypeptide (Rubnitz *et al.*, 1994), it was shown that the C-terminal segment of ENL has a transactivation capacity in lymphoid and myeloid cells. Together with the recent finding that the yeast homologue of AF-9 and ENL is found in a complex with yeast RNA polymerase II (Werel *et al.*, submitted for publication), this transcriptional transactivation experiment suggests that AF-9, ENL, and possibly AF-4 polypeptides interact with the transcriptional machinery of the cell. Possibly these polypeptides would affect the transcription regulatory function of the ALL-1 moiety. Currently there is no evidence that the other three partner polypeptides—AF-6, AF-17, and AF-1P—are involved in transcription. In fact, AF-1P and probably AF-6 are cytoplasmic or cytoskeleton-associated proteins and presumably do not play a role in RNA synthesis in their normal context. These three polypeptides, though, contain domains which are presumably involved in protein–protein interactions (dimerization): the leucine zipper in AF-17, the α-helical coiled coil domain of AF-1P, and the region with homology to the tail domain of myosin heavy chain (which includes the GLGF motif) in AF-6.

The fact that in the six 11q23 chromosome translocations studied the products encode chimeric proteins and not a truncated ALL-1 protein indicates that the partner polypeptides have a significant function. On the other hand, the identification of several AML patients in whom the ALL-1 protein undergoes partial duplication with no involvement of a

partner polypeptide (Schichman *et al.*, 1994) indicates that in certain circumstances the latter is dispensable. These last results can be perhaps explained by invoking a loss of function model. According to this, ALL-1 rearrangements result in inactivation of the ALL-1 protein. The partner polypeptide augments this loss perhaps by blocking the activity of the normal ALL-1 protein presumably present in the leukemic cells. Partner polypeptides such as AF-9, ENL, and AF-4 might interact avidly with and sequester transcription factors necessary for activity of the normal protein. Partner polypeptides such as AF-17, AF-6, and AF-1P might act through their dimerization domains to form homodimers or heterodimers which could efficiently occupy ALL-1 DNA target sites and prevent transcription of these targets. This model would explain the requirement for the product of the der 11 chromosome by its role in inhibiting the activity of the normal ALL-1 protein. In this model, tandem duplication of an N-terminal segment of the ALL-1 protein is sufficient for inactivation of the latter, as well as for blocking of the normal species. It is also possible that in cells with that last abnormality the normal allele of *ALL-1* is not present or is not expressed, but this has yet to be investigated.

Although chromosome translocations are usually associated with overexpression or activation of oncogenes, there is a recent example for a translocation which apparently involves loss of function and a dominant negative effect. Thus, in the t(15;17) chromosome translocation associated with acute promyelocytic leukemia, a major effect of the fusion protein PML/RAR is sequestering of the normal PML protein and inhibiting its organization into nuclear macromolecular organelles (Dyck *et al.*, 1994; Weis *et al.*, 1994).

VIII. Conclusions

Following the cloning of the *ALL-1* gene it became apparent that the latter is rearranged in the vast majority of 11q23 abnormalities. These rearrangements usually fuse *ALL-1* to one of a series of genes positioned on many chromosomes and result in synthesis of chimeric RNAs and most likely of chimeric proteins. The fused RNA encoded by the derivative 11 chromosome has been identified in all patients and cell lines examined so far, and is therefore thought to constitute the critical product of the aberrations. Among the six partner polypeptides characterized, only two show sequence homology. The absence of a common function motif in these polypeptides allows only speculations with regard to their role in the chimeric ALL-1 proteins. A major related question is whether *ALL-1* rearrangements result in gain or loss of function

of this gene. The homology of ALL-1 to *Drosophila trithorax* makes it very likely that *ALL-1* controls expression of a variety of genes, possibly including one or more involved in hematopoiesis. *ALL-1* rearrangements probably result in overexpression or loss of expression of such genes. The identification of *ALL-1* target genes and the determination whether ALL-1 acts (in conjunction with other proteins) through local changes in chromatin structure will be challenging issues during the next years. On the clinical side, the development of PCR-based tests will now enable examination of residual disease in patients in remission. In addition, results from initial studies suggest that it will be beneficial to test for ALL-1 rearrangements in infant acute leukemias which do not show 11q23 karyotypic alterations. Finally, it remains to be shown whether ALL-1 is involved in cancers other than acute leukemia.

ACKNOWLEDGMENTS

Studies done in the laboratories of the authors and cited in this review were supported by NCI Grants CA 39860, CA 50507, and CA 42232 and by grants from the Israeli Academy of Sciences and Israel Cancer Research Fund.

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ROLE OF SCATTER FACTOR AND THE *c-met* PROTOONCOGENE IN THE PATHOGENESIS OF AIDS-ASSOCIATED KAPOSI'S SARCOMA

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I. Introduction

Kaposi's sarcoma (KS) is a complex mesenchymal neoplasm of suspected vascular endothelial cell origin that until recently was reported to develop almost exclusively in elderly males of Jewish and Italian ancestry. With the emergence of the acquired immune deficiency syndrome (AIDS) in the early 1980s, a highly lethal and disseminated form of KS emerged among populations of homosexual and bisexual men suffering from AIDS. As more cases of this particularly virulent form of KS were reported, two obvious questions arose. First, what is the mechanistic link between HIV infection and the increased prevalence of this hither-to-for rare neoplasm? Second, what are the histogenetic and pathogenetic factors that contribute to the development of KS in this particular setting of AIDS? In recent years, much emphasis has been placed in searching for an etiologic link between the HIV virus as well as other opportunistic infectious agents with transforming potential and AIDS-associated KS. Also of interest and equal importance is elucidating the role of aberrant cytokine networks operative in the evolution of AIDS-

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associated KS. Despite much intensive research activity directed at HIV-related diseases, this unique form of KS continues to be an enigma.

In this review we will address both of these issues and outline recent experimental evidence that suggests a potential role for a novel cytokine termed scatter factor (SF), also known as hepatocyte growth factor, and its receptor the c-*met* protooncogene, in the pathogenesis of KS. Although SF/c-*met* has only recently been identified as a major growth factor produced by HIV-infected T cells and KS tumor cells, several lines of evidence suggest that conversion of SF/c-*met* from a normal paracrine to an aberrant autocrine growth regulatory system may play a central role in orchestrating a key series of pathogenetic events in the evolution of KS.

II. Epidemiology of AIDS-Associated Kaposi's Sarcoma

KS was first brought to attention in 1872 as a rare cutaneous malignancy that developed predominantly in elderly Mediterranean or Eastern European Jewish and Italian men (Kaposi, 1872; Oettle, 1962, Davies and Loethe, 1976; Flotte *et al.*, 1984; Rothman, 1962; Templeton, 1976). This form of the disease now referred to as "classical" KS often presents initially as solitary or multicentric violaceous macules and nodules that affect the extremities (Friedman-Kien and Saltzman, 1990; Buchbinder and Friedman-Kien, 1991). The disease typically runs a relatively benign course although after many years it may progress to involve more distant skin sites as well as internal organs (Friedman-Kien *et al.*, 1989; Friedman-Kien and Ostreicher, 1984). Another more aggressive form of the disease affecting predominately young adult African males termed "African endemic KS" was first recognized in 1940 in Central Africa (Oettle, 1962; Rothman, 1962; Loethe and Murray, 1962; Olweny, 1984). Unlike classical KS, this "endemic" form can run the gamut from a benign localized nodular lesion to a lethal, rapidly progressive disseminated disease with local invasion and early visceral and lymph node involvement (Davies and Loethe, 1976; Taylor *et al.*, 1971; Templeton and Bhana, 1975; Friedman-Kien *et al.*, 1989). A third form of KS, first reported in the 1970s, develops in a small percentage of immunosuppressed transplant patients, particularly in individuals treated with corticosteroids (Harwood *et al.*, 1979; Penn, 1979; Klepp *et al.*, 1978; Gange and Jones, 1978, Kapadia and Krause, 1977; Klein *et al.*, 1974). These KS lesions are for the most part limited to the skin and oral mucosa, but on occasion, may disseminate widely in the skin and involve internal organs. Interestingly, it has been reported that when

immunosuppressive therapy was discontinued the KS lesions regressed in some of these patients (Klein *et al.*, 1974; Leung *et al.*, 1981).

In 1980 it soon became apparent that KS that developed in patients with AIDS was a very different disease. It was a highly aggressive and lethal disease in this patient population, especially in homosexual and bisexual men with AIDS where it was reported to account for up to 95% of all AIDS-associated malignancies (Friedman-Kien, 1981; Friedman-Kien *et al.*, 1981; Gottlieb *et al.*, 1981; Hymes *et al.*, 1981; Buchbinder and Friedman-Kien, 1991; Tappero *et al.*, 1993). The clinical course of KS in HIV-infected men is often more aggressive than in patients with other non-HIV-related forms of KS. The lesions are often multicentric in the skin and oral mucosa, but may also involve internal organs and lymph nodes. Approximately 20% of patients suffer significant morbidity from their disease and succumb to complications due to KS. Although all available treatments help control the lesions, none have been shown to effectively lengthen survival (Lemp *et al.*, 1990; Gill *et al.*, 1990; Bacchetti *et al.*, 1988; Rabkin *et al.*, 1990; Errante *et al.*, 1991; Payne *et al.*, 1990).

III. Agents Implicated in the Etiology of Kaposi's Sarcoma

The unusual prevalence of KS in men with sexually acquired AIDS has prompted much speculation regarding the possible role of environmental, genetic, or infectious cofactors unique to this patient population (Beral *et al.*, 1990; Friedman-Kien *et al.*, 1990; Beral *et al.*, 1991). Perhaps the most compelling data are those emerging from epidemiological studies that suggest a role for an infectious agent distinct from HIV-1 but which is more readily transmitted during homosexual than heterosexual activity (Friedman-Kien *et al.* 1990; Biggar *et al.*, 1989). Although AIDS–KS is rare in females with AIDS, it is more common in female sexual partners of bisexual men than among heterosexual drug abusers (Biggar *et al.*, 1989; Beral *et al.*, 1990; Biggar *et al.*, 1985). Several candidates have been proposed as the putative KS agent(s).

Cytomegalovirus (CMV), a well-known cause of immunosuppression in humans and animals, has been implicated previously in the classical and African endemic forms of KS (Giraldo *et al.*, 1972; Filia *et al.*, 1975; Fenoglio and McDougall, 1984). A high prevalence of serum antibodies to CMV has been reported in HIV-infected men and immunosuppressed renal transplant patients. More recently, however, studies have failed to detect CMV DNA sequences in KS tumor tissue samples, nor was CMV or its DNA detected by *in situ* hybridization, immunostaining,

or electron microscopy in tumor biopsies from African's with endemic KS (Delli Bovi *et al.*, 1986). This was despite the fact that they had elevated serum antibodies to CMV (Haverkos, 1987; Ambinder *et al.*, 1987).

Anogenital condyloma accuminata which develops in association with human papillomavirus (HPV) is one of the most prevalent sexually transmitted viral infections (Huang *et al.*, 1992a; Tappero *et al.*, 1993). Certain strains of HPV, in particular strains 16 and 18, are closely associated with cervical intraepithelial neoplasia and cervical carcinoma (Vousden, 1989; Braun, 1994). It has been recently reported that women with HIV have a higher rate of cervical abnormalities and the prevalence of HPV infection appears to be higher in HIV-seropositive women than in seronegative women (Provencher *et al.*, 1988; Schrager *et al.*, 1989; Schafer *et al.*, 1991; Vermund *et al.*, 1991; Johnson *et al.*, 1992). Huang *et al.* (1992a) have demonstrated by PCR analysis the presence of the highly conserved E6 region of HPV-16 in up to 30% of homosexual AIDS patients and in two continuous cell cultures derived from AIDS–KS lesions (Huang *et al.*, 1992a). Nickoloff *et al.* (1992) have also demonstrated in paraffin-embedded archival specimens positive immunostaining for HPV in dermal dendrocytes in up to 70% of KS lesions.

There is also evidence that the HIV-1 virus itself may play a role in the development of AIDS–KS, although this is still controversial (Delli Bovi *et al.*, 1986; Kovacs *et al.*, 1989; Huang *et al.* 1992a). HIV-1 transcripts have been detected in AIDS–KS lesions in which it has been localized to factor XIIIa-positive dermal dendritic cells (Mahoney *et al.*, 1991). It has also been shown that KS-derived cells can be readily infected in culture (Nakamura *et al.*, 1988). Of great interest are recent studies with a transgenic mouse model that contains the HIV-1 tat sequence. These animals spontaneously develop hepatocellular carcinoma and dermal tumors that bear striking resemblance to human KS (Vogel *et al.*, 1988; Ensoli *et al.*, 1990; Vogel *et al.*, 1991). Furthermore, the HIV tat protein has been shown to stimulate the proliferation of cultured spindle cells derived from KS lesions and that anti-tat antibodies can partially block the mitogenic effect of HIV-infected T cell conditioned media (Ensoli *et al.*, 1990). Despite these compelling, albeit circumstantial data, the etiologic agents responsible for AIDS-associated KS still remains uncertain.

IV. The Kaposi's Sarcoma Phenotype

The histopathological features of AIDS-associated KS are complex, ranging from early-stage lesions that resemble reactive granulation tis-

sue to late-stage lesions that more closely resemble angiosarcomas and/or fibrosarcomas (Friedman-Kien, 1981; Gottlieb and Ackerman, 1982; McNutt *et al.*, 1982). The KS lesion is generally classified as a "spindle cell" neoplasm that consists of a mixture of fibroblasts, endothelial cells, and inflammatory cells (Friedman-Kien, 1981; Gottlieb and Ackerman, 1982; McNutt *et al.*, 1982). Given the complex nature of the KS lesion it is not unexpected that the origin of the KS cell remains controversial. Recently, however, studies from several laboratories concerned with the immunobiology of KS have begun to shed some light on the KS lineage. With the ability to grow KS cells *in vitro* using CM from HIV-infected T lymphocytes, new evidence has emerged revealing the histogenic origin and phenotype of KS tumor cells (Nakamura *et al.*, 1988). All KS cells express factor XIIIa, CD14, VCAM-1, and ICAM-1 (Modlin *et al.*, 1983; Rutger *et al.*, 1986; Bechstead *et al.*, 1986; Jones *et al.*, 1986; Nickoloff and Griffiths, 1989a,b; Yang *et al.*, 1994; Huang *et al.*, 1994; Schaumburg-Lever *et al.*, 1994). Other reports suggest that KS cells can also express CD34, ELAM-1, and factor VIII (Zhang *et al.*, 1994).

In addition to expression of these surface antigens on KS spindle cell populations, these same markers serve to identify another prominent component of KS lesions, the dermal dendrocyte (Nickoloff and Griffiths, 1989a,b, 1991; Gray *et al.*, 1991). This has lead some investigators to speculate that the putative KS cell may be the dermal dendritic cell or another cell of monocyte/macrophage lineage (Nickoloff and Griffiths, 1989a,b). In a more recent study Zhang *et al.* (1994) examined antigen expression in KS tumors by immunocytochemistry and mRNA by *in situ* hybridization. These investigators found expression of the endothelial cell markers ELAM-1, thrombomodulin, and tissue factor in KS lesions from AIDS and non-AIDS patients, thus implicating a vascular origin for KS. Indirect evidence has also been provided by O'Connell *et al.* (1991) and O'Connell and Rudmann (1993) who showed that a murine line of SV40-transformed endothelial cells when transplanted to nude mice grew as spindle cell neoplasms that bore striking resemblance to human KS tumors.

In addition to the histological and immunophenotypic complexity associated with KS lesion, these tumors have been shown to produce a wide array of cytokines (Ensoli *et al.*, 1989, 1990, 1991a,b, 1992). Many of these mediators have been shown to function as autocrine and paracrine growth factors and are thus suspected of contributing either directly or indirectly to the growth and progression of KS cells *in vivo*. KS cells constitutively express high levels of mRNA for basic fibroblast growth factor, IL-1β and IL-6, and oncostatin-M, moderate levels of

GM-CSF, TGF-β, PDGF-A and -B, vascular endothelial growth factor, and lower levels of acidic FGF and IL-1α (Ensoli *et al.*, 1992; Huang *et al.*, 1992b, 1993; Li *et al.*, 1993). Many of these cytokines have been shown to have potent growth-promoting effects for not only KS tumor cells but also for normal host cells. These host cell targets include among others, vascular endothelial cells, smooth muscle cells, and fibroblasts in which one or more of these mediators have been shown to stimulate their proliferation and chemotaxis *in vitro* and neovascularization *in vivo* (Salahuddin *et al.*, 1988). Another potential growth factor for KS tumor cells is the HIV-tat protein. Studies with the tat transgenic mouse model have demonstrated that these animals progressively develop dermal tumors that closely resemble human KS. Although neither the *tat* protein nor its mRNA transcripts were detected in these tumors there is nevertheless strong evidence that the *tat* gene product may play a central role, perhaps in a paracrine manner in stimulating the growth of KS tumors (Vogel *et al.*, 1988; Ensoli *et al.*, 1990; Vogel *et al.*, 1991).

V. Endothelial Cells Undergo Phenotypic Conversion to Kaposi's Sarcoma-like Tumor Cells When Exposed to HIV-Infected T Lymphocyte-Conditioned Media

During the course of our studies of KS tumors we asked whether the culture media used to propagate KS cells would have any influence on normal vascular endothelial cells. We found that when HTLV-II conditioned media was added to cultures of human umbilical vein endothelial cells (HUVEC), within 24 hr the normal epithelioid cobble stone morphology of HUVEC changed to a "spindle"-shaped appearance with some cells demonstrating prominent dendritic processes. We refer to this striking morphological alteration as "phenotypic conversion" (Naidu *et al.*, 1994). Not only did this "transdifferentiation" process involve a change in the appearance of HUVEC, but there also occurred several molecular alterations including induction of factor XIIIa, appearance of ICAM-1, and the production of several cytokines unique to KS cells. Figure 1 is a series of photomicrographs demonstrating the morphological and immunophenotypic alterations (phenotypic conversion) induced in cultures of normal HUVEC following their exposure to KSGM.

We found that KS tumor expressed IL-1β, IL-6, IL-8, TGF-α, TGF-β, and ICAM-1 mRNAs, results consistent with those previously reported. Interestingly, HUVECs grown in conventional media failed to express IL-1β, IL-6, IL-8, ICAM-1, and IL-10, GM-CSF, and TGF-α mRNAs, but when transferred to KS growth medium (KSGM) for 18 hr the

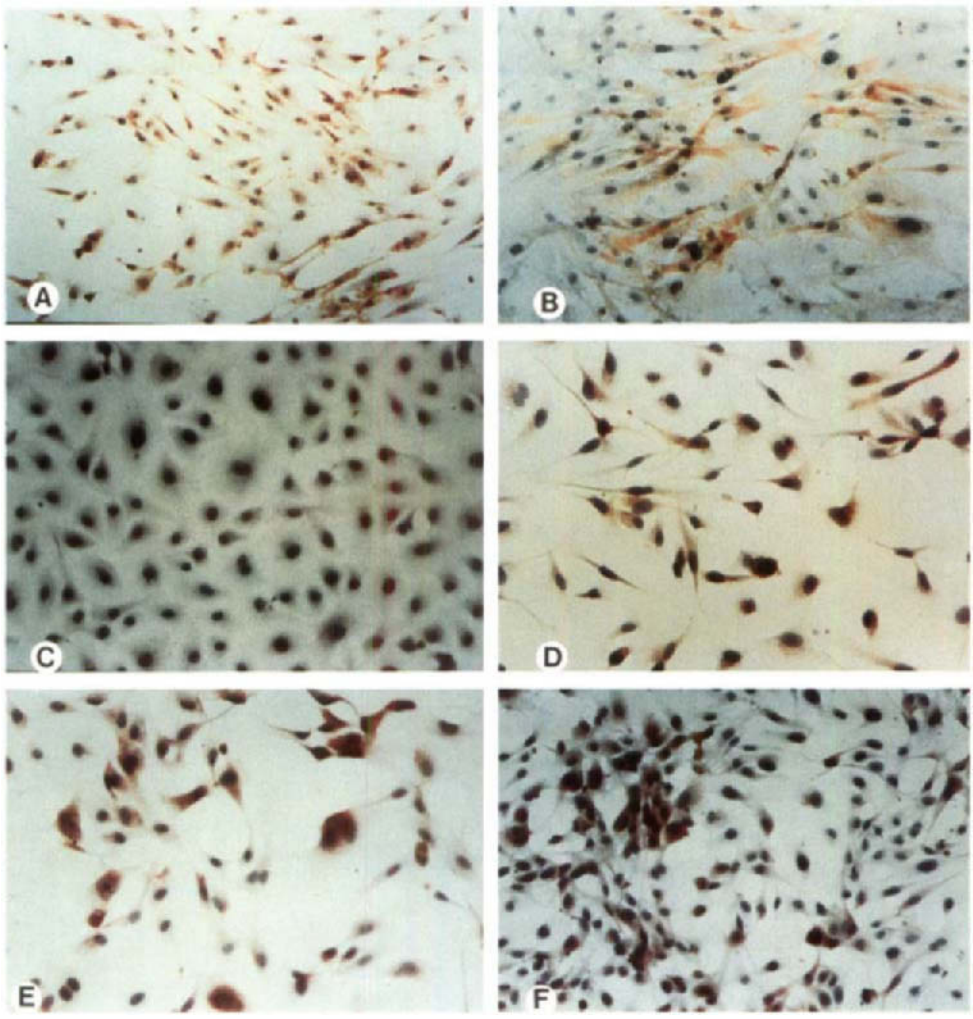

FIG. 1. Conversion of human endothelial cells to a KS tumor cell-like phenotype. Cell cultures were immunostained as described previously (Naidu *et al.*, in press). Positive cytoplasmic reactivity is indicated by red (alkaline phosphatase) staining. KS tumor cells grown in KSGM were stained for factor XIIIa (1A) and for VCAM-1 (1B). HUVECs were stained for factor XIIIa before (1C) and after (1D) a 24-hr incubation in KSGM. HUVECs exposed to 250 units/ml of purified native murine scatter factor (SF) (1E) or recombinant human SF (1F) for 24 hr were stained for factor XIIIa. Magnification, ×60.

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transdifferentiated HUVECs began to express IL-1β, IL-6, IL-8, ICAM-1, and IL-10, GM-CSF, and TGF-α mRNAs, the molecular signature of KS tumor cells. The process of phenotypic conversion was, however, not entirely complete as discordance was observed for IL-10, GM-CSF, and TGF-α. Taken together, the expression of these KS-related phenotypes was remarkable for demonstrating the ability of HTLV-II CM to induce normal HUVECs to express morphological, immunologic, and cytokine phenotypes reminiscent of authentic KS tumors. Table I summarizes the immunological and cytokine profiles of cultured KS cells and KS tumors *in vivo* and those expressed by normal HUVEC and phenotypically converted HUVEC.

We then sought to identify components in KSGM responsible for this conversion phenomenon. Our initial screening of KSGM revealed high titers of SF, a serum-derived mitogen with potent growth-promoting and *in vivo* angiogenic activity (Bussolino *et al.*, 1992; Grant *et al.*, 1993). Our interest in SF and c-*met* stemmed from our ongoing studies of the mechanisms underlying the aberrant neovascularization that accompanies the angiogenesis-dependent and HIV-1-associated disease psoriasis (Grant *et al.*, 1993). Our studies revealed positive immunoreactivity for SF in dermal dendrocytes and at sites of developing new capillary blood vessels in psoriatic lesions. Furthermore, as we contemplated the morphological changes by endothelial cells during neovascularization, it occurred to us that SF may be important in our studies of KS.

We examined two different batches of HTLV-II CM and found that it contained 218 and 154 scatter units/ml (i.e., ability to scatter Madin–Darby canine kidney cells) and ELISA results indicated high levels of SF antigen (10.5 ng/ml). SF production rates for T cells infected with HTLV-II were estimated to be 120 units (U)/10^6 cells /48 hr compared to 20–80 U/10^6 cells /48 hr for six different fibroblasts lines. PCR analysis confirmed that the HTLV-II-infected T cells expressed SF mRNA (Naidu *et al.*, in press). Neither resting peripheral blood T cells nor the HUT 78 T cell line produced detectable SF by bioassay or ELISA. Interestingly, when HUT T cells were infected by the HIV-1 IIIb virus PCR analysis revealed induction of SF mRNA. Furthermore, when KSGM was assayed in the rat corneal bioassay for angiogenic activity, it induced prominent neovascularization. This angiogenic activity was almost completely abrogated by preincubating KSGM with neutralizing antibody (Ab) to SF indicating that SF was the major angiogenic mediator in KSGM (Table II and Figure 2). Also, the phenotypic conversion phenomenon induced by KSGM could be mimicked with the addition of highly purified SF or recombinant HGF to HUVEC growth medium. Thus, it became clear to us that SF present in KSGM was responsible for

TABLE I

IMMUNOPHENOTYPE, CYTOKINE, AND c-*met* RECEPTOR PROFILE OF KAPOSI'S SARCOMA CELLS, DERMAL DENDRITIC CELLS, PHENOTYPICALLY CONVERTED (PC) ENDOTHELIAL CELLS, (EC) AND NORMAL (N) EC

Marker	KS tumor cells	Dendritic cells	PC EC	N EC
Immunological				
Factor XIIIa	+	+	+	−
Factor XIIIs	−	−	−	+
Factor VIII	−	−	−	+
CD 4	+	+	ND[a]	−
CD 14	+	+	+	−
CD 31	+	+	+	+
CD 34	+	+	+	+
E-selectin	−	ND	ND	±[b]
ELAM-1	±	+	−	±
ICAM-1	+	−	+	+
Thrombomodulin	+	ND	ND	+
Tissue factor	+	ND	ND	+
VCAM-1	+	+	+	±
Cytokines				
aFGF	+	−	ND	−
bFGF	+	ND	+	+
GM-CSF	+	+	−	−
IL-1α	+	ND	ND	−
IL-1β	+	ND	+	−
IL-6	+	ND	+	−
IL-8	+	ND	+	−
IL-10	+	ND	−	−
Onco-M	+	ND	ND	−
PDGF-A and B	+	ND	ND	+
TGF α	+	ND	−	−
TGF β	+	ND	+	+
SF (HGF)	+	+	+	−
Receptor				
c-*Met*	+	−	+	−

[a] ND, not determined.

[b] Expressed either at low levels or after exposure to cytokines.

both the angiogenic activity in KSGM and the phenotypic conversion of normal of HUVECs to KS-like tumor cells.

To explore the *in vivo* relevance of these findings we analyzed biopsies of KS tumors for expression of SF and its c-met receptor (Naidu *et al.*, 1994). We observed positive immunostaining of SF in lymphoid cells, perivascular dendritic cells, and interstitial spindle cells. Samples

TABLE II
NEOVASCULAR RESPONSES INDUCED BY KAPOSI SARCOMA GROWTH MEDIUM (KSGM) AND RECOMBINANT HUMAN HEPATOCYTE GROWTH FACTOR (rhHGF) AND THEIR INHIBITION BY ANTI-SCATTER FACTOR ANTIBODIES

Content of hydron pellet	Corneal neovascularization positive responses (%)
Controls	
PBS	0/3 (0)
bFGF (100 ng)	3/3 (100)
rhHGF (100 ng)	2/2 (100)
KSCM	4/4 (100)
Chicken anti-HGH Ab	0/4 (0)
Rabbit anti-HGF Ab	0/3 (0)
bFGF (150 ng) + rabbit Ab	0/3 (0)
KSGM + Ab	
rhHGF (100 ng) + chicken Ab (1:20)	1/3 (33)
rhHGF (100 ng) + rabbit Ab (1:200)	1/5 (20)
KSCM + chicken Ab	2/5 (40)
KSCM + rabbit Ab	1/4 (25)

showed strong positive c-met staining of pili-erector smooth muscle bundles, as well as pericytes, HUVECs, dermal dendritic cells, and interstitial spindle-shaped tumor cells. Cytospin preparations of KS tumor cells and HUVEC were also strongly positive for c-met but not for Ab control. After verifying that KS cells expressed c-met mRNA we asked whether SF had any mitogenic effects on KS cells. Three KS tumor lines were examined and all were stimulated to proliferate when either highly purified SF or recombinant HGF was added to the culture media. Even when compared to optimal concentrations of the two other well-characterized mitogens, IL-6 and oncostatin-M, SF was as least as potent if not slightly better in stimulating KS tumor cell growth.

Taken together, these results suggest that SF may play an important role in the pathogenesis of KS. As previously mentioned, mice that have been genetically engineered to overexpress the HIV–tat protein spontaneously develop cutaneous KS-like lesions and exhibit a high incidence of hepatocellular carcinoma in the absence of detectable HIV–tat transcripts or protein in the tumors. These investigators proposed that the liver tumors were likely mediated by extrahepatic growth factors (Vogel *et al.,* 1991). We now propose that SF/c-met is a likely candidate for these experimental findings. However, before describing how we envision SF

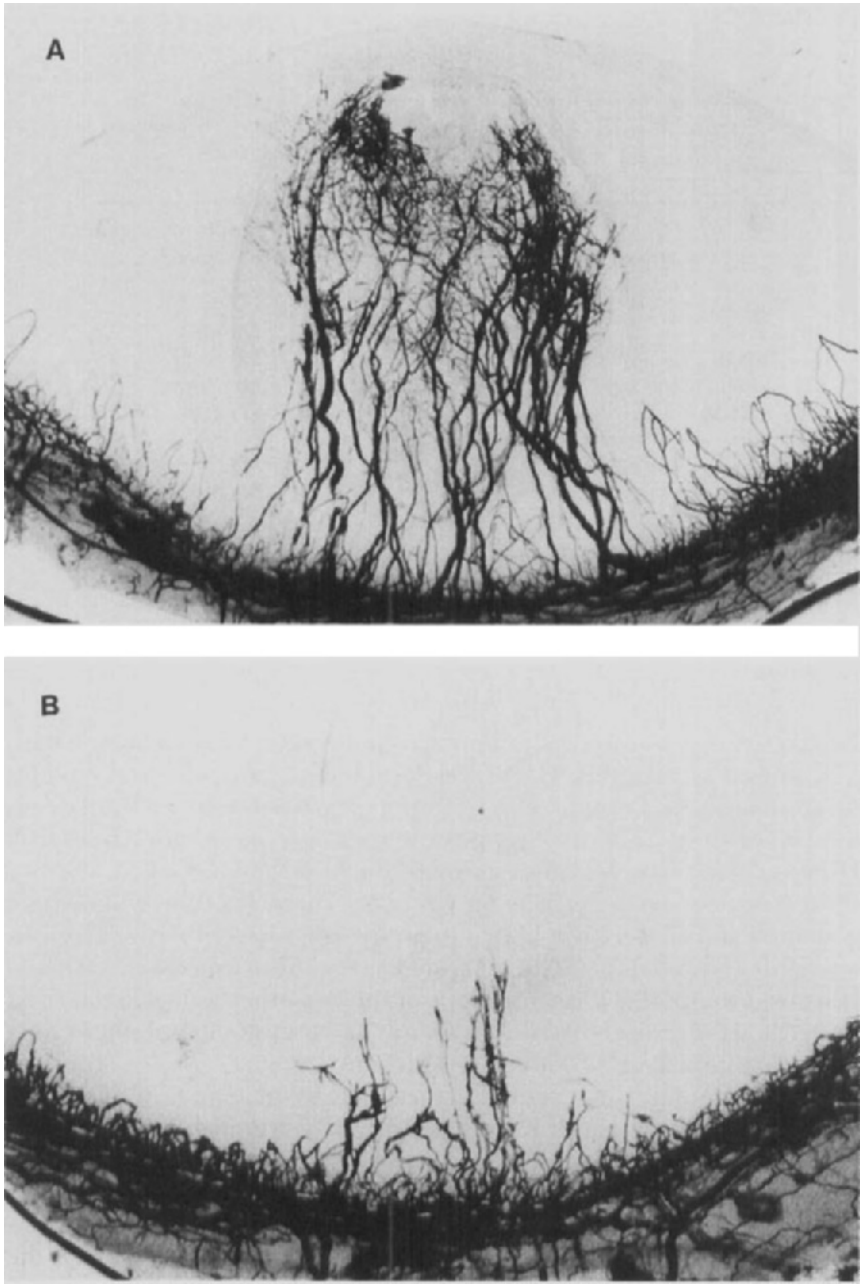

FIG. 2. Angiogenic responses induced in rat corneas by KS CM. Colloidal carbon perfused corneas 7 days after implantation of Hydron pellet containing 20X concentrated KS CM (A) and KS GM with rabbit Ab to HGF (B). Note the vigorous neovascular response in A compared with the markedly suppressed response in B. Magnification, ×15.

is involved in the pathogenesis of KS, we will provide a brief summary of some of the function of SF and c-met and describe some recent work that implicates this cytokine and its receptor in tumor development.

VI. Scatter Factor and the c-*met* Receptor: A Paracrine and Autocrine Growth Signaling System

SF was first identified by Stoker and Perryman (1985) as a fibroblast-derived cytokine that induced dispersion, spreading, and enhanced the motility of normally cohesive epithelial cell populations (Stoker, 1984; Stoker and Perryman, 1985; Stoker *et al.*, 1987; Rosen *et al.*, 1989). It is a heparin-binding glycoprotein consisting of a 60-kDa heavy α-chain and a 30-kDa light β-chain linked by disulfide bonds (Gherardi *et al.*, 1989; Rosen *et al.*, 1990; Weidner *et al.*, 1990). SF belongs to a class of kringle-containing proteins and exhibits about 38% amino acid sequence homology to the proenzyme plasminogen (Nakamura *et al.*, 1989). SF is also closely related to macrophage-stimulating protein (MSP), a serum protein that renders macrophages responsive to chemoattractants (Yoshimura *et al.*, 1993). It has been demonstrated by functional, biochemical, and sequence analysis that SF and hepatocyte growth factor are one and the same protein and are indistinguishable ligands for the c-*met* protooncogene tyrosine kinase receptor (Furlong *et al.*, 1991; Weidner *et al.*, 1991; Bottaro *et al.*, 1991; Naldini *et al.*, 1991; Bhargava *et al.*, 1992).

In addition to stimulating cell motility, SF can influence the growth and differentiation of a variety of epithelia including mammary and renal tubular epithelial cells, keratinocytes, bronchial epithelia, and biliary epithelial cells (Rubin *et al.*, 1991; Kan *et al.*, 1991; Rosen and Goldberg, in press). SF has also been shown to function as a morphogen where it induces kidney and mammary epithelial cells grown on collagen gels to rapidly organize into branching tubules and mammary duct-like structures, respectively (Montesano *et al.*, 1991; Tsarfaty *et al.*, 1992). When vascular endothelial cells are grown on the reconstituted basement membrane Matrigel, SF rapidly induces endothelial cells to organize into capillary-like tubes (Rubin *et al.*, 1991; Grant *et al.*, 1993). *In vivo* SF is a potent mediator of angiogenesis (Bussolino *et al.*, 1992; Grant *et al.*, 1993; Naidu *et al.*, 1994).

The SF receptor, the c-*met* protooncogene, is a tyrosine kinase growth-factor receptor that is found predominantly on epithelial cells and on some mesenchymal cells, i.e. endothelial cells (Park *et al.*, 1987; Rosen and Goldberg, in press). Although mesenchymal in origin, endothelial cells display certain features characteristic of epithelial cells including the formation of gap and tight junctions, a flattened squamous-like

like morphology, and the ability to organize into tubular structures. Thus, endothelial cells have the potential of serving as both a source and a target of SF. Binding of SF to c-met induces phosphorylation of an intracellular tyrosine acceptor site, activation of tyrosine kinase, formation of a signaling and transduction of the signal to the nucleus. The molecules that transduce the c-met signal and the target genes induced by that signal are largely unknown (Rosen and Goldberg, in press).

Both SF and the c-met receptor normally function in a paracrine signaling system in which mesenchymal cells produce the SF ligand that binds to its receptor on epithelial cells. This would suggest that SF/c-*met* plays an important role in epithelial/mesenchymal communication. Recent work from the Vande Woude laboratory has revealed how alterations in the expression of c-*met* can convert a normal paracrine signaling system to an autocrine pathway leading to dramatic alterations in cell differentiation and neoplasia (Faletto *et al.*, 1991; Rong *et al.*, 1992, 1993a,b; Tsarfaty *et al.*, 1994). It has been demonstrated that c-*met* protooncogene is overexpressed in most spontaneously transformed NIH/3T3 and can mediate NIH/3T3 tumorigenicity (Cooper *et al.*, 1986; Hudziak *et al.*, 1992). While c-*met* is normally expressed at low levels in primary fibroblasts in the presence of endogenously expressed SF, this results in an autocrine interaction and disregulated cell growth. Rong *et al.* (1993) showed that c-*met* overexpression occurs frequently in various types of human sarcomas thus implicating c-*met* in tumorigenicity. In a more direct test of this hypothesis Tsarfaty *et al.* (1994) demonstrated that when c-*met* and SF were coexpressed in NIH/3T3 fibroblasts the cells became tumorigenic in nude mice. Furthermore, they found that the resultant tumors displayed a lumen-like morphology, contained carcinoma-like focal areas with intercellular junctions resembling desmosomes, and coexpressed the epithelial and mesenchymal cytoskeletal markers cytokeratin and vimentin. Table III lists some of the more well-known sources of SF and c-*met*. Thus, SF and c-*met* can have prominent growth-promoting as well as transdifferentiating activity on a number of different cell types. In the next section we explore this important ligand–receptor pair in KS and present our novel hypothesis for the pathogenesis of KS.

VII. Proposed Role for Scatter Factor and c-*met* in Kaposi's Sarcoma Carcinogenesis

We hypothesize that the unique pattern of distribution of SF and c-*met* in KS tumors and the transient morphological, functional, and immu-

TABLE III
CELLULAR DISTRIBUTION OF SCATTER FACTOR AND *c-met*

Paracrine signaling (normal cells) cells expressing		Autocrine signaling (tumors)[a] cells expressing SF and c-*met*
SF	c-*met*	
Fibroblasts	Biliary epithelium	Chondrosarcoma
Macrophages	Bronchial epithelium	Fibrosarcoma
Platelets	Endothelial cells	Kaposi's sarcoma[b]
Smooth muscle	Hepatocytes	Leiomyosarcoma
T lymphocytes	Keratinocytes	Osteogenic sarcoma
	Mammary epithelium	Rhabdomyosarcoma
	Renal tubular epithelium	Synovial sarcoma
		Melanoma

[a] Data from Rong *et al.*, 1993.
[b] Data from Niadu *et al.*, 1994.

nophenotypic alteration that are induced in normal endothelial cells in response to SF/c-*met* reflect alterations that are central to the pathogenesis of AIDS-associated KS. It is well established that neoplasms, including KS, evolve through a series of sequential steps that by convention have been defined operationally as initiation, promotion, and progression (Nowell, 1976; Farber and Cameron, 1980). When viewed from this perspective the increased incidence of KS in patients with sexually acquired AIDS can perhaps be more readily explained. Figure 3 depicts our model of how we believe SF and c-*met* participate in the pathogenesis of AIDS-associated KS.

In adult organisms endothelial cells (EC) normally exhibit a low turnover rate; usually on the order of several months or years (Engerman *et al.*, 1967). Under these conditions the chances of a somatic mutation occurring in this quiescent cell population are exceedingly rare. This is the mechanism that we believe best explains the sporadic incidence of KS observed in the "classical" form of the disease (Model 1). In contrast, in AIDS-associated KS (Model 2), T cells infected with the HIV virus would provide a constant supply of SF that could function as a potent "promoting" agent for carcinogen (HPV or HIV)-initiated EC. This would be the scenario if the EC of an HIV-infected individual acquired a carcinogenic strain of an organism such as HPV (HPV E6/E7) or the HIV virus itself (HIV–tat). In these instances mutation might result directly or indirectly in production of SF along with overexpression of c-*met*. EC would now be both a source of and a target for SF. What is

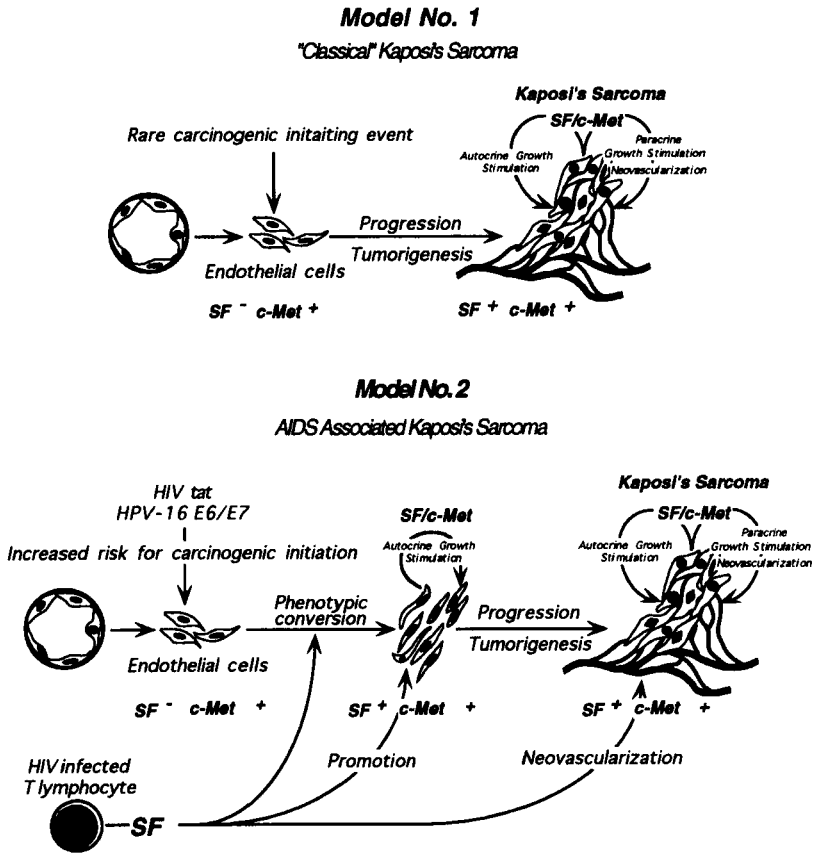

FIG. 3. Model depicting the role of SF and the c-*met* protooncogene in the pathogenesis of AIDS-associated KS.

normally a tightly regulated paracrine system would now be converted to an disregulated autocrine signaling system. These events would drive the carcinogenic process by promoting the growth of "phenotypically converted," "initiated" endothelial cells. A similar mechanism could be envisioned for an individual whose EC already contained a transforming viral sequences and who then acquired the HIV virus. In this setting the HIV-infected T cells would provide a rich source of SF that would promote the growth of initiated, mitoticly dormant EC. As a result of coexpressing SF and c-*met* the phenotypically converted, initiated endothelial cells would be able to stimulate their own growth (autocrine) and promote the growth and recruit adjacent host cells (paracrine) into the

developing KS tumor. Not only is SF able to act directly on endothelial cells to promote their growth but it is also able to act indirectly by stimulating neovascularization. We speculate that the acquisition of SF-mediated angiogenic activity by EC occurs early in this process, at the stage of phenotypic conversion. In this instance, EC would behave as "preneoplastic cells;" they would display angiogenic activity, a trait that is necessary although not sufficient for tumor formation (Gimbrone *et al.*, 1976a,b; Brem *et al.*, 1977, 1978; Folkman, 1985; Moroco *et al.*, 1990).

VIII. Conclusions and Summary

Kaposi's sarcoma is a highly lethal tumor in patients with sexually acquired AIDS. A number of etiologic agents have been implicated in the development of this disease in this patient population and there is ample evidence that aberrant production of and responsiveness to KS tumor and host cell-derived cytokines plays a central role in the pathogenesis of AIDS–KS. In this review we propose that aberrant expression SF and c-*met* is central to the pathogenesis of KS. KS is a serious and life-threatening consequence for many patients with AIDS. Unfortunately, current therapeutic strategies for the treatment of this complex neoplasm have met with only limited success. In view of the poor survival rates for AIDS–KS patients which continue to decline at an alarming rate, it is eminently clear that a better understanding of the etiology and pathogenesis of this form of KS is needed if novel therapeutic strategies designed to successfully combat this disease are to be developed. If our hypothesis is validated, one could envision several approaches whereby the modulation of SF/c-*met* function or production might lead to a reduction in the incidence and severity of KS lesions. Antibody therapy directed against either SF-producing tumor cells or against the c-*met* receptor might decrease the incidence of new tumors by limiting their clonal expansion and lead to regression of established tumors by blocking SF-mediated tumor cell proliferation and neovascularization. It might also be possible to suppress production of SF or accessory cytokines involved in the induction SF production and thus short circuit SF/c-*met* growth-promoting effects. We have outlined a novel hypothesis for understanding the mechanism underlying the development of AIDS-associated KS. This is most certainly not the whole story, however. Clearly, other cytokines and alterations in natural host defenses and the immune system contribute significantly to the development of AIDS-associated KS. We believe, however, that recognition of SF/c-*met* as a participant in this disease is necessary if we are to more fully understand the pathogenesis of AIDS-associated KS.

ACKNOWLEDGMENTS

This work was supported by Grants HL39926 and CA64416 (P.J.P.) and AR0065, AR01823, and AR40488 (B.J.N.) from the National Institutes of Health.

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REL/NF-κB/IκB STORY

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I. Introduction

Cells respond to external stimulus by generating second messengers in the cytoplasm which send specific signals to the nucleus, culminating in the expression of a specific set of genes. Thus, transcriptional regulation can determine the growth property, differentiation status, development, cell fate, or even pathological status such as cancer. Gene expression is regulated by *cis* elements in genes which are recognized by transcription factors whose activity in turn is modulated by the incoming signals. Activity of transcription factors can be regulated by a number of distinct mechanisms, transcription, post-translational modification, interaction with other factors, or subcellular localization.

NF-κB is a transcription factor first identified as a lymphoid-specific protein that binds to a decameric oligonucleotide (GGGACTTTCC) present in the κ-light chain gene intronic enhancer (Sen and Baltimore, 1986a). The activity of NF-κB is regulated by its subcellular localization.

NF-κB is present as a dormant complex with an inhibitor IκB in the cytoplasm (Fig. 1). A wide variety of external or internal signals modify the NF-κB/IκB complex with poorly understood mechanisms, leading to the release of IκB and nuclear translocation of NF-κB, where it binds to a specific DNA motif and regulates transcriptional activity of the target genes. Molecular cloning of the genes encoding NF-κB and IκB revealed a family of related genes from fly to man. In flies, NF-κB-like factors regulate embryonic development and adult immunity. In man, these factors are involved in immune and acute phase responses, cell cycle control, differentiation, and pathogenesis of acquired immunodeficiency syndrome and cancer.

In this review, we will attempt to summarize our current understanding of the regulation of the NF-κB family of proteins and IκB proteins. The reader is encouraged to consult many excellent reviews on various

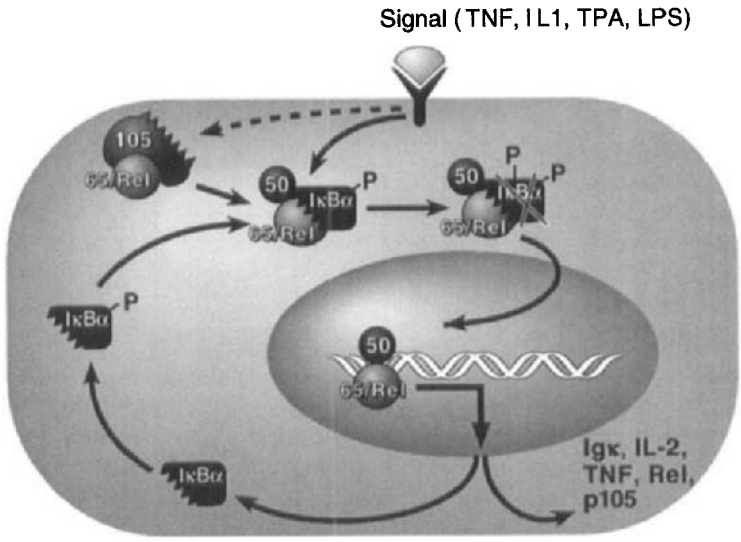

FIG. 1. Regulation of Rel/NF-κB transcription factors: A model. The inactive Rel/NF-κB–IκBα complex (p50-p65-IκBα or p50-cRel-IκBα) is present in the cytoplasm of resting cells. IκBα is basally phosphorylated. Following stimulation with a variety of agents, (signal) IκBα is rapidly phosphorylated and degraded with a poorly understood mechanism. Signal-induced phosphorylation of IκBα does not result in its dissociation from Rel/NF-κB complex and degradation of IκBα is likely to occur while in association. Once IκBα is degraded, free Rel/NF-κB complex migrates to the nucleus and regulates transcription of target genes including those encoding the inhibitor IκBα and the p50 precursor p105. Augmented expression of IκBα results in the termination of Rel/NF-κB activity, unless persistent stimulation with inducers is provided. p105 processing to p50 is also accelerated upon stimulation with external signals and likely to involve phosphorylation event(s).

aspects of this subject (Baeuerle, 1991; Baeuerle and Henkel, 1994; Beg and Baldwin, 1993; Blank *et al.*, 1992; Bose, 1992; Gilmore, 1991; Grilli *et al.*, 1993; Grimm and Baeuerle, 1993; Liou and Baltimore, 1993; Nolan and Baltimore, 1992; Pahl and Baeuerle, 1994; Schmitz *et al.*, 1991).

II. κB-Binding Factors

Since NF-κB was originally identified only in B cells (mature B and plasma cells) and was crucial for κ enhancer function (Atchison *et al.*, 1987; Lenardo and Baltimore, 1987), it was considered as a B cell-specific transcription factor involved in the activation of the Igκ gene. However, it soon became evident that NF-κB activity is inducible by phorbol esters (TPA) in pre-B cells as well as non-B cells (Sen and Baltimore, 1986b). In addition, DNA motifs closely related to the Igκ–κB site were found in multiple genes that are regulated by NF-κB, and are collectively called "κB sites" (Table I gives a few examples). Thus, the inducible NF-κB is observed in many cell types, defining NF-κB as a ubiquitous transcription factor involved in the regulation of a wide variety of genes through their κB sequences.

Multiple related and unrelated proteins can bind to κB sites. These include the Rel/NF-κB family of proteins (Fig. 2), zinc-finger proteins, and RκB. In this review, any protein complex that can bind to a κB site is referred to as a "κB-binding factor or complex." We will discuss κB-binding factors and their regulation in both the vertebrate systems (Sections II–V) and *Drosophila* (Section VI).

A. Rel/NF-κB Family

1. *NF-κB Complex: NFKB1 (p50/p105) and RelA (p65)*

The inducible NF-κB complex was originally purified from human cell lines, rabbit lung tissues, and human placenta (Baeuerle and Baltimore, 1989a; Ghosh *et al.*, 1990; Kawakami *et al.*, 1988; Zabel *et al.*, 1991). NF-κB complex is composed of two proteins of molecular weights 50 and 65 kDa, referred to as p50 and p65, respectively. It was shown to be heterodimeric by glycerol gradient centrifugation (Urban *et al.*, 1991). However, gel filtration analysis demonstrated that higher-order complexes also form (Baeuerle and Baltimore, 1989). Purified NF-κB binds the Igκ–κB site stably with a half-life of 45 min and with an affinity of $\sim 4\times10^{-13} - 5.7\times10^{-12}$ *M* (Urban and Baeuerle, 1990; Fujita *et al.*,

TABLE I
κB ENHANCER ELEMENTS

Target gene	κB site	Reference
Immunoreceptors		
Igκ light-chain gene	GGGACTTTCC	Sen and Baltimore (1986a)
IL-2Rα	GGGAATCTCC	Ballard *et al.* (1988)
TCRβ	GGGAGATTCC	Jamieson *et al.* (1989)
MHC-I (H-2Kb)	GGGGATTCCC	Baldwin and Sharp (1988)
Cytokines		
IL-2	GGGATTTCAC	Lenardo *et al.* (1988)
G-CSF	GGGGAATCTC	Nishizawa *et al.* (1990)
TNF-α	GGGGCTTTCC	Shakhov *et al.* (1990)
IFN-β	GGGAAATTCC	Lenardo *et al.* (1989)
Rel/NF-κB members		
p50/p105	GGGGCTTCCC	Ten *et al.* (1992)
	GGGAGCGCCC	
c-Rel (chicken)	GGGAAATTCC	Hannink and Temin (1990)
C-Rel (mouse)	GGGAACCACC	Grumont *et al.* (1993)
	GGGATTTCTC	
	GGGAAATCCC	
IκB members		
IκBα (mouse)	GGGAATTTCC	Chiao *et al.* (1994)
IκBα (pig)	GGGAATTTCC	deMartin *et al.* (1993)
	AGGACTTTCC	
Viruses		
HIV	GGGACTTTCC	Nabel and Baltimore (1987)
SV40	GGGACTTTCC	Kanno *et al.* (1989)
	GGGAAGTACC	
CMV	GGGACTTTCC	Sambucetti *et al.* (1989)
	GGGGATTTCC	
Adenovirus	GGGACTTTCC	Williams *et al.* (1990)
Others		
c-myc	GGGAAAACCC	Duyano *et al.* (1990)
H-*ras*-1	GGGACGCCAC	Trepicchio and Krontiris (1992)
serum amyloid A	GGGACTTTCC	Edbrooke *et al.* (1989)
vimentin	GGGGCTTTCC	Lilienbaum *et al.* (1990)
Consensus	GGGRNNYYCC	

1992; Kretzschmar *et al.*, 1992). The half-life of NF-κB on a κB site, however, may depend on the source of the complex as p50/p65 heterodimers observed in lipopolysaccharide (LPS)-stimulated 70Z/3 cells, a murine pre-B cell line, have a half-life of less than 30 sec on the Igκ–κB site (Miyamoto *et al.*, 1994a). Upon binding to the κB site, purified

NF-κB activates transcription *in vitro* (Kawakami *et al.*, 1988). Thus, NF-κB is a sequence-specific transcription factor composed of a dimer of p50 and p65 subunit proteins.

The genes encoding p50 and p65 subunits of NF-κB are related to each other (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991; Nolan *et al.*, 1991; Ruben *et al.*, 1991) and other multiple genes including the oncogene v-*rel* (Stephens *et al.*, 1983; Wilhelmsen *et al.*, 1984) and the gene encoding a *Drosophila* morphogen, *dorsal* (Steward, 1987) (see Fig. 2). v-*rel* is a resident oncogene in the reticuloendotheliosis virus strain T (Rev-T) which causes fatal lymphomas in birds (see review by Bose, 1992). *Dorsal* is a maternal morphogen which determines the dorsal–ventral polarity in a developing *Drosophila* embryo (see review by Govind and Steward, 1991). The proteins encoded by these genes share an extensive sequence homology at the N-terminal ~300 aa, referred to as the Rel homology domain (RHD; Gilmore, 1990), which contains a dimerization domain, a DNA-binding motif, and a nuclear localization signal. Thus, homologous sequence within this family of proteins encodes important functional domains.

Unlike most other transcription factors, p50 is synthesized as a precursor protein of a molecular weight 105–110 kDa (p105) (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991). Ubiquitin-dependent proteasome generates the N-terminal p50 subunit (Palombella *et al.*, 1994). A protease encoded by immunodeficiency virus (HIV) enhances the proteolysis of p105 to p50 (Riviere *et al.*, 1991). Since the agents which activate NF-κB also enhance the proteolysis of p105 (Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann and Scheidereit, 1994), processing of p105 provides a regulatory step controlling the level of p50 production in the cell.

p50 alone does not generally activate transcription through κB sites in transient transfection assays (Franzoso *et al.*, 1992; Perkins *et al.*, 1992; Ruben *et al.*, 1992; Ryseck *et al.*, 1992; Schmitz and Baeuerle, 1991). Furthermore, p50 has been shown to repress transcription induced by NF-κB (Franzoso *et al.*, 1992; Kang *et al.*, 1992; Schmitz and Baeuerle, 1991). Thus, it was suggested that p50 is a repressor rather than an activator of κB-dependent transcription. p50 homodimers can, however, activate κB-dependent transcription *in vitro* (Fujita *et al.*, 1992; Kretzschmar *et al.*, 1992). In addition, conformational changes induced by specific κB sites directly correlates with p50-dependent transactivation potential (Fujita *et al.*, 1992). Thus, under certain conditions, p50 homodimers can activate transcription depending on the κB sites.

Unlike p50, the p65 subunit of NF-κB is not produced as a precursor protein (Nolan *et al.*, 1991; Ruben *et al.*, 1991). p65 can form homodimers

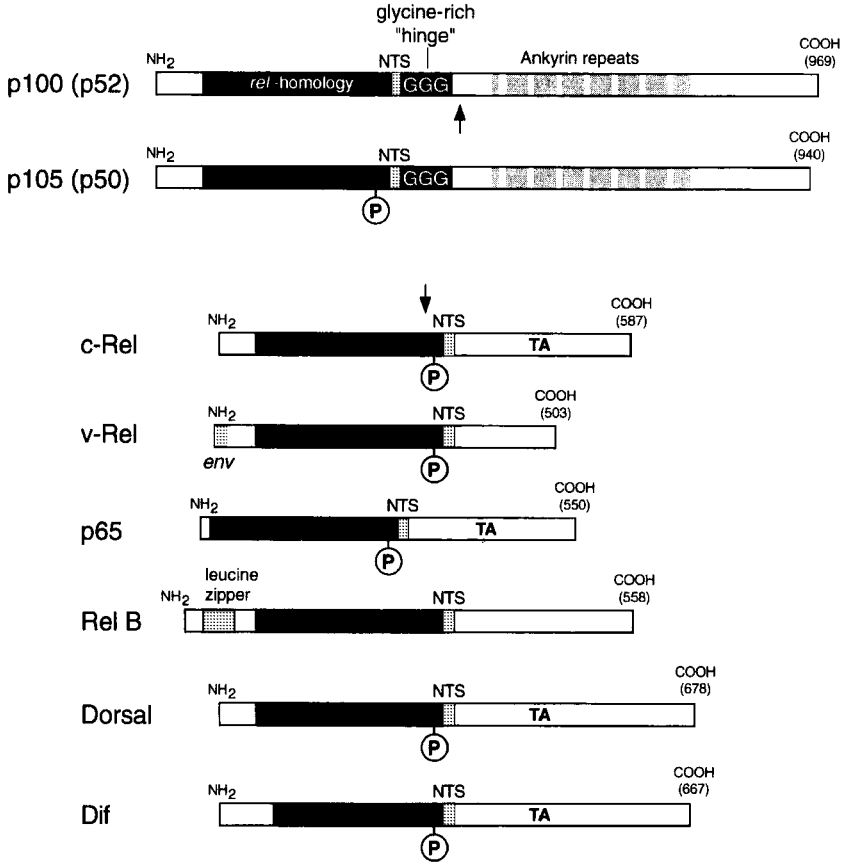

FIG. 2. Rel/NF-κB/Dorsal family of proteins. Rel/NF-κB/Dorsal proteins contain well-conserved N-terminal ~300aa sequences (rel-homology domain, shown in dark box) which include the DNA binding, dimerization domains, and nuclear transport signal (NTS). There is a well-conserved protein kinase A site in the Rel homology domain of most of the family members. p50 and p52 are produced by proteolysis of the precursor proteins, p105 p105 and p100, respectively. Both p105 and p100 contain a glycine rich "hinge" region followed by the C-terminal ankyrin repeats which are also present in the IκB family of proteins (see Fig. 3). Some members, such as c-Rel, RelB, p65, Dorsal, and Dif (*Drosophila* immunity factor), contain transactivation domain (TA) in addition to Rel homology domain. RelB contains two TA domains which are not shown in the figure.

and bind κB sites directly. It can cause κB-dependent transactivation due to the presence of a potent C-terminal transactivation domain (Ballard *et al.*, 1992; Schmitz and Baeuerle, 1991). Since p50/p65 combination leads to marked transcriptional activation, while p50 alone does not, the transactivation potential of NF-κB seems to be provided primarily by the p65 subunit.

The DNA-binding domain of NF-κB is likely to be a novel type, having no sequence similarity to previously described structures. A conserved motif, RXXRXRXXC, has been described as a potential DNA-binding domain because mutation in this region abolishes DNA-binding activity (Kumar *et al.*, 1992). However, it is not clear whether this is due to the disruption of DNA-binding contact points or to the three-dimensional structure. Toledano *et al.*, (1993) demonstrated that replacement of the N-terminal 58 aa of p65 with the corresponding 82 aa of p50 (both of which contain the conserved motif) lead to a chimeric p65 protein which binds to κB sites with p50 specificity (see Section V for DNA-binding specificity of Rel/NF-κB complexes). Coleman *et al.*, (1993) provided an intriguing observation that only 4 aa substitution in p65 with the corresponding residues of p50 in this region changed the DNA-binding specificity of p65 to that of p50. Domain swapping with other well-characterized DNA-binding domains may demonstrate whether this putative region of NF-κB is sufficient for direct DNA-binding activity.

The structure of the NF-κB dimerization domain is also undefined. A dimerization domain has been localized within ~100 aa at the C-terminus of the ~300 aa RHD because deletion of N-terminal 201 aa of p50 does not affect dimerization (Logeat *et al.*, 1991). There are several aa substitution mutants in the C-terminal RHD which also block dimerization (Bressler *et al.*, 1993). The naturally occurring splice variant of p65, p65Δ, with 10 aa deletion in this region cannot dimerize with p50 although it can still form a complex with p65 (Ruben *et al.*, 1992). This type of change in dimerization specificity has also been shown with p65 point mutants in this domain (Ganchi *et al.*, 1993). Thus, subtle sequence alterations can confer partner specificity for p50 and p65 proteins. The identification of the precise nature of both the DNA-binding and dimerization domains may require structural analyses using X-ray crystallography of the RHD.

2. *v-Rel and c-Rel*

v-*rel* is an oncogene present in the replication-defective avian Rev-T which causes fatal lymphomas in infected chickens (for review see Bose, 1992). It is derived from the turkey protooncogene c-*rel*. v-Rel protein is

generated by deletions of 2 aa at the N-terminus and 118 aa at the C-terminus of c-Rel which are replaced by 11 aa and 18 aa at each terminus with the env sequence of the Rev-A virus, respectively (Capobianco *et al.*, 1990; Stephens *et al.*, 1983; Willhelmsen *et al.*, 1984). In addition, it contains multiple point mutations internally (Wilhelmsen *et al.*, 1984). v-Rel is missing the C-terminal transactivation domain of c-Rel and thus generally does not transactivate from κB sites even though it can bind to κB sites directly (Ballard *et al.*, 1990; Bull *et al.*, 1990; Inoue *et al.*, 1991; Kabrun *et al.*, 1991; Richardson and Gilmore, 1991). Instead, it can repress transcription mediated by NF-κB or Rel proteins, (Ballard *et al.*, 1990; Inoue *et al.*, 1991). Thus, it was hypothesized that v-*rel* may induce transformation as a dominant-negative oncogene. However, addition of a C-terminal transactivation domain to v-Rel creates a transactivating v-Rel fusion protein which can still transform chicken spleen cells (Hannink and Temin, 1989). Thus, loss of transactivation potential *per se* is not necessary for v-Rel transforming activity. Recently, it was shown that the transactivation potential of v-Rel is context dependent, as it can activate transcription from κB sites in undifferentiated F9 cells (Walker *et al.*, 1992). It appears that the DNA-binding activity of v-Rel is necessary for transforming activity (Walker *et al.*, 1992). The conserved PK-A site in the RHD is critical for transformation and transcriptional repression (Mosialos *et al.*, 1991). Therefore, transformation by v-Rel may involve multiple events, including DNA-binding which alters transcriptional regulation by c-Rel proteins.

In addition to DNA binding, v-Rel proteins also form complexes with a number of cellular proteins in transformed spleen cells. Immunoprecipitation of v-Rel proteins coprecipitates associated proteins p124, p115, p75, p70, and pp40 (Davis *et al.*, 1990a,b; Kochel *et al.*, 1991; Lim *et al.*, 1990; Morrison *et al.*, 1989; Simek and Rice, 1988). In untransformed cells, c-Rel is also complexed with the same set of proteins (Morrison *et al.*, 1989; Davis *et al.*, 1990a; Kochel *et al.*, 1991). These proteins are identified as a chicken homolog of mammalian p105, p100 (see below), c-Rel, heat-shock protein 70, and IκBα (inhibitor of NF-κB, see Section III), respectively (Capobianco *et al.*, 1992; Davis *et al.*, 1991; Lim *et al.*, 1990; Sif and Gilmore, 1993; Simek and Rice, 1988). Thus, in transformed cells, the overproduction of v-Rel protein causes association with proteins that are otherwise bound to c-Rel.

Disruption of the c-*rel* gene is associated with several types of lymphomas. The expression pattern of c-Rel is cell-type specific, unlike p50 or p65, which are more ubiquitous. Enhanced expression can be seen only in lymphoid cells (Brownell *et al.*, 1987, 1988; Grumont and Gerondakis 1990; Moore and Bose, 1989). It is of particular interest to note that

those cell types which normally show high expression of c-Rel are the very targets of v-Rel-induced transformation in chicken (Barth *et al.*, 1990; Barth and Humphries, 1988a; Zhang *et al.*, 1991). An alteration of the c-*rel* locus can be seen in lymphomas derived from these cell types as well. For example, a c-*rel* alteration is observed in a chicken B cell lymphoma which is induced by insertional mutagenesis by avian leukosis virus (Kabrun *et al.*, 1990). This results in the production of truncated c-Rel proteins whose functional properties are not clearly understood. c-*rel* alteration can also be observed in a human B cell lymphoma cell line (Lu *et al.*, 1991). In this case, a chromosomal translocation of c-*rel* generates a fusion protein with only the RHD of c-Rel and Nrg, a product of a non-*rel*-related gene. The fusion protein can bind to κB sites and is located in the nucleus but does not transactivate due to the lack of C-terminal transactivation domain of c-Rel (J. Chen, unpublished observations). Thus, deregulation of tissue-specific functions of c-Rel may result in transformation of lymphoid cells.

3. Other Rel/NF-κB Members

The Rel/NF-κB family is composed of many members in addition to p50/p105, p65, v-Rel, and c-Rel. These include p52/p100, RelB, and p65Δ. The gene encoding p52/p100 was cloned by several investigators (Bours *et al.*, 1992; Mercurio *et al.*, 1992; Neri *et al.*, 1991; Schmid *et al.*, 1991). This gene encodes a protein of 90–100 kDa (p100) which contains RHD in its N-terminus. The N-terminal DNA-binding protein is referred to as p52 and can be generated in the cell by alternate splicing (Schmid *et al.*, 1991), by proteolysis (Bours *et al.*, 1992; Mercurio *et al.*, 1992), or as a fusion protein generated by a chromosomal translocation involved in a B cell lymphoma (Neri *et al.*, 1991). p52 alone does not generally transactivate but it can heterodimerize with p65 and become a potent activator of HIV-LTR (Schmid *et al.*, 1991). Recently, H2TF-1, another κB-binding protein, has been shown to be p100 (Scheinman *et al.*, 1993; Potter *et al.*, 1993).

Another member of this family is RelB, whose gene was cloned as an immediate early gene in NIH3T3 cells stimulated with serum (Ryseck *et al.*, 1992). This protein contains the RHD and two transactivation domains, one in the RHD with a leucine-zipper (LZ)-like motif and the other at the C-terminus (Dobrzanski *et al.*, 1993; Ryseck *et al.*, 1992). It can heterodimerize with p50 or p52. Although one of the transactivation domains of RelB is sufficient for transactivation potential with p50 or p52 proteins, the combination of the two domains is required for full activity. It is possible that LZ-like motif could interact with other pro-

teins. Because RelB is induced by serum, it may perform functions in cell cycle control.

PCR analysis identified a splice variant form of p65, p65Δ, which is missing 10 aa (aa 222–231) in the RHD of p65 (Ruben *et al.*, 1992). It cannot homodimerize or heterodimerize with p50. It can, however, complex with p65, which results in reduced DNA-binding activity. Thus, p65Δ may negatively regulate p65 activity *in vivo*. It is of interest to note that p65Δ has been shown to transform rat embryonic fibroblast (Naranayan *et al.*, 1992), suggesting that selective inhibition of p65 activity by p65Δ may be directly involved in the transformation event. Although transforming activity of p65Δ is not readily reproducible (Grimm and Baeuerle, 1994), p65Δ remains the only member of the Rel/NF-κB family which has been shown to transform mammalian cells in cell culture.

4. Mechanisms for Transformation by Rel/NF-κB Proteins

We suggest that deregulation of Rel/NF-κB activity may be directly involved in cellular transformation. This can come about either by the loss of the transactivation domain or by overproduction of an altered protein. In general, the transforming proteins of the Rel/NF-κB family (v-Rel, Rel-nrg, lyt-10, and p65Δ) do not transactivate, further strengthening the notion that transformation by the Rel/NF-κB family may be related to the loss of transactivation potential. In fact, deletion of the C-terminal transactivation domain of c-Rel creates a transforming protein (Kamens *et al.*, 1990). However, as discussed earlier, a v-Rel fusion protein containing the transactivation domain of c-Rel still transforms chicken spleen cells (Hannink and Temin, 1989). This provides evidence that transformation by v-Rel is not due to a simple loss of transactivation capacity. However, since the v-Rel protein contains multiple mutations compared to that of c-Rel, addition of a transactivation domain may not exert a fully negative effect on transformation. Thus, it is still possible that the loss of transactivation activity may be directly involved in transforming activity by this family of proteins.

An alternate hypothesis is that a disruption of the homeostatic balance of Rel/NF-κB proteins by overproduction of the transforming members leads to transformation. The formation and the levels of Rel/NF-κB complexes may be regulated in a temporal and spatial manner in normal cells. Since in all cases the transforming proteins are overexpressed, it may cause alteration of the balance of Rel/NF-κB complexes leading to unregulated Rel/NF-κB activity and eventual

transformation. Development of a reproducible transformation assay for mammalian cells by Rel/NF-κB genes would greatly facilitate dissection of the underlying mechanism(s).

B. κB-Binding Factors That Are Not Related to Rel/NF-κB Members

There are other classes of κB-binding proteins which lack discernable structural homologies with Rel/NF-κB family of proteins. One of them is a large zinc-finger-containing protein called MBP-1/PRDII-BF-1/HIV-EP1/αA-CRYBP1 which was cloned by screening λgt11 libraries using radiolabeled κB sites of the H-2Kb MHC-I gene, the PRDII motif of β-interferon gene, or the HIV enhancer (Baldwin *et al.*, 1990; Fan and Maniatis, 1990; Nakamura *et al.*, 1990; Nomura *et al.*, 1991; Singh *et al.*, 1988). The DNA-binding domain in this protein is located within a 118-aa region containing two zinc-finger motifs. This gene is inducible in T cells by TPA and PHA and in fibroblasts by serum (Baldwin *et al.*, 1990; Fan and Maniatis, 1990; Nomura *et al.*, 1991), suggesting a role in T cell activation and cellular proliferation.

There are other κB-binding proteins which also contain zinc-finger motifs; AT-BP1 and AT-BP2 (Mitchelmore *et al.*, 1990), HIV-EP2 (Nomura *et al.*, 1991), MBP-2 and KBP-1 (Rustgi *et al.*, 1991), and AGIE-BP1 (Ron *et al.*, 1991). In general, the zinc-finger proteins that bind κB sites share a motif, **RGKYICE**, which is similar to the putative DNA-binding domain of the Rel/NF-κB members, **RFRYXCE** (Toledano *et al.*, 1993). This similarity may underlie their common DNA recognition properties. As mentioned previously, the precise DNA-binding domain of Rel/NF-κB family is undefined. Since DNA-binding activity of NF-κB also requires zinc ions (Zabel *et al.*, 1991), the Rel/NF-κB DNA-binding motif may also involve a novel type of zinc chelation.

RκB is another gene encoding a κB-binding factor which does not resemble either Rel/NF-κB family or zinc-finger proteins (Adams *et al.*, 1991). It was isolated by screening a λgt11 library using a radiolabeled κB site of the IL-2Rα gene. RκB encodes a protein of 107 kDa with no known DNA-binding motif. The *in vivo* function or relevance of this factor is not clearly understood.

The presence of various κB-binding factors generates diversity, and consequently a complex system of κB-dependent transcription of various genes. A variety of parameters are crucial in determining the contribution of different κB-binding complexes for the transcriptional regulation of specific target genes. These include the relative concentration of each factor, relative affinity to the target κB site, conformational changes

induced by the κB site, and the presence of other interacting factors (see below). The most abundant form of a κB-binding complex in many cells seems to be NF-κB as this is the major factor purified from a variety of cellular extracts (Baeuerle and Baltimore, 1989b; Ghosh *et al.*, 1990; Kawakami *et al., 1988, Urban and Baeuerle, 1991; Zabel et al.*, 1991). However, the levels of active κB-binding complexes may vary at different time points following stimulation as some of the κB-binding factors are inducible (see Table I). The distinct complexes may be distributed in a tissue-specific manner, such as in murine mature B cell lines where p50/c-Rel is constitutively activated (Liou *et al.*, 1994; Miyamota *et al.*, 1994a; Rice and Ernst, 1993). In addition, a κB site may be preferentially recognized by different κB-binding factors (see Section V), adding further complexity to κB-dependent transcriptional regulation. The κB site can induce conformational changes of these complexes upon binding (Fujita *et al.*, 1992) and conversely NF-κB causes DNA bending (Schreck *et al.*, 1990). Furthermore, the overall transcriptional activity of a target gene depends on the presence of other interacting transcription factors (see Section VI). Thus, the presence of multiple κB-binding factors may provide the specificity required for regulating a wide variety of κB-dependent genes.

III. IκB Family of Proteins

IκB (inhibitory protein) was originally identified as a labile factor which inhibited the activity of NF-κB by a direct protein–protein interaction (Baeuerle and Baltimore, 1988b). In pre-B cells, transcription of the Igκ gene is inducible by LPS without the requirement for *de novo* protein synthesis (Nelson *et al.*, 1985; Wall *et al.*, 1986). In fact, the addition of a protein synthesis inhibitor can activate Igκ transcription, indicating that a labile inhibitor is present. Because NF-κB is inducible by the same agents that activate Igκ gene transcription and the activation of NF-κB does not require new protein synthesis, it was postulated that a labile factor regulates activity of NF-κB post-translationally (Sen and Baltimore, 1986b).

The presence of a latent NF-κB/IκB complex is widespread. The DNA binding activity of NF-κB can be induced *in vitro* by treating cytoplasmic extracts with deoxycholate or formamide, which disrupts the IκB interaction (Baeuerle and Baltimore, 1988b). Thus, the activation of NF-κB can be induced by the release of IκB molecule. Subsequently, a number of proteins capable of inhibiting the DNA-binding activity of NF-κB were identified and are referred to as the IκB family. This family

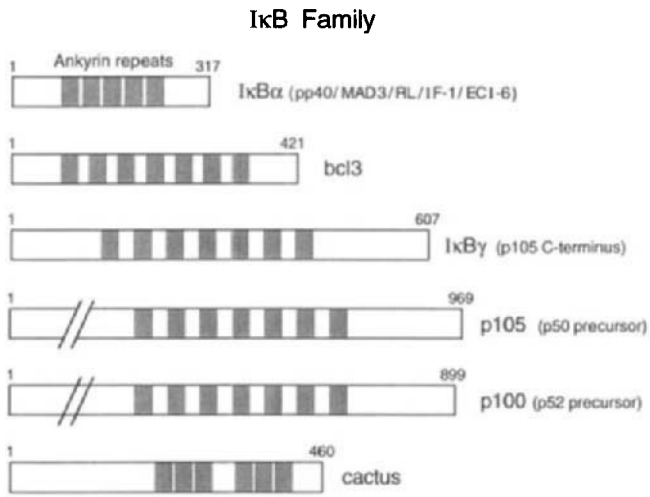

FIG. 3. IκB family of proteins. IκB proteins contain conserved ankyrin repeat motifs (shaded bars) which are essential for the IκB activity. The number of repeats varies from five (IκBα) to seven (Bcl3, IκBγ, p105, and p100). p105 and p100 are the precursor proteins for p50 and p52, respectively. IκBγ is produced as an alternate mRNA from the gene encoding p105. Cactus is the functional homologue of IκB protein in *Drosophila*.

includes IκBα, IκBβ, IκBγ, p105, p100, and Bcl-3 (Fig. 3). The genes encoding all of these proteins except IκBβ have been cloned (Davis *et al.*, 1991; Ghosh *et al.*, 1990; Haskill *et al.*, 1991; Inoue *et al.*, 1992a; Kieran *et al.*, 1990; Neri *et al.*, 1991; Ohno *et al.*, 1990) In this review we will discuss only those members whose genes are known.

All IκB proteins contain a conserved region, referred to as the ankyrin repeat domain (ARD). An ankyrin repeat is a 30–34 aa motif also present in a number of other proteins, such as ankyrin, CDC10, SW16, SW14, GABP-β, Notch, and TAN-1 (for review see Bennett, 1992). The ankyrin repeat domain provides an interface for protein–protein interaction such as those involved in ankyrin with anion exchanger/tubulin and in GABP-β with GABP-α. The ARD of IκB proteins is also essential for interaction with NF-κB (Inoue *et al.*, 1992b). Although the aa sequence of the ARD of the IκB family is well conserved, it is quite divergent from that found in non-IκB proteins (Nolan and Baltimore, 1992). Thus, the inhibition of NF-κB activity is specific to the IκB family of proteins with the distinct ARD.

A. Molecular Cloning Of Genes Encoding IκB Proteins

1. κBα: *MAD-3, pp40, RL-IF1, and ECI-6*

The gene encoding the IκBα protein was fortuitously cloned as an immediate-early gene, *mad-3*, which is induced when monocytes attach to plastic substrate (Haskill *et al.*, 1991). The homologous gene was also cloned from chicken (pp40; Davis *et al.*, 1992), rat (RL-IF1; Tewari *et al.*, 1992), pig (ECI-g; de Martin *et al.*, 1993), and mouse (P. Chiao, unpublished). IκBα proteins contain internal five complete and a sixth incomplete ankyrin repeat motifs followed by an acidic region. The region N-terminal to the ARD does not appear to be required for its inhibitory activity (Inoue *et al.*, 1992b). Notably, association with c-Rel or p65 requires five ankyrin repeats (Leveillard and Verma, 1993). However, association *per se* is not sufficient for inhibition of DNA-binding activity. It additionally requires the C-terminal region which contains the incomplete sixth ankyrin repeat and the acidic region (Hatada *et al.*, 1993; Inoue *et al.*, 1992b; Leveillard and Verma, 1993). IκBα can sequester p65 or c-Rel in the cytoplasm and to a lesser extent p50 (Ganchi *et al.*, 1992; Inoue *et al.*, 1991; Zabel *et al.*, 1993).

The association of IκBα with p65 requires the nuclear localization signal (NLS) of p65 proteins (Beg *et al.*, 1992; Ganch *et al.*, 1992). Masking of the NLS is most likely the mechanism for the inhibition of nuclear translocation of p65 by IκBα proteins. It is not defined how IκBα interaction leads to the inhibition of p65 DNA-binding activity but p65 C-terminus is required (Ganchi *et al.*, 1992). In contrast, IκBα interacts with both the putative DNA-binding domain and the NLS of Rel proteins (Beg *et al.*, 1992; Kerr *et al.*, 1991; Kumar and Gelinas, 1993). The C-terminus of Rel is dispensable for IκBα association. Thus, in this case IκBα seems to simultaneously cover both the NLS and the DNA-binding domain, inhibiting nuclear translocation and DNA binding at the same time.

In addition to sequestering NF-κB in the cytoplasm, IκBα can also displace NF-κB bound to its cognate DNA sequence (Zabel and Baeuerle, 1990). Binding of p65 to IκBα or DNA seems to be mutually exclusive (Ganchi *et al.*, 1992). Hence, IκBα can terminate NF-κB-dependent transcription *in vitro* (Kretzschmar *et al.*, 1992). IκBα can be localized in the nucleus when overexpressed (Cressman and Taub, 1993; Zabel *et al.*, 1993) and in v-Rel-transformed cells, some IκBα (pp40) can also be detected in the nucleus (Davis *et al.*, 1990a). These observations suggest roles for IκBα not only as a cytoplasmic retention molecule but also a terminator of

transcription by migrating to the nucleus (perhaps by passive diffusion) and displacing active NF-κB complex from κB sites. IκBα has also been shown to be capable of transcriptional transactivation when fused to a heterologus DNA-binding domain (Morin and Gilmore, 1992).

2. p105/p100: Precursors of p50 and p52

Another member of the IκB family is the p50 precursor protein p105. It contains seven ankyrin repeats at its C-terminus which display extensive homology to the ARD of IκBα. The p50 region is at its N-terminus (see Fig. 2). Despite the presence of the DNA-binding domain in the N-terminus, p105 does not generally bind κB sites (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991). However, deletion or disruption of the ARD leads to DNA binding, directly demonstrating that the ARD inhibits this activity. p105 can also inhibit the DNA binding activity of NF-κB in *trans* (Rice *et al.*, 1992). Thus, intra- or intermolecular masking of the N-terminal Rel homology domain by the C-terminal ARD causes the inhibition of the DNA-binding activity.

Despite the presence of a nuclear localization signal, the subcellular location of p105 is exclusively cytoplasmic (Blank *et al.*, 1991; Henkel *et al.*, 1992; Inoue *et al.*, 1992a; Rice *et al.*, 1992, Rice and Ernst, 1993). The NLS is inaccessible to antibody specific for this region of p105 unless it is denatured or its C-terminal ARD is removed, suggesting that p105 is in a conformation in which NLS is masked by its C-terminal ARD in its native state (Henkel *et al.*, 1992). In addition, p105 causes cytoplasmic retention of other Rel/NF-κB members in *trans* (Hatada *et al.*, 1993; Henkel *et al.*, 1993). The p50 precursor, therefore, is an IκB protein involved in its own cytoplasmic retention and other Rel/NF-κB members by intra- or intermolecular mechanisms.

The precursor of p52, p100, has also been shown to function as an IκB protein by similar criteria *in vivo* (Mercurio *et al.*, 1993). p124 and p115, two of the v-Rel-associated proteins, have been shown to be the chicken homologues of p105 and p100 proteins, respectively (Capabianco *et al.*, 1991; Sif and Gilmore, 1993). Taken together, the ankyrin repeat domains of p105 and p100 regulate the cytoplasmic/nuclear partition of Rel/NF-κB proteins *in vivo*.

3. IκBγ/CTD: C-Terminal Half of p105

Another member of the IκB family, IκBγ (also referred to as CTD), is generated from the same gene coding for the precursor of the p50 protein (Inoue *et al.*, 1992a; Liou *et al.*, 1992). This gene contains an alternative transcription start site in its intron, generating a protein

containing only the C-terminal 607 aa of p105 which includes seven ankyrin repeats (Inoue *et al.*, 1992a; J. I. Inoue, personal communication). The IκBγ protein can inhibit the DNA-binding activity of NF-κB in addition to that of other κB-binding complexes (see Section III,B). It can also inhibit the nuclear translocation of c-Rel. IκBγ interacts with p50 through the NLS (Inoue *et al.*, 1993). Therefore, one gene codes for a transcription factor and its inhibitor at the same time.

Interestingly, the distribution of IκBγ mRNA is cell-type specific, unlike p105, which is ubiquitous. It is only detected in murine lymphoid cells (Inoue *et al.*, 1992a). Furthermore, the level of IκBγ mRNA decreases during the differentiation of pre-B to mature B cells (Liou *et al.*, 1992; S. Miyamoto, unpublished observation). These data suggest a cell-type specific role for IκBγ in murine lymphoid cells specifically during the differentiation of B cells.

4. Bcl-3: IκB, Coactivator and a Putative Oncogene

Another IκB member, Bcl-3, was identified as a putative oncoprotein in a human B cell chronic lymphocytic leukemia which displays somal translocation t(14;19)(q32;q13.1) (Ohno *et al.*, 1990). This protein of 446 aa contains proline-rich regions in both N- and C-termini and the central seven ankyrin repeat motifs similar to the ARD of other IκB members (Fig. 3). Unlike other IκB proteins, its inhibitory activity is specific for p50 or p52 homodimers (Franzoso *et al.*, 1992; Inoue *et al.*, 1993; Kerr *et al.*, 1992; Naumann *et al.*, 1993; Nolan *et al.*, 1993; Wulczyn *et al.*, 1992; see Section III.B). In addition, its subcellular location is nuclear in NTera-2, NIH3T3, and HeLa cells (Bours *et al.*, 1993; Franzoso *et al.*, 1993; Kerr *et al.*, 1993; Nolan *et al.*, 1993). Since Bcl-3 also interacts with the NLS of p50 (Inoue *et al.*, 1993; Leviellard and Verma, 1993), it is not clear how $(p50)_2$–Bcl3 complex enters the nucleus. One group has reported that Bcl-3 in Cos-7 cells is cytoplasmic (Naumann *et al.*, 1993). Thus, Bcl-3 has an ARD related to that of IκB members, but the specificity of inhibition and subcellular location appear distinct.

Surprisingly, Bcl-3 can also activate rather than inhibit transcriptional activity through κB sites (Bours *et al.*, 1993; Fujita *et al.*, 1993). This is contrary to the conventional function of IκB proteins. There are two possible explanations: (a) Antirepressor activity of Bcl-3. Since Bcl-3 can displace p50 homodimers which can act as a repressor of κB-dependent transcription, it indirectly allows NF-κB to activate transcription (Franzoso *et al.*, 1992; Inoue *et al.*, 1993); (b) Bcl-3 as a coactivator of κB-dependent activation. Contrary to the above observations, Bcl-3 can form ternary complexes with homodimers of p50 or p52 on DNA (Bours *et al.*, 1993; Fujita *et al.*, 1993). In addition, Bcl-3 enhances the

transactivation potential of p52 in transient transfection assays and that of p50 homodimers in *in vitro* transcription assays. Thus, these data suggest that Bcl-3-induced transactivation is a result of its coactivation potential. These two models involve opposite effects when Bcl-3 interacts with p50 and p52 homodimers: displacement from a κB site or ternary complex formation on the target DNA. The exact action of Bcl-3 in the cell probably depends on its concentration, post-translational modifications, and the target κB sequences.

Bcl-3 is inducible during T cell activation (Ohno *et al.*, 1990) and a high level of Bcl-3 is observed in mature B cells (Bhatia *et al.*, 1991; Liou *et al.*, 1994). These findings suggest a possible role for Bcl-3 in proliferation and differentiation of lymphoid cells. Deregulated production of putative oncoprotein Bcl-3 could, therefore, cause cellular transformation by altering the activity of p50- or p52-mediated functions.

B. Subunit Specificity of IκB Inhibition

The presence of multiple forms of IκB proteins is intriguing. Are they specific for different κB-binding complexes? The earlier studies using the partially purified IκB demonstrated that the p65 subunit was the receptor for IκB protein because it only inhibited p50/p65 complex but not p50 homodimers (Baeuerle and Baltimore, 1989; Nolan *et al.*, 1991; Urban and Baeuerle, 1990; Urban *et al.*, 1991). It is now clear that the inhibition of independent κB-binding complexes can be accomplished by many forms of IκB proteins. The list may increase as more proteins with the characteristic ankyrin repeat domain (Nolan and Baltimore, 1993) are discovered and tested for their inhibitory activity. It is likely that the need for different forms of IκB proteins in the cell is to selectively attenuate various κB-binding complexes composed of different members of Rel/NF-κB proteins.

κB-binding complexes have varying sensitivity to different IκB members (Table II). For example, the NF-κB complex (p50/p65) can be inhibited by IκBα, IκBγ, p105, and p100 (Davis *et al.*, 1991; Kerr *et al.*, 1991; Inoue, *et al.*, 1992; Mercurio *et al.*, 1993; Rice *et al.*, 1992; Wulczyn *et al.*, 1992; Zabel *et al.*, 1993). κB-binding complexes containing p65 or c-Rel are generally inhibited by IκBα and p105/p100 (Davis *et al.*, 1991; Kerr *et al.*, 1991; Mercurio *et al.*, 1993; Rice *et al.*, 1992), whereas homodimers of p50 or p52 are more sensitive to Bcl-3 (Franzoso *et al.*, 1992; Inoue *et al.*, 1993; Nolan *et al.*, 1993). The inhibitory activity of IκBγ (or CTD) on complexes containing p65, c-Rel, or p52 is controversial, although the inhibitory effect on p50 homodimers is consistent (Grumont *et al.*, 1993; Inoue *et al.*, 1992a; Leveillard and Verma, 1993; Liou *et al.*,

TABLE II
SPECIFICITY OF INHIBITION OF κB-BINDING COMPLEXES BY IκB MEMBERS

κB-binding complex	IκB Member	Reference
p50/p65	IκBα, IκBγ, p105, p100	Baeuerle and Baltimore (1989); Nolan *et al.* (1991); Davis *et al.* (1991); Kerr *et al.* (1991); Haskill *et al.* (1991); Tewari *et al.* (1992), Inoue *et al.* (1992a,b, 1993); Wulczyn *et al.* (1992); Liou *et al.* (1992); Naumann *et al.* (1993)
p52/p65	IκBα	Duckett *et al.* (1993)
p50/Rel	IκBα, IκBγ	Davis *et al.* (1991); Tewari *et al.* (1992); Miyamoto *et al.* (1994)
p50/RelB	IκBα	Tewari *et al.* (1992)
p65/p65	IκBα, IκBγ, p105	Davis *et al.* (1991); Wulczyn *et al.* (1992); Liou *et al.* (1992); Ganchi *et al.* (1992); Beg *et al.* (1992); Inoue *et al.* (1992b, 1993); Leveillard and Verma (1993); Hatada *et al.* (1993); Naumann *et al.* (1993); Scheinman *et al.* (1993)
Rel/Rel	IκBα, IκBγ, p105	Kerr *et al.* (1991); Davis *et al.* (1991); Inoue *et al.* (1992a); Rice *et al.* (1992); Hatada *et al.* (1993); Gerondakis *et al.* (1993)
p50/p50	Bcl3, IκBγ, p105	Inoue *et al.* (1992a, 1993); Wulczyn *et al.* (1992); Franzoso *et al.* (1992, 1993); Liou *et al.* (1992); Kerr *et al.* (1992); Nolan *et al.* (1993); Leveillard and Verma (1993); Hatada *et al.* (1993; Naumann *et al.* (1993)
p52/p52	Bcl3, IκBγ	Kerr *et al.* (1992); Nolan *et al.* (1993)

1992; Naumann *et al.*, 1993). Thus, these studies demonstrate differential sensitivity of Rel/NF-κB complexes to IκB proteins. It will be important to identify the concentrations of different inhibitors *in vivo* to determine their relevance to specific κB-binding complexes. In addition, systematic studies addressing the relative affinities of the inhibitory complexes will facilitate the understanding of the specificity of inhibition.

The IκB members can be grouped into two classes based on their subcellular location. The first group includes p105, p100, and IκBγ

(CTD) which reamin in the cytoplasm (Inoue *et al.*, 1992a; Mercurio *et al.*, 1993; Rice *et al.*, 1992). IκBα and Bcl-3 comprise the second group, which can be found both in the cytoplasm and the nucleus (Davis *et al.*, 1990; Kerr *et al.*, 1991; Zabel *et al.*, 1993). IκBα and Bcl-3 may enter the nucleus by passive diffusion or perhaps through yet unidentified nuclear localization signals. This second group of IκB proteins not only inhibit DNA-binding activity of κB-binding complexes, but also displace them from DNA. Potential nuclear localization of these IκB's suggests that they may act as repressor (IκBα) or antirepressor (Bcl-3) of NF-κB activity.

IV. Regulation of Rel/NF-κB Activity

There are two types of Rel/NF-κB activities, inducible and constitutive. The activity of inducible Rel/NF-κB complexes is regulated at multiple levels. First, there are a wide variety of inducers, extracellular or intracellular (Table III). Second, many signaling systems are involved in the pathway, either directly or indirectly. Finally, different members of the IκB family can serve as targets for the incoming signals generated. Thus, the Rel/NF-κB/IκB complexes are strategically placed to integrate and respond to various signals required for selective gene activation.

Since the major form of the latent cytoplasmic κB-binding complex in many cell types appears to be NF-κB/IκBα, we will first discuss its regulation, and later consider the regulation of other κB-binding complexes and IκB members. Constitutive forms of κB-binding complexes are discussed below (see Section IV,D).

A. Regulation of NF-κB/IκBα Complex

Activating signals must result in modification of IκBα rather than NF-κB. This hypothesis is based on several observations made by Baeuerle and Baltimore (1988a,b). First, the dormant NF-κB has an intrinsic DNA-binding activity because mild detergents can lead to IκBα dissociation resulting in NF-κB DNA binding. Second, detergent induced NF-κB DNA binding activity is comparable to that which appears in the nucleus following stimulation. Third, nuclear NF-κB is still sensitive to exogenously added IκBα, demonstrating that NF-κB maintains its ability to bind to IκBα. Finally, IκBα activity is not recovered in the cytoplasm following addition of inducers. These observations indicate that the activating signals modify IκBα to release NF-κB activity.

The modification of IκBα, however, may not be the only pathway for NF-κB activation. Hayashi *et al.*, (1993) demonstrated the presence of a

p65-associated serine kinase which activates NF-κB by directly phosphorylating p65 subunit. Naumann and Scheidereit (1994) have shown that not only IκBα but p65 also is phosphorylated following treatment of HeLa and Namalwa cells with tumor necrosis factor. In this case, the DNA binding activity of NF-κB was greatly enhanced by p65 phosphorylation. Dorsal also undergoes phosphorylation prior to nuclear translocation (Norris and Manley, 1992; Whalen and Steward, 1993). These studies suggest that a direct modification of Rel/NF-κB may also lead to its activation. Since more information is available on the modification of IκBα proteins at present, we will consider the NF-κB activation pathway involving IκBα modifications in more detail.

Modification of IκBα can be induced by a variety of apparently unrelated agents, such as TPA, LPS, cytokines, viral infection, uv irradiation, or H_2O_2 (Table III). Inspection of inducers does not offer a simple known second messenger system that might be involved in IκBα modification. Thus, these stimuli lead to distinct multiple pathways which either independently modify IκBα or converge into common pathways. Since the activation of NF-κB is associated with common events—

TABLE III
INDUCERS OF NF-κB ACTIVITY

Type of inducer	Example	Reference[a]
Growth factors/cytokines	TNF-α	Griffin *et al.* (1989)
	IL-1	Freimuth *et al.* (1989)
	PDGF	Olashaw *et al.* (1992)
T-cell mitogens	Phorbol esters	Sen and Baltimore (1986b)
	Lectins	Bohnlein *et al.* (1988)
	Calcium ionophors	Novak *et al.* (1990)
Bacterial lipid	Lipopolysaccharide	Sen and Baltimore (1986b)
Protein synthesis inhibitor	Cycloheximide	Sen and Baltimore (1986b)
DNA-damaging agents	uv light	Stein *et al.* (1989)
	X rays	Brach *et al.* (1991)
Viral transactivators	X (HBV)	Staddiqui *et al.* (1989)
	tax (HTLV-1)	Leung and Nabel (1988)
	E1A (Adenovirus)	Shurman *et al.* (1989)
Double-stranded RNA	Poly (I-C)	Visvanathan and Goodbourn (1989)
Oxidative stress	H_2O_2	Schreck *et al.* (1991)
Others	Nitric oxide	Lander *et al.* (1993)
	Okadaic acid	Thevenin *et al.* (1990)

[a] The reference is not meant to be complete and provides only an example.

phosphorylation of IκBα, degradation of IκBα, and the activation of antioxidant-sensitive pathway(s) (see below)—it is likely that different signaling pathways may converge into some common pathway(s). However, it is not clear how these events are interconnected to activate NF-κB. It is generally assumed that the phosphorylation of IκBα is an intermediate step for its degradation. An antioxidant-sensitive step is likely to be present upstream of the NF-κB/IκBα complex integrating multiple incoming signaling pathways.

1. Phosphorylation of IκBα

Phosphorylation by specific kinases inactivates IκBα *in vitro* (Ghosh and Baltimore, 1990; Kerr *et al.*, 1991). These kinases include PK-C, PK-A, and a heme-regulated eIF-2 which likely phosphorylate independent residues. Thus, inactivation of IκBα may be accomplished by phosphorylation at multiple sites.

In vivo phosphorylation of IκBα is induced by a variety of activators, such as TNF-α, TPA, and IL-1 (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Mellits *et al.*, 1993). Since TPA activates PK-C, which can directly phosphorylate and inactivate IκBα *in vitro* (Ghosh and Baltimore, 1990), it is possible that PK-C directly phosphoryates IκBα *in vivo*. Other phosphorylation pathways, such as tyrosine kinases, are also important for NF-κB activation. Treatment with tyrosine kinase inhibitors, herbimycin A or genistein, inhibits IL-1- or radiation-induced NF-κB activation (Iwasaki *et al.*, 1992; Uckun *et al.*, 1993), suggesting the involvement of a tyrosine kinase pathway. IκBα is tyrosine phosphorylated following exposure to hypoxia (Koong *et al.*, 1994) and c-Rel and p105 are also phosphorylated on tyrosine residues in T cell lines (Neumann *et al.*, 1992). Induction of NF-κB by a variety of activators can be inhibited by dominant-negative Ras or Raf protooncoproteins (Devary *et al.*, 1993; Finco and Baldwin, 1993), suggesting that this phosphorylation cascade may also be involved in the activation process. Double-stranded (ds) RNA-dependent kinase can phosphorylate IκBα *in vitro* (Kumar *et al.*, 1994) and is important for dsRNA activation of NFκB *in vivo* (Maran *et al.*, 1994). Thus, it is conceivable that different kinase pathways phosphorylate IκBα on different sites thereby inactivating its activity. This scenario predicts that there are multiple phosphorylation sites on IκBα specific for different kinases as predicted from *in vitro* studies. It is also possible that different kinase pathways converge into a common phosphorylation event by an as yet unidentified IκBα-specific kinase. In any case, phosphorylation events are likely to be an integral component of the NF-κB activation pathway. Therefore, identification

of kinase(s) and the new IκBα phosphorylation site(s) will greatly facilitate the understanding of NF-κB regulation.

2. *Degradation of IκBα*

One common event that can be observed during the activation of NF-κB in many cell types is the rapid degradation of IκBα proteins (Brown *et al.*, 1993; Chiao *et al.*, 1994; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*, 1993; Miyamoto *et al.*, 1994c; Rice and Ernst, 1993; Scott *et al.*, 1993; Sun *et al.*, 1993). It is of importance to note that the degradation is rapid (as short as a few minutes) and extensive (no obvious intermediate proteolytic products can be seen). Apparently, IκBα degradation is an obligatory event because a number of protease inhibitors, such as TPCK and TLCK, can inhibit IκBα degradation and consequently inhibit NF-κB activation (Chiao *et al.*, 1994; Henkel *et al.*, 1993; Miyamoto *et al.*, 1994a). In some cases, newly phosphorylated forms of IκBα can be observed transiently prior to degradation indicating that a phosphorylation event may be an intermediate step for degradation (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Mellits *et al.*, 1993; Miyamoto *et al.*, 1994c). If IκBα phosphorylation leads to dissociation of IκBα from NF-κB, then inhibition of IκBα degradation is expected to have little effect on NF-κB activation. However, protease inhibitors inhibit not only the degradation of IκBα but also the activation of NF-κB, suggesting that phosphorylation of IκBα may not result in dissociation.

Mellits *et al.*, (1993) showed that TPCK treatment results in not only the inhibition of IκBα degradation, but also the loss of phosphorylation event, suggesting that TPCK may also inhibit kinase(s). We have recently observed that both TPCK and TLCK blocked IκBα phosphorylation induced by TNFα and LPS (Miyamoto *et al.*, 1994c). Inhibition of NF-κB activation by these inhibitors is likely the result of the inhibition of IκBα phosphorylation rather than direct inhibition of IκBα protease(s). Calpain inhibitors I and II, however, blocked degradation of IκBα without affecting its phosphorylation. Under these conditions, NF-κB was still sequestered in the cytoplasm complexed with the phosphorylated form of IκBα. Thus, IκBα does not dissociate from NF-κB following phosphorylation induced by stimulation with TNFα or LPS (Fig. 1). Furthermore, these results suggest that the substrate for IκBα protease(s) is not free IκBα but rather IκBα associated with NF-κB. However, dissociation of IκBα from NF-κB may occur under some *in vivo* conditions. It is of interest to note that IκBα contains a PEST-like sequence at its C-terminus (Rechsteiner, 1990). Deletion of this sequence results in a

more stable IκBα protein when transiently transfected into 293 cells, suggesting that the PEST sequence may function as a degradation signal (D. VanAntwarp, unpublished data). This sequence may be masked in NF-κB–IκBα complex and phosporylation of IκBα may induce conformational changes, making it accessible for a protease. The precise nature and regulation of IκBα protease(s) is not clearly understood, but Palombella *et al.*, (1994) recently suggested that ubiquitin-dependent proteasome may be involved. Study of the regulation of IκBα degradation is likely to provide new insights into the regulatory mechanisms involved in orchestration of various signaling inputs involved in NF-κB activation.

3. Sensitivity to Antioxidants

NF-κB activation pathways are extremely sensitive to changes in oxidation state of the cells (for review see Baeuerle and Henkel, 1994; Pahl and Baeuerle, 1994). The redox sensitive step must lie downstream and possibly at the converging pathway because antioxidants can effectively inhibit NF-κB activation induced by a variety of inducers (Israel *et al.*, 1992; Meyer *et al.*, 1993; Mihm *et al.*, 1991; Schreck *et al.*, 1991, 1992; Staal *et al.*, 1990). In fact, addition of H_2O_2 can induce IκBα phosphorylation (Naumann and Scheidereit, 1994) and activate NF-κB (Schreck *et al.*, 1991). An antioxidant, pyrrolidinedithiocarbamate, has been shown to inhibit IκBα degradation (Sun *et al.* 1993). These studies strongly suggest that the antioxidant-sensitive step lies upstream of IκBα kinase(s) and downstream of multiple signal transduction pathways. Thus, it is of great interest to identify the molecular target(s) of reactive oxygen intermediates leading to IκBα phosphorylation.

B. Regulation of Other κB-Binding Factors and IκB Proteins

The activation pathway involving other IκB members is less defined. So far, p105 and p100 have been shown to be processed to p50 and p52, respectively, following stimulation (Mercurio *et al.*, 1993; Rice *et al.*, 1992). Thus, the activating signals lead to the increased concentration of available p50 and p52 proteins. The activation of proteolysis of these precursor/IκB proteins not only decreases the overall IκB level but increases new κB-binding subunits at the same time. Transient phosphorylation of p105 can be seen during NF-κB induction (Mellits *et al.*, 1993; Naumann and Scheidereit, 1994). Interestingly, p50 generated from phospho-p105 is not phosphorylated, indicating that p105 phosphorylation site(s) is at the C-terminus (Naumann and Scheidereit,

1994). Like IκBα, p105 processing seems to require phosphorylation event.

The activation pathway involving IκBγ or Bcl-3 is not known. Dephosphorylation of Bcl-3 causes loss of its p50/p50 inhibitory activity, suggesting a potential role for phosphorylation in Bcl-3 regulation (Nolan *et al.*, 1993). Since p105 undergoes phosphorylation at its C-terminus, IκBγ protein may also be phosphorylated upon stimulation.

C. Autoregulation of Rel/NF-κB and IκB

Activation of NF-κB enhances transcription of genes encoding some members of the Rel/NF-κB and the IκB families, such as c-Rel, IκBα, p105, and Bcl-3 (Bours *et al.*, 1990; Chiao *et al.*, 1993; Grumont *et al.*, 1993; Ohno *et al.*, 1990; Paya *et al.*, 1992; see Table I). This is most notable in lymphoid cells like mature B cells, where κB-binding activity is constitutive (Miyamoto *et al.*, 1994a,b). This is because their promoters contain κB sites and are inducible by NF-κB (Chiao *et al.*, 1994; Cogswell *et al.*, 1993; Grumont *et al.*, 1993; Hannink and Temin, 1990; Ten *et al.*, 1992).

Transcriptional regulation of the inhibitor, IκBα, by NF-κB provides an elegant means to autoregulate its activity. In this way, only continuous stimulation leads to persistent activation of NF-κB as a result of enhanced degradation of IκBα proteins. As soon as the activating signal is terminated, the newly synthesized IκBα would quickly turn off additional NF-κB activation. Furthermore, some portion of IκBα may enter the nucleus and rapidly terminate NF-κB-mediated transcription (Zabel *et al.*, 1991). Because the basal half-life of IκBα is much shorter than that of NF-κB proteins (Miyamoto *et al.*, 1994b; Rice and Ernst, 1993), a large increase in IκBα production ensures the termination of NF-κB. Any excess IκBα would be quickly degraded, as free IκBα is unstable (Nolan *et al.*, 1993; Sun *et al.*, 1993). Because NF-κB/IκBα complex is available as soon as the stimulation is terminated, this system allows rapid reactivation upon secondary stimulation. Since the overall level of Rel/NF-κB/IκB complexes is increased, the secondary stimulation may result in augmented activation. Even in the system in which κB binding is constitutively active, such as in mature B cells, further activation over the constitutive level can be achieved by surface IgM crosslinking, incubation with T helper cells, TPA, or LPS (Dobrzanski *et al.*, 1994; Lalmanach-Girard *et al.*, 1993; Liou *et al.*, 1994; Liu *et al.*, 1991). Further increase in κB-binding factors may be needed for activation of additional genes which are involved in the response to antiviral or

antigen stimulation. Thus, coordinated IκBα induction by NF-κB ensures that the system is always under negative regulation to tightly control NF-κB activity in the cell.

D. Molecular Mechanism for Constitutive κB-Binding Activity during B Cell Differentiation

κB binding activity is inducible in most cell types. However, during differentiation of B cells, Rel/NF-κB is constitutively activated concomitant with the transcriptional activation of the Igκ gene (Sen and Baltimore, 1986a). κB binding activity is inducible in pre-B cell lines and is composed of p50/p65 dimer (Baeuerle and Baltimore, 1988; Liou *et al.*, 1994; Miyamoto *et al.*, 1994b; Rice and Ernst, 1993). The constitutive form is observed in nature B cell lines, but the κB binding complex is not p50/p65 but rather a p50/c-Rel dimer (Liou *et al.*, 1994; Miyamoto *et al.*, 1994b; Rice and Ernst, 1993). Thus, inducible and constitutive forms are distinct in this system. The heterodimeric p50/c-Rel was also observed in mature B cells isolated from mouse spleen (Liou *et al.*, 1994; S. Miyamoto, unpublished data), but a p50/RelB dimer can also be seen (Lernbecher *et al.*, 1993). Interestingly, the constitutive κB-binding complex further changes from p50/c-Rel in mature B cells to p52/RelB in plasma cells (end-stage B cell differentiation, Liou *et al.*, 1994). Thus, the nature of constitutive κB-binding complexes changes as a pre-B cell undergoes a multistage differentiation process. Since many of the Rel/NF-κB members, such as p50/p105 and c-Rel, are inducible by κB-binding activity (Table 1), sequential induction of Rel/NF-κB members may modify the complex partners. In fact, prolonged treatment of pre-B cells with LPS alters κB-binding complexes (Liou *et al.*, 1994; Miyamoto *et al.*, 1994a). However, activation of p50/c-Rel depends on the continuous presence of LPS, because it is quickly lost once LPS stimulation is removed. Thus, change of complex alone is not sufficient to induce constitutive activation during pre-B to B transition.

The mechanism for constitutive activation of p50/c-Rel is not clear. Enhanced IκBα degradation is seen in a mature B cell line and seems causally involved in p50/c-Rel activation (Miyamoto *et al.*, 1994a). Constitutive phosphorylation of IκBα may be the reason for its rapid degradation in the absence of external stimulus, indicating a kinase may be constitutively activated in B cells (Naumann and Scheidereit, 1994). In contrast, the mechanism for constitutive activation of RelB-containing complexes (p50/RelB or p52/RelB) is recently characterized; IκBα has a low affinity for RelB in B or plasma cells resulting in constitutive nucelar

transport of RelB complexes, while p65-or c-Rel-containing complexes are kept inducible (Dobrzanski *et al.*, 1994; Lernbecher *et al.*, 1994). IκBα can, however, inhibit p50/RelB in non-B cells. Since constitutive activation of κB-binding complex is also tissue specific (Lernbecher *et al.* 1993), an appropriate combination of tissue or nontissue-specific transcription factors is necessary to maintain tissue- and differentiation stage-specific activation of Rel/NF-κB complexes. This type of transcriptional regulation may ensure induction of c-Rel during pre-B to mature B cell transition and reduction during mature B to plasma cell differentiation (Brownell *et al.*, 1988; Grumont and Gerondakis, 1990). Similarly, the induction of RelB, p50/p105 and p52/p100 must be tightly controlled during B cell differentiation to ensure production of stage-specific κB-binding complexes. The biological consequences of the presence of the distinct κB-binding complexes in B cell differentiation remain elusive, but the target genes important for B cell differentiation may be dictated by the nature of the κB-binding complexes (see also Section V). Gene knock-out of different Rel/NF-κB members will likely resolve some of the roles of distinct κB-binding complexes in B cell differentiation.

V. Rel/NF-κB Regulation of the Target Gene Transcription

There are numerous genes which contain κB site(s) in their regulatory elements (Table I; see review by Grilli *et al.*, 1993). The majority of the target genes are involved in immune responses, acute response, or viral replication. Transcription of these genes is regulated by transcription factors including Rel/NF-κB complexes. How Rel/NF-κB contributes to the transcriptional regulation of these genes depends at least on two parameters; the specific κB sequence which is preferentially recognized by specific Rel/NF-κB factors and their interaction with other transcription factors. Thus, we will discuss the κB site specificity and interactions with other transcription factors.

A. κB Site Specificity

The κB sites of the target genes are quite divergent with the consensus sequence GGGRNNYYCC (Baeuerle, 1991; Table I). It has been shown that p50 binds to the 5′ half, whereas p65 prefers the more divergent 3′ half (Urban *et al.*, 1991). Homodimers of p50 subunit prefer a palindromic site, such as that of the MHC-IκB site (Kierran *et al.*, 1990; Kretzchmar *et al.*, 1993). The more divergent Igκ-κB site is

generally recognized more efficiently by p50/c-Rel, p50/p65, or p52/p65 than by $(p50)_2$ or $(p52)_2$ (Duckett *et al.*, 1993; Fujita *et al.*, 1992; Mauxion and Sen, 1989; Miyamoto *et al.*, 1994a; Perkins *et al.*, 1992). The κB site in the interferon-γ intronic region is recognized by $(c\text{-}Rel)_2$ but not by $(p50)_2$ (Sica *et al.*, 1992). p65/c-Rel heterodimers bind to the urokinase κB site (Hansen *et al.*, 1992). PCR-based selection of κB sites using bacterially expressed p50, p65, and Rel proteins demonstrated that (1) three-G's in the 5′ half site are essential for p50 binding, (2) two C's at 3′ end are required for p65 binding, (3) sites that are not recognized by either p50 or p65 alone can be recognized efficiently by p50/p65 heterodimer, and (4) Rel is the least specific in that it can bind to any κB motif that was selected by p50 or p65 (Kunsch *et al.*, 1992). Thus, interaction between the κB site and the κB-binding complex follows a set of rules which are dictated by the sequence of κB sites.

Transactivation by κB-binding complexes is not solely determined by the DNA-binding affinity. p52/p65 or p52/c-Rel can efficiently bind to the κB sites of Igκ, HIV-LTR, IL-2R, or MHC-I gene, but only activates transcription through Igκ and HIV-LTR κB sites (Perkins *et al.*, 1992). Both c-Rel and p65 bind to the HIV-LTR κB site, but only p65 activates, whereas c-Rel represses transcription through this LTR (Ballard *et al.*, 1990; Doerre *et al.*, 1993). p50 homodimers can activate transcription *in vitro* from κB sites of MHC-I, Igκ, and HIV-LTR, but not from INF-β (Fujita *et al.*, 1993; Kretzchmar *et al.*, 1993). As mentioned earlier, the conformational change of κB-binding proteins induced by different κB sites is correlated with the efficiency of transcativation. Since NF-κB can bend DNA through κB sites (Schreck *et al.*, 1990), differential DNA bending may be important for correct alignment of κB-binding factors for productive interaction with the basal transcription machinery. Thus, the variation in κB sites could provide specificity by several mechanisms: (1) different affinities to κB-binding complexes composed of distinct subunits, (2) κB site-induced conformation on κB-binding complexes, and (3) extent of DNA bending induced by κB-binding complexes.

B. Cooperation with Other Transcription Factors

Most eukaryotic promoters or enhancers contain binding sites for multiple transcription factors. In general, κB sites are also present with other *cis* elements. For example, Igκ intronic enhancer contains multiple regulatory elements besides the κB site, including binding sites for E12/47 basic helix–loop–helix proteins, C/EBP, and silencer-binding

factors (for review see Staudt and Lenardo, 1991). Mutagenesis studies show that all of these elements contribute to maximal activity of the Igκ enhancer. The enhancer activity is minimal when expression vectors coding p50/c-Rel or E47 are transfected individually with reporter gene linked to the κ enhancer and a minimal promoter (S. Miyamoto, unpublished results). However, when these genes are transfected together, marked increase in transcription can be observed, suggesting that p50/c-Rel and E47 synergize in activating this enhancer. Thus, the presence of interacting *cis* elements ensures maximal efficiency and specificity of transcriptional regulation.

Synergistic or antagonistic interactions between transcription factors may involve direct protein–protein associations. The AP-1 and glucocorticoid receptor physically interact to mediate antagonistic effects (Jonat *et al.*, 1990; Schule *et al.*, 1990; Yang-yen *et al.*, 1990). MyoD and c-Jun also mutually inhibit transcription by direct contacts (Bengal *et al.*, 1991). This type of direct interaction between NF-κB and other transcription factors has been documented in a wide variety of gene regulation. The transcription factors that interact directly with NF-κB include AP-1, Sp-1, C/EBP, ATF family, NF-IL6, SRF, glucocorticoid receptor, and components of the basal transcription factors TBP and TFIIB (Kaszubska *et al.*, 1993; Kerr *et al.*, 1993; Kuang *et al.*, 1993; LeClair *et al.*, 1992; Perkins *et al.*, 1993; Stein and Baldwin 1993; Stein *et al.*, 1993; Xu *et al.*, 1993). Other factors, such as HMGI/Y, have been shown to directly interact with NF-κB to induce effecient transcriptional activation through PDR-II at the INF-β gene (Du *et al.*, 1993). The direct interaction of NF-κB with other transcription factors may enhance or diminish transcription by different mechanisms; (1) increase or decrease of the affinity to κB sites, or (2) conformational changes which allow enhanced or decreased interaction with coactivators and/or the basal transcriptional machinery. The interaction of NF-κB with other cell type-specific transcription factors also provides an opportunity for cell-type specific transcriptional regulation by an otherwise ubiquitous NF-κB transcription factor.

VI. *Drosophila* System: Dorsal, Dif, and Cactus

Dorsal is a maternal protein which regulates dorsal–ventral axis development in the *Drosophila* embryo (Govind and Steward, 1991). The activity of Dorsal is regulated by its sucellular localization: Dorsal is uniformly cytoplasmic in an early embryo, but about 90 min following fertilization it translocates into the nuclei only in the ventral cells. Dorsal

shares sequence similarity with Rel-related proteins in chickens and mammals (Steward, 1987; Fig. 2). The homology is the highest in the N-terminal RHD, but is otherwise unrelated. It contains a transactivation domain in its C-terminus, like c-Rel. Upon nuclear translocation, Dorsal activates transcription of *twist* and *snail,* leading to a mesodermal phenotype, and represses *zerknüllt* and *decapentaplegic,* inhibiting dorsalization in the ventral region (Irish and Gelbart, 1987; Ray *et al.,* 1991; Roth *et al.,* 1989; Rushlow *et al.,* 1987). Thus, the activity of Dorsal protein, a member of the Rel/NF-κB family, is regulated by cytoplasmic/nuclear partioning, much like its vertebrate homologue.

Dorsal is sequestered in the cytoplasm by Cactus, a IκB family member (Geisler *et al.,* 1992; Kidd, 1992; Fig. 3). *Cactus* was identified as one of the 11 maternal genes that regulate the activity of Dorsal (Govind and Steward, 1991). Cloning of the gene for Cactus demonstrated that it contains six ankyrin repeat motifs which are related to those found in the mammalian IκB family members (Geisler *et al.,* 1992; Kidd, 1992). Cactus directly associates with Dorsal and inhibits its DNA-binding activity. Dissociation of Cactus is an integral component of Dorsal activation during embryogenesis. Cactus does not inhibit the DNA binding of NF-κB/Rel proteins nor do mammalian IκB proteins inhibit that of Dorsal (Nolan *et al.,* 1993; Wulczyn *et al.,* 1992; S. Miyamoto, unpublished observation).

One of the upstream regulators of Dorsal is Toll, a receptor molecule which activates Dorsal through a PKA-dependent pathway (Norris and Manley, 1992). PKA directly phosphorylates Dorsal at the well-conserved PKA site in the RHD which is an essential event for Dorsal nuclear translocation. Another maternal upstream regulator of Dorsal activity is Pelle, a kinase which most resembles mammalian Raf and Mos protooncoproteins (Shelton and Wasserman, 1993). It is not defined whether Pelle directly phosphorylates the Cactus/Dorsal complex or it regulates Dorsal activity in an indirect manner. However, Pelle can phosphorylate Cactus *in vitro* (S. Wasserman, personal communication). Since some NF-κB activation requires Raf proteins, there seems to be a direct analogy in the signaling systems used in activation of Dorsal and NF-κB (Fig. 4). It is, however, not known whether Cactus is degraded during Dorsal activation or antioxidants have any effect on Dorsal activity in *Drosophila.*

Another member of the c-*rel* family of genes, *dif,* was recently identified and cloned from *Drosophila* (Ip *et al.,* 1993). Dif is expressed in stages past embryonic development and is involved in *Drosophila* immunity against pathogens such as bacteria. Its activity is also regulated by cytoplasm to nuclear localization and it activates transcription of genes, such

as the *CecA1* cecropin gene, involved in insect immunity. Because Cactus has also been implicated in functions in adult fly, Cactus may also be involved in Dif regulation.

Since *Drosophila* genetics has identified 11 regulators of Dorsal activity, it is likely that the mammalian Rel/NF-κB system may also involve similar pathways. Cloning of homologous gens for Pelle kinase from mammalian cells may help in dissecting the activating pathways. The conserved PK-A site of Rel/NF-κB members may also play an essential role in nuclear translocation as in the case for Toll-induced Dorsal phosphorylation. Parallel studies of these different systems will greatly facilitate the understanding of the signaling pathways involved in Rel/-NF-κB/Dorsal activation (Fig. 4).

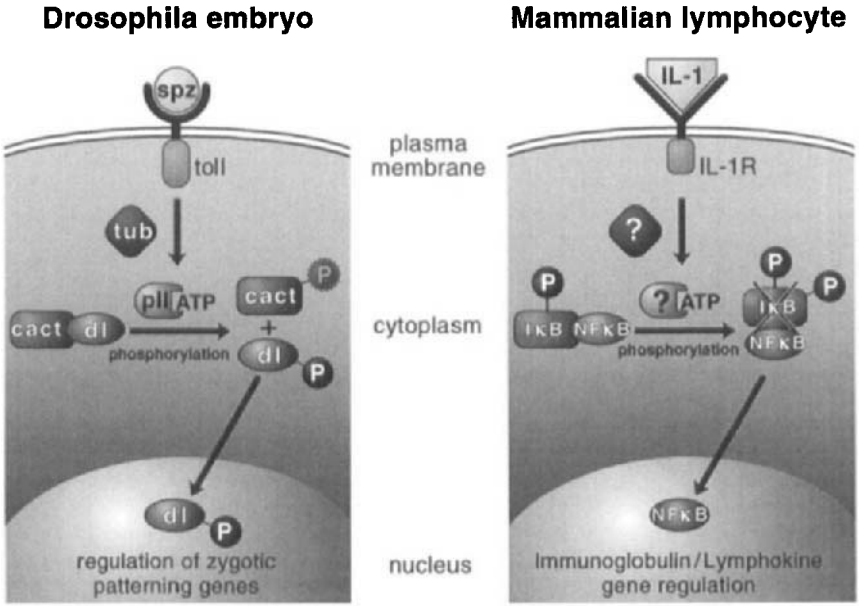

FIG. 4. Parallel Rel/NF-κB/Dorsal systems in *Drosophila* and mammals. Nuclear localization of Dorsal (dl) is activated by maternal proteins including spatzel (spz), Toll (a receptor for spz), and Pelle (pl1, a kinase). Phosphorylation of the conserved protein kinase A site of Dorsal is critical for its nuclear localization. It is not clear whether Cactus is phosphorylated and/or degraded by activation of Toll. In mammalian lymphoid cells, interleukin-1 receptor (IL-1R) activated by IL-1 generates second messengers which eventually lead to degradation of IκBα proteins. It is not known what kinase might be involved in IκBα phosphorylation. NF-κB may also undergo phosphorylation prior to nuclear translocation (modified from Shelton and Wassermann, 1993).

VII. Concluding Remarks

Discovery of NF-κB transcription factors generated a new concept for gene activation: the regulation by cytoplasmic inhibitor molecules. This system is ubiquitous and evolutionally well conserved. A wide variety of exogenous and endogenous agents generate a multitude of regulatory cascades which converge on this cytoplasmic complex, resulting in the release of the active transcription factor.

Despite great interest, the signaling pathways are not yet understood, but post-translational modification is involved. Clearly, one of the rate-limiting steps is inhibitor modification by phosphorylation and degradation. Thus, one of the urgent goals is the identification of kinases that directly phosphorylate IκB proteins and protease system(s) involved in IκB degradation *in vivo*. The intermediate pathways include receptors, oxygen radicals, tyrosine kinases, and/or the Ras–Raf system. How do these signaling pathways act on Rel/NF-κB/IκB? How are related κB-binding complexes selectively induced? The answers will help, for example, in designing a specific inhibitor for HIV replication without conpromising cellular functions.

The targets of the NF-κB system extend to general functions in cell cycle control and specific functions such as the immune response, differentiation, or development. Unveiling how a ubiqutous factor differentially regulates key cellular functions requires future investigations. Identification of target genes involved in cell cycle or differentiation may provide a new way to understand how deregulation of this system can lead to lymphoid and nonlymphoid cancers.

Acknowledgments

We are very grateful to Drs. J.-I. Inoue, Nik Somia, and Elaine Alarid for critical reading of the manuscript, we are also indebted to other members of the Verma laboratory and multiple colleagues in the field for helpful discussions. We thank Pat McClintock for preparation of the manuscript. We could not refer to all the relevant publications due to the vastness of literature in the field and we apologize to those whose contributions were not directly mentioned in this review. I.M.V. is an American Cancer Society Professor of Molecular Biology. This work was supported by grants from the National Intitutes of Health, American Cancer Society, the H. N. and Frances C. Berger Foundation, and the Council for Tobacco Research–USA, Inc.

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RADIATION LEUKEMIA VIRUS-INDUCED LEUKEMOGENESIS: A PARADIGM OF PRELEUKEMIA AND ITS CONTROL BY PREVENTIVE THERAPY

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I. Introduction

The transition of a cell from normal to neoplastic growth involves the sequential accumulation of genotypic and phenotypic alterations over a long period of time, often many years (Klein and Klein, 1985; Weinberg, 1989; Vogelstein and Kinzler, 1993). Thus, the appearance of a gross malignant disease is preceded by a long period of premalignancy, during which small numbers of potentially neoplastic cells exist subclinically and are, as yet, under host control (Wakefield and Sporn, 1990; Gale and Butturini, 1992; Haran-Ghera *et al.*, 1992; Peterson *et al.*, 1993).

It is plausible to expect that therapeutic intervention during premalignancy would be more efficacious than treatment begun after frank disease is already evident. This is because the number of aberrant cells is limited; these have not yet fully acquired the ability to involve normal tissues and spread metastatically; no clinical manifestations of illness have developed. Recognition of the premalignant state thus affords

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opportunities for preventive therapy at a time when it might be categorically effective and curative. To realize these opportunities, it is imperative to explore means for identifying and characterizing the premalignant state and to develop new therapeutic modalities specifically designed to eliminate the premalignant cells. These goals could be advanced by exploiting experimental systems in which premalignancy can be reproducibly induced, identified, and manipulated.

II. Tumorigenesis Induced by Chronic Retroviruses

A suitable model for studying the evolving nature of host–tumor relationships during the course of tumor progression is the lengthy premalignant phase of oncogenesis induced by chronic retroviruses (Bishop, 1987; Teich *et al.*, 1982, 1985; Lazo and Tsichlis, 1990; Coffin, 1990). Because such viruses do not carry an oncogene, their integration into the cellular genome is not accompanied by direct neoplastic transformation. Nevertheless, they initiate a complex process of continuous and dynamic evolution which eventually leads to tumor development. Multiple molecular and cellular events underlying this process have been identified and characterized (Varmus, 1988; Tsichlis and Lazo, 1991). Some of these (e.g., insertional mutagenesis) occur toward the end of the premalignant phase, whereas others (e.g., transactivation of cellular genes or receptor-mediated stimulation) take place shortly after the initial infection. Although extremely heterogenous with regard to their genome, susceptible host, tissue tropism, and type of tumor induced, chronic retroviruses share a mandatory common feature. They are nondefective, replication-competent viruses whose oncogenic potential depends on immediate reproductive infection (Teich, 1982).

In some cases, repeated cycles of infection and replication ensure eventual stochastic integration of the provirus in the vicinity of an oncogene whose activation results in malignant transformation (Coffin, 1990). Hyperexpression or modulation of a cellular oncogene product is dictated by insertion of promoter (Teich *et al.*, 1985, 1982; Neel *et al.*, 1981; Hayward *et al.*, 1981; Fung *et al.*, 1983), enhancer (Cuypers *et al.*, 1984; Steffen, 1984; Tschilis *et al.*, 1983; Payne *et al.*, 1982; Corcoran *et al.*, 1984; Teich *et al.*, 1985), leader (Nilsen *et al.*, 1985), or terminator (Nusse and Bern, 1988) elements located within the LTR region of the retroviral genome (Coffin, 1990). It is thus obvious that the LTR plays a cardinal role in the oncogenic competence of chronic retroviruses which transform their target cells through insertional mutagenesis (Westaway *et al.*, 1984). In other cases, primary infection triggers a cascade of events leading to the transformation of a cell initially infected by the virus

(Famulari, 1983; Davis *et al.*, 1987; Ben-David and Bernstein, 1991). Since the prelude to infection is specific binding of the viral envelope (Env) to the target cell membrane, it has been assumed that the Env glycoprotein is involved in eliciting the early cellular responses that promote oncogenesis. Indeed, recent studies employing sequence and recombination analysis of MuLVs demonstrated that the integrity of the Env is essential for induction of the early leukemogenic steps (Holland *et al.*, 1989). The latter scenario implies that shortly after viral infection, a population of potentially malignant (i.e., premalignant) cells emerges and persists until eventual oncogenic transformation has occurred.

III. Radiation Leukemia Virus (RadLV)-Induced Leukemogenesis

The existence of premalignant cells has been most convincingly demonstrated during the induction of leukemogenesis in C57BL mice by RadLV. RadLV was first isolated from a T cell lymphoma induced in a C57BL/Ka mouse by fractionated irradiation (Lieberman and Kaplan, 1959). The original isolate was weakly leukemogenic upon intrathymic (it) injection into C57BL/Ka mice (Kaplan, 1964). However, serial passage of the virus in newborn mice yielded a highly leukemogenic virus that induced thymic lymphomas in adult mice (Kaplan, 1967; Lieberman *et al.*, 1978; Decleve *et al.*, 1974, 1978).

Another RadLV isolate was generated by Haran-Ghera (1966) from the bone marrow of a C57BL/6 mouse exposed to fractionated irradiation. This virus, designated D-RadLV, exhibited low leukemogenicity and induced thymic lymphomas in only 10% of the mice inoculated. However, when the mice were irradiated 4 Gy prior to virus injection, the incidence of lymphoma was increased to nearly 100% (Haran-Ghera, 1971). Several passages of D-RadLV in C57BL/6 mice resulted in the selection of a highly leukemogenic virus variant (A-RadLV) that induced high-incidence lymphomas in nonirradiated adult mice (Haran-Ghera *et al.*, 1977). All the isolated RadLV appeared to be FV-1^b tropic (Haran-Ghera, 1980).

The establishment of lymphoma cell lines producing RadLV enabled genetic and molecular analysis of the virus. Two questions were raised by the molecular biologists: (1) how could a highly leukemogenic RadLV have evolved from a nonleukemogenic, endogenous virus?, and (2) what are the genetic elements in the virus genome which determine its unique biological properties? Ben-David *et al.* (1987a) demonstrated that the A-RadLV-induced lymphoma cell line 136.5 (Haas, 1974) produces a mixture of N–, B–, NB– and xenotropic, fibrotropic viruses, in addition

to the highly leukemogenic virus. The fibrotropic viruses were not oncogenic and their RNA fingerprinting resembled that of the endogenous virus counterpart. *Ex vivo* infection of T lymphocytes with the 136.5 virus mixture yielded a lymphoma (TIM-1) which produced a single, thymotropic, highly leukemogenic virus with a unique genomic structure bearing ecotropic and xenotropic RNA sequences (Ben-David *et al.*, 1987b).

Rassat *et al.*, (1986) cloned and compared nonleukemogenic endogenous MuLV, low leukemogenic primary RadLV, and high leukemogenic passaged RadLV produced by the BL/VL3 cell line (Lieberman *et al.*, 1979). The biological properties of the cloned viruses and their sequence analysis indicated that RadLV had emerged from an N-tropic, fibrotropic virus which had undergone serial recombination with xenotropic and B-tropic viruses. The highly leukemogenic virus of BL/VL3 cells bore xenotropic sequences in its LTR and *env* and had acquired 43 base-pair tandem repeats in the LTR-U3 region, as well as additional point mutations (Rassat *et al.*, 1986; Merregaert *et al.*, 1985; Gorska-Flipot *et al.*, 1992).

The unique sequences common to the RadLV LTR may confer tissue tropism; the tandem repeats of 43 base pairs could function as transcriptional enhancers. The finding that in 10% of RadLV-induced rat lymphomas the provirus integrates in the vicinity of c-*myc* supports this notion (Janowski *et al.*, 1986). By using the electrophoretic mobility shift assay, Flipot and Joulicoeur (1990) identified three factors which bind to the LTR region of RadLV. One of them, Rad-1, is present in extracts of T cells but not of fibroblasts. Rad-1 binds to a unique sequence located immediately downstream of the core consensus region, which has a sequence motif in its minus DNA strand. It is possible that Rad-1 interacts with other factors bound to the LTR core sequence.

The U3 of RadLV produced by BL/VL3 cells induces synthesis in fibroblasts of suppressive factor(s) which act to block the replication of RadLV as well as of other MuLVs introduced into the cells by superinfection (Rassart *et al.*, 1988). Thus, one function of U3 is to restrict the tropism of RadLV by inducing intracellular resistance in nonlymphoid cells which interferes with virus replication at a later stage of infection (Gorska-Flipot *et al.*, 1992).

The RadLV *env* gene is highly homologous to the *env* of the Akv-MuLV but contains 139 scattered point mutations (Merregaert *et al.*, 1985, 1987). A strikingly variable region of a 21 amino acid length (designated R) is located at the carboxy end of the Env precursor. This region is homologous in length and sequence to the corresponding region in xenotropic retroviruses (Merregaert *et al.*, 1987). It was sug-

gested that the 21 amino acid-long R peptide is cleaved from the Env precursor by the viral-encoded protease late in the virus maturation process (Shinnick *et al.*, 1981).

The Env protein has been shown to play a role in the pathogenesis of certain MuLV-induced malignancies (Holland *et al.*, 1985, 1989; Oliff *et al.*, 1984; Ruscetti and Wolff, 1984; Teich *et al.*, 1982, 1985). However, sequence analysis of the RadLV *env* gene and its comparison with other ecotropic retroviruses did not reveal any unique motifs that could be responsible for the observed tropism and leukemogenesis. Poliquin *et al.*, (1992) constructed a series of recombinant viruses between RadLV and a clone of nonleukemogenic endogenous virus derived from a Balb/c mouse. The biological properties of the recombinant viruses indicated that the thymotropism of RadLV is conferred by the *env* region of its genome. However, the endogenous Balb/c retrovirus could be rendered thymotropic by replacement of its *env* or LTR with those of RadLV. It was concluded that it is the complementarity between *env* and LTR which determines the thymotropism of the virus.

The Env glycoprotein, which mediates virus binding to the target cell membrane, undergoes extensive genetic alterations leading to antigenic modifications (Coffin, 1990). Since the thymus is populated by heterogenous T cell subsets expressing different membrane proteins (Fowlkes and Pardoll, 1989), it is likely that a specific combination of *env*, LTR, and target cell is required for productive infection and subsequent transformation within the thymus. The induction of thymic lymphomas by RadLV might accordingly be dependent on the formation of a particular *env*–LTR–target cell makeup in the thymic microenvironment.

IV. Mechanism of Transformation by RadLV

Activation of a cellular protooncogene via proviral integration has been implicated in the induction of malignancies by a number of chronic retroviruses (Peters, 1990). Thus, c-*myc*, *lck*, *gin-1*, *pim-1*, and *mlvi 1–4* have been identified as common integration sites for MuLVs, and the genomic rearrangement of these regions was found to correlate with the development of T cell lymphomas and leukemias (Steffen, 1984; Corcoran *et al.*, 1984; Li *et al.*, 1984; Selten *et al.*, 1984; O'Donnel *et al.*, 1985; Cuypers *et al.*, 1984; Tsichlis *et al.*, 1983, 1984; Graham *et al.*, 1985; Lemay and Jolicoeur, 1984; Villeneuve *et al.*, 1986). However, integration of RadLV in the vicinity of these genetic regions in murine lymphomas has not been demonstrated. On the other hand, Tremblay *et al.* (1992) detected a novel gene, designated *vin-1*, which was rearranged by insertion of proviral RadLV in some RadLV-induced lymphomas. The

vin-1 gene was later identified by Hanna *et al.* (1993) as coding for the Cyclin-D2 protein which regulates cell transition from the G1 to the S phase of the cell cycle (Hunter and Pines, 1991). These investigators suggested, therefore, that constitutive activation of Cyclin-D2 by insertional mutagenesis may contribute to oncogenesis by driving the cells to continuous proliferation. It is unlikely, however, that Cyclin-D2 expression per se could account for the malignant transformation of a T cells by RadLV; the process apparently requires additional genetic events (Tremblay *et al.*, 1992). Moreover, proviral integration at the *vin-1/cyclin-D2* locus occurred in only 5% of the RadLV-induced lymphomas tested, indicating that *cyclin-D2* activation is neither a mandatory nor a common genetic alteration occurring during RadLV-induced leukemogenesis.

Involvement of c-*myc* and *mlvi 1–2* in RadLV leukemogenesis was indicated by the high frequency (>60%) of RadLV-induced lymphomas displaying chromosome 15 trisomy (Wiener *et al.*, 1978a). The important segment involved in chromosome 15 duplication was localized to the distal region, in which c-*myc* and *mlvi 1–2* are located (Wiener *et al.*, 1978b). Acquisition of chromosome 15 trisomy is a late event in the genesis of T cell lymphomas and occurs independently of insertional mutagenesis or c-*myc* rearrangements (Wirschubsky *et al.*, 1984). The genetic overdose of c-*myc* due to the chromosomal duplication apparently has a decisive influence on the expression of the malignant phenotype (Wirschubsky *et al.*, 1986).

Weissman and McGrath (1982) proposed receptor-mediated lymphomagensis as a mechanism by which a chronic retrovirus transforms T (and B) lymphocytes in the absence of insertional mutagenesis. They found that virus particles produced by the RadLV-induced lymphoma C6VL bound selectively to the membrane of C6VL cells (McGrath *et al.*, 1978). The binding ability was mapped to the Env (O'Neill and Weissman, 1989), and antibodies inhibiting the binding suppressed lymphoma cell proliferation (O'Neill, 1988). In another study, O'Neill *et al.* (1987) showed that a clonotypic MoAb to the T cell receptor αβ heterodimer of C6VL cells inhibited RadLV binding and that the CD4 was also involved in virus-cell recognition. They suggested that RadLV (and perhaps other retroviruses as well) transforms T cell clones expressing TCR specific for the virus Env glycoprotein. Binding of the virus or its Env products to a clonotypic TCR could induce antigenic stimulation resulting in continuous proliferation. A major drawback of this model is the fact, borne out by ample evidence, that TCR does not recognize an antigen unless it is processed and presented by MHC molecules of antigen-presenting cells (Germain, 1993). The establishment of lympho-

mas transformed by RadLV which express TCR specific for virus-unrelated proteins, such as KLH (Finn *et al.*, 1979) and OVA (Yefenof *et al.*, 1982), poses an additional difficulty.

V. RadLV-Induced Preleukemic (PL) Cells

The multiplicity of mechanisms presumed to underlie RadLV-induced leukemogenesis, and the controversy regarding their relative contribution to lymphoma progression, suggest the existence of multiple alternative pathways of cellular and molecular alterations following primary infection which eventually converge to produce a common malignant phenotype. This concept is reinforced by data demonstrating that, regardless of the eventual transforming event, RadLV-induced lymphomas emerge from a population of potentially malignant (preleukemic) cells which appear shortly after virus inoculation and persist in the thymus during the entire premalignant phase (Lonai *et al.*, 1982; Goffinet *et al.*, 1983; Boniver *et al.*, 1981; Haran-Ghera and Rubio, 1977; Haran-Ghera, 1980; Haran-Ghera *et al.*, 1978; Ben-David *et al.*, 1987b; Haran-Ghera, 1978; Haran-Ghera and Peled, 1979; Lieberman *et al.*, 1987a; Yefenof *et al.*, 1988; Ben-David *et al.*, 1987a; Yefenof and Ben-David, 1983; Yefenof *et al.*, 1980a).

Detection of such cells is based on a transplantation bioassay in which thymocytes of virus-injected mice are transferred to the thymus of genetically marked recipients. Inoculation of C57BL/6, Thy 1.2, or female cells into (C57BL/6xBALB/c) F_1, Thy 1.1 cogenic, or male mice, respectively, allows distinction between donor and recipient-derived cells (Hanan-Ghera, 1978; Lieberman *et al.*, 1987; Ben-David *et al.*, 1987b). Development of donor-type thymic lymphomas in the recipients indicates the existence of preleukemic (PL) cells in the donor at the time of transfer. Using this assay, Haran-Ghera (1980) demonstrated that as early as 10 days after inocluation of a highly leukemogenic RadLV (A-RadLV), PL cells appeared in the thymus. Inoculation of low leukemogenic RadLV (D-RadLV) resulted in the induction of PL cells within the bone marrow (Haran-Ghera, 1980). These findings reemphasize the central role of the thymic microenvironment in the leukemogenic process, which calls for persistent interaction between RadLV-infected PL cells and the thymic stroma (Decleve *et al.*, 1974). Lymphoma development following inoculation of a low leukemogenic RadLV will occur only if PL cells emerging in the bone marrow are able to migrate to the thymus (Haran-Ghera, 1980).

In a later study, Gokhman *et al.* (1990) found that PL cells induced by

RadLV reside predominantly within the premature, double-positive ($CD4^+8^+$), continuously dividing thymocyte population. These cells constitute the major subpopulation of thymic lymphocytes undergoing the positive and negative selection which shapes the T cell repertoire (Fowlkes and Pardoll, 1989; Von Boehmer, 1990).

We have used the PL transplantation assay to estimate the number of PL cells in the thymus of RadLV-inoculated mice (Yefenof *et al.*, 1991). Graded numbers of thymocytes from mice inoculated 3 weeks earlier with RadLV were transferred to a number of recipients. It was found that the minimal number of PL thymocytes required to transfer lymphomagensis was 10^3/mouse. Since the average number of thymic lymphocytes in a C57BL/6 mouse is 8×10^7, the finding implies that 3 weeks after virus inoculation, the thymus contains some 8×10^4 "leukemogenic units," each capable of independent lymphoma progression if transferred to a susceptible recipient.

Ben-David *et al.* (1987b) used a MoAb against the RadLV Env glycoprotein (gp70) to identify virus-producing cells in the PL thymus. Reactivity to the antibody was first detected 10–15 hr after virus inoculation, the timing corresponding to the kinetics of retroviral infection, integration, and replication (Panet, 1980). The percentage of positive cells continuously increased, constituting one-third of the total thymic cell population after 1–4 days. The frequency of positive cells then gradually decreased to 2 or 3% in the third week following virus inoculation. The low level of virus-positive cells remained constant during the remainder of the premalignant latency and until the appearance of overt lymphoma, when the thymus was repopulated by lymphoma cells infected by the virus (Ben-David *et al.*, 1987b). These results imply that immediately after it inoculation, RadLV infects a large, polyclonal population of thymic lymphocytes, the majority of which are subsequently eliminated due to thymic lymphocyte turnover (Fowlkes and Pardoll, 1989) and the elicitation of antivirus immunity (Yefenof *et al.*, 1980b). However, lymphoma progression is carried on by a small number of virus-infected cells that are maintained in the thymus over extended periods of time and which eventually give rise to a thymic lymphoma.

Collectively, the data suggest that during most of the premalignant latency, the thymus contains $1.6–2.9\times10^6$ virus-infected premalignant cells (2 or 3% of the 8×10^7 thymic lymphocyte population) and that the minimal number of infected cells required to transfer lymphomagenesis to a naive mouse is 20–30 (2 or 3% of the 10^3 thymic lymphocytes which constitute a leukemogenic unit).

VI. PL Cell Cultures

Haas *et al.* (1984, 1987) developed a procedure for *in vitro* propagation of factor-dependent, early T cell lymphomas induced by irradiation or RadLV. Such cells proliferated when cocultivated with adherent splenic stromal cells. Attempts to culture RadLV-induced PL cells on splenic or thymic stroma proved unsuccessful. However, growth could be provoked by the addition of conditioned supernatant from a cultured, RadLV-induced lymphoma (SR4) (Yefenof *et al.*, 1991). Since SR4 lymphoma cells constitutively secrete IL-4 (Yefenof *et al.*, 1992b), it was assumed that the lymphokine stimulated T cell growth in the thymic–stromal coculture. However, replacement of SR4 supernatant with recombinant IL-4 was not sufficient to maintain lymphoid cell growth. Nonetheless, cell proliferation in cultures supplemented with SR4 supernatant was inhibited by the anti-IL-4 MoAb 11B11 (Yefenof *et al.*, 1991). These results suggested that although PL lymphoid cells respond to IL-4, additional factor(s) are necessary for their continuous growth. Interaction of RadLV particles released from SR4 cells with the thymic-derived lymphocytes may be required to stimulate their *in vitro* growth (O'Neill *et al.*, 1987).

Lymphocytes proliferating in the coculture were found to be infected with RadLV. Such *in vitro* growth did not occur when thymic cells were explanted from mock-infected mice. Moreover, the majority of the cells in the culture were Thy1$^+$, CD3$^+$, CD4$^+$, and RadLV$^+$, although the lines had been initiated from thymocytes of which no more than 3% were infected by the virus (Ben-David *et al.*, 1987b). Hence, there was enrichment of the RadLV-infected cells in the culture, whereas the non-infected cells gradually disappeared.

RadLV-induced PL cells have been defined as thymocytes lacking malignant competence but capable of progressing into mature lymphomas in the thymic milieu (Haran-Ghera *et al.*, 1978). To ascertain whether the cultured, virus-infected cells fit these criteria, we compared their malignant properties with those of established RadLV-induced lymphoma lines. The latter are able to form local tumors when injected subcutaneously into syngeneic mice (Yefenof and Ben-David, 1983) and grow in colonies when seeded in soft agar (Epsztein, 1990). All the PL lines tested were devoid of these properties (Yefenof *et al.*, 1991), suggesting their nonmalignant character.

On the other hand, injection of the *in vitro* propagated PL cells into the thymus of C57BL/6 mice resulted in the development of lymphomas after a latency of 6–8 weeks (Yefenof *et al.*, 1991). These observations

indicated that cultured PL cells resemble normal thymic lymphocytes phenotypically and are not malignant. However, they can be distinguished from normal T cells by their ability to grow *in vitro*, as well as by their expression of RadLV and leukemogenic potential, which is manifested only when the cells are allowed to progress in a susceptible thymus.

VII. Clonal Nature of PL Cells

The relatively large number of potentially malignant cells in the thymus of RadLV-inoculated mice led to the assumption that they comprise a polyclonal population of T cells. The monoclonal origin of mature, RadLV-induced lymphomas has been established by the detection of unique rearrangements in the gene coding for the β-chain of the T cell receptor (Tβ) (Ben-David *et al.*, 1987b; Yefenof *et al.*, 1988). To determine the clonal nature of PL cells, a split transfer assay was developed in which thymocytes from a single, RadLV-inoculated mouse are injected into the thymus of several recipients. Donor-type lymphomas are then analyzed by Tβ Southern hybridization using a Tβ-specific probe. The clonal makeup of the PL cells can be inferred, since different Tβ rearrangements within the array of generated lymphomas reflect the clonal heterogeneity of PL progenitors at the time of transfer. Using this assay, it was found that when recipient mice were injected with limited numbers of PL thymic cells (10^3 or 10^4 corresponding to 1–10 leukemogenic units) explanted 3–6 weeks after virus inoculation, each of the donor-derived lymphomas, developing 3–5 months later, displayed a unique and distinct Tβ rearrangement (Yefenof *et al.*, 1991). This pattern indicated that the PL cells comprise a pleioclonal population of thymic lymphocytes, with a single cell becoming the sole progenitor of a malignant lymphoma. When the PL cells remain within the thymus of the virus-inoculated mouse, a clonal lymphoma eventually arises due to selection of one PL cell that has acquired a fully malignant phenotype. However, a split injection of the PL thymic cell content into several mice results in independent progression of leukemogenesis in each recipient, yielding clonal lymphomas derived from different PL precursors.

VIII. IL-4 Dependency of PL Cells

The persistence of PL cells in the thymus over a latent period of several months was enigmatic in view of the short-term thymic residence of maturing T cells (Fowlkes and Pardoll, 1989). Studies of population dynamics revealed that thymic lymphocytes either die or migrate to

secondary lymph nodes within several days (Huesmann *et al.*, 1991). Indeed, most of the cells primarily infected by RadLV were eliminated from the thymus in the first week of virus inoculation (Ben David *et al.*, 1987). However, several million virus-infected cells remained in the thymus over a period of several months. It was postulated, therefore, that infection by RadLV endows a selected pleioclonal population of thymic lymphocytes with the ability to survive in the thymus until one of the clones is transformed and becomes tumorigenic. Since RadLV-induced PL cells have been characterized as activated, dividing T lymphocytes (Gokhman *et al.*, 1990), it is reasonable to assume that the continuous proliferation of RadLV-infected cells results in the maintenance of a PL cell population in the thymus.

Haas *et al.* (1984, 1987) reported that *in vitro* propagation of early detected T cell lymphomas in RadLV-inoculated mice is dependent on a growth factor of undefined nature, continuously secreted by the cells. The factor-dependent cells could be converted into factor-independent cells by passage in the thymus of a syngeneic mouse. In a subsequent study, we found that some, but not all, RadLV-induced lymphomas secrete IL-4 (Yefenof *et al.*, 1992b). Secretion of the factor was inhibited by anti-RadLV antibodies, albeit without influencing cell growth. Since none of the lymphomas tested produced IL-2, it was concluded that RadLV preferentially transforms lymphocytes belonging to the T_{H2} subset which, upon stimulation with antigen or mitogen, releases IL-4, but not IL-2 (Mosmann *et al.*, 1986).

RadLV did not infect or transform T cells *in vitro* (Yefenof *et al.*, 1982). However, exposure of normal thymic cells to RadLV *in vitro* resulted in IL-4 secretion, a response inhibited by anti-RadLV antibodies (Yefenof *et al.*, 1992). Whereas untreated, normal thymic cells did not secrete IL-4, PL thymocytes constitutively produced the factor. Secretion was enhanced by RadLV and inhibited by anti-RadLV antibodies, suggesting that interaction with the virus triggered the cells to release the factor.

O'Neill *et al.* (1987) showed that RadLV particles cross-ligate the CD3 and CD4 expressed on the membrane of T_H cells. These molecules are directly involved in relaying activation signals to T_H cells recognizing an antigen presented with MHC class II molecules (Saizawa *et al.*, 1987). Upon presentation to T_{H2} cells, an IL-4-dependent autocrine growth-stimulation loop begins to operate, bringing about the clonal expansion of cells bearing receptors specific for the stimulating antigen (Fernandes-Botran *et al.*, 1986).

Collectively, the data suggest that through its binding to CD3 and CD4, RadLV mimics the action of antigen on T_{H2} cells and induces the

autocrine growth stimulation mediated by IL-4. There are, however, two basic differences between antigen- and RadLV-driven stimulation: (1) whereas antigen activates an autocrine growth loop for clonal expansion of antigen-specific T cells, RadLV acts on a pleioclonal population of T cells; and (2) in the ontogeny of the immune response to antigen, IL-4 secretion is limited and comes to a halt as soon as antigen is eliminated. On the other hand, autocrine activation by RadLV is perpetual, since the virus is integrated and replicates within the infected cells. Continuous autocrine response to IL-4 enables the cells to proliferate in the thymus and to maintain a population of pleioclonal PL cells of which one is eventually transformed.

The *in vitro* growth of PL cells is dependent on exogenous IL-4 (Yefenof *et al.*, 1991). In contrast, the growth of RadLV-induced lymphomas, even those that secrete IL-4, is autonomous. It is therefore conceivable that IL-4 secretion in mature lymphomas is a vestige of their PL progenitors whose long-term survival is dependent on an IL-4-driven autocrine stimulus. Thus, progression from PL to the leukemic state involves selected outgrowth of a PL cell liberated from IL-4 dependency. Such a cell will become the sole progenitor of a clonal lymphoma which is no longer dependent on the factor for continuous growth.

The proposed scenario for RadLV-induced proliferation of PL cells ought to be compared with the mode of action of two other retroviruses, HTLV-1 and Friend erythroleukemia virus, which induce polyclonal proliferation of tissue-specific cells. HTLV-1 evokes T cell lymphomas in adult humans (Uchiyama *et al.*, 1977; Wong-Staal and Gallo, 1985). Infection of T lymphocytes and proviral integration initiate IL-2-dependent autocrine growth, triggered by transactivation of the *IL-2* gene with the TAX viral protein (Siekevits *et al.*, 1987; Gazzolo and Duc-Dodon, 1987). Friend virus, on the other hand, induces growth of erythroid cells because its Env glycoprotein (gp55) binds to the erythropoietin receptor (EPO-R), thus mimicking the action of a tissue-specific growth factor (erythropoietin) (Li *et al.*, 1990; Ben-David and Bernstein, 1991). This interaction involves the binding of intracellular gp55 to EPO-R in the endoplasmic reticulum of virus-infected cells (Yoshimura *et al.*, 1990). Hence, growth stimulation following HTLV-1 and Friend virus infection is initiated within the cells and does not require chronic interaction with exogenous virus particles. Proliferation of RadLV-infected T cells is not induced by transactivation of cellular genes nor by intracellular binding of the virus to a growth factor receptor. It could, therefore, represent a class of retroviruses which induce autocrine growth stimulation by extracellular binding to membrane mol-

ecules involved in the transduction of activation signals to lymphocytes during the development of the immune response.

This model may be construed as a modification of the "receptor-mediated lymphomagenesis" model proposed by McGrath and Weissman (discussed under Section V). Our model proposes interaction of the transforming retrovirus with the antigen receptor as a growth-promoting event, but excludes the elements of specific recognition of virus antigens by clones expressing a unique antigen receptor.

IX. Active Intervention in RadLV Leukemogenesis

The recognition of PL cells and the properties underlying their long-term persistence and progression has enabled the development of therapeutic modalities for prophylactic intervention in preleukemia. The rationale for these studies is that treatments which arrest the growth of PL cells or eliminate them from the thymus before a clone is selected for expression of a malignant phenotype may rescue the premalignant mouse from a full-blown lymphoma. Since maintenance of PL cells in the thymus is dependent on autocrine IL-4 stimulation, agents antagonizing IL-4 activity should presumedly be effective in preventing lymphoma development. A suitable drug for this purpose is cyclosporin-A (CSA), an immunosuppressive cyclic peptide widely used to prevent rejection of allogenic transplants (White and Calne, 1982).

The mechanism by which CSA mediates its immunosuppressive effects is not entirely clear. However, one of its pronounced biological activities is the inhibition of lymphokine secretion by activated T lymphocytes (Wagner, 1983). Normally, CSA has no role in cancer therapy, in which host immunity should be stimulated rather than suppressed. However, since the RadLV-induced premalignant state is perpetuated by secretion of and response to IL-4, treatment with CSA could prove beneficial.

Before embarking upon *in vivo* experiments, the effect of CSA was studied *in vitro* (Yefenof *et al.*, 1992a). CSA markedly inhibited IL-4 secretion by RadLV-induced lymphomas and PL thymocytes, as well as normal and PL thymocytes stimulated by RadLV *in vitro*. These results substantiated the rationale for using the drug *in vivo* during premalignant latency. Administration of CSA 3–6 weeks after virus inoculation resulted in a significant delay in lymphoma onset, the most effective regimen being biweekly intraperitoneal injection of 50 mg/kg CSA for 2 consecutive weeks (Yefenof *et al.*, 1992a). Although CSA treatment was able to prolong survival of the virus-inoculated mice by 4–7 weeks, it did

not reduce the incidence of lymphomas. Thus, nonspecific inhibition of IL-4 secretion by CSA delays progression of PL cells to frank lymphoma but does not abort it.

Another tactic was the specific targeting of PL cells with an immunotoxin (IT) composed of a ricin A-chain and a monoclonal antibody directed against the RadLV envelope glycoprotein (gp70). The antibody chosen (2F10) was highly reactive with RadLV-infected lymphoma and PL cells, whereas normal thymic cells did not bind it (Yefenof *et al.*, 1991). Two ITs were constructed, one with an intact A-chain (2F10-A), the other with a deglycosylated A-chain (2F10-dgA). The two ITs were equally specific and effective in inhibiting protein synthesis of RadLV-induced lymphomas *in vitro*. However, when administered to mice undergoing RadLV-induced leukemogenesis, a clear difference emerged between the two preparations. Treatment with 2F10-A was ineffective, whereas 2F10-dgA significantly delayed lymphoma development (Yefenof *et al.*, 1992a).

Thorpe *et al.* (1985) showed that ITs containing an intact A-chain administered *in vivo* are rapidly removed from the circulation. This nomenon was attributed to trapping by hepatic Kupffer cells, which express receptors to the mannose-rich carbohydrates of the A-chain. ITs prepared with deglycosylated A-chain exhibit an improved antitumor effect *in vivo* (Blakey *et al.*, 1987; Fulton *et al.*, 1988). Indeed, experiments with radiolabeled ITs revealed that the uptake of 2F10-dgA by the thymus of PL mice was 30-fold greater than that of 2F10-A (Abboud, 1991). This may explain the inability of 2F10-A to exert an anti-lymphoma effect *in vivo*.

On the other hand, 2F10-dgA strikingly prolonged survival of PL mice. When administered biweekly during the fourth and fifth week after inoculation of the virus, iv injection of 1mg/kg 2F10-dgA delayed mortality by 40–45 days (Yefenof *et al.*, 1992a). Like CSA treatment, the administration of IT did not prevent tumorigenesis and most of the animals eventually died of thymic lymphomas. Hence, treatment with IT or CSA alone is insufficient for total eradication of PL cells. However, since the inhibitory effects of the two drugs are induced by two entirely different mechanisms, it was postulated that they may act in concert. Indeed, when administered simultaneously in an optimal therapeutic regimen, CSA and 2F10-dgA synergized in protecting the majority of the mice (80%) from lymphoma development (Yefenof *et al.*, 1992a). The mice remained tumor-free up to 12 months after virus inoculation, at which time the experiment was terminated. In addition, the few animals which did develop lymphomas following this dual treatment sur-

vived longer than animals treated with either drug alone. One may thus postulate that when administered during PL latency, 2F10-dgA eradicates the great majority of RadLV-infected cells in the thymus. This treatment only acts to delay the premalignant process because as few as 20–30 surviving PL cells are still capable of progressing and developing into a lymphoma (Yefenof *et al.*, 1991). However, if CSA is also administered, it presumably arrests the proliferation of escaping PL cells whose growth is dependent on IL-4 secretion. These complementary activities should, therefore, exert a synergistic effect and prevent lymphoma development.

CSA is a nonspecific biomodulator with a variety of immunosuppressive activities. A more specific IL-4 antagonist is the 11B11 monoclonal antibody which binds to murine IL-4 and neutralizes its activity (O'Hara and Paul, 1985). This antibody inhibited proliferation of PL cells *in vitro* (Yefenof *et al.*, 1991). When provided at doses of 10–20 mg/kg, 11B11 substituted for CSA in delaying lymphoma induction and synergizing with 2F10-dgA in lymphoma prevention (Yefenof *et al.*, 1992a). Increasing the dose of 11B11 to 60 mg/kg resulted in 50% protection of the PL animals without concomitant treatment with 2F10-dgA. These results demonstrated that antagonizing the autocrine response of PL cells either by reducing IL-4 production (CSA) or by neutralizing its activity (11B11) can be of prophylactic value.

One might question to what extent these observations in a murine model are relevant to human malignancies in general and to human lymphomas in particular. Although it is widely accepted that most human neoplasms evolve through a long, multistep process of tumor progression (Klein and Klein, 1985; Weinberg, 1989; Vogelstein and Kinzler, 1993), a premalignant stage in most cancers in humans has not been clearly identified. Once techniques are developed to accurately define and characterize premalignant conditions in humans, prophylactic intervention adapted to the unique features of the premalignant cells may be feasible.

A human cancer somewhat resembling the model of RadLV leukemogenesis is adult T cell lymphoma/leukomia (ATLL) (Uchiyama *et al.*, 1977; Wong-Staal and Gallo, 1985). ATLL is caused by a chronic retrovirus (HTLV-1) that transactivates IL-2-dependent autocrine growth stimulation of infected T cells (Siekevits *et al.*, 1987; Gazzolo and Duc-Dodon, 1987). However, since only 2% of HTLV-1-positive individuals eventually develop ATLL (Dalgleish and Malkovsky, 1988), attempts at prophylactic intervention in the preleukemic state are impractical. Identification of additional predisposing cofactors may facilitate the

prognosis of tumor development in preleukemic individuals (Ikeda *et al.*, 1990). Such individuals would then be likely candidates for prophylactic treatment with IL-2 antagonists and antivirus ITs.

X. Conclusion

In the first part of this article we have reviewed the unique properties of RadLV as a chronic transforming retrovirus and addressed the mechanisms implicated in the ability of RadLV to provoke thymic lymphomas, with emphasis on events taking place during the premalignant latency.

The identification of premalignant cells shortly after inoculation of the virus and their persistance in the thymus points to RadLV-induced leukemogenesis is a suitable experimental system for studying cancer prevention by active intervention during the premalignant state. These investigations are described in the second part of this paper and demonstrate that the lengthy course of the premalignant process can be obviated by applying chemopreventive regimens which interfere with its further progression.

The singular interplay of events in the induction and progression of RadLV leukemogenesis relates to the particular relationship between an etiologic agent, an affected cell, a target organ, and a susceptible host. Nonetheless, the lessons to be learned from our review extend beyond their relevance to a specific murine disease. They emphasize the importance of developing assays for identification of preneoplastic cell populations and tactics for decreasing their size and growth whenever feasible. An important caveat of this approach would be not to employ mutagenic pharmacologic agents, since these might offset benefits accruing from a reduction of the premalignant cell population. Soluble cytokine receptor, ITs, anti-cytokine antibodies, and growth arrest factors are particularly attractive candidates for such clinical interventions. Hopefully, chemotherapeutic trials of this nature will eventually lead to the primary prevention of malignant diseases whose subclinical, precancerous state permits identification, characterization, and targeting.

Acknowledgments

We are indebted to Drs. David Weiss, Jonathan Uhr, Ellen Vitetta, and Alexandra Mahler for their comments and suggestions on the manuscript and to Ms. Bat-Sheva Geffen for secretarial assistance. The authors were supported by the U.S.–Israel Binational Science Foundation, Concern II, Israel Cancer Research Fund, the Society of Research Associates of the Lautenberg Center, the Wakefern/Shoprite Endowment, and the Israel Science Foundation.

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THE INSERTION OF FOREIGN DNA INTO MAMMALIAN GENOMES AND ITS CONSEQUENCES: A CONCEPT IN ONCOGENESIS

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I. Scope of Review

In this chapter, I propose to examine the consequences of foreign DNA insertion into established mammalian genomes. To what extent can these insertions contribute to the transformation of a cell from the growth-regulated to the oncogenic phenotype? Of course, the term "established" represents an unwarranted simplification. Genomes are in a constant state of flux. However, for the relatively short life span of an individual, its genome may be considered an established entity. It is

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unknown how frequently insertions of foreign DNA into the genome of an organism can occur. From the outcome of experiments in cell culture, it appears that any foreign DNA, when offered under appropriate ionic conditions and in a stabilized configuration, can be ingested by cells, transported to the nucleus, and integrated into the cellular genome. The efficiency and frequency of these events have not yet been sufficiently quantitated. Since the days that temperent phage have been discovered, it is common knowledge that the integration of foreign DNA into a cellular genome introduces new genetic potential into the cells' repertoire. In addition, insertion can lead to the disruption of cellular genetic information. It is likely that only those insertion events can be survived by the cell in which (i) damage to cellular genes by integration of foreign DNA has been kept to a minimum and (ii) expression of foreign genes potentially detrimental to the cell can be curtailed. In mammalian cells, the ubiquitous repetitive DNA sequences possibly constitute a reservoir of insertion targets whose disturbance may have less immediate functional consequences. Of course, when the functions of repetitive DNA sequences are discussed, we argue with considerable ignorance.

Among the consequences of the insertion of foreign DNA into established genomes and of the transformed cell phenotype are (i) the *de novo* methylation of the foreign DNA and (ii) alterations in the existing patterns of DNA methylation in the preexisting cellular genome. These alterations have been observed both in mammalian and in plant genomes. I have proposed that the *de novo* methylation of integrated foreign DNA can be interpreted as a cellular defense mechanism against the activity of foreign genes haphazardly integrated into an established eukaryotic genome (Doerfler, 1991b). This cellular defense hypothesis has to be understood in the context of the selection of cells surviving the insertion event. For unknown reasons and by an as yet undetermined mechanism possibly related to the transformed phenotype of the cells, foreign DNA insertion can destabilize the patterns of cellular DNA methylation. We do not yet know how far reaching and how general these alterations can be. Changes in the methylation of cellular genes, increases or decreases, can entail the shutoff or the activation, respectively, of cellular genes. There is considerable evidence for the notion that promoter methylation in eukarotes is associated with the long-term inactivation of genes (for reviews see Doerfler, 1981, 1983a, 1989, 1993). Hence, alterations in the spectrum of cellular gene expression upon insertion of foreign DNA, e.g., of viral genes, in virus-transformed cells can be viewed as a consequence of alterations in the extent of methylation in cellular DNA. Many questions remain, however, to be answered before the mechanisms of these complex interdependencies between

foreign DNA integration, transformation of the cell, alterations in methylation patterns of cellular DNA, and changes in cellular gene expression can be fully explained.

Motivated by the discovery of adenovirus oncogenesis in newborn hamsters (Trentin *et al.*, 1962), I started in 1966 to investigate the interaction of adenovirus type 12 (Ad12) with hamster cells. We have studied problems of Ad12 DNA integration, viral and cellular gene expression, and alterations in DNA methylation in the integrated viral and the cellular genomes (for recent reviews, see Doerfler, 1991a, 1992, 1993). Although its direct relevance for human oncogenesis remains unproven, the adenovirus system continues to be evaluated as an excellent model to identify new basic mechanisms in molecular biology (Doerfler and Böhm, 1995).

Recently, we have made a discovery that sheds new light on the importance and the fate of foreign DNA in the mammalian organism (Schubbert *et al.*, 1994). Foreign DNA ingested by mice with their diet survives the digestive regime of the gastrointestinal tract in fragments of considerable lengths and can be recovered from the bloodstream of the animals. The DNA of bacteriophage M13 has been used as the tracer molecule in these model experiments. Small amounts of this DNA have been retrieved from the animals' feces with a length of about 20% of that of the intact phage genome which measures 7250 nucleotide pairs. In the bloodstream, $<0.1\%$ of the diet-ingested M13 phage DNA has been detected with lengths of maximally about 12% of that of the phage genome. This DNA persists, again transiently between 2 and 8 hr. after feeding, predominantly in the blood leukocytes (R. Schubbert and W. Doerfler, manuscript in preparation).

It will be necessary to investigate the fate of food-ingested foreign DNA in the organism. Our recent data documented by experiments on >100 experimental animals point to the gastrointestinal tract of mammals as the portal of entry for foreign DNA. This DNA is derived from the daily diet, is only partly degraded, and can enter in the form of highly recombinogenic fragments into the animals' bloodstream. It appears unlikely that these DNA fragments would not occasionally find their way into the genomes of cells in the host. What impact can the daily uptake of foreign DNA into the bloodstream have on the stability of an established genome?

I submit that these unexpected findings will be of importance in human cancer research. Diet-ingested foreign DNA could play an important role in eliciting mutagenic and oncogenic events in mammals. In the light of these findings, the postulated cellular defense mechanism by methylation of integrated foreign DNA receives plausibility.

There are additional reasons to investigate the insertion of foreign DNA and its consequences in the mammalian genome. The technique to generate transgenic organisms, in which specific genes have been inactivated by site-specific recombination, has been widely applied for a multitude of experimental purposes. On the basis of the available evidence, the question arises whether the insertion of genes into an existing genome would not cause alterations in segments of this genome which lie adjacent to or remote from the site of integration. If such alterations occurred, the interpretation of many results obtained with transgenic organisms might have to be reexamined.

In part, current concepts in gene therapy are also premised on the integration of foreign genes into established genomes and the continued regulated expression of these artificially introduced genes. Prospects for gene therapy will have to be carefully examined considering the poorly understood consequences of foreign DNA insertion for the entire preexisting genome.

Thus, for many reasons it would be desirable to understand mechanisms and consequences of integrative recombination of artificially introduced foreign genomes with the host genome, the mode of *de novo* methylation of integrated foreign DNA, and changes in DNA methylation in the recipient cell's genome.

II. The Adenovirus System as a Model

With the notion that practically any foreign DNA can be incorporated into preexisting mammalian genomes, I need to explain why we have chosen the adenovirus system as a model. The prime motivation for this choice was the well-established oncogenic nature of the virus in rodents, mainly in newborn hamsters. In that respect, adenovirus work has been intimately connected to the biological questions of cell transformation by oncogenic viruses and to the underlying mechanisms. Moreover, for several decades of intensive research, adenoviruses have proven themselves as impressive tools to study the molecular biology of mammalian cells. It was with the help of this viral system that many fundamental mechanisms in the molecular biology of mammalian cells have been recognized and at least partly unraveled. One may also argue that in studies on the fate of foreign DNA in mammalian cells, any choice of DNA will prove somewhat arbitrary. Thus, the selection of a well-characterized viral genome, whose biochemistry and molecular biology are understood in considerable detail, has obvious advantages. Of course, the use of DNA from an oncogenic virus will involve selection of cells that have been transformed by this virus and exhibit very specific

biological properties. This choice was intended and aimed at contributing to our understanding of the transformation mechanism of cells by oncogenic viruses.

Nevertheless, in the more general interpretation of the data adduced with the adenovirus system, it will be prudent to keep this selection and its limitations in mind. On the other hand, any DNA molecule chosen for similar studies might have entailed different, but similarly selective mechanisms which would have been only less apparent for a less well-characterized DNA molecule. I recognize that all the more generalizing conclusions in this review will have to be prefaced by the thought that we have made a deliberate choice which will reflect on all data obtained.

Details on the molecular biology of adenoviruses cannot be reviewed within the scope of this chapter. Several books on this subject have been published (Doerfler, 1983b, 1984b; Ginsberg, 1985; Doerfler and Böhm, 1995). The reader is referred to these volumes for a basic introduction into the adenovirus system.

A. Site Selection in the Integration of Adenovirus DNA

With the possibility that the insertion of adenovirus DNA into the mammalian genome could fundamentally alter the transcriptional program of infected and transformed cells, it was important to investigate whether the foreign DNA was inserted randomly at many different or at highly specific sites in the genomes of the affected cells. We have spent considerable time and effort in analyzing the sites of adenovirus DNA integration in a large number of virus-transformed or Ad12-induced tumor cells from rodents, mainly from hamsters (for reviews see Doerfler, 1982, 1991a; Doerfler *et al.*, 1983). We have studied integrated genomes in rodent cells transformed in cell culture by infection with adenovirus (adenovirus-transformed cells), in Ad12-induced tumors, or in cells cultured from these Ad12-induced tumors. It is not known whether these different types of cells can be considered as similar with respect to their tumorigenic phenotype.

Three types of analytical approaches were chosen to investigate and to proof the integrated state of the adenovirus genomes in the cellular genomes.

(i) The DNA from transformed or tumor cells was cleaved with different restriction endonucleases, the fragments were transferred to membranes, and the distribution of adenovirus-specific DNA fragments was determined by DNA–DNA hybridization to adenovirus DNA or to the cloned terminal fragments of virion DNA. In this way, the internal and

the terminal viral DNA fragments could be localized in the cellular genome and in their relation to cellular DNA fragments. In many instances, the viral DNA, particularly Ad12 DNA, was found to be integrated in an orientation colinear with that in the virion genome, i.e., the viral genome inside purified virus particles. Consequently, the terminal viral DNA segments thus linked to flanking cellular DNA sequences and excised jointly with them did, therefore, not comigrate on gel electrophoresis with any of the known virion DNA fragments, but appeared instead in "off-size" positions. From the analysis of at least 80 different transformed and tumor cell lines performed since 1976 in my laboratory, it appeared that adenovirus DNA was never found free in any of these transformed or tumor cells, but always integrated into the host genome. In several instances the off-size fragments also contained rearranged viral DNA sequences (Sutter *et al.*, 1978; Stabel *et al.*, 1980; Kuhlmann and Doerfler, 1982; Kuhlmann *et al.*, 1982; Orend *et al.*, 1991, 1994, 1995). When analyzed at the nucleotide sequence level (see below), these integration patterns have proved to be very complicated in individual instances. Additional complexities of the system will be described below (cf. Table I).

(ii) In order to prove the covalent linkage (integration) of adenoviral to cellular DNA sequences, it was necessary to molecularly clone and determine the nucleotide sequence of some of the junction sites between viral and cellular DNAs. From the data available to date, all the cellular DNA sequences flanking integrated viral DNA sequences were different from each other (Deuring *et al.*, 1981a; Gahlmann *et al.*, 1982; Gahlmann and Doerfler, 1983; Stabel and Doerfler, 1982; Deuring and Doerfler, 1983; Schulz and Doerfler, 1984; Lichtenberg *et al.*, 1987; Jessberger *et al.*, 1989a). These data provided no evidence for the notion that adenovirus DNA had integrated at highly specific cellular DNA sequences. However, only a relatively small number of such junction sites have so far been investigated. Moreover, the extent of DNA sequences determined in the flanking cellular DNA has been limited. In addition, many of the cells, in which junction sequences between adenovirus DNA and cellular DNA were analyzed, had been cloned cell lines and had been maintained in culture for many years. It was thus possible that the integrated viral genomes could have been rearranged, transposed, or altered in other ways after the original integration event. In a few instances (see Section II.B), evidence was obtained for the occurrence of selective sites of viral DNA integration in different cell lines.

(iii) Recently, we have initiated studies on Ad12-transformed cell lines and on Ad12-induced tumor cell lines to determine the chromosomal

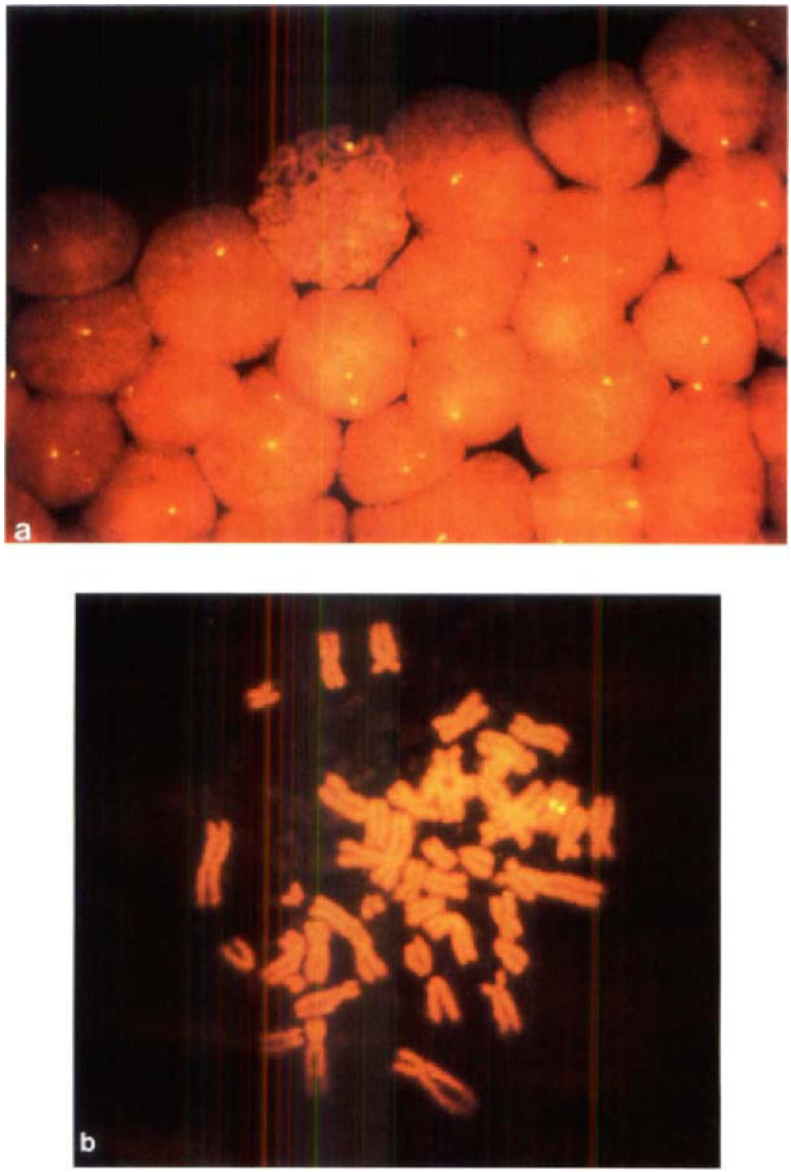

FIG. 1. Integration of Ad12 DNA into one of the hamster chromosomes in the Ad12-transformed cell line T637 as demonstrated by the fluorescent *in situ* hybridization (Lichter and Cremer, 1992). The hamster cell line T637 contains in an integrated form about 20–26 copies of Ad12 DNA (Sutter *et al.*, 1978; Stabel *et al.*, 1980; Orend *et al.*, 1994a). The integrated Ad12 genomes were visualized by using the biotinylated *Pst*I-D fragment of Ad12 DNA as the hybridization probe and fluorescent-labeled avidin for detection under uv light. (a) Interphase nuclei, (b) chromosome spread. This experiment was performed by Petra Wilgenbus, Köln.

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locations of the integrated viral genomes. The data adduced so far on cell lines T637, HA12/7, H191, and H281 using fluorescent *in situ* hybridization methods with biotinylated Ad12 probes (P. Wilgenbus, G. Meyer zu Altenschildesche, M. Lutze, S. T. Tjia, and W. Doerfler, unpublished results) indicate that the Ad12 genomes are indeed chromosomally located and that the bulk of the integrated viral genomes lies at a single chromosomal site (Fig. 1), which is different for each of these cell lines.

B. Modes of Adenovirus DNA Integration—Synopsis of Data

Under the premise that we have necessarily studied only a limited set of adenovirus-transformed cell lines or Ad12-induced tumor cell lines, the following conclusions can presently be drawn (Table I).

(i) In evaluating the outcome of an infection with adenoviruses with respect to cellular transformation, the biology of the virus–host cell system has to be considered. In contrast, transfection of viral DNA fragments presents yet a very different situation which must affect the outcome of the transformation event. Lastly, although almost any experimental system can be criticized as having nonnatural properties, I consider the induction of tumors in living animals to be as close to the full range of complexities as possibly attainable. Nevertheless, one can rightfully argue that Ad12 in reality might not often have had the opportunity to infect *Mesocricetus auratus,* the Syrian gold hamster, before Trentin and colleagues performed their pioneering experiments in 1961 and 1962.

Ad12 infects hamster cells nonproductively; the infection is completely abortive with failure of Ad12 virion production, of Ad12 DNA replication, and of late gene transcription. Newly assembled viral particles can never be found. The basis for this nonpermissive interaction is complex and multitiered (for reviews see Doerfler, 1991a; Zock and Doerfler, 1993). We have been able to document that the major late promoter of Ad12 DNA carries a mitigator element in the downstream sequence, which at least in part appears responsible for the inability of this promoter to function in hamster cells (Zock and Doerfler, 1990; Zock *et al.,* 1993). Upon the experimental removal of this mitigator element, the major late promoter of Ad12 DNA becomes functional in hamster cells, and its activity in the permissive human cells is enhanced. As a consequence of the nonpermissive interaction of Ad12 with hamster cells, there is no selective pressure against the persistence of the entire Ad12 genome in hamster cells, and we have thus frequently found that Ad12 DNA can be integrated into the hamster cellular

TABLE I
ELEMENTS OF ADENOVIRUS DNA INTEGRATION INTO THE MAMMALIAN GENOME: A PLIABLE MECHANISM

Chromosomal location of adenovirus integrates
Intact genomes and/or fragments can be integrated
Rearranged parts of the viral genome can be integrated in the form of fragments close to intact viral DNA molecules
Sites of linkage exhibit frequently but not always patchy sequence homologies between recombination partners
Integrated adenovirus DNA can be intact, rearranged, or carry deletions
Mode of viral DNA persistence depends on the permissivity of the virus–host system used
At sites of linkage, viral nucleotides can be deleted
Adenovirus DNA integration can entail deletions of cellular DNA at site of insertion or can be effected without the loss of a single cellular nucleotide
Cellular DNA sequences at sites of insertion have been found to be transcriptionally active
Integrated adenovirus DNA can be partly or completely lost from the cellular genome: Morphological revertants of transformed cells can arise
Upon cultivation of Ad12-induced tumor cells, cells can somehow be selected that carry integrated viral genomes at selective cellular sites
Integrated adenovirus genomes become *de novo* methylated
Patterns of *de novo* methylation depend on site of adenovirus DNA integration, perhaps also on other factors
Integration of foreign (adenovirus) DNA into established mammalian genomes can be associated with extensive changes in the methylation patterns of cellular genes
Integration/recombination of adenovirus DNA into/with cellular DNA can be imitated in a cell-free system with purified components from hamster nuclear extracts
The *in vitro*-generated recombinants exhibit similarities to the *in vivo*-observed integrates: Patch homologies

genome nearly intact and colinear with the arrangement of Ad12 DNA sequences as is found in the DNA extracted from the virus particle. Realistically, it must be added that in some Ad12-transformed cell lines, which carry multiple Ad12 DNA copies, e.g., in cell line T637, certain parts of the integrated Ad12 genomes can be very markedly rearranged (Eick and Doerfler, 1982; Orend *et al.* 1995).

In contrast, adenovirus type 2 (Ad2) infects hamster cells productively and can replicate to appreciable titers. As a consequence, the persistence of the intact viral genome in hamster cells seems to be selected against or else the intact Ad2 genome would be replicated, the infected

cells would all be killed, and transformed cells could probably not arise. In keeping with this line of reasoning, in Ad2-transformed hamster cells, we have mainly found fragments of integrated Ad2 genomes with varying lengths of internal deletions. Thus, patterns of persistence and integration can be decisively influenced by the biology of the virus–host system. At least that is one possible interpretation of the data. In some of the cell lines investigated, the Ad2 genome, usually with internal deletions, is integrated in an orientation colinear with the arrangement of the Ad2 DNA sequence in the virion. In some instances, the orientation has been rearanged (Vardimon and Doerfler, 1981).

In most cases, certainly in cells carrying Ad12 genomes in an integrated form, linkage of the viral sequences to the adjacent cellular DNA sequences was via the terminal viral DNA sequences. At most junction sites between viral and cellular DNAs analyzed so far, a number of viral nucleotides were deleted in the process. This number of deleted nucleotides ranged from 0 to 174 in different cell lines. At the left end of integrated Ad12 DNA in the Ad12-transformed hamster cell line HA12/7, not a single viral nucleotide was found to be deleted. On the other end of the spectrum, viral DNA integration could proceed without the deletion of a single cellular nucleotide at the site of linkage, or large segments of cellular DNA could be lost in the process of inserting foreign (viral) DNA. Most frequently, multiple copies of viral DNA molecules became integrated upon infection of cells or hamsters with adenoviruses. We were able to demonstrate that these multiple copies were not integrated as true tandems but that other nucleotide sequences, cellular DNA or rearranged viral DNA sequences, had become interspersed between adjacent viral DNA molecules. Particularly when multiple copies of viral DNA were integrated, viral DNA termini were found to be rearranged or partly inverted in some of the integrated DNA molecules.

When cell lines carrying integrated viral DNA molecules were passaged in culture for longer periods of time, the loss of all or part of the viral genomes from the transformed cell lines was occasionally observed (Groneberg *et al.*, 1978; Eick *et al.*, 1980). This loss was accompanied by changes in cell morphology. However, in a few instances the loss of Ad12 DNA sequences, including the left terminal segment, which was considered to be essential in the transformation of cells by adenoviruses, did not affect the tumorigenic phenotype of these revertants (Kuhlmann *et al.*, 1982). Thus, at least in these cell lines, persistence of the viral genome could not be considered an absolute prerequisite for the maintenance of the tumorigenic cell phenotype. It was conceivable that, as a consequence of viral infection and/or integration of Ad12 DNA into the

cellular genome and the subsequent loss of the integrated foreign DNA, the organization of the cellular genome or the expression patterns of cellular genes were changed thus that the affected cells were transformed to malignant cells. In the cells that had lost the Ad12 genomes, Ad12 DNA could not be detected any more by the very sensitive Southern blotting technique and subsequent hybridization to ^{32}P-labeled, cloned fragments of Ad12 DNA. We are currently reexamining whether traces of Ad12 DNA can still be detected in these revertants by the polymerase chain reaction. For this purpose, the revertant cell lines will have to be rigorously recloned to ascertain absence of a few cells from the original Ad12-transformed cells.

Junction sequences between adenoviral and cellular DNA sequences were cloned and their nucleotide sequences were determined from the following cell lines: The Ad12-transformed hamster cell lines T637 (M. Lutze, B. Schmitz, and W. Doerfler, manuscript in preparation), HA12/7 (Jessberger *et al.*, 1989a), from the Ad2-transformed hamster cell line HE5 (Gahlmann *et al.*, 1982; Gahlmann and Doerfler, 1983), the Ad12-induced hamster tumor cell lines CLAC1 (Stabel and Doerfler, 1982) and CLAC3 (Deuring *et al.*, 1981a), the tumor T1111/2 (Lichtenberg *et al.*, 1987), and H191 (M. Lutze, B. Schmitz, and W. Doerfler, manuscript in preparation), from the Ad12-induced mouse tumor line CBA12/T1 (Schulz and Doerfler, 1983), and from the symmetric recombinant (SYREC2) of Ad12 DNA (Deuring and Doerfler, 1983).

In the Ad12-encapsidated SYREC2 DNA molecule, the DNA consisted of the left terminal 2081 nucleotide pairs of Ad12 DNA positioned at both SYREC termini and a large palindrome of human cellular DNA of partly unique and partly repetitive DNA sequences to make up a molecule that had about the length of Ad12 DNA. This recombinant DNA molecule generated in cell culture could be packaged into Ad12 virions due to the presence of terminal Ad12 DNA sequences that might be akin to known packaging sequences identified at the left terminus of other adenoviral genomes. Upon denaturation and reannealing of SYREC2 DNA, molecules were generated with about half the length of Ad12 DNA, attesting to the proposed structure of palindromic DNA molecules (Deuring *et al.*,1981b). In addition, restriction analyses of these DNA molecules revealed that they contained the left terminus of Ad12 DNA at both termini. These recombinant molecules, of course, required wild-type Ad12 as a helper for their replication in cell culture. It will be interesting to evaluate these SYREC molecules for their potential as adenovirus vectors for the encapsidation and transfer of large segments of foreign DNA. With an original length similar to that of Ad12 DNA

with 34,125 nucleotide pairs (Sprengel *et al.*, 1994), the SYREC DNA molecule might accomodate up to 30 kbp of foreign DNA.

Another aspect raised by the structure and composition of the SYREC2 DNA molecule was that of recombination between Ad12 DNA and human cellular DNA in productively infected human cells. After the infection of permissive human cells with Ad12 or Ad2 virions, transformed human cells were never obtained. The few adenovirus-transformed human cell lines available were generated by transfecting viral DNA fragments into human cells. The existence of SYREC2 DNA molecules proved that, even in human cells productively infected with Ad12, recombination could proceed between Ad12 DNA and cellular DNA. It could not be decided whether the SYREC molecules were generated as a consequence of viral DNA integration and excision or in the course of recombination between Ad12 DNA and fragments of cellular DNA produced during viral infection. By cytogenetic and *in situ* hybridization methods, the preferential association of Ad12 DNA with human chromosome 1 in Ad12-infected cells was demonstrated early and late after Ad12 infection of permissive human cells (McDougall *et al.*, 1972, 1973; Rosahl and Doerfler, 1988).

Although in many cell lines, in which the junctions between Ad12 DNA and cellular DNA were analyzed, there was no evidence for site-specific integration, there were a few examples suggesting selectivity in certain integration events. Our conclusions on the absence of site-specific integration of viral DNA in adenovirus-transformed or Ad12-induced tumor cells were based on results adduced with cloned cell lines that had been kept in culture for long periods. It was unknown to what extent these experimental parameters might have selected for cells with the foreign viral DNA integrated in a particular manner, whether the integrated viral DNA could have been rearranged in the course of cell culturing, or whether the results obtained were actually representative of the primary integrative behavior of adenovirus genomes.

We recently isolated and characterized a set of five Ad12-induced hamster tumor cell lines from Ad12-induced tumors by maintaining uncloned tumor cells in culture for longer periods of time. Subsequently, the patterns of Ad12 DNA integration were analyzed by using five different restriction endonucleases and the Southern blotting procedure (Orend *et al.*, 1994). Terminal Ad12 DNA fragments on these blots were identified by hybridizing the DNA fragments to the cloned terminal Ad12 DNA fragments. In these experiments, the patterns of Ad12 DNA integration appeared very similar, if not identical, for all five hamster tumor cell lines which were derived from different oncogenesis

experiments. We do not yet understand what selective procedure during cell culture might have led to the isolation of these five cell lines, one of which was isolated about 10 years prior to the time of isolation of the four other cell lines (Kuhlmann and Doerfler, 1982). Integration patterns of Ad12 DNA had also been determined at early passage levels after explantation of the cells from the tumor-bearing animals, and at that time, the integration patterns were different in these different cell lines. Upon prolonged cultivation, cells might have been selected that had growth advantages and integration patterns which might have changed under the selective conditions of cell culture employed. Such changes might not occur very frequently, because in most cloned cell lines analyzed for integration patterns over a long period of time, such changes were not apparent. It was probably significant that the cell lines with similar integration sites of Ad12 DNA originated from uncloned populations of Ad12-induced hamster tumor cells. In cell lines generated from cloned tumor cells, the selection of cells with preferred integration sites of Ad12 DNA might have been prevented by excluding a cell population capable of rearranging integrated viral DNA during cell cultivation.

III. On the Mechanism of Integrative Recombination

A. Insertion of Foreign DNA by a Versatile Mechanism

The results of studies on the model of adenovirus DNA integration in mammalian cells suggested that the insertion mechanism had to be rather pliable. Usually, many copies of viral DNA were integrated, apparently often not in a true tandem fashion. Individual viral DNA molecules could be separated by cellular or rearranged viral DNA segments. Intact or nearly intact viral genomes or additional viral DNA fragments were integrated, sometimes in proximity to the intact viral genomes. At the sites of junction to cellular DNA, viral nucleotides could be deleted; occasionally, the terminal viral nucleotide sequence was preserved. At several of the nucleotide sequences linking viral and cellular DNAs, patchy homologies were observed between either the linked viral and cellular nucleotide sequences or between the deleted terminal viral sequences and the cellular segments replacing them. However, we also saw junctions devoid of such patchy sequence homologies. The mechanism of integrative recombination operative in mammalian cells could apparently take advantage of patchy homologies but did not have to depend upon their availability. Recombinants with patchy homologies at the sites

of junction were perhaps more frequently found than those without patchy homologies. When taking into account the flexibility of the integrative recombination mechanism with respect to the necessity for nucleotide sequence homologies between the reaction partners, one appreciated that it was logically difficult to categorize this mechanism as being akin to homologous or to nonhomologous recombination.

The sometimes frustrating strife for neat, simple classifications neglects to recognize the fact that integrative recombination in mammalian cells tends to utilize pliable mechanisms that cannot easily be subsumed under idealized categories. The mechanism can work under a variety of molecular constellations and, perhaps for that reason, has proved to be quite efficient and successful. I suspect that this mechanism may have evolved over long periods during evolution and has been playing a major role in permitting cells to incorporate foreign DNA very efficiently and subsequently to select or counterselect for the persistence and the continuing expression of certain foreign DNA sequences or for their persistence and permanent silencing by sequence-specific DNA methylation, respectively.

There is an additional element to consider in investigations on the mechanism of insertional recombination in mammalian cells. We have reported that all the cellular DNA sequences at the junction sites between adenovirus DNA and cellular DNA, that we have examined in detail, are transcriptionally active (Gahlmann *et al.*, 1984; Schulz *et al.*, 1987). Transcriptional activity for these sequences was found in hamster, mouse, or human cells that had never been exposed to adenoviruses, in the Ad12-induced tumor cells, and in adenovirus-transformed cells. The transcription products derived from these cellular sites vary in length and quality. They can constitute short RNAs probably without coding capacity or represent transcription products exhibiting open reading frames. We have proposed the working hypothesis that the cellular DNA sequences which are actively transcribed have assumed a chromatin configuration and are occupied by cellular proteins that render them particularly amenable to the machinery for integrative recombination with foreign DNA. For foreign genes whose informative content seeks to be realized in transcription, it may appear advantageous to have access to active cellular transcription processes. Although this argument is merely one of plausibility, it is still supported by the fact that integrative recombination has been found to be directed toward cellular sites of transcriptional activity also in other viral and cellular systems (Mooslehner *et al.*, 1990; Scherdin *et al.*, 1990).

In the adenovirus integration system, the question is still unresolved whether cellular factors alone suffice to expedite adenovirus DNA

integration or whether viral gene products participate as essential components of the integrative recombination machinery or merely modify cellular factors. Since adenovirus DNA, like any other foreign DNA, transfected into mammalian cells can be integrated into the cellular genome in the absence of viral infection, the bias lies in favor of a mechanism of integrative recombination supplied by the recipient cell. However, it is likely that, upon adenovirus infection and subsequent adenovirus DNA integration, viral gene products can exert an auxiliary or modifying role in integrative recombination between viral and cellular DNAs. Integrative recombination may not be dependent on adenoviral functions but they could render the event more efficient or alter essential parameters to render the reaction more specific. It is prudent to keep in mind that the mechanism under investigation has a high degree of flexibility. A number of years ago, we began to study this mechanism further by using a cell-free system from nuclear extracts of uninfected hamster cells (Jessberger *et al.*, 1989b; Tatzelt *et al.*, 1992, 1993; Fechteler *et al.*, 1995).

B. Studies on the Mechanism of Integrative Recombination in a Cell-Free System

High salt extraction of isolated nuclei from BHK21 hamster cells generates nuclear, cell-free extracts that facilitate the *in vitro* recombination between fragments of Ad12 DNA and preinsertion sequences from hamster cells (Jessberger *et al.*, 1989b). In designing this experimental approach, it has been reasoned that, in the absence of information about the actual requirements for the integrative recombination reaction, a cellular DNA sequence should be chosen as recombination partner with Ad12 DNA that had previously already served as an integration target in a living organism. We have cloned and sequenced the preinsertion sequence from BHK21 hamster cells that corresponds to the insertion sequence of Ad12 DNA in the Ad12-induced hamster tumor cell line CLAC1 (Stabel and Doerfler, 1982). This cellular preinsertion sequence has been designated p7. Of course, it is impossible to predict what nucleotide sequence requirements a cellular DNA segment must exhibit to qualify as integrative recombination partner with foreign DNA. Thus, a previously identified cellular DNA segment as target of Ad12 DNA integration has appeared to be a qualified choice.

In a series of such cell-free recombination experiments, we have observed that the segment of Ad12 DNA comprising nucleotides 20,885 to 24,053 in the complete Ad12 DNA sequence (Sprengel *et al.*, 1994)

recombines *in vitro* more frequently with the p7 hamster preinsertion DNA sequence than other Ad12 DNA segments (Jessberger *et al.*, 1989b). At present, this phenomenon cannot be explained, but it is not due to obvious nucleotide sequence homologies between p7 hamster DNA and the nucleotide 20,885–24,053 fragment of Ad12 DNA. This and other Ad12 DNA segments recombine much more frequently with p7 hamster DNA sequence than with the adjacent pBR322 vector sequence in the construct used. With a second Ad12 preinsertion DNA segment p16 from hamster cells (Lichtenberg *et al.*, 1987), similar results of enhanced recombination with the Ad12 DNA fragment have been adduced. We have also investigated the possibility whether randomly selected hamster cell DNA sequences would recombine with Ad12 DNA fragments in the cell-free system, but have so far not found recombinants (Jessberger *et al.*, 1989b; Tatzelt *et al.*, 1992). It is, therefore, likely that the p7 or the p16 preinsertion sequence contains motifs that are preferentially recognized by the recombination machinery and are used as recombination targets for Ad12 DNA. Nonpreinsertion hamster DNA sequences may thus be utilized much less efficiently.

By using standard gel filtration and chromatographic procedures, we have been able to purify components from the nuclear extracts to a considerable extent. The most highly purified preparations, that still catalyze *in vitro* recombination of the selected p7 and Ad12 DNA (nucleotide 20,885–24,053 fragment) partners, exhibit a limited number of proteins when analyzed by SDS–polyacrylamide gel electrophoresis followed by silver staining of the polypeptides (Tatzelt *et al.*, 1993). More recent experimental work has led to further purification of the components. The most highly purified fractions exhibit four or five major protein bands when analyzed as described (Fechteler *et al.*, 1995). The functional characterization of these components has been initiated. I surmise that the recombination machinery consists of several proteins, perhaps combined in a complex. Thus, further purification may, at some step, lead to the disruption of the necessary ensemble of cellular proteins. The fact that we have been able to demonstrate recombination between p7 hamster cell DNA and Ad12 DNA in fractionated nuclear extracts from uninfected hamster cells demonstrates that, at least for the imitation model reaction described here, cellular factors do suffice. Of course, our model system may still not catalyze the true integration reaction of Ad12 DNA. We have yet to analyze whether viral-encoded functions participate in the reaction qualitatively or quantitatively.

As assay systems for the identification of recombinants, we have applied an *Escherichi coli* transfection test using recA− bacterial strains and

have documented in a large number of control experiments that the recombination between p7 hamster DNA and Ad12 DNA fragments has not been effected after transfection into *E. coli* but in the cell-free extracts (Jessberger *et al.*, 1989b; Tatzelt *et al.*, 1992). This interpretation has been corroborated by the finding that a completely different assay system has also successfully identified cell-free-generated recombinants: The polymerase chain reaction method (PCR) (Saiki *et al.*, 1988) reveals recombinants when used with DNA reextracted directly from the fractionated nuclear protein mixture, and these recombinants resemble those identified by the transfection method (Tatzelt *et al.*, 1993). This assay obviates the involvement of *E. coli* transfection in the isolation of recombinants. Again, the results of a large number of control experiments have verified our interpretations and ruled out the possibility of PCR artefacts. In particular, when the reaction partners were separately incubated with fractionated nuclear extracts, subsequently reextracted, mixed, and then subjected to PCR, recombinants were not found. With the improved purification of the system, it may become feasible in the future to identify recombinants directly by Southern blotting without the use of additional procedures.

An appreciable number of *in vitro*-generated recombinants have been analyzed for their nucleotide sequences at the sites of linkage between Ad12 DNA and the p7 hamster DNA sequence. It is striking that, as described for the linkage sites from adenovirus-transformed cells or Ad12-induced tumor cells, patchy homologies are apparent at the linkage sites of the recombinants generated in the cell-free system as well (Jessberger *et al.*, 1989b; Tatzelt *et al.*, 1992). This finding encourages expectations that this cell-free system simulates to some extent elements of the *in vivo* integration reaction.

The data obtained so far need to be complemented in the future by work examining the activity of extracts from nuclei of Ad12-infected hamster cells. Moreover, we have initiated experiments utilizing cosmid constructs with cellular preinsertion sequences and the intact Ad12 genome, either as isolated DNA or complexed with authentic viral core proteins, in the hope of approaching the actual integration reaction more realistically.

We have also explored a cell-free system from nuclei of *Spodoptera frugiperda* insect cells which catalyze the *in vitro* recombination between adenovirus DNA and *Autographa californica* nuclear polyhedrosis virus DNA fragments (Schorr and Doerfler, 1993). Apparently, the capacity for this type of nonhomologous recombination is also inherent in insect cells.

IV. *De novo* DNA Methylation of Integrated Foreign DNA

A. *De novo* Methylation of Integrated Foreign DNA: A Cellular Defense Mechanism?

In our investigations on the structure of integrated Ad12 genomes in transformed hamster cells, we have found early on that these genomes have become de novo methylated in very specific patterns (Sutter *et al.*, 1978; Sutter and Doerfler, 1979, 1980; Vardimon *et al.*, 1980; Kuhlmann and Doerfler, 1982; Orend *et al.*, 1991, 1995). We had previously shown that the virion DNA from purified Ad12 particles was not detectably methylated (Günthert *et al.*, 1976; Wienhues and Doerfler, 1985; Kämmer and Doerfler, 1995). Thus, it was possible to unequivocally demonstrate that the previously unmethylated Ad12 DNA was *de novo* methylated after integration into the cellular genome.

An inverse correlation has been observed in many parts of integrated adenovirus genomes between the extent of DNA methylation and the level of transcription (Sutter and Doerfler, 1979, 1980). This correlation has subsequently been refined for the promoter regions of integrated adenovirus genes (Kruczek and Doerfler, 1982). These observations initiated a decade of research on the role that sequence-specific promoter methylation can play in the long-term silencing of eukaryotic genes. In these studies (for reviews see Doerfler, 1981, 1983a, 1984a, 1989, 1992, 1993; Doerfler *et al.*, 1993), we have mainly, but not exclusively, used viral promoters. I shall not duplicate here the contents of earlier reviews. DNA methylation has more recently been recognized in its importance for long-term gene inactivation also in developmental biology and in human genetics. The earlier work on eukaryotic viral and cellular promoters has conceptually opened the path for studies on complex genetic phenomena, e.g., that of genomic imprinting in mammalian genomes (Surani *et al.*, 1984; Sapienza *et al.*, 1987; Reik *et al.*, 1987; Swain *et al.*, 1987; Li *et al.*, 1993). The observation that integrated foreign DNA molecules, like the Ad12 genome, can be *de novo* methylated and consequently partly or completely inactivated has not been restricted to adenovirus genomes. Other integrated viral genomes or, for that matter, any foreign DNA integrated into established genomes, e.g., after transfection and selection in mice (Lettmann *et al.*, 1991) or in plants (Linn *et al.*, 1990), have been subjected to the same, apparently ubiquitous control mechanism and have become extensively *de novo* methylated and inactivated. There are also examples in which *de novo* methylation has not ensued.

The genome of Epstein–Barr virus (EBV), a member of the herpesvirus group, which can persist in virus-transformed cells predominantly in a nonintegrated, circular, episomally free form, can also become *de novo* methylated in specific patterns (Ernberg *et al.*, 1989; Li-Fu *et al.*, 1991). Thus, *de novo* methylation of foreign genomes in mammalian cells is not solely associated with the integrated state of the newly acquired DNA. Persisting EBV genomes continue to be replicated in synchrony with the cycle of the cellular genome, presumably by cellular DNA polymerase systems. It is challenging to consider the possibility that the cellular replication machinery may be intimately associated with the apparatus for the *de novo* methylation of DNA which would then be responsible for the methylation of the EBV DNA. In contrast, free adenovirus DNA replication is self-sufficient and provides its own replication system which lacks, however, a DNA methyltransferase system. Perhaps, for that reason, intracellular, free adenovirus DNA has never been found to become *de novo* methylated (Wienhues and Doerfler, 1985; Kämmer and Doerfler, 1995).

Since the insertion of foreign DNA into established genomes and its continued transcription constitutes a major goal of many, though not all, strategies in gene therapy, the mechanism of *de novo* methylation and subsequent long-term inactivation of integrated foreign genomes requires serious consideration and detailed investigations. *De novo* methylation may represent a major obstacle on this frequently considered path toward the successful repair of genetic defects in mammalian cells. Alternate approaches, like the presentation of foreign genes in free nonintegrated form, e.g., in free adenovirus genomes (Ragot *et al.*, (1993), under conditions in which they do not predominantly integrate, may have a better chance of providing means for the long-term, nonobstructed expression of foreign genes designed to substitute for missing genetic functions in a cell or an organism. Even if one could replace a defective gene exactly by the wild-type allele, the question has to be raised whether this replaced gene or DNA segment will also be subject to the defense mechanism of *de novo* methylation because, due to the lack of the authentic cellular methylation pattern, it will be recognized as foreign by the *de novo*-methylation system of the cell.

As mentioned earlier, the *de novo* methylation of integrated foreign DNA in established genomes can be viewed as an ultimate cellular defense mechanism which apparently can operate selectively. Possibly by survival and selection of cells with an optimized pattern of *de novo* methylated and inactivated or nonmethylated and continually expressing foreign set of genes, specific patterns of *de novo*-methylated genes persist and contribute to the constellation of newly introduced genes in a thus

altered genome. Adenovirus-transformed cells provide an example for this mechanism. Frequently, the early viral genes, mainly the E1 and E4 genome segments, do not succumb to this cellular defense mechanism (Orend *et al.*, 1995), probably because by selection they can escape inactivation and contribute to the transformed state of those cells in which they continue to be expressed.

It appears that the cells have developed several mechanisms for their defense against the insertion of foreign DNA and genes. Under experimental conditions, a variety of options have become available for the introduction of foreign DNA into cells in culture. It is unknown how frequently cells of an intact organism are exposed to, take up, and chromosomally integrate foreign DNA. It is likely that the cytoplasmic membrane is a first barrier against the penetration of foreign DNA molecules. Once that barrier is penetrated, foreign DNA can be nucleolytically degraded in the cytoplasm or in its organelles. Nevertheless, foreign DNA can be transported to the nucleus and become integrated. Such integrated genomes can be lost again from the cellular genome, as exemplified by the existence of morphological revertants of Ad12-transformed cells in which viral genomes, in part or *in toto*, have been excised (Groneberg *et al.*, 1978; Eick *et al.*, 1980). Finally, in case all these possibilities to eliminate foreign genes had failed, they could be *de novo* methylated and thus become inactivated.

B. Initiation of *de novo* Methylation in Mammalian Cells Is Not Predominantly Dependent on the Nucleotide Sequence of Foreign DNA

Integrated Ad12 DNA in hamster tumor cells or Ad12 DNA fixed in the hamster cell genome by transfection and selection is not immediately *de novo* methylated. It requires an unknown number of cell generations — and other unknown factors — to initiate *de novo* methylation. We have investigated where in the colinearly integrated Ad12 genome *de novo* methylation is initiated. It commences in two paracentrally located regions of Ad12 DNA and not at the termini of Ad12 DNA, which are contiguous with cellular DNA sequences that probably present an established methylation pattern. The graphs in Fig. 2 (Orend *et al.*, 1995) present schematically the sites of initiation and progression of *de novo* methylation in Ad12-induced tumor cells and the final state of methylation of the integrated viral genomes in the tumor-derived cell lines.

There is evidence from several different systems in which *de novo* methylation has been studied that certain nucleotide sequences may be

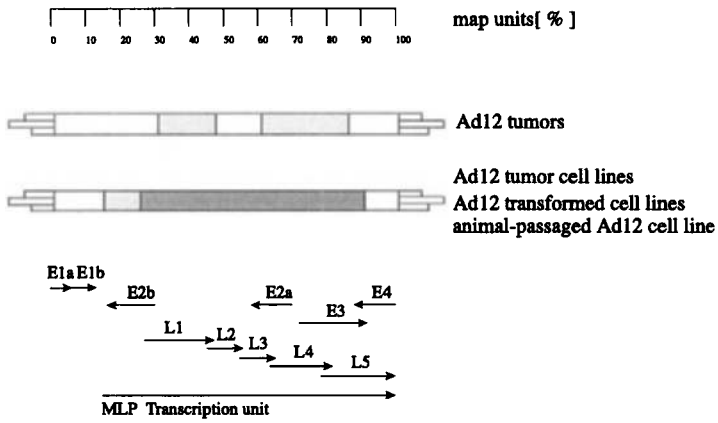

FIG. 2. Initiation and progression of *de novo* methylation in integrated Ad12 genomes in Ad12-induced tumors and in cell lines derived from these tumors. The lightly shaded areas indicate regions in which the *de novo* methylation of integrated Ad12 DNA is initiated (map units of the linearly inserted Ad12 genome). The darker shaded area designates the state of *de novo* methylation in established cell lines. This graph was taken with modifications from Orend *et al.* (1995). MLP, major late promoter.

preferentially and initially *de novo* methylated (Szyf *et al.*, 1990; Mummaneni *et al.*, 1993; Hasse and Schulz, 1994). While nucleotide sequence may play a certain role in selecting sites for the initiation of *de novo* methylation, the results from investigations on *de novo* methylation in the adenovirus system argue that nucleotide sequence by itself cannot be the sole determining parameter that characterizes sites of initiation of *de novo* methylation.

(i) In integrated Ad12 genomes, *de novo* methylation is initiated in the paracentrally located nucleotide 20,885–24,053 fragment of the viral genome; more precisely in an internal segment of this region (Orend *et al.*, 1995). When the same viral DNA segment is transposed, e.g., to the left end of the integrated Ad12 genomes in cell line T637, the transposed Ad12 DNA segment with the same nucleotide sequence is not methylated or at least not to the same extent as the internally located segment.

(ii) When fragments of Ad12 DNA, such as the nucleotide 1-5574 *Eco*RI-C fragment or the nucleotide 20,885–24,053 (*Pst*I-D) fragment, are transfected into and fixed by integration in the genomes of mammalian cells, these DNA fragments become methylated in some cell lines, but remain unmethylated in others, possibly dependent on the site of foreign DNA integration (Orend *et al.*, 1995). In contrast, Ad12-

transformed cells, which have been transformed by the infection with Ad12 virions, carry the entire Ad12 genomes in an integrated form and the 1-5574 nucleotide fragment remains hypomethylated and the internal *Pst*I-D fragment becomes heavily methylated.

(iii) In the Ad2-transformed hamster cell line HE1 (Cook and Lewis, 1978), the late E2A promoter in the Ad2 genome is completely methylated at all 5′-CG-3′ sequences as determined by genomic sequencing. The exact same nucleotide sequence is not at all methylated in another Ad2-transformed hamster cell line, HE2 (Toth *et al.*, 1989, 1990). In HE1, the E2A gene has been silenced; in HE2, it is transcribed and translated into the DNA-binding protein (Johannsson *et al.*, 1978).

(iv) The large segment of human cellular DNA sequences in the symmetric Ad12 DNA recombinant SYREC2 (Deuring *et al.*, 1981b; Deuring and Doerfler, 1983) is not methylated in its 5′-CCGG-3′ sequences in the SYREC2 genome isolated from purified virions. The same cellular nucleotide sequences are, however, very heavily methylated in the 5′-CCGG-3′ sequences inside the human cellular genome in cells growing in culture.

I, therefore, tentatively conclude that the *de novo* methylation mechanism is not predominantly regulated simply by a specific nucleotide sequence but that additional parameters like location in different intranuclear compartments, DNA structure, the type of proteins bound at such structures, the site of foreign DNA integration, and the replicative state of the cell may all have an important influence. For future experimental designs in related projects, it is important to recognize that one must not select for the same foreign DNA segments whose *de novo* methylation one intends to follow.

Next to nothing, unfortunately, is known about the enzymatic mechanism of *de novo* methylation. It is not clear whether *de novo* and maintenance methylations are effected by the same, by different, or by one enzyme in conjunction with different cofactors. It still can not be determined how many DNA methyltransferases exist in mammalian cells (Li *et al.*, 1993).

In our own work on DNA methyltransferases, we have turned to studies on frog virus 3 (FV3), a member of the iridovirus group (Willis *et al.*, 1989). The FV3 genome in the virion is heavily methylated (Willis and Granoff, 1980). By the genomic sequencing method, we have been able to demonstrate that, in the viral DNA segments investigated, all 5′-CG-3′ sequences are methylated and that 5-methyldeoxycytidine (5-mc) occurs exclusively in these dinucleotide sequences (Schetter *et al.*, 1993). By the use of several methylation-sensitive restriction enzymes, it

has also been rendered likely that all 5′-CG-3′ sequences in the genome are methylated (Willis and Granoff, 1980; Schetter *et al.*, 1993). There is evidence that, after infection of cells with FV3, the newly synthesized viral DNA is not immediately methylated after replication, but becomes rapidly and completely *de novo* methylated shortly thereafter. This system thus offers the possibility to study characteristics of the enzyme(s) involved in this *de novo* methylation reaction. Such studies have been initiated (Schetter *et al.*, 1993).

C. A Fully 5′-CG-3′ but Not a 5′-CCGG-3′ Methylated Late Frog Virus 3 Promoter Retains Activity

Several lines of evidence demonstrate that the DNA of the iridovirus FV3 is methylated in all 5′-CG-3′ sequences both in virion DNA and in the intracellular viral DNA at late times after infection. The 5-mC residues in this viral DNA occur exclusively in 5′-CG-3′ dinucleotide positions. We have cloned and determined the nucleotide sequence of the L1140 gene and its promoter from FV3 DNA. The gene encodes a 40-kDa protein. The results of transcriptional pattern analyses for this gene in fat-head minnow (FHM) fish cells document that this gene is transcribed exclusively late after FV3 infection. The L1140 gene and its promoter are fully methylated at late times after infection. We have been interested in resolving the apparent paradox that the methylated L1140 promoter is methylated and active late in FV3-infected cells. Of course, the possibility cannot be excluded that one or a few 5′-CG-3′ sequences outside restriction endonuclease sites might have escaped *de novo* methylation after FV3 DNA replication. A construct has been devised that places the chloramphenicol acetyltransferase (CAT) gene construct under the control of the L1140 promoter. Upon transfection, this construct exhibits activity only in FV3-infected BHK21 hamster cells, but not in uninfected BHK21 cells. For technical reasons, FHM cells have proved less suitable for transfection experiments. The fully 5′-CG-3′ or 5′-GCGC-3′ (*Hha*I) methylated, *Hpa*II-mock-methylated, or unmethylated L1140 promoter–CAT gene construct is active in FV3-infected BHK21 cells, whereas the same construct 5′-CCGG-3′ (*Hpa*II) methylated has lost all activity. Apparently, complete methylation of the late L1140 promoter in FV3 DNA is compatible with activity. However, a very specific 5′-CCGG-3′ methylation pattern, that does not naturally occur in authentic FV3 DNA in infected cells, abrogates promoter function. These results further support the notion that very specific patterns of methylation are required to inhibit or inactivate eukaryotic and viral promoters (Munnes *et al.*, 1995).

D. Methylation of Triplet Repeat Amplifications in the Human Genome: Manifestation of the Cellular Defense Mechanism?

In human genetic diseases, apparently autonomous amplifications of naturally present triplet repeats in the human genome have been recognized to be associated with diseases like myotonic dystrophy, the fragile *X* syndrome (FRAXA), Kennedy disease, Huntington's disease, mental retardation found with the fragile site FRAXE on the human X chromosome, spinocerebellar ataxia type I, or hereditary dentatorubropallidoluysian ataxia (Caskey *et al.*, 1992; Richards and Sutherland, 1992; Riggins *et al.*, 1992; The Huntington Disease Collaborative Research Group, 1993; Knight *et al.*, 1993; Orr *et al.*, 1993; Koide *et al.*, 1993; Nagafuchi *et al.*, 1994). These amplifications can lie either in the coding sequence of genes or in their 3′ or 5′ located noncoding regions. It is not understood in detail how this new class of mutations can be linked to the pathogenetic mechanisms of these ailments frequently involving the central nervous system. Moreover, it is still a puzzle by what (enzymatic) mechanism the amplifications are generated.

We have observed that synthetic oligodeoxyribonucleotides, like $(CGG)_{17}$, $(GCC)_{17}$, $(CG)_{25}$, but not $(TAA)_{17}$ or $(CAGG)_{13}$, by themselves can be *in vitro* expanded by *Taq* polymerase (PCR conditions) or by Klenow polymerase (without cycling) to chains of up to 2000 bp. This *in vitro* amplification, which apparently requires a certain nucleotide sequence and, dependent on it, a specific structure, can be inhibited, though not obliterated, by the methylation of the C residues in the oligodeoxyribonucleotides, in that much shorter chains are then synthesized *in vitro* (Behn-Krappa and Doerfler, 1994).

In some of the amplified sequences in human genetic diseases, like in the FMR-1 gene in the fragile X syndrome, an increase of DNA methylation in these sequences has been observed (Oberlé *et al.*, 1991). We have suggested (Behn-Krappa and Doerfler, 1994) that these *de novo* methylations may represent another manifestation of the cellular defense mechanism against foreign DNA mentioned above. According to this reasoning, the amplified triplet repeats amounting in some cases to >2000 nucleotide pairs in excess over the original sequence at this site with many 5′-CG-3′ dinucleotides could be recognized as foreign by the DNA methyltransferase system of the host cell and could thus become *de novo* methylated, possibly as a shield against further expansion. This prediction is consistent with the results of our *in vitro* amplification studies and the effect of 5′-CG-3′ methylation which inhibits amplification.

V. Alterations in Patterns of Cellular DNA Methylation and Gene Expression as Consequences of Foreign DNA Insertions into Mammalian Genomes?

We have investigated the possibility that the insertion of foreign DNA into an established mammalian genome can lead to far-reaching alterations in patterns of cellular DNA methylation and gene expression. These alterations might contribute to the oncogenic transformation of cells at least as significantly as some of the viral gene products thought to be involved in the process.

In the pursuit of this concept, we have initially been able to demonstrate that the methylation state in a hamster cell DNA sequence of about 1 kb length that immediately abuts the site of insertion of Ad12 DNA in the Ad12-induced tumor T1111/2 has been altered in that all the sequences that are completely methylated in normal hamster DNA have lost the methyl groups (Lichtenberg *et al.*, 1988).

More recently, we screened the hamster cell genome with several different, randomly selected genomic DNA or cDNA probes by Southern blot hybridization after cutting the hamster cell DNA with *Hpa*II, *Msp*I, or *Hha*I. Cellular DNA was extracted from the following cell types: (i) a number of Ad12-transformed hamster cell lines; (ii) Ad12-induced tumore cell lines; (iii) BHK21 cells that carried integrated Ad12 genomes fixed by neomycin (neo) gene cotransfection and neo selection in the hamster genome but did not show the Ad12-transformed phenotype. In these cells the E1 region of the Ad12 genome was not detectably transcribed; (iv) Normal BHK21 hamster cells; and (v) Ad12-infected BHK21 hamster cells at 30 hr and several weeks postinfection.

Among the different cellular hybridization probes, several showed very striking increases in DNA methylation in cellular genes in some of the Ad12-transformed cells, in some of the Ad12-induced tumor cells, and in BHK21 cells with integrated Ad12 DNA lacking the Ad12-transformed phenotype; others revealed no changes (Heller *et al.*, 1995). It was shown by the technique of fluorescent *in situ* hybridization that the hybridization probes used in these experiments were located on different hamster chromosomes (Heller *et al.*, 1995). One of the cellular DNA hybridization probes used in these studies, the intracisternal A particle (IAP) DNA, which exhibited a very striking increase in DNA methylation in cell line T637 compared to BHK21 hamster cells, was present on most hamster chromosomes.

Several possible interpretations can be offered to account for these changes in patterns of cellular gene methylation: (i) transformation by Ad12; (ii) insertion of foreign DNA into the established hamster cell

genome; and (iii) The action of early Ad12 gene products synthesized in Ad12-transformed or Ad12-induced tumor cells. We have tried to distinguish between these possibilities and analyzed cellular DNA isolated from BHK21 cells at 30 hr or several weeks after infection with Ad12. In these cells none of the aforementioned alterations in cellular gene methylation have been observed. Two BHK21 cell lines, HAd12-neo2 and HAd12-neo5 (Orend *et al.*, 1995), which contained multiple copies of integrated Ad12 DNA, were also investigated. These cell lines did not show the transformed phenotype typical of Ad12 but were indistiguishable from BHK21 cells. An increase of DNA methylation for the IAP probe was also observed in these cells. Recently, we found that BHK21 cells with integrated plasmid DNA devoid of Ad12 DNA sequences also exhibited increases in DNA methylation in the IAP sequences (Heller *et al.*, 1995).

We therefore favor the interpretation that, perhaps in conjunction with the transformed state of the cells, the insertion of the Ad12 genomes or of any foreign DNA into the established hamster genome contributes to the increase in DNA methylation in many regions of the cellular genomes. The mechanism by which these changes are effected is not known. Since the patterns of early Ad12 DNA expression are similar in Ad12-infected and in Ad12-transformed hamster cells it is unlikely that Ad12 gene products play the decisive role in rapidly changing the patterns of methylation in cellular genes of transformed cells. It should be emphasized that there are considerable differences in the extent and locations of these changes in different Ad12-transformed cells or Ad12-induced hamster tumor cells. Moreover, only a subset of genes seems to be affected. The cellular genes with altered methylation patterns are located on hamster chromosomes definitely different from the chromosome on which Ad12 DNA is integrated. Since we have used only a relatively small number of randomly selected hamster gene probes and found changes in DNA methylation in a high proportion of them, these alterations must be extensive and widely distributed.

It has been shown in many different systems that changes in DNA methylation are associated with changes in patterns of gene expression. In Ad12-transformed cells, we have demonstrated earlier that among 40 different genes tested, the expression of 5 genes has been altered in comparison to non-Ad12-transformed BHK21 cells (Rosahl and Doerfler, 1992). Although the ratio, number of genes with alterations in expression to number of genes tested, argues for frequent changes, more work will be required to support these interpretations. We continue to pursue the possibility that the integration of foreign DNA into the hamster genome is associated with widespread changes in DNA methylation and consequently in expression patterns among hamster cellular

genes. These findings may have significance for the mechanism of viral oncology, for gene therapy, and for the interpretation of results obtained with transgenic organisms.

VI. Uptake of Foreign DNA through the Gastrointestinal Tract

We have set out to explore the possibility that traces of foreign DNA that are constantly ingested with the routine food intake might be taken up by the cells of an organism and become integrated at random into the cellular genome. By subsequently eliciting alterations in the methylation and expression patterns of the affected cells, targets might be thus generated in which these changes lead to the oncogenic transformation of individual cells. This — as yet hypothetical — mechanism of oncogenic transformation is related to the daily exposure of the animals' gastrointestinal tract to foreign DNA.

We investigated whether foreign DNA taken up by mammals with the food supply could, at least in part, survive the digestive regimen of the gastrointestinal tract and eventually enter into the bloodstream (Schubbert *et al.*, 1994). In model experiments, 3- to 6-month-old mice were fed bacteriophage M13 DNA (Hofschneider, 1963) in amounts between 10 and 50 μg. The DNA was supplied in the double-stranded supercoiled circular or *Eco*RI-linearized form directly by pipette feeding to the animals' oral cavity or with the food pellets. M13 DNA was chosen as traceable food additive because we failed to find any homologies between this phage DNA and the DNA repurified from the feces of control mice that had never received this DNA. Moreover, the entire nucleotide sequence of this viral DNA had been determined (Yanisch-Perron, 1985). At various times after feeding M13 DNA, DNA was extracted (i) from the feces, either extracorporeally or taken from the animals rectum; or (ii) from whole blood, from isolated blood cells, or from the serum. These preparations were subsequently analyzed for the presence of M13 DNA sequences by electrophoresis and Southern (1975) blot hybridization, by dot blot hybridization, or by the PCR (Saiki *et al.*, 1988).

The results of these analyses (Schubbert *et al.*, 1994) demonstrated that (i) M13 DNA sequences were detected in the animals' feces between 1 and 7 hr after feeding, and (ii) M13 DNA sequences were present in the bloodstream 2 to 8 hr after feeding. From the feces, a few percent of the ingested DNA was recovered, in the bloodstream $\leq 0.1\%$. The bulk of the feces-excreted M13 DNA was in the size range between 100 and 400 nucleotide pairs. By PCR, M13 DNA molecules of up to a length of 1692 nucleotide pairs were discovered. The PCR-amplified M13 DNA was resequenced, and apart from occasional, nonsystematic deviations,

was found to be identical to the published nucleotide sequence. The results of these studies were identical regardless of whether DNA was extracted from extracorporeally deposited feces or from feces removed from the terminal gut of the animals. The latter precaution precluded the possibility that the feces extracorporeally deposited might have been externally contaminated by unnoticed oral contacts by the animals.

Similarly and surprisingly, M13 DNA sequences were also detected in DNA extracted from total blood or from peripheral leukocytes, but not from the serum of M13-fed animals. The maximal lengths of M13 DNA fragments observed were 976 nucleotide pairs. Upon resequencing this PCR-amplified DNA, authentic M13 DNA was found with occasional rare deviations in nucleotide sequence. DNA isolated from the bloodstream of animals that had not been fed M13 DNA was consistently found free of M13 DNA by any of the analytical techniques applied.

These data were confirmed with DNA preparations from >50 different animals (fecal samples) plus 16 buffer-fed (0.01 Tris–HCl, pH 7.5; 1 m*M* EDTA) controls and from 105 different animals (blood samples) plus 30 buffer-fed controls with essentially identical results (Schubbert *et al.*, 1994). We are currently investigating whether integrated M13 DNA sequences can be cloned by suitable vector systems from the DNA of organs (leukocytes, liver) taken from M13-fed animals.

The results described (Schubbert *et al.*, 1994) verify that food-ingested foreign DNA is not completely degraded in the gastrointestinal tract and can reach the bloodstream, although in minute amounts and in fragmented form. Of course, it is known that fragments of DNA are highly recombinogenic. Since the exposure of many organ systems to recombinogenic DNA fragments is continuous over the entire life span of an organism, it will be very interesting to consider their possible contributions to mutagenic and oncogenic events which likely are cumulative over the duration of the individuals life span.

VII. A Concept of Oncogenesis — Implications for Gene Therapy and Research on Transgenic Organisms

The classical concept of insertional mutagenesis relates to damage caused to cellular functions or genes that are encoded at the sites of foreign (adenoviral) DNA insertion into the host genome. Since a very sizeable part of the mammalian genome consists of repetitive sequences with essentially unknown functions, insertion might frequently be non-consequential to the repertoire of cellular functions. However, we have adduced evidence that the insertion of adenovirus DNA into the hamster

cell genome, possibly in conjunction with the cells' transformed phenotype, can be associated with extensive changes in the methylation patterns of cellular genes and of their expression. In that way, foreign DNA insertion at a restricted region on one chromosome (cf. Fig. 1) could have important sequelae for the expression profile of the afflicted cell involving a large but so far unknown number of remote cellular locations. It is likely that each insertion event generates a different pattern of changes and, in that sense, a unique disturbance in the recipient nucleus. The overall consequences for cellular survival will probably range over a wide continuum from cell death to the absence of detectable functional changes. For oncogenic transformation to ensue with derailed growth control, very specific subsets of alterations in methylation and expression patterns may be required.

Does insertion of foreign DNA into an established genome elicit a signal to the DNA methyltransferase system of the cell? How could such a signal be transmitted to distant parts of the genome involving DNA on different chromosomes? Could the nuclear matrix play a part in the transmission events? Obviously, we do not yet understand these mechanisms, and a great deal of experimental work will be required to research these and other possibilities.

By linking the observation of apparently frequent alterations in patterns of DNA methylation in cellular genes upon insertion of and transformation by the adenovirus genome to the discovery that food-ingested DNA does reach the bloodstream of mammals with the potential of dissemination to many organs of the animal, a concept for oncogenic transformation arises. How frequently do food-ingested DNA fragments obtain access to cells of the organism and become integrated into their genome? How specific or variable are the changes in DNA methylation and expression patterns and do they cause cell transformation or various stages of loss of growth control? One can envisage a very wide gamut of possibilities that will be difficult to prove or disprove in an individual tumor incidence in which it will be impossible to differentiate between primary and secondary events, e.g., changes in DNA methylation and expression patterns, which may be consequences rather than the cause of oncogenesis. This dilemma has accompanied decades of research in tumor biology.

In the future, we will extend research to the basic mechanisms of foreign DNA insertion in animals, to its frequency and sites of insertion, and to changes in patterns of DNA methylation. For this latter aspect, it would be advisable to use foreign DNA as a model that does not have coding capacity or cannot be expressed in eukaryotic cells in order to circumvent the difficulty of having to differentiate between effects of the

insertion event and of gene products of biologically active DNA, such as Ad12 DNA. A wide field of research will have to be addressed here. These projects will be relevant for oncogenesis, but also for gene therapy and the interpretation of experiments in which transgenic animals or plants are utilized. In gene therapy and in transgenic organisms, foreign DNA can possibly affect and alter many parts of an established genome at sites remote from the targeted site of insertion via changes in DNA methylation. Although these ideas will complicate the interpretation of some experiments, these concepts will have to be carefully weighed in the design of future projects.

ACKNOWLEDGMENTS

I am indebted to Petra Böhm for expert editorial work. Research in the author's laboratory was supported by the Deutsche Forschungsgemeinschaft (SFB274-A1 and Schwerpunkt Virulenzfaktoren und Wirtstropismus animaler Viren), the Bundesministerium for Forschung und Technologie (Project 2.03) (Genzentrum Köln), and the Fritz Thyssen-Stiftung.

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ISBN 0-12-006666-1