ADVANCES IN MOLECULAR AND CELLULAR ENDOCRINOLOGY

Editor: DEREK LEROITH

Volume 1 • 1997

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Bethesda, Maryland

VOLUME 1 • 1997


JAI PRESS INC.

Greenwich, Connecticut *London, England*

55 Old Post Road No. 2
Greenwich, Connecticut 06836

JAI PRESS LTD.
38 Tavistock Street
Covent Garden
London WC2E 7PB
England

ISBN: 0-7623-0158-9

Manufactured in the United States of America

CONTENTS

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PREFACE

Historically the field of endocrine research has always been at the forefront of scientific endeavors. The investigators of these important breakthroughs in research have been rewarded by numerous Nobel awards. In the field of diabetes alone, Nobel prizes have been awarded to researchers who discovered insulin, characterized the protein and invented radioimmunoassays using insulin as a paradigm. Not surprisingly, biomedical researchers have always been attracted by the endocrine system and other similar systems of intercellular communication.

Over the past two decades, endocrine research has developed rapidly and adapted modern molecular and cellular biology techniques for its specific use. These changes have allowed researchers in the field to maintain their edge. Thus, endocrine disease-related genes have been characterized and mutations in these genes have helped explain common and less common endocrine disorders. Our understanding of the regulation of gene expression has been greatly enhanced by molecular techniques.

In an attempt to bring investigators up to date with the recent advances in this exploding field we have decided to publish a yearly series entitled *Advances in Molecular and Cellular Endocrinology*. Internationally famous investigators have agreed to participate and their contributions are appreciated. Each volume will include reviews on different aspects of endocrinology.

Volume 1 has focused on aspects of the hypothalamic-pituitary axis including GnRH and GH gene regulation, molecular aspects of insulin, insulin-like growth factors and glucagon. In addition, reviews on the recently cloned calcium receptor

and steroid receptor interactions with DNA are presented. We have tried to include articles covering many of the major subsections of endocrine research and hope that the readers get as much out of these articles as I have, while editing the volume.

Derek Le Roith
Editor

Chapter 1

Molecular Aspects Of GnRH Gene Expression

MELODY E. CLARK, MARK A. LAWSON,
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Advances in Molecular and Cellular Endocrinology
Volume 1, pages 1-30.

ISBN: 0-7623-0158-9

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) resides at the top of the hypothalamic-pituitary-gonadal axis that controls reproductive function. It is secreted from the hypothalamus in a pulsatile pattern to regulate pituitary synthesis and secretion of the reproductive hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn act on the testes and ovaries to stimulate gametogenesis and induce the secretion of sex steroids. Alterations in the pulsatility of GnRH secretion occur during puberty, the menstrual cycle, pregnancy, and in menopause (Yen and Jaffe, 1991). In addition, changes in pulse pattern are circadian and seasonal. Dysregulation of the GnRH neuron has been implicated in conditions such as hypogonadotropic hypogonadism, precocious puberty, and infertility. Furthermore, the negative effects of stress, excessive exercise, and malnutrition on reproduction are also linked to altered GnRH pulsatility (Yen and Jaffe, 1991). Thus, understanding the regulation of GnRH in the hypothalamus is critical for understanding the physiological basis of a number of endocrine and reproductive diseases.

While GnRH is clearly an important hormone, study of its synthesis and regulation have been very difficult due to the cellular complexity of the mammalian brain and the post-mitotic nature of the mature, differentiated GnRH-releasing neurons. Furthermore, it is estimated that there are less than 800 GnRH-expressing neurons present in the adult mouse, making these neurons a rare cell type in the brain (Wray et al., 1989). In addition, GnRH-expressing neurons have a unique developmental origin: they are born in the olfactory placode at day 11 in the mouse and then migrate through the cribriform plate at the base of the skull, into the forebrain, and towards the hypothalamus. While most GnRH neurons in the adult are located in the preoptic area of the anterior hypothalamus, several of these cells remain scattered along the migratory path, forming a continuous distribution that extends along the course of the vomeronasal and terminalis nerves, from the nasal septum to the hypothalamus (Schwanzel-Fukuda and Pfaff, 1989). Thus the scattered location and low number of GnRH-expressing neurons in the hypothalamus

has made molecular studies of GnRH synthesis and regulation particularly challenging.

THE GENE STRUCTURE OF THE GnRH GENE

The cDNA for the human GnRH gene was first isolated by Seeburg and Adelman (1984). These investigators used degenerate oligonucleotides based on the amino acid sequence of the purified GnRH decapeptide to probe a human placental genomic library. The resulting partial clone was used to probe a human placental cDNA library. A full length cDNA clone was isolated that coded for a GnRH precursor protein of 92 amino acids in which the GnRH decapeptide was preceded by a signal peptide of 23 amino acids and followed by a Gly-Lys-Arg sequence for enzymatic cleavage of the decapeptide from its precursor and amidation of the C-terminus of GnRH. The remaining 56 amino acids encodes the GnRH- associated peptide (GAP). Although the results are still somewhat controversial, GAP has been synthesized in bacteria and shown to cause gonadotropin release similar to GnRH, and also to possess potent prolactin secretion inhibitory activity (Adelman et al., 1986). The genomic clone of the GnRH gene is fairly simple, containing four exons and three introns (Hayflick et al., 1989). The first exon codes for part of the 5′ untranslated region (5′ UTR) of the gene (Figure 1). The second exon codes for the rest of the 5′ UTR, the signal peptide, the GnRH peptide, and the first part of GAP. The third and fourth exon codes for GAP, while the fourth exon also codes for the 3′ untranslated region (Seeburg and Adelman, 1984). Interestingly, the opposite strand of DNA from the GnRH coding strand is also transcribed into a polyadenylated RNA that shares significant exonic sequences with GnRH. This RNA hybridizes specifically with rat heart RNA, however its function and putative protein product are not known (Adelman et al., 1987).

Transcriptional Start Sites

The human GnRH cDNA from the placental cDNA library contained an unusually long 5′ UTR sequence, however when the GnRH cDNA was cloned from a hypothalamic library, a much shorter 5′ UTR was present (Adelman et al., 1986). The major GnRH mRNA species found in the hypothalamus is 600 nucleotides while the major species from the placenta is 1,500 nucleotides. This larger mRNA is the result of retention of the intron 1 sequences in the placenta suggesting the utilization of an upstream transcriptional start site (Adelman et al., 1986). Recently, Roberts and co-workers have used primer extension and reverse transcription-polymerase chain reaction (RT-PCR) assays to identify a transcriptional start site 579 bases upstream of the hypothalamic site in a placental cell line (Dong et al., 1993). This placental upstream start site lacks the usual TATA and CAAT elements present in most RNA-polymerase II promoters, however a sequence found in many

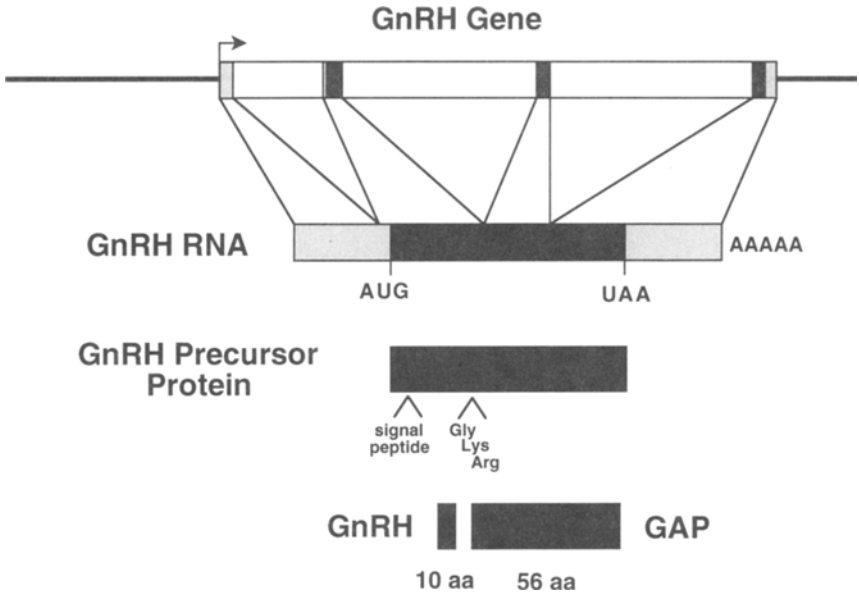

Figure 1. Genomic structure of the rat GnRH gene. The gene spans 4.5 kb of DNA and consists of four exons (filled boxes) separated by three introns (white boxes). The GnRH precursor protein contains a signal peptide, the GnRH decapeptide, a Gly-Lys-Arg cleavage sequence, and the 56 amino acid GAP protein.

TATA-less promoters, known as the initiator element (Smale and Baltimore, 1989), is present.

Conservation of the Human, Mouse, and Rat GnRH Genes

The GnRH genomic DNA has now been cloned from the human (Adelman et al., 1986; Radovick et al., 1990), rat (Adelman et al., 1986; Bond et al., 1989; Kepa et al., 1992), and mouse (Mason et al., 1986). Transcriptional start sites differ slightly between the rat and human or mouse gene (Mason et al., 1986; Radovick et al., 1990; Kepa et al., 1992) (see GnRH Promoter section, below) suggesting the possibility of species-specific regulation of the GnRH gene. The overall homology between the rat and human genes is about 72% with the GnRH decapeptide and the Gly-Lys-Arg sequence following it completely conserved between the two species. Because of a shorter signal peptide, the mouse gene codes for a protein of 90 amino acids which is 2 amino acids smaller than the rat and human gene. The overall homology between the human and mouse GnRH genes is about 75%. There is also strong homology between mouse and rat GnRH precursor proteins with no

changes in the GnRH decapeptide. Interestingly, by cloning the mouse GnRH gene from both normal and a hypogonadal strain of mice, Mason et al. showed that hypogonadism in the *hpg* mouse is caused by deletion of at least 33.5 kb that includes the distal half of the GnRH precursor protein gene (Mason et al., 1986). Furthermore, introduction of mouse GnRH genomic DNA into transgenic *hpg* mice restored reproductive function (Mason et al., 1986).

TISSUE SPECIFICITY

While GnRH is primarily detected in the hypothalamus, and to a lesser extent in the placenta, small amounts have been detected in other tissues as well. GnRH has been detected in the milk of several species, which implies that the mammary gland is either a site of synthesis for GnRH or it is concentrated in plasma from the mammary gland. Breast carcinomas and breast carcinoma cell lines are known to have GnRH binding sites and GnRH analogues have been used successfully to inhibit the growth of these cells. This raised the possibility that GnRH is produced locally by the breast tumor cells themselves. Harris et al. (1991) found immunoreactive GnRH in extracts of two breast carcinoma cell lines MDA-MB-231 and ZR-75-1 and using S1 nuclease protection assay, primer extension studies, and PCR, they showed that the GnRH gene is expressed in these cells and that both the hypothalamic and placental transcriptional start sites are utilized. In addition, GnRH has been detected using RT-PCR in mouse mammary glands (Ikeda et al., 1995) and in the mammary glands of lactating rats (Palmon et al., 1994). GnRH-like activity has also been detected in cytosolic preparations from DU145 human prostatic cell lines and by radioimmunoassay implying a possible autocrine loop (Qayum et al., 1990).

The RT-PCR technique has also been used to detect GnRH mRNA in the cerebral cortex and testes (Dong et al., 1993), in the rat anterior pituitary (Pagesy et al., 1992), in human granulosa-luteal cells (Peng et al., 1994), and in the rat ovary (Oikawa et al., 1990; Goubau et al., 1992). In the rat ovary Goubau et al. (1992) found GnRH mRNA by RT-PCR, although in ovaries some of the transcripts contained intronic sequences and utilized a different start site. These investigators find the predicted 0.6 kb transcript as in the hypothalamus, but find a 3.3 kb transcript in the ovary using Northern blot analysis. Roberts et al. have proposed that upstream start sites will be used only in reproductive tissues, such as placenta, testes, ovary, and mammary gland, suggesting tissue-specific regulation at this site (Dong et al., 1993). However, further studies will be needed to support this hypothesis. Surprisingly, GnRH has also recently been detected in rat thymic and splenic lymphocytes and human peripheral T and B cells implying the existence of a hypothalamic-pituitary-lymphocyte axis (Wilson et al., 1995). GnRH gene expression in most of these tissues awaits confirmation by methods other than PCR. This review will focus on GnRH gene expression in the GnRH-expressing neurons of the hypothalamus as this is clearly the major source of GnRH in the animal.

IMMORTAL, CULTURED CELL MODEL SYSTEMS

The GT1 Cell Lines

The scattered distribution of GnRH neurons has made it difficult to study GnRH gene regulation and other aspects of GnRH physiology. Therefore, Mellon et al. (1990) created an immortalized cell line of GnRH-expressing neurons, GT1 cells, to serve as a model for the GnRH system. These cells were created using the technique of targeted oncogenesis. Briefly, a hybrid gene containing regulatory 5′ flanking regions of the rat GnRH gene fused to the coding sequences for the oncoprotein SV40 T antigen was introduced into transgenic mice. These mice specifically expressed T antigen within differentiated GnRH neurons in the hypothalamus, because this was the cell population in which the GnRH regulatory sequences were active. This targeted expression of the oncogene led to the formation of hypothalamic tumors from which the GT1 cells were cultured. GT1 cells maintain their differentiated phenotype *in vitro* probably because the availability of the transforming oncoprotein T antigen is coupled to the expression of GnRH which is the marker of differentiation. Thus, those cells that dedifferentiate to lose expression of GnRH would also cease to synthesize T antigen, and would therefore no longer replicate in cell culture.

GT1 cells closely resemble their *in vivo* counterparts in many ways. They are clearly neuronal in their physiology, morphology, and histology. They are excitable and depolarize in response to veratridine and K^+; extend neurites to form synapses with one another; express neuronal (but not glial) markers, including neurofilament and synaptosomal proteins. More specifically, they manifest traits that are appropriate to their neuroendocrine lineage, including expression of chromogranin B, abundant rough endoplastic reticulum and golgi, and neurosecretory granules (Mellon et al., 1990; Liposits et al., 1991). In addition, they transcribe and translate the GnRH gene, appropriately process the prohormone, and secrete high levels of the mature peptide (Wetsel et al., 1991). Remarkably, isolated GT1 cells in culture secrete GnRH in a pulsatile manner as does the intact hypothalamus *in vivo* (Krsmanovic et al., 1991; Martinez de la Escalera et al., 1992a; Wetsel et al., 1993). Indeed, intrahypothalamic injection of these cells is sufficient to rescue the hypogonadal phenotype of mutant mice that lack GnRH expression (Silverman et al., 1992).

Since their development, GT1 cells have been extensively used in investigations of GnRH cellular physiology, particularly in studies identifying agents capable of directly regulating secretion of GnRH. Specifically, peptide hormones such as activin (Gonzalez-Manchon et al., 1991), neuropeptide Y (Besecke et al., 1994), and endothelin (Krsmanovic et al., 1991), and the neurotransmitters dopamine (Martinez de la Escalera et al., 1992c), norepinephrine (Martinez de la Escalera et al., 1992b), NMDA (N-methyl-D-aspartic acid) (Mahachoklertwattana et al., 1994b), nitric oxide (Moretto et al., 1993; Rettori et al., 1993), gamma-ami-

nobutyraic acid (Martinez de la Escalera et al., 1994) , and histamine (Noris et al., 1995), stimulate secretion of GnRH, while prolactin represses secretion (Milenkovic et al., 1994). Furthermore, GnRH secretion from GT1 cells might be regulated in an autocrine manner (Krsmanovic et al., 1993), potentially providing a simple mechanism for the observed pulsatility of secretion.

The GN Cell Line

A second cell line of GnRH neurons has been subsequently developed in a similar manner to the GT1 cells. Radovick et al.(1991) targeted oncogenesis in transgenic mice with a smaller fragment of the human GnRH regulatory region (–1131 to +5 bp) and found migratory arrest of the GnRH neurons. A tumor from the olfactory bulb was cultured and a cell line established. These cells, called GN cells, secrete only a small amount of GnRH in comparison with the GT1 cells. They produce both the full length GnRH mRNA and a more abundant alternatively spliced proGnRH mRNA species which does not contain exon 2 of the GnRH gene (Patriquin et al., 1994). Since exon 2 encodes the GnRH decapeptide and the N-terminal domain of GAP, this may account for the decreased secretion of GnRH in this cell line. The GN cells contain glucocorticoid receptors and estrogen receptors (Radovick et al., 1994; Wierman et al., 1995), but to date remain less characterized than the GT1 cell line. Because the GnRH neurons migrate from the olfactory placode to the hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989), the GN cell line may represent a developmentally earlier version of the GT1 cells. This may suggest that developmental expression of the GnRH gene is under transcriptional control (Patriquin et al., 1994). A comparison of GnRH gene expression between these two cell lines may provide insight into the developmental control of GnRH gene expression.

THE GnRH NEURON-SPECIFIC ENHANCER

The GT1 cell model system has made it possible to study regulation of the GnRH gene in its natural cellular context. Using transient transfection into a variety of cell lines, Whyte et al. (1995) demonstrated high expression of 3 kb of the rat GnRH 5′ flanking DNA in GT1 cells, while expression in other cell types, including glioma, fibroblast, choriocarcinoma, gonadotrope, pheochromocytoma, neuroblastoma, and another neuronal cell line (Suri et al., 1993) is dramatically lower (Lawson et al., 1994; Whyte et al., 1995) (Figure 2). Truncation of the 5′ flanking sequences from –2258 to –1571 sharply decreased expression. This corroborated the results of Kepa et al. (1992), who found that deletion of 2 kb from –3026 to –1031 of the GnRH 5′ DNA from a different rat allele decreases expression 50-fold in GT1-7 cells, and Chandran et al. (1994), who found a 20-fold decrease after deletion from –2281 to –1580.

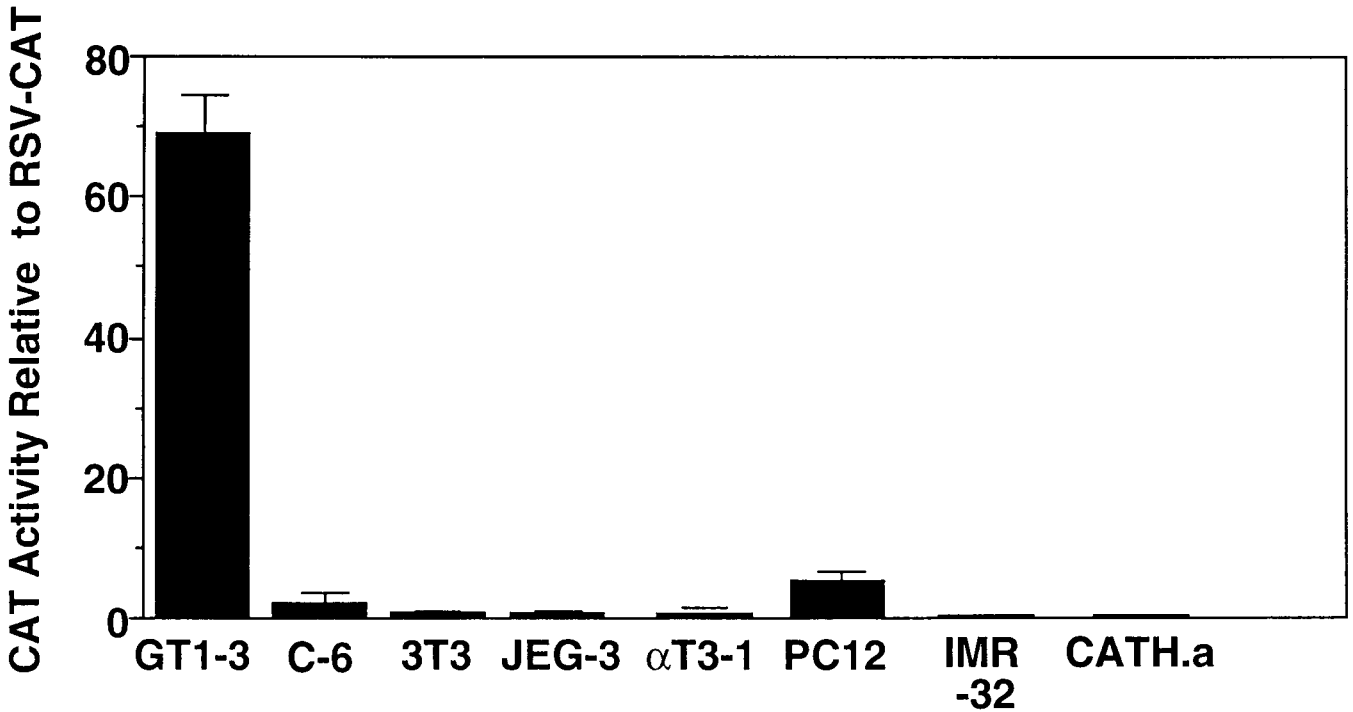

Figure 2. Specific expression of the rGnRH gene in the GT1 cell line. A variety of cell lines derived from different tissues were transiently transfected with a GnRH-CAT plasmid and an internal control plasmid. C-6 is a rat glioma-derived cell line, 3T3 is a mouse fibroblast cell line, JEG-3 is a human placental cell line, αT3-1 is a pituitary gonadotrope cell line, PC12 is a pheochromocytoma-derived cell line, IMR-32 is a neuroblastoma-derived cell line and CATH.a is a CNS neuronal cell line. Each value is shown ± SEM corrected for the internal control plasmid and expressed relative to the level of RSV-CAT activity (set to 100%). Adapted from Whyte et al., 1995, with permission.

In addition, Whyte et al. (1995) determined that GT1 cell specificity is conferred by a 300 bp enhancer (–1863 to –1571) that restores expression from the –173 truncated GnRH promoter to the level of the intact 3 kb 5′ flanking sequence (a 50-fold increase) only in GT1 cells. On a heterologous herpesvirus thymidine kinase (TK) promoter, the enhancer (in either orientation) specifically stimulates expression four-fold in GT1 cells (Whyte et al., 1995). Further, the GnRH enhancer induces activity on a weak, truncated RSV-LTR (Rous Sarcoma Virus Long Terminal Report) promoter by 25-fold in GT1 cells while activity was not induced in other cell types (Huang and Mellon, unpublished observations).

The enhancer binds multiple nuclear proteins as detected by DNase I footprinting (Whyte et al., 1995). Two strong central footprints (GATA-a and GATA-b) cover duplicate 8 bp sequence elements (CTATCATT) that contain the conserved GATA transcription factor binding site TGATAG on the lower strand (Orkin, 1990). Several other footprints upstream and downstream of the duplicated element contain highly AT-rich elements with a conserved ATTTT sequence motif as a repeated element. Protected regions, indicated by boxes (Figure 3) are decoded into separate footprints. Surprisingly, 5′ deletion to –1833 increases activity despite a lack of apparent protein binding in the deleted region. However, deletion of more than 30 base pairs from either end decreases expression (Whyte et al., 1995). Smaller fragments, each of which is missing a footprinted region, also show reduced expression. Activities of these fragments are independent of orientation. The smallest internal fragment with full enhancer activity is 240 bp (–1833/–1594) which contains all of the footprinted regions. Thus, the GnRH neuron-specific enhancer requires the coordinate action of a number of elements which have little or no activity even in groups of four or five.

Block replacement mutations in the 5′- and 3′-most elements reduce enhancer activity (Whyte et al., 1995). Replacements in the AT-a region have the strongest effect, eliminating enhancer activity (Figure 3). Mutation of the GATA-a and AT-b element have no effect. However, a replacement mutation (–1711/–1704) which overlaps the GATA-b footprint (mutating the T of the TGATAG consensus) reduces expression 30-40%. Both the deletion analysis and the replacement mutations demonstrate that multiple elements of the enhancer are involved in the generation of transcriptional activity. Thus, the GnRH gene, like other genes that integrate temporal and environmental cues, contains a complex multicomponent regulatory region (Robertson et al., 1995). The high degree of interdependence between GnRH enhancer elements for activity could also be a quality adapted for specifying expression of a gene to a very rare cell type, since the simultaneous presence of multiple interdependent DNA-binding proteins is required before significant activation can occur (Whyte et al., 1995).

AT-Rich Regions

Clark and Mellon further investigated the role of the two AT-rich regions centered in the enhancer, AT-a and AT-b. These two sites share a 6 base pair

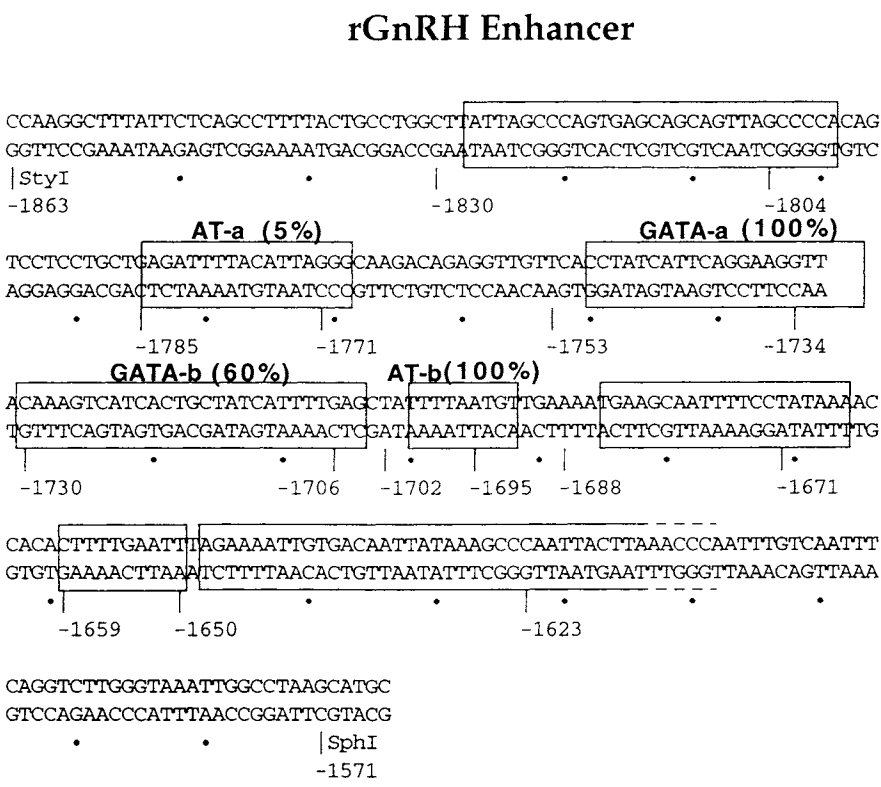

Figure 3. Sequence of the rGnRH enhancer with the AT-a, GATA-a, GATA-b, and AT-b elements highlighted. The sequence from –1863 to –1571 of the GnRH 5′-flanking region is illustrated with boxes enclosing the elements footprinted by GT1 nuclear extract. The percent of transcriptional activity of block replacement mutants in the AT-a, GATA-a, GATA-b, and AT-b regions when transfected into GT1 cells compared to the wild type sequence is indicated above the boxes. Adapted from Whyte et al., 1995, with permission.

conserved sequence motif of ATTTTA, but mutation of AT-a decreases basal transcription to 5%, while mutation of AT-b has no effect (Figure 3). The AT-a and AT-b sequences are both 6/8 matches (bottom strand) (Bendall et al., 1993) to the octamer consensus sequence (ATGCAAAT), a conserved binding site for POU-homeodomain proteins. The expression of members of the POU-homeodomain transcription factor family is restricted to lymphoid cells, regions of the nervous system, and the developing embryo, although one family member, Oct-1, is expressed in almost all tissue types (Clerc et al., 1988; He et al., 1989; Monuki et al., 1989).

To identify the protein(s) binding to the AT-a and AT-b sites, electrophoretic mobility shift assays (EMSAs) were performed using GT1 nuclear extracts. A complex of similar mobility was detected using an AT-a, AT-b, or consensus octamer oligonucleotide probe (Clark and Mellon, 1995). The AT-a and AT-b complexes are cross-competed and competed by a consensus octamer but not a mutant octamer oligonucleotide. To determine if the specific complex formed on the AT-a and AT-b probes was unique to GT1 cells, an EMSA was performed with both probes and nuclear extracts from a number of different cell lines. The complexes which formed on both the AT-a and AT-b probes with GT1 cell nuclear extract were identical to those observed with nuclear extracts from a variety of different cell lines, indicating that the protein(s) bound to the AT-rich regions in the GnRH enhancer is common to cells derived from a number of different tissues (Clark and Mellon, 1995).

POU-homeodomain family members all contain a highly related DNA-binding domain that allows a number of the proteins in this family to bind to the consensus octamer motif with various affinities. Using RNase protection assays, Oct-1 (expressed in several tissues but rare in the brain (He et al., 1989)), Oct-2 (expressed in the CNS (central nervous system) and lymphoid cells (Stoykova et al., 1992)), Brain-3 (expressed in the CNS and sensory ganglia (Gerrero et al., 1993)), and SCIP/Testes-1 mRNAs (expressed in the CNS and testes (Monuki et al., 1989)) were detected in GT1 cells. Brain-2 (Fujii and Hamada, 1993) and Brain-4 (Mathis et al., 1992b) mRNAs have also been detected in GT1 cells using RT-PCR (Clark and Mellon, unpublished data). The AT-a/AT-b protein complex is found in many cell lines; thus, a more common POU-homeodomain protein such as Oct-1 is likely to be binding to these regions. An antibody specific to Oct-1 (Santa Cruz Biotech.) supershifted the majority of the specific complex formed on the AT-a, AT-b, and octamer probes demonstrating that the complex contains Oct-1 (Clark and Mellon, 1995).

To determine whether binding to the octamer in the AT-a region was essential to enhancer activity, specific mutations were created in two base pairs in the AT-a sequence known to be required for POU protein binding (ATGTAAAA → CTGTACAA; the AT-a mOCT probe) and in the flanking region (AT-a flank probe) (Figure 4). The AT-a mOCT mutation eliminates binding to the AT-a probe and when an enhancer carrying this mutant is transfected into GT1 cells, it retains only 5% of wild-type activity (the same as the AT-a block mutations; Figure 3), while the AT-a flank mutation has little effect on binding or transcription (Figure 4) (Clark and Mellon, 1995). Thus, these experiments identify Oct-1 as the POU-homeodomain transcription factor binding the AT-a and AT-b sequences in the GnRH enhancer *in vitro*, and demonstrate a critical role for Oct-1 in the neuron-specific enhancer of the GnRH gene.

GATA Motifs

The linker replacement mutagenesis mentioned above also implied a role for at least one of the central GATA-factor binding motifs of the enhancer in activating

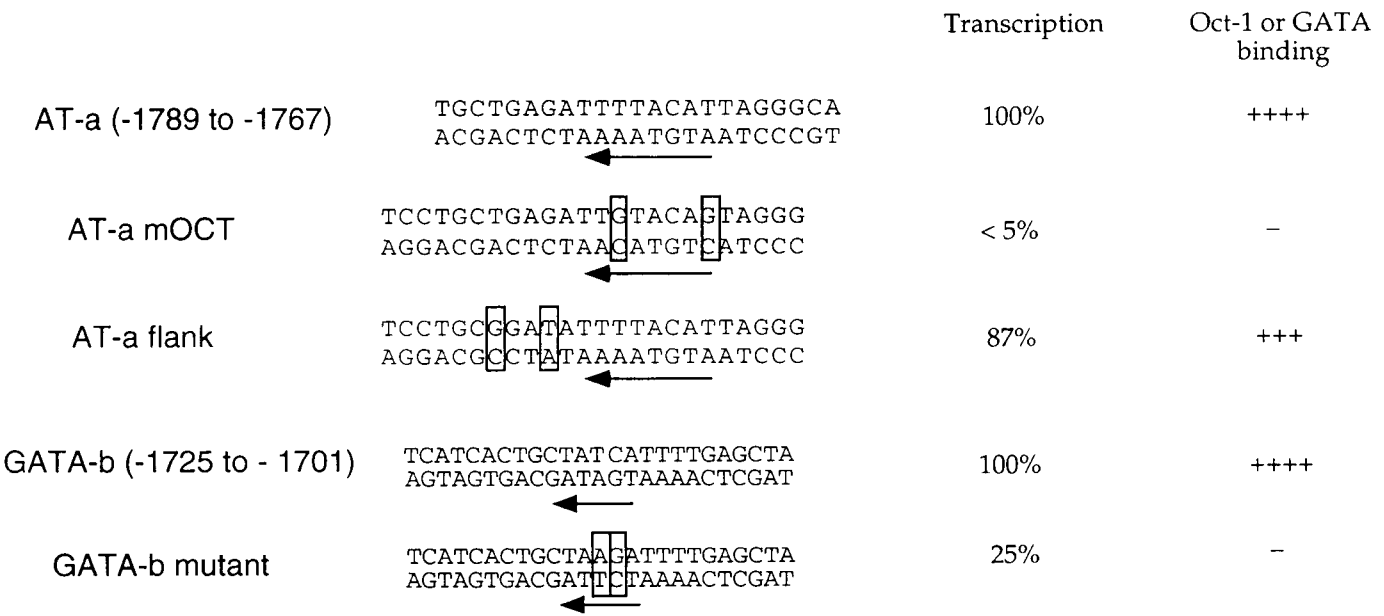

Figure 4. Site-directed mutation of the AT-a and GATA-b regions of the rGnRH enhancer. Arrows indicate homology to the octamer consensus sequence and the GATA consensus sequence. Bases changed by site-directed mutagenesis are boxed. The transcriptional activity of these constructs when transfected into GT1 cells, and the binding of GT1 cell nuclear extract to these oligonucleotide sequences using electrophoretic mobility shift assays is indicated. Summarized results from Clark and Mellon, 1995, Whyte et al; 1995; and Lawson et al., 1996.

GnRH gene transcription. Replacement mutagenesis of the GATA-a motif shows little effect on enhancer activity. However, a replacement mutation that alters flanking residues of the GATA-b motif decreases enhancer activity to approximately 60% of the wild type enhancer (Figure 3). The role of the GATA-b site was further examined by evaluating the activity of the enhancer bearing site-directed mutations of the GATA motif only. Specific mutation of the GATA-b sequence (TGATAG to TCTTAG) decreased activity to approximately 25% that of wild type (Figure 4) (Lawson et al., 1996). Interestingly, electrophoretic mobility shift assays of an oligonucleotide probe representing the GATA-b and flanking sequences indicated more than one protein in GT1 nuclear extracts interacts with this site. Competition with an oligonucleotide representing the preproendothelin 1 gene GATA motif, a well characterized GATA-factor binding element (Wilson et al., 1990; Dorfman et al., 1992), indicated that at least one of the factors forming a complex with the GATA-b oligonucleotide probe is a classical GATA-binding factor. Northern blot analysis of total RNA isolated from GT1 cells indicated the presence of GATA-2 and GATA-4, but not GATA-1 or GATA-3. To determine whether one of these factors was binding GATA-b, antibody supershift assays using antiserum directed against human GATA-2 and mouse GATA-4 were used to demonstrate that GATA-4 can interact with the GATA-b motif of the GnRH enhancer *in vitro*. No binding of GATA-2 or GATA-4 to the GATA-a site of the enhancer could be detected *in vitro*. To demonstrate that GATA-4 could activate the GnRH enhancer—a CAT gene reporter plasmid containing the GnRH enhancer on the thymidine kinase promoter cotransfected with a mouse GATA-4 expression plasmid (Arceci et al., 1993) was dependent on both enhancer GATA motifs when analyzed in NIH 3T3 cells. In GT1 cells, however, no significant transactivation of a GnRH enhancer-containing reporter gene plasmid cotransfected with a human GATA-2 (Dorfman et al., 1992) or mouse GATA-4 expression plasmid was detected. A role for GATA factors in GT1 cells was further demonstrated in cotransfection studies of GnRH enhancer activity using a dominant negative chicken GATA-3 (Smith et al., 1995). An approximately 30% decrease of reporter gene activity was dependent on the presence of intact GnRH enhancer GATA motifs. These observations provide the most compelling evidence for the role of GATA factor interaction in GnRH enhancer-mediated activation of GnRH gene expression.

THE GnRH PROMOTER REGION

Sequence alignment of the regions around the transcriptional start site of the rat (Bond et al., 1989), mouse (Mason et al., 1986), and human (Hayflick et al., 1989) GnRH genes reveals a zone of homology located between –185 and –5 of the rat gene. Within this region approximately 80% of the nucleotides are identical between the three species, while flanking sequences manifest little homology. This

specific evolutionary conservation indicates that the promoter sequence is critical for appropriate regulation of GnRH gene expression, probably because it contains recognition motifs for transcription-regulating proteins. The conserved sequence is AT-rich, with both deoxyadenosine and thymidine appearing in homopolymeric stretches. Interestingly, the rat GnRH enhancer also contains multiple instances of AT-rich repeats (Whyte et al., 1995). However, the human and mouse gene sequences of this upstream region remain unavailable for comparison. The downstream border of the conserved promoter sequence approximately coincides with a TATA box located at –19 of the rat gene (Kepa et al., 1992). This element has the sequence TTTAAA, which differs by one base from the ideal TATA box motif, TATA(A/T)A (Corden et al., 1980). The human gene utilizes the consensus TATA sequence TATAAA, situated approximately 90 bp further downstream (Radovick et al., 1990). The rat gene contains a deoxycytidine insertion in the homologous region, mutating the downstream TATA box to the sequence TCATAAA. The transcription start site has not been determined for the mouse gene; however, the downstream TATA box is preserved, suggesting that the mouse gene might utilize a start site similar to that of the human, rather than the rat gene (Radovick et al., 1990).

GT1 Cell Nuclear Proteins Bind the Rat GnRH Promoter

Eraly and Mellon (1995) performed DNase I protection assays to identify the particular elements within the rat GnRH promoter-proximal region that interact with the sequence-specific DNA-binding proteins present in GT1-7 cell nuclei. The evolutionarily conserved sequence is bound along its entire length by GT1-7 nuclear proteins, as indicated by contiguous nucleotide stretches (footprints) protected from DNase I cleavage. These footprints have been numbered in ascending order of their distance from the transcription start site (Figure 5). Footprints 1 through 7 fall within the conserved sequence, while footprint –1 is downstream of the start site within a region that has not been conserved. Footprint 3, an extremely narrow region of protection flanked by multiple hypersensitive sites, could only be demonstrated on the sense strand. While footprint 2 contains sequences that are completely protected from cleavage under normal conditions, the other footprints are only partially protected. However, partially protected sequences manifested highly specific binding to GT1-7 nuclear proteins in mobility shift assays (Eraly and Mellon, 1995). Transfection of expression vectors with sequentially truncated GnRH promoters into GT1 cells show that the footprinted regions are responsible for basal transcriptional activity of the promoter (Figure 5). In particular, the footprint 2 region of the promoter is critical for basal promoter activity since specific deletion of these sequences reduces promoter activity by approximately 20-fold. Because the GnRH promoter on its own has very little activity, each of these promoter constructs is assayed with the GnRH enhancer.

Since footprint 2 is 51 nucleotides long, extending from –76 to –26, it probably encompasses more than one protein binding site. Two clusters of hypersensitive

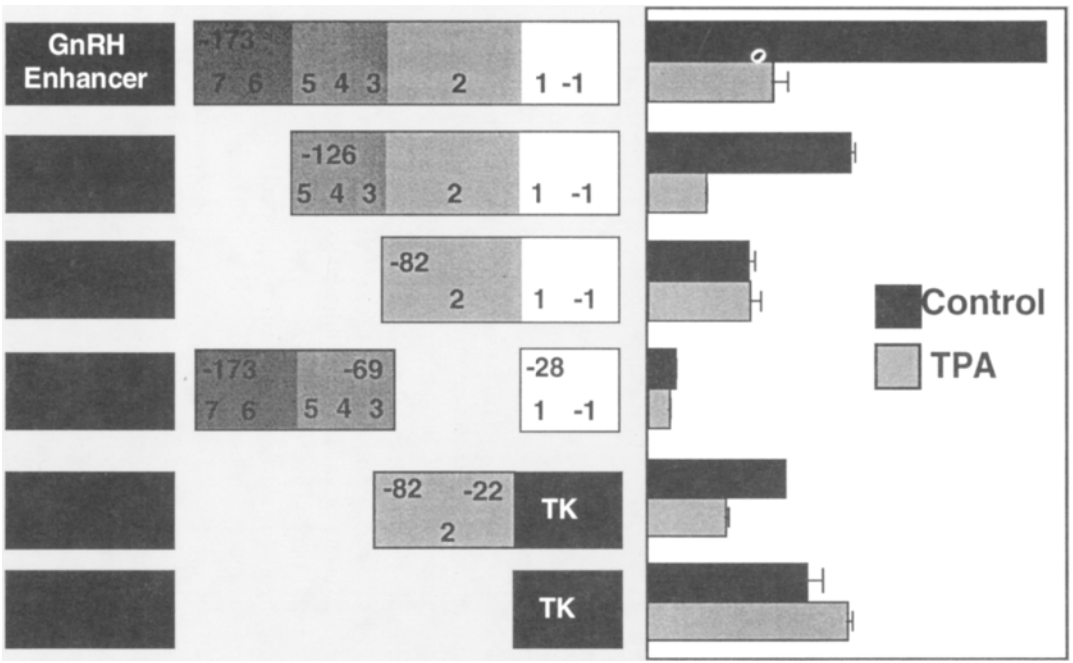

Figure 5. PKC regulation and deletion analysis of regions of the rGnRH promoter footprinted with GT1 cell nuclear extract. Deletions of the GnRH promoter and the full-length promoter or TK promoter are schematically illustrated by the rectangles to the left of the figure. The numbers within the rectangles correspond to the footprinted regions retained in the construct. Note that all constructs contain the GnRH enhancer. These plasmids were transiently transfected into duplicate GT1 cell cultures that were then incubated with either 100 nM TPA or control vehicle for 24 hours before harvesting and determination of CAT activity. CAT activity was normalized to the activity of the vector containing the GnRH enhancer on the GnRH promoter (set to 1). Adapted from Eraly and Mellon, 1995, with permission.

sites, distributed on both strands, fall within the downstream half of this footprint, one around –45, and the other around –35, and might indicate boundaries between distinct protein binding sites. The upstream half of this footprint contains an E box (CAGGTG; consensus motif for helix-loop-helix transcription factors (Murre et al., 1989)) at –64, and a CAAT box (CCAAT; recognized by CCAAT/enhancer-binding protein family members (Umek et al., 1991)) at –56. Footprint 1 includes a TATA box and 10 additional downstream nucleotides. The upstream footprints (3 through 7) are uniformly AT-rich (~60%), but do not otherwise manifest any obvious sequence similarities to one another. By contrast, the footprint downstream of the transcription start site (–1), which falls outside the conserved sequence, has a relatively high GC content (~60%). While some of the footprinted regions of the GnRH promoter have sequence homology to known transcription factor binding sites, the proteins that bind to the promoter have yet to be characterized.

REGULATION OF GnRH GENE EXPRESSION BY HORMONES, NEUROTRANSMITTERS, AND SECOND MESSENGERS

The development of the GT1 cell line has allowed a number of laboratories to address the role of second messengers, protein hormones, steroid hormones, and neurotransmitters in GnRH gene expression. Supporting the previous speculation of a short feedback loop regulatory mechanism, the GT1 cells express GnRH receptors and addition of GnRH to the culture medium has been found to repress GnRH mRNA levels in these cells (Krsmanovic et al., 1993). An increase in prolactin levels, due to physiological conditions such as pregnancy and lactation, has been associated with a suppression of GnRH (Yen, 1991). Prolactin receptors are present in the GT1 cells and prolactin has been found to repress secretion and GnRH mRNA levels in GT1 cells (Milenkovic et al., 1994).

Steroid hormones play a central role in mammalian reproduction. Glucocorticoids have been shown to repress mouse and rat GnRH gene transcription (Kepa et al., 1992; Chandran et al., 1994), and the levels of glucocorticoid receptors (GRs) are detectable, but low, in the GT1 cells. Although gonadal steroids have been found to influence both GnRH secretion and gene expression *in vivo*, GT1 GnRH neurons do not appear to express estrogen (Herbison and Theodosis, 1992) or androgen (Huang and Harlan, 1993) receptors, and it is unclear whether progesterone receptors are present (Fox et al., 1990). However, there has been one report indicating that the GT1-1 subclone contains specific binding sites for estrogens, androgens, and progesterone (Poletti et al., 1994). Radovick et al. (1992) have measured specific estrogen receptor binding in the developmentally earlier GN cell line. Using transient cotransfection experiments in placental JEG cells and the avidin-biotin DNA-binding assay, an estrogen response element has been localized to the 5′ flanking region of the human GnRH gene between –525 and –521 bp (Radovick et al., 1994).

The signal transduction pathways found to influence GnRH gene expression and secretion include those acting through protein kinase C, adenylate cyclase, and cGMP-dependent protein kinase. Chronic exposure to phorbol esters has opposite effects on GnRH secretion and gene expression in the GT1 cells (Bruder et al., 1992; Mellon et al., 1992; Wetsel et al., 1993), acutely stimulating secretion while repressing gene expression in the longer term. Repression appears to occur by downregulation of protein kinase C (PKC) (Yeo et al., 1991, 1993; Bruder et al., 1992; Wetsel et al., 1993), affecting transcription (Wetsel et al., 1993; Bruder et al., 1992), turnover (Yeo et al., 1993), and translational efficiency (Gore and Roberts, 1994) of the GnRH mRNA. On the other hand, an activator of adenylate cyclase, forskolin, has been found to increase GnRH secretion while having no effect on GnRH gene expression (Wetsel et al., 1993; Bruder et al., 1992), although one group has reported a repression of GnRH mRNA levels (Yu et al., 1994). GnRH gene expression is also repressed by the cGMP-dependent protein kinase signal transduction pathway involving the glutamate agonist, NMDA, and nitric oxide (NO) (Belsham et al., 1995). Furthermore the calcium ionophore, ionomycin, has been shown to repress GnRH mRNA levels after a long-term chronic exposure of calcium to the cells (Yu et al., 1994), but since calcium has diverse effects in many second messenger pathways, further study will be required to determine the mechanism of action in this case.

Thus far, the actions of the various second messengers that have been investigated are consistent and may reflect a unified mechanism. In acute administration, secretion of GnRH is induced. Over the long term (4-24 hours), activation of the same pathways causes downregulation of the GnRH gene. This may represent compensation whereby sustained stimulation of secretion utilizing second messenger pathways causes desensitization of the GnRH neuron through decreased synthesis of GnRH mRNA and hence decreased GnRH available for release.

Downregulation by Phorbol Esters

Treatment of GT1-7 cells with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), causes a progressive decline in GnRH mRNA levels that is apparent after 8 hours and maximal between 16 and 24 hours (Bruder et al., 1992; Wetsel et al., 1993). Phorbol esters typically produce a sharp, but transient, increase in PKC activity. However, prolonged treatment of cells with phorbol esters can lead to the depletion of at least some PKC isozymes by a proteolytic mechanism, a phenomenon referred to as PKC downregulation. Wetsel et al. (1993) used the highly specific PKC inhibitor, NPC 15437 (Sullivan et al., 1991a, b, 1992; Mathis et al., 1992a) to investigate whether repression of GnRH mRNA by TPA was due to the activation of PKC, or to its subsequent downregulation. Treatment of GT1-7 cells with the inhibitor abolishes the induction of *c-fos* mRNA by TPA, but fails to antagonize the repression of GnRH mRNA. Instead, administration of the inhibitor alone causes a decrease in GnRH mRNA, indicating that TPA-repression of GnRH

is due to the downregulation of PKC. Thus, continued PKC activity is required for the maintenance of expression of the GnRH gene. Additionally, PKC regulation of GnRH occurs, at least in part, at the level of transcription, as treatment with either the inhibitor or TPA represses the activity of a transfected reporter-expression construct that contains 3 kb of 5′ flanking sequence from the rat GnRH gene (Wetsel et al., 1993).

Investigations by Bruder et al. (Bruder and Wierman, 1994) demonstrate that repression of the GnRH gene by TPA is maintained with truncation to –126, but lost following truncation to –73, indicating that the intervening region contains sequences important for PKC regulation of transcription. The region between –175 and –13 is conserved in the rat, mouse, and human genes and the rat gene has seven distinct protein binding sites occupying the entire conserved region (Eraly and Mellon, 1995) (Figure 5). Consistent with the previous findings, Eraly et al. (1995) observe that excision of footprints 3, 4, and 5 (–126 to –73), lead to loss of TPA repression of GnRH. However, specific internal deletion of footprint 2 alone (–76 to –26), also abolish repression by TPA (Figure 5). Thus, within the GnRH promoter, at least two elements are required to establish PKC regulation of GnRH: one within footprint 2, and one from among footprints 3, 4, and 5. On the other hand, footprint 2 alone is sufficient to impose TPA-repression on the heterologous TK promoter, while a fragment containing footprints 3, 4, and 5 is insufficient to confer regulation (Figure 5) (Eraly and Mellon, 1995). Thus PKC regulation might be constituted by interactions between proteins binding footprint 2 and those binding either the TK promoter or footprints 3, 4, and 5.

Repression by Glutamate and Nitric Oxide

The release of GnRH from nerve terminals is regulated by a number of neurotransmitters and modulators. The excitatory amino acid, glutamate, regulates GnRH secretion through the NMDA receptor *in vivo* (Bourguignon et al., 1989a, 1989b; Donoso et al., 1990; Lopez et al., 1992; Bonavera et al., 1993). Furthermore, NMDA activates the release of GnRH from GT1 cells in culture (Mahachoklertwattana et al., 1994b). NO also stimulates secretion of GnRH *in vivo* (Bonavera et al., 1993; Rettori et al., 1993); however, in GT1 cells the results are conflicting, with two reports of NO activating (Moretto et al., 1993; Rettori et al., 1993), and one of NO repressing GnRH release (Sortino et al., 1994). The stimulatory effect of NMDA on GnRH secretion in GT1 cells is mediated by neuronal NO synthase (NOS) (which is present in these cells), since inhibitors of NOS block NMDA-induced secretion of GnRH (Mahachoklertwattana et al., 1994a). Thus, the neurotransmitters NMDA and NO regulate GnRH secretion.

One of the major signal transduction pathways involving the excitatory amino acid glutamate utilizes NO as a second messenger (Figure 6). Glutamate released from nerve terminals binds to the NMDA receptor on the postsynaptic membrane and activates an influx of calcium, which in turn binds to calmodulin (Nakanishi,

1992). The binding of the calcium/calmodulin complex to the enzyme, NOS, increases its conversion of arginine to NO (Bredt and Snyder, 1990). NO, a freely diffusible gaseous molecule, acts either intracellularly or in a paracrine fashion, diffusing through cell membranes. NO binds guanylyl cyclase and allows an accumulation of intracellular cGMP (Bredt and Snyder, 1989, 1992). cGMP effects cGMP-gated ion channels and cGMP-regulated phosphodiesterases (Butt et al., 1993), but also activates specific cGMP-dependent kinase which phosphorylates target proteins at serine and threonine residues.

In the GT1 hypothalamic neuronal cell line, Belsham et al. (1995) have demonstrated the action of this signal transduction pathway on the transcription of the GnRH gene. In the presence of calcium, the glutamate agonist NMDA represses GnRH mRNA levels after four hours. This action is mimicked by NO released from the compound sodium nitroprusside (SNP). Blockade of the endogenous synthesis of NO (by inhibition of NOS) prevents the action of NMDA, demonstrating an obligate role for NOS in the action of NMDA on GnRH gene expression. Furthermore, NO then acts through guanylyl cyclase, since an analogue of cGMP, the next molecule in the cascade, also mimics the effects of NMDA (and NO) and blockade of guanylyl cyclase prevents the actions of NMDA and NO on GnRH gene expression. Inhibition of cGMP-dependent protein kinase also prevents repression by NMDA, NO, and cGMP, thereby demonstrating the linearity of the signal transduction pathway mediating the repression of the GnRH gene and its obligate action through cGMP-dependent kinase.

Though the actions of NMDA, NO, and cGMP, on GnRH gene expression are now described, the next links in the cascade remain to be fully understood. It has been demonstrated that protein synthesis is required for the repression of GnRH mRNA levels by this neurotransmitter pathway (Belsham et al., 1995). This indicates that activation of cGMP-dependent protein kinase may then induce the synthesis of one or more proteins involved in repression of GnRH gene expression. However, the targets of cGMP-dependent protein kinase in GnRH neurons are as yet unknown.

The repression of GnRH gene expression by this neurotransmitter pathway occurs at the level of transcription, since the downregulatory effect has also been observed using a reporter gene controlled by 3 kb of the 5′ regulatory region of the GnRH gene (Belsham et al., 1995). Furthermore, by transfecting truncated regions of the GnRH 5′ flanking region into GT1 cells, it has been found that the NMDA, NO, cGMP pathway appears to repress transcription through the well-defined neuron-specific enhancer previously described(Belsham et al., 1995). Since repression occurs through the neuron-specific enhancer of the GnRH gene, the elements necessary for repression that lie within this region can be defined. Thus, identifying the proteins that bind to the GnRH regulatory sequences conferring NMDA and NO responsiveness may allow the elucidation of a complete pathway from the neurotransmitter at the cell surface to regulation of transcription at the GnRH gene.

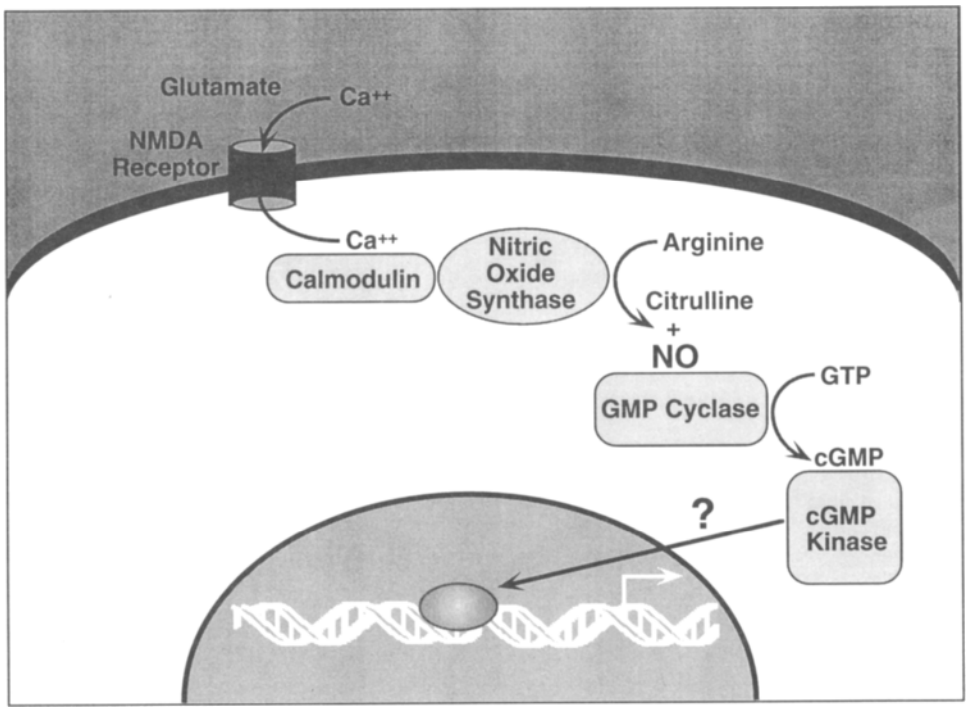

Figure 6. The nitric oxide (NO) signal transduction pathway. Glutamate released from nerve terminals binds to the NMDA receptor and activates an influx of calcium, which in turn binds calmodulin. The binding of this complex to the enzyme NOS increases the conversion rate of arginine to NO. NO binds guanylyl cyclase (GMP cyclase) resulting in an increase in cGMP which activates cGMP kinase. This kinase may phosphorylate transcriptional regulatory proteins.

DISCUSSION

A number of issues concerning the regulation of GnRH secretion and gene expression have been addressed using the GT1 cell model system, however, there remain many open questions. Particularly intriguing is the question of the mechanism by which the activity of the rat GnRH enhancer is restricted to GnRH neurons. Thus far, the proteins found to bind the enhancer have also been detected in other cell types in which the enhancer is inactive. A second major issue is the developmental activation of the GnRH gene during the unique migration and differentiation pattern of GnRH neurons. Finally the intracellular targets of the signal transduction pathways known to effect GnRH transcription have not yet been elucidated.

Neuron-Specific Activation by the GnRH Enhancer

In the simplest cases, tissue-specific gene expression is conferred by a binding protein restricted to one cell type. In GT1 cells, the GnRH enhancer is dependent on an Oct-1 binding site (AT-a), yet the enhancer is inactive in other cell lines, many of which contain Oct-1. One hypothesis to explain this is that GT1 cells may contain a neuron-specific Oct-1 coactivator restricted to GnRH neurons. Oct-1 is not known to be a strong transcriptional activator by itself; however, in conjunction with coactivators, the Oct-1 complex can promote potent transactivation of target genes. The herpesvirus VP16 protein is one such coactivating protein that binds to Oct-1 (Walker et al., 1994). Recently, a B cell specific coactivator protein OBF-1/Bob-1 has been identified (Gstaiger et al., 1995; Strubin et al., 1995). This protein does not bind DNA directly, instead it binds specifically to the POU domain of Oct-1 and Oct-2 *in vitro* (though it cannot be detected as a supershift). When OBF-1 is transfected into non-B cells, they become competent to transcribe immunoglobulin genes. Thus, in B cells, tissue specificity is provided not by the DNA-binding component (Oct-1), but by a tissue specific coactivator protein (OBF-1). It is therefore possible that a neuron-specific coactivator is present in GT1 cells that binds to Oct-1 and contributes to the specificity of the GnRH enhancer. It is also possible that a neuron-specific splice variant of Oct-1 may lend specificity to the enhancer. Alternatively, unique neuron-specific DNA-binding proteins may yet be identified binding to the GnRH enhancer.

Transcriptional Regulation During Development

The unique ontogeny of the GnRH neurons poses several questions concerning the molecular aspects of GnRH gene regulation. GnRH expression is detected in the medial olfactory pit as early as day 11 of gestation (Schwanzel-Fukuda and Pfaff, 1989). Multiple neuronal cell types arise from this area, indicating that the GnRH neurons are already committed to their developmental program prior to the

appearance of GnRH gene expression. It is not necessarily the case that transcription factors regulating GnRH gene expression in the more differentiated cell are those that are also responsible for the initial activation of gene expression. Studies of GATA-factor gene expression during development of definitive adult hematopoietic progenitors have suggested that transient bursts of transcription factor gene expression are an integral part of differentiation programs (Whitelaw et al., 1990). Therefore the factors involved in maintenance of gene expression may not perform the same tasks during development of the cell type. Indeed, hematopoietic progenitors rely on the interplay of GATA-1 and GATA-2, which overlap in their functional activity, to regulate differentiation while only GATA-1 is required for transcriptional activity in the mature cell (Weiss and Orkin, 1995).

The presence of GATA-2 and GATA-4 transcripts in cells derived from the pituitary gonadotropes has been recently reported(Steger et al., 1994). It is of interest to note that multiple tissues of the reproductive axis, gonads, pituitary, and GnRH neurons, express common GATA-binding proteins, GATA-2 and GATA-4. However this is more likely to be a reflection of common early embryological origins of the tissues rather than a specific, coordinated differentiation. GATA factors are expressed before the first 10 days of gestation, whereas distinct differentiation of the reproductive endocrine tissues does not occur until later. Cardiac, gonadal, and hematopoietic precursor cells which express GATA factors arise from the splanchnopleural mesoderm or aorta/gonad/mesonephros region (AGN) (Godin et al., 1993; Medvinsky et al., 1993; Weiss and Orkin, 1995). In addition to a high level of expression in the developing heart, GATA-4 has also been reported in the head and pharyngeal arch mesoderm at day 8-9 post conception prior to restriction of Rathke's pouch, the pituitary anlagen, and initial GnRH neuron differentiation (Heikenheimo et al., 1994). The presence of these factors in GT1 cells may be a reflection of their non-CNS origin in development. It also suggests that other neuronal cell types arising from the same region may also contain GATA-factor target genes which are important in their developmental programs.

Nevertheless, as with Oct-1, it is not likely that GATA-binding factors are solely responsible for GnRH neuron-specific expression. Many of the questions regarding the mechanism of GnRH neuron differentiation may be addressed by using the already defined targeting elements of the GnRH gene, the enhancer and/or the promoter, to create new transgenic mouse lines expressing non tumorigenic reporter genes. Analysis of the targeting elements *in vivo* may provide greater insight into the mechanism of the initiation of gene expression and possibly direct us to other elements not defined in the cultured cell model system.

Targets of Signal Transduction Pathways

The signaling molecules that cause acute stimulation of GnRH secretion have also been consistently found to repress GnRH gene expression over the longer term. A specific example is the induction of GnRH secretion by NMDA or NO and a repression of gene expression by these same neurotransmitters. This apparent

paradox can be viewed as an endocrine damping mechanism. Many signals that are normally received intermittently or as short pulses, when applied for long periods (hours), will downregulate the system at a fundamental level to protect cells from overstimulation.

The GT1 cells contain a number of voltage-activated ion channels, including tetrodoxin-sensitive Na^+ channels, transient and sustained Ca^{2+} channels, and both inward and outward rectifying K^+ channels (Bosma, 1993). Furthermore, the GT1 cells contain GABA-gated Cl^- channels (Hales et al., 1992), and Ca^{2+} channels, including P- or Q-type, T-type, L-type, and R-type channels (Shcherbatko et al., 1993). All of these channels have been implicated in the release of GnRH, yet little is known how they might be involved in various second-messenger cascades changing GnRH gene expression. Further, Ca^{2+} has been shown to be required for secretion (Krsmanovic et al., 1992; Weiner et al., 1992; Wetsel et al., 1992) and gene expression (Belsham et al., 1995) in the GT1 cells, but the precise mechanism of calcium action in these neurons is not yet known. Since the hypothalamic neuron releases GnRH in a pulsatile manner, as does the GT1 cells, the effects of the numerous signal transduction pathways already shown to affect GnRH secretion and expression may change the frequency and/or amplitude of the GnRH pulses. Many diverse signals and pathways may be necessary to generate the preovulatory surge of GnRH and may account for the fact that numerous signal transduction pathways change GnRH gene expression in the hypothalamic neuron.

SUMMARY

In this review we have assembled the recent literature concerning the molecular aspects of GnRH gene regulation. Since investigation of the molecular mechanisms of gene expression requires an appropriate cell model system, we have focused on experiments performed in the GT1 hypothalamic neuronal cell line. Important regions of the GnRH gene, including a neuron-specific enhancer region and a proximal promoter, have been determined and proteins that bind to these regions are now being identified. Thus far, GATA transcription factors and the Oct-1 transcription factor are known to bind the GnRH gene. Several other proteins have been detected but their identities are still unknown. Understanding how these proteins are involved in a neuron-specific enhancer will increase both our knowledge of GnRH regulation and the mechanisms underlying tissue specificity in general. Some of these proteins may be targets of the PKC and cGMP-dependent protein kinase signal transduction pathways known to effect GnRH transcription. Thus far agents that affect these pathways have been found to induce secretion of GnRH in the short term, but cause downregulation of the GnRH gene in the long term. It is hoped that by understanding the molecular mechanisms underlying GnRH synthesis, rational treatments for endocrine and reproductive diseases involving GnRH will result.

ACKNOWLEDGMENTS

Our research contibutions to this review were supported by NIH grant R01 DK44838 to P.L.M.. Dr. Clark is a fellow of the American Cancer Society (PF-3904), Dr. Lawson was a Presidents and Ford Foundation Fellow, Dr. Belsham was a postdoctoral fellow of the Medical Research Council of Canada, and Dr. Eraly was supported by the NIH T32 AG00216 training grant.

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Chapter 2

MOLECULAR ASPECTS OF HORMONE DEFICIENCY CAUSED BY PIT-1 GENE MUTATIONS

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INTRODUCTION

Pit-1, also known as growth hormone factor-1 (GHF-1), is a member of a family of transcription factors, POU, responsible for mammalian development. POU is an

Advances in Molecular and Cellular Endocrinology
Volume 1, pages 31-50.

ISBN: 0-7623-0158-9

acronym for Pit-1, Oct-1 which is widely expressed, Oct-2 which is expressed in B lymphocytes and in certain areas of the brain, and Unc-86 which functions in *Caenorhabditis elegans* neuronal cell development (Mangalam et al., 1989). Over 20 POU-specific factors have been identified (Haugen et al., 1995). Pit-1 expression is restricted to the anterior pituitary gland (Bodner et al., 1988) and was identified by its specific binding to AT-rich cell-specific elements in the rat (r) prolactin (Prl) and growth hormone (GH) genes (Ingraham et al., 1988).

The human (h) Pit-1 cDNA is 96% identical to other mammalian Pit-1s. Most of the differences are found in the N-terminal region (93% identity), whereas the POU-domain is 97 to > 99% identical to other mammalian species (Lew and Elscholtz, 1991). The human Pit-1 gene is greater than 14 kb in size and is located on chromosome 3p11 (Ohta et al., 1992a), while the mouse Pit-1 gene is located on chromosome 16 (Li et al., 1990).

PIT-1 PROTEIN

Pit-1 contains two protein domains, termed POU-specific and POU-homeo (Figure 1). The 60 amino acid POU-homeodomain (POU-HD) is a region near the C-terminus which has considerable homology to the homeobox, a conserved sequence motif identified in genes that regulate development in the fruit fly *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae* (Anderson and Rosenfeld, 1994). The POU-HD is predicted to form a helix-turn-helix motif similar to the DNA binding domain of prokaryotic repressors (Ingraham et al., 1990). It is required and sufficient for low affinity DNA binding with relaxed specificity, while it is the 75 amino acid POU-specific domain (POU-S) which is necessary for high affinity site-specific binding to natural Pit-1 response elements. The POU-S also

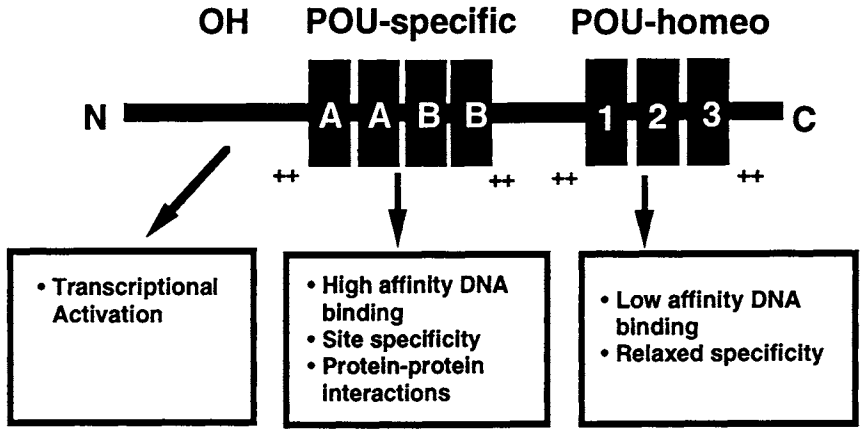

Figure 1. Pit-1 protein domains.

contributes to DNA-dependent Pit-1-Pit-1 interactions (Anderson and Rosenfeld, 1994; Ingraham et al., 1990; Theill et al., 1989). Chou-Fasman algorithms predict the presence of 2 α-helices in the POU-S and 3 α-helices in the POU-HD (Ingraham et al., 1990), although recent crystallographic evidence in Oct-1 suggests there are 4 α-helices present in the POU-S (11). The first α-helix (A) of the POU-S seems necessary for DNA binding, suggesting this may be the region responsible for mediating protein-protein interactions, while the second α-helix (B) appears involved in site specificity (Ingraham et al., 1990). In between the POU-S and POU-HD is a non-conserved linker of 15 amino acids (Anderson and Rosenfeld, 1994). Transcriptional activation on enhancer elements is mediated primarily by a separate N-terminal domain containing 21% hydroxylated amino acid residues (serine- and threonine-rich) (Ingraham et al., 1990; Theill et al., 1989).

Pit-1 is monomeric in solution but associates as a dimer on most DNA response elements (Ingraham et al., 1990). Some Pit-1 response elements are able to only bind Pit-1 homeodimers (such as the distal site in the rGH promoter), while heterodimers of Pit-1 and other factors bind preferentially to some Pit-1 response elements (including the proximal site in the rat GH promoter) (Voss et al., 1991a). However, on certain sites, Pit-1 binds preferentially and with high affinity as a monomer (Rosenfeld, 1991).

SPLICE VARIANTS

The major Pit-1 protein species migrates as a doublet of 31 and 33 kDa, depending on which translation initiation start site in the Pit-1 mRNA is used. The two forms differ in the presence or absence of a 27 amino acid sequence between the first and second methionine residues of the primary amino acid sequence (Ingraham et al., 1988; Voss et al., 1991b). Both forms are similar in their transactivation ability (Voss et al., 1991b).

Other splice variant Pit-1 proteins have been identified in rodent species (Figure 2). One variant referred to as Pit-1β, Pit-1a, or Pit-2 is a 35.8 kDa protein containing a 26 amino acid insert in the transactivation domain due to alternative splicing of the Pit-1 gene transcript at the end of intron one (Konzak and Moore, 1991; Morris et al., 1992; Delhase et al., 1995). Transactivation of the Prl promoter by Pit-1β has been reported as undetectable (Morris et al., 1992) or as significantly lower than that by Pit-1 (Konzak and Moore, 1991). However, induction of the rGH promoter by Pit-1β is similar or somewhat less than by Pit-1, suggesting that the less abundant Pit-1β is a more potent inducer of the GH promoter (Konzak and Moore, 1991). Indeed, Pit-1β mRNA is present at a level of 14% of Pit-1 mRNA, and Pit-1β protein is present at a level of less than 3% of Pit-1 (Konzak and Moore, 1991), so the significance of this variant *in vivo* is not clear at this time.

Another variant, Δ4Pit-1, has been detected in all Pit-1 producing rat pituitary-derived cell lines studied. Due to alternative RNA splicing, there is fusion of exon 3 to exon 5 with deletion of the POU-S domain. Δ4Pit-1 can not bind to the Prl-1P site, nor can it transactivate the Prl promoter, but DNA binding to other sites is not affected.

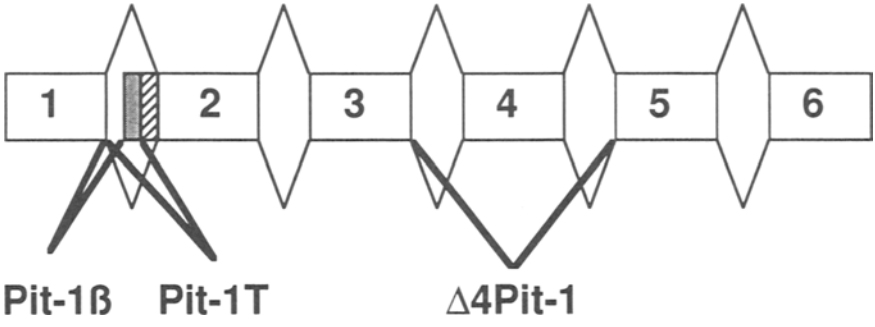

Figure 2. Pit-1 splice variants. Thin lines represent Pit-1 splice events. Thick lines represent variant Pit-1 splice events: Pit-1T has the addition of the diagonally shaded area; Pit-1β had the addition of both the diagonally and dot shaded areas.

Thus, the POU-S is an essential, but nonmodular component of the Pit-1 trans-activation domain (Voss et al., 1993). In gene transfer studies, D4Pit-1 inhibits Prl promoter activity and, therefore, it may be a potential mediator of Prl gene expression (Day and Day, 1994).

A third variant, Pit-1T, has been identified in thyrotroph-derived cells. It contains a 14 amino acid insert in the transactivation domain due to an alternate 3′ splice acceptor site. It appears necessary for thyroid stimulating hormone β (TSHβ) promoter stimulation, as transiently transfected Pit-1T increases TSHβ promoter activity in TtT-97 thyrotropic tumor cells (express an endogenous TSHβ gene), whereas additional Pit-1 has no effect. α-TSH cells (derived from a pituitary thyrotropic tumor), which have lost the ability to express the TSHβ gene and lack all Pit-1 proteins, require both isoforms in order to stimulate TSHβ promoter activity (Haugen et al., 1993). When added to GH_3 cells which lack only the Pit-1T isoform, Pit-1T selectively stimulates the TSHβ promoter and not the GH or Prl promoters, suggesting that the thyrotroph-specific Pit-1T exhibits a promoter-specific effect (Haugen et al., 1994).

PITUITARY DEVELOPMENT

During development, Pit-1 transcripts are initially detected in the rat neural tube and neural plate on embryonic day (e) 10-11 and then disappear by e13 (Figure 3). They reappear exclusively in pituitary cells on e15 (Simmons et al., 1990). Pit-1 protein is detected in the somatotrophs and lactotrophs, preceding GH and Prl gene expression on e16 and 17 respectively, suggesting that Pit-1 is the major cell-specific activator of hormone expression from these cell types (Simmons et al., 1990). Additional nuclear factors, however, appear to be necessary for full expression of these genes. For example, a zinc-finger transcription factor, Zn-15, synergizes with Pit-1 to activate the GH promoter (Lipkin et al., 1993), with maximal levels of GH expression by e19-20. The estrogen receptor (ER), in a synergistic effect with Pit-1,

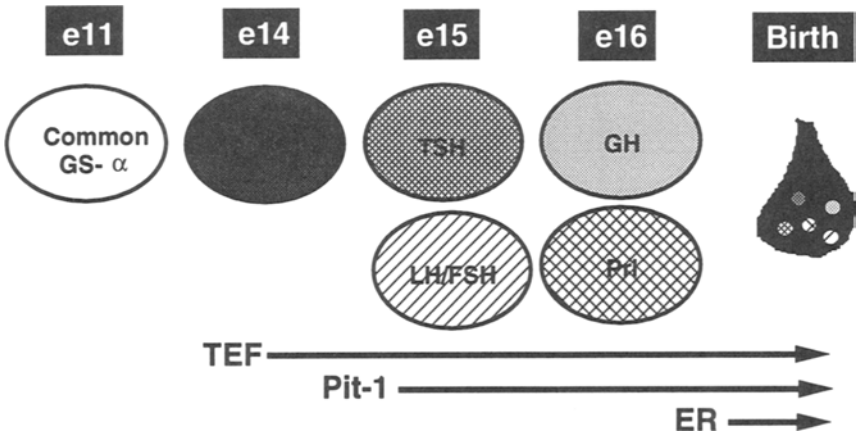

Figure 3. Anterior pituitary gland development in the rat.

appears to be capable of mediating the progressive increase in Prl gene expression characteristic of the mature lactotroph phenotype, with full Prl gene activation appearing developmentally after birth (Simmons et al., 1990).

Pit-1 protein is also expressed in the thyrotrophs (Simmons et al., 1990). Thyrotrophs appear to arise from two independent cell populations in mice. The first population is Pit-1 independent and appears on e12 in the rostral tip of the developing anterior pituitary gland prior to the first detectable expression of Pit-1 on e14.5, but phenotypically disappears by the day of birth. The second population is Pit-1-dependent and arises subsequently in the caudomedial portion of the developing pituitary gland on e15.5, following the initial expression of Pit-1 in this area. Pit-1 appears necessary for the appearance of these precursors of the mature thyrotroph cell type, as the caudomedial thyrotroph cells are not present in the Snell dwarf mouse which is Pit-1 defective, and Pit-1 can bind to and transactivate the TSHβ promoter (Lin et al., 1994). A PAR-bZIP protein, thyrotroph embryonic factor (TEF), may be involved in the initial expression of the mouse TSHβ promoter, as it is first selectively expressed in the rostral tip cells of the anterior pituitary concomitantly with the activation of TSHβ gene expression. TEF binds to and can effectively transactivate the TSHβ promoter (Drolet et al., 1991).

Pit-1 transcription is detected before the appearance of Pit-1 protein and therefore, Pit-1 can not be responsible for developmental activation of its own gene (Lew et al., 1993). Immortalized somatotropic progenitor cells (GHFT1 cells) express Pit-1 but not GH or Prl. This cell line has an active regulatory element that is not present in more committed cells, an enhancer located at -3.2 to -5.3 kb on the Pit-1 gene. The enhancer appears to be stage specific and functions only in somatotropic progenitor cells. Activation is likely to occur on or before e13 and may signal

divergence of somatotropic progenitor cells from cells destined to express glycoprotein hormones (Lew et al., 1993).

Once the amount of Pit-1 protein has reached a critical threshold, Pit-1 transcription is likely maintained by autoregulation and additional transcriptional regulation. For example, retinoic acid (RA) induction of the Pit-1 gene requires both the retinoic acid receptor (RAR) and Pit-1 (Rhodes et al., 1993). P-Lim, a member of a different family of homeodomain transcription factors (which contain a cysteine-rich domain and two adjacent zinc-coordinate structures) acts synergistically with Pit-1 on Prl, TSHβ, and Pit-1 gene regulatory regions (Bach et al., 1995).

In analysis of the Snell dwarf mouse, the Pit-1 gene appears at the normal time in the expected region of the pituitary gland. Pit-1 expression is detectable until postnatal days 0-5; however, by e18.5 there is a significantly decreased level compared to wild type mice. Thus, *in vivo* studies corroborate in vitro experiments showing that functional Pit-1 protein is not required for the initial activation of the Pit-1 gene, but Pit-1 is subsequently required to maintain Pit-1 gene expression (Rhodes et al., 1993).

GENE ACTIVATION AND REGULATION

Tissue-specific enhancers in the 5′ flanking regions of both the GH and Prl genes appear to control their pituitary-specific expression (Nelson et al., 1986). The expression of both the hGH and rGH genes is controlled by a pituitary-specific promoter which contains two binding sites for Pit-1, GH-I (–96 to –70) and GH-II (–134 to –106) (Nelson et al., 1988) (Figure 4). Both sites are essential for GH promoter activity *in vitro* and *in vivo* (Bodner et al., 1988). All cell-specific transcription of the rat Prl gene depends on a distal enhancer segment (–1830 to –1530) containing four Pit-1 binding sites (1D to 4D) and a proximal promoter region (–422 to –36) containing four Pit-1 binding sites (1P to 4P) (Figure 4). The distal region accounts for 99% of activity, and the proximal region accounts for

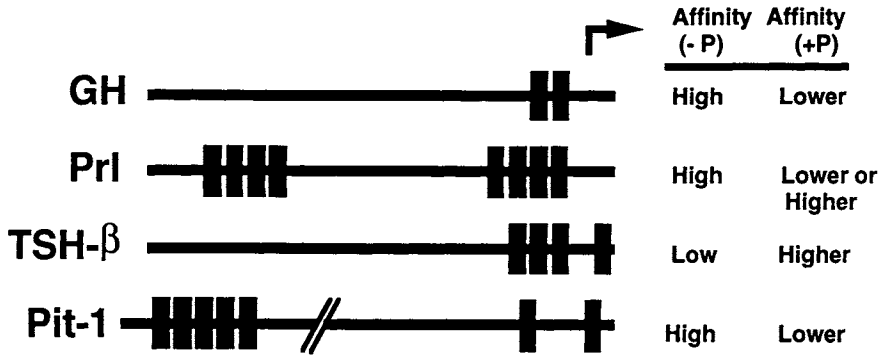

Figure 4. Pit-1 binding sites in target genes.

1-2% of maximal activity (Nelson et al., 1988). Prl-1P and 3D and both GH-1 and GH-2 are strong competitors for Pit-1 binding. They have a consensus sequence with a core of A(A/T)(A/T)TATNCAT (Nelson et al., 1988). The level of Pit-1 in pituitary cell lines is sufficient to activate the minimal elements in both the GH and Prl promoters necessary for cell-specific expression of these genes (Fox et al., 1990). However, other nuclear factors are also necessary for activation of the GH and Prl genes.

Zn-15, a novel member of the Cys/His zinc finger superfamily, syngergizes with Pit-1 to activate the GH promoter. The Zn-15 DNA-binding domain is composed of three zinc fingers separated by unusually long linker sequences. The Zn-15 binding site, the GH Z box, of the rGH promoter is at -110 to -95, between the proximal and distal Pit-1-binding sites and is unusually well conserved across species (Lipkin et al., 1993).

Thyroid hormone (T3) response on the rGH promoter may require Pit-1 binding (Ye et al., 1988). The T3 dependent effect seems to primarily occur through binding of the T3 receptor (T3R) to a thyroid hormone response element (TRE) located at -162 to -182 within the GH promoter, upstream of the two Pit-1 binding sites. TRE occupancy enhances the interaction of Pit-1 with its DNA binding sites (Schaufele et al., 1992; Glass et al., 1987; Brent et al., 1989; Lira et al., 1993; Force and Spindler, 1994). rGH gene is almost inactive in the presence of either Pit-1 or T3R alone; however, it is strongly activated by both Pit-1 and T3R in cultured cells (Schaufele et al., 1992). In addition to the T3R, Pit-1 effectiveness requires activators of protein kinase A (PKA) and protein kinase C (PKC) (Schaufele et al., 1992). However, the Pit-1 and T3R synergy is controversial, as other investigators have not confirmed this finding (Suen and Chin, 1993). In addition, the sequence of the TRE is not conserved in the hGH promoter (Lipkin et al., 1993), and T3 has been found to negatively regulate human GH (Zhang et al., 1992).

Growth hormone releasing hormone (GHRH), the stimulus for GH release, increases intracellular cyclic AMP (cAMP) levels in normal pituitary cells with consequent activation of PKA (Barinaga et al., 1985; Bileikjian et al., 1983). Pit-1 has been suggested to be involved in the cAMP regulation of the rGH promoter (Brent et al., 1988; Copp and Samuels, 1989; Dana and Karin, 1989). In addition, the GHRH receptor transmits its signal for cell growth and GH gene expression through Pit-1 (Lin et al., 1992).

In the hGH gene, Sp1 binds at a site separated from the Pit-1 binding site GH-II by 10 bp. Sp1 binding activates transcription from the GH promoter. Pit-1 binding at GH-II does not stimulate the expression of hGH; however, it interferes with Sp1 binding and may modulate activity indirectly. Pituitary-specific expression is directed by Pit-1 binding to site GH-I (Tansey et al., 1991; Schaufele et al., 1990). Negative regulation may also be achieved through activin. In somatotrophs, activin suppresses the biosynthesis and secretion of GH and cellular proliferation (Bilezikjian et al., 1990; Billestrup et al., 1990). With activin treatment, the binding of Pit-1 to the GH promoter is lost (Struthers et al., 1992).

Multiple factors interact synergistically with Pit-1 to regulate the Prl gene. The distal enhancer element (-1713 to -1495) of the Prl gene permits cAMP, thyrotropin releasing hormone (TRH), epidermal growth factor (EGF), and estradiol to stimu-

late expression. The proximal element (-292 to -39) of the Prl gene permits regulation in response to cAMP, TRH, EGF, and phorbol esters (Day and Maurer, 1989). Normal transcription of the Prl gene involves strong cooperative interactions between individual DNA elements (d'Emden et al., 1991).

Estradiol stimulates transcription of the Prl gene through activation of an intracellular estrogen receptor (ER). The ER interacts directly with a distal enhancer element, an estrogen response element (ERE), in the 5′ flanking region of the Prl gene (Maurer, 1989; Maurer and Notides, 1987; Waterman et al., 1988). The ERE is distinct from, but may interact cooperatively with, the other hormone response elements (Day and Maurer, 1989). Pit-1 binding sites and the ER are required for distal enhancer activation by estradiol *in vitro* (Day et al., 1990). The most important Pit-1 binding site appears to be the site adjacent to the ER binding site. Other Pit-1 binding sites also contribute to the estrogen response of the Prl gene, so communication between the distal enhancer and proximal promoter elements is important (Nowakowski and Maurer, 1994). Labeled ER can bind to Pit-1 in a protein interaction assay, but most of the interaction between Pit-1 and the ER appears to be DNA-dependent (Nowakowski and Maurer, 1994). However, estrogen treatment does not increase Pit-1 mRNA in the rat, while it does stimulate lactotroph proliferation and expression of the Prl gene. Therefore, Pit-1 mRNA may not be required for estrogen-induced lactotroph proliferation or Prl gene expression *in vivo* (Tsukahara et al., 1994).

Three Pit-1 binding sites of the proximal region (–250 to –42) are involved in both TRH and EGF regulation. Thyrotropin releasing hormone response elements are located at -75 to +38 near the the Prl-1P site and at -164 to -113 near the Prl-3P site. Pit-1 transduces the TRH signal to site 1P and may also transduce a TRH signal to site 3P (Yan et al., 1991). The first and second Pit-1 binding sites together with the sequence between them (-115 to -85) can transfer TRH and EGF regulation. This area of Pit-1 binding sites (sequence A) and the intervening sequence (fragment A), which contains a TGACG motif, are involved in cAMP regulation (Peers et al., 1992). Fragment A binds Pit-1 monomer and also a ubiquitous factor that is neither cAMP-responsive element-binding protein (CREB) nor activator protein-1 (AP-1) (Peers et al., 1991). Although TRH and EGF activate mostly different intracellular pathways, their mediation of transcriptional induction of the Prl promoter is via identical cis elements (Berwaer et al., 1993).

Other transcription factors interact with Pit-1 in Prl gene expression. Oct-1 also binds to the Prl-1P site (Elscholtz et al., 1990). Pit-1 and Oct-1 proteins form heterodimers on the Prl-1P site (Voss et al., 1991a), as well as in the absence of DNA through the POU-S and POU-HD (Voss et al., 1991a; Verrijzer et al., 1992). Like Pit-1, Oct-1 can induce Prl gene expression, and together, Pit-1 and Oct-1 act synergistically.

Dopamine inhibits Prl synthesis. The minimal promoter region of Pit-1 containing a Pit-1 binding site exhibits dopamine responsiveness. Dopamine's inhibition of Prl synthesis may in part be through the regulation of the DNA binding or transactivating functions of Pit-1 (Elscholtz et al., 1991).

rPit-1 can interact with the 5′ flanking region of the TSHβ gene at a fragment of the hTSHβ promoter from –128 to –92 (Steinfelder et al., 1991). This area is

necessary for TRH and cAMP responsiveness in transient transfections in nonthyrotropic Pit-1 containing GH_3 cells (Steinfelder et al., 1992a). However, Pit-1 is not limiting for cell-specific expression of the TSHβ gene in thyrotroph-derived cells, as exogenously added Pit-1 is not capable of reconstituting cell-specific mTSHβ promoter activity in α-TSH cells (Gordon et al., 1993). Following stimulation by phorbol esters, forskolin, or TRH, an AP-1-like factor interacting with a TGGGTCA element at –1 to +6, together with Pit-1 bound at -128 to -61, mediates the induction of the hTSHβ promoter. This induction requires that both factors bind to their own binding sites, but Pit-1 neither increases the binding of the TGGGTCA-specific factor to its target sequences nor associates with this factor to form a heterodimer (Kim et al., 1993). In addition, three more upstream regions within the 5′ flanking region of the rTSHβ promoter at –274 to –258 (TSH A), at –336 to –326 (TSH B), and at – 402 to –385 (TSH C) contain sequences similar to Pit-1 consensus binding sites and bind Pit-1 (Mason et al., 1993) (Figure 4). TSH A and TSH C are able to confer TRH, cAMP, or PKC-stimulated responses to a heterologous promoter in transient expression assays in GH_3 cells. TSH C can confer basal enhancer activity as well (Shupnik et al., 1992). TSH B does not confer a significant TRH response or enhancer activity (Mason et al., 1993). TSH A and TSH C also form several additional DNA-nuclear protein complexes not observed with TSH B. Some of these complexes may contain Pit-1 as their formation is inhibited by the addition of Pit-1 antibody. Thus, Pit-1 may play a role in the basal and TRH-stimulated expression of the rTSHβ gene along with other factors (Mason et al., 1993).

The transcription initiation site of the Pit-1 gene is located 120 bp upstream of the translation initiation codon. Pit-1 transcription is controlled in at least three ways. First, the Pit-1 promoter regions contain two possible binding sites for CREB, and Pit-1 is regulated by cAMP. Since CREB does not activate the Pit-1 promoter, the response to CREB requires an interaction with pituitary-specific factors (Chen et al., 1990). Second, the Pit-1 promoter is positively autoregulated as a consequence of Pit-1 binding to two Pit-1 binding elements, PitB1 and PitB2 (Figure 4), with sequence similarity to the consensus sequence of (T/A)/(T/A)TATNCAT (Chen et al., 1990; McCormick et al., 1990). Occupancy of the 5′ Pit-1-binding site, PitB1, markedly stimulates Pit-1 promoter transcription (Chen et al., 1990), and mutation of this site abolishes positive autoregulation (McCormick et al., 1990). Occupancy of the PitB2 site (3′ to the Cap site), in the context of the intact Pit-1 promoter, acts to attenuate the stimulatory effects of the PitB1 site (Chen et al., 1990), and mutation of this element markedly increases expression of the Pit-1 promoter (McCormick et al., 1990), reflecting either decreased efficiency of transcriptional initiation or attenuation of nascent transcripts (Chen et al., 1990). Thus, PitB1 is a positive autoregulatory element, and PitB2 is an inhibitory regulatory element.

Expression of the Pit-1 gene requires the actions of a cell-specific 390-bp enhancer located 10 kb 5′ of the Pit-1 transcription start site, within sequence that proves essential for effective pituitary targeting of transgene expression during murine development. The Pit-1 gene enhancer contains five binding sites for Pit-1. Four conform to the

previously observed TATNCAT/A consensus. Three of these Pit-1 binding sites contribute significantly to enhancer activity. Enhancer activity is stimulated by RA and 1,25-dihydroxyvitamin D_3. RA and 1,25-dihydroxyvitamin D_3 act at the RAR and vitamin D receptor, respectively; they are members of the nuclear receptor superfamily of ligand-dependent transcription factors that control critical aspects of development as a consequence of binding to response elements on target genes. The Pit-1 enhancer contains a retinoic acid response element which has been named the PRE. The PRE requires both Pit-1 and RAR for induction by RA. Thus, a cell-specific transcription factor (Pit-1) and a morphogen receptor (RAR) have been shown to act together (Rhodes et al., 1993).

PHOSPHORYLATION

Pit-1 is phosphorylated at two major sites, serine (ser) 115 and threonine (thr) 220, and one minor site, thr 219 through the PKA and PKC pathways. Casein kinase II and type II calcium-calmodulin-dependent protein kinase do not phosphorylate Pit-1. Thr 219 and thr 220 are contained in a phosphorylation consensus site ($K/R_4RT(S/T)I$ which is conserved between the homeodomains of other POU proteins. After phosphorylation, the binding affinity of Pit-1 is decreased for GH-I, Prl-1D -2D, and -4D, slightly lower for Prl-1P, about the same for PitB1 and PitB2, and increased for Prl-3P (Kapiloff et al., 1991). However, in other studies, phosphorylation of Pit-1 enhances binding to Prl-1P, so the effects may be variable in different systems in different laboratories (Okimura et al., 1994). Unphosphorylated Pit-1 contacts two adjacent major grooves on the same face of the DNA duplex, and results suggest that phosphorylation alters the conformation of Pit-1 on DNA which could result in differential presentation of the transactivation domain to the cellular transcriptional apparatus at specific sites (Kapiloff et al., 1991). Unlike the GH and Prl elements that appear to bind less well to phosphorylated Pit-1, phosphorylation by PKA or PKC enhances Pit-1 binding to TSHβ elements. This might be explained by variation within the consensus sequence for Pit-1 binding: A(A/T)(A/T)AATNCAT in the TSHβ gene and A(A/T)(A/T)TATNCAT in the Prl and GH genes. Mutation of the Pit-1 binding DNA elements in the TSHβ gene reduce basal expression and TRH and forskolin induction (Steinfelder et al., 1992b).

Mutations of ser 115 and thr 220 block the ability of cAMP to induce phosphorylation in nonpituitary COS-7 cells, but the Prl-1P promoter is still induced (Okimura et al., 1994). A Pit-1 protein with mutations of the ser 115, thr 219, and thr 220 sites is less effective than wild type Pit-1 in transactivating basal expression of the rPrl promoter, but it has similar expression after induction by forskolin and 12-0-tetradecanoyl phorbal-13-acetate (TPA) (Fischberg et al., 1994). Thus, although PKA and PKC pathways are important in Pit-1 target gene activation, Pit-1 is not necessarily the target. In addition, TRH induces phosphorylation of Pit-1 maximally at five minutes with return to basal levels at 30 minutes, while cAMP effects on phosphorylation persist longer than 30 minutes after treatment. Treatments that acutely deplete cellular PKC do not effect TRH-mediated stimulation

of the Prl promoter, but chronic depletion of PKC does decrease TRH stimulation (Howard and Maurer, 1994). Therefore, PKA and PKC activation may not be responsible for the TRH response. Although it has been suggested that these data conclude that phosphorylation of Pit-1 may not be necessary for TRH-mediated enhancement of Prl gene transcription, kinase systems other than PKA and PKC have not been evaluated. Cotransfected PKA-β or Pit-1 alone each activate the rPrl promoter to an equal extent, whereas together they result in a marked synergistic effect (Rajnarayan et al., 1995). Thus phosphorylation is important in Pit-1's effect on TRH signaling, but may not be through the phosphorylation of Pit-1 itself.

MUTATIONS OF THE PIT-1 GENE

Naturally occurring mutations in the Pit-1 gene have confirmed that Pit-1 is essential for the development of certain anterior pituitary cells (Figure 5, Table 1). The Jackson dwarf mouse has a gross structural alteration of the Pit-1 gene with either an inversion or insertion of a greater than 4 kb segment of DNA. These animals have hypoplastic anterior pituitaries, combined pituitary hormone deficiency (CPHD) of GH, Prl, and TSH, and no Pit-1 gene expression (Li et al., 1990). Snell dwarf mice also have hypoplastic anterior pituitaries and CPHD, but they have a low level of Pit-1 gene expression. In these mice, a G-to-T point mutation in both alleles of the Pit-1 gene alters a tryptophan (W) to a cysteine (C) in codon 261 in the putative recognition helix of the POU-HD. This mutant Pit-1 does not bind to the high affinity Pit-1 site, Prl-1P (7).

A number of humans with CPHD and Pit-1 gene mutations have also been described. Interestingly though, the inheritance pattern and phenotypic presentation is quite different among these patients. A C-to-T sporadic mutation altering an arginine (R) to tryptophan (W) in codon 271 in one allele of the Pit-1 gene, has

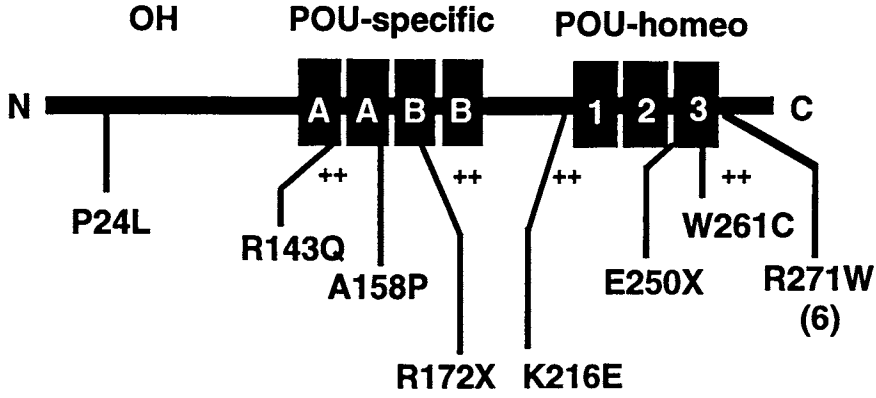

Figure 5. Point mutations in the Pit-1 gene resulting in CPHD.

Table 1. Phenotypic variation due to Pit-1 gene point mutations

Mutation	*GH Deficiency*	*Prl Deficiency*	*TSH Deficiency*	*Pituitary Size*	*Genetics*
P24L	+	partial	partial	?	heterozygous
R143Q	+	+	+	decreased	autosomal recessive
A158P-I	+	+	after GH treatment	normal	autosomal recessive
A158P-II	+	+	+	decreased	compound heterozygous
R172X	+	+	+	decreased	autosomal recessive
K216E	+	partial	partial	?	sporadic
W261C	+	+	+	decreased	autosomal recessive
E250X	+	+	+	?	autosomal recessive
R271W-I	+	partial	+	decreased	sporadic
R271W-II	+	+	partial	normal	sporadic
R271W-III	+	+	partial	decreased	sporadic
R271W-IV	+	+	partial	normal	heterozygous biallelic
R271W-V	+	?	+	decreased	?
R271W-VI	+	+	+	decreased	heterozygous

been described in several unrelated patients of different ethnic backgrounds (Radovick et al., 1992; Cohen et al., 1995; Ohta et al., 1992b; Okamoto et al., 1994; de Zegher et al., 1995). They had varying degrees of CPHD, which may reflect age-related differences or some other factor. Patient I had GH deficiency, partial Prl deficiency (baseline 3 μg/L), and complete TSH deficiency at 21 years of age (Rogol and Kahn, 1976). He was recently reevaluated, because his original studies were performed more than 27 years ago. Thyroid hormone levels were low (T4 28.3 nmol/L), and baseline TSH was undetectable and had no response to TRH stimulation; however, his thyroid hormone replacement could only be discontinued for two weeks. Prl was also unresponsive to TRH. GH was minimally detectable after administration of arginine and insulin-induced hypoglycemia. His pituitary is hypoplastic on magnetic resonance imaging (MRI) at 48 years of age (Cohen et al., 1995). Patient II had failure to thrive in the neonatal period. Thyroid hormone levels were low (free T4 6.4 pmol/L) and TSH was normal (2.3 mU/L). The TSH response to TRH stimulation was delayed (2.8 mU/L at 0 minutes, 12.8 mU/L at 30 minutes, 13.7 mU/L at 60 minutes). Prolactin was undetectable (<0.1 μg/L) and did not respond to TRH stimulation. MRI of the head at two months of age revealed a normal pituitary size (Cohen et al., 1995). Patient III had GH and Prl deficiency,

detectable TSH but a blunted response to TRH stimulation, and a hypoplastic pituitary gland on MRI at 19 months of age (Ohta et al., 1992b, personal communication). Patient IV was determined to have GH and Prl deficiencies, hypothyroidism with a small response of TSH to TRH stimulation, and a normal anterior pituitary on MRI around 6 months of age (Okamoto et al., 1994). The phenotypic variation in these patients may be explained by the mutant Pit-1 affecting pituitary cell survival, not development. As the cells atrophy, TSH deficiency and pituitary hypoplasia may occur. (Cohen et al., 1995). The severity of this problem is exemplified by a case of genetic transmission. Patient V is a female who was noted to be GH and TSH deficient at 7 years old. An MRI at 19 years of age revealed a hypoplastic pituitary. However, the genetic nature of the deficiency was not appreciated. Due to poor compliance of the patient with thyroid hormone replacement coupled with her infant's (patient VI) hypopituitarism due to the same R271W Pit-1 gene mutation, the infant was born with multisystem failure and suffered severe sequalea (de Zegher et al., 1995).

The R271W mutant, by substituting a tryptophan to an arginine, reduces the positive charge in a basic amino acid region of Pit-1. An arginine residue is strictly conserved at this location among Pit-1 gene products from several species and other related POU proteins (Radovick et al., 1992). Moreover, either arginine or lysine is found at this position in all other homeobox proteins analyzed. Based on the protein conformation model of Ingraham et al. (Ingraham et al., 1990), codon 271 is 3′ to the α-helical domains thought to be important for DNA-binding in the Pit-1 homeodomain. Mutant Pit-1 bearing the R271W mutant has been shown to bind normally to DNA. However, the mutant protein acts as a dominant inhibitor of transcription (Radovick et al., 1992). Thus, the mutation need only be present in one allele to cause CPHD. Okamoto et al. noted biallelic expression of the R271W mutant Pit-1 in an affected patient and monoallelic expression in unaffected relatives (Okamoto et al., 1994). The relevance of this finding is unclear at this point.

Two Dutch kindreds have been noted to have a C-to-G mutation altering an arginine (A) to a proline (P) in one Pit-1 allele (Pfaffle et al., 1992). Both kindreds have GH and Prl deficiency. Family I had normal thyroid function and only developed central hypothyroidism after GH treatment. They had normal size pituitary glands on MRI. Family II had TSH deficiency and hypoplastic pituitary glands (Wit et al., 1989). Whereas family I shows autosomal recessive inheritance, members of family II are compound heterozygotes with one absent Pit-1 allele. The A158P mutant Pit-1 has a minimal decrease in binding to GH and Prl binding sites and to the PitB1 site, but selective loss of dimeric species. It is incapable of activating transcription from the GH-I site and has low activation of transcription of the PitB2 site, the distal enhancer and proximal promoter sites for Prl (PrlDE/P), and 320 bp of the 5′ GH promoter sequence (GH320). Thus, the A158P mutant protein does not interfere with the development of somatotrophs, lactotrophs, and thyrotrophs, but does interfere with gene activation (Pfaffle et al., 1992).

A patient with selective dysregulation of anterior pituitary hormone secretion has also been evaluated. He initially had normal thyroid function which progressed to secondary hypothyroidism over the first two years of life, as evidenced by low thyroid hormone levels and a blunted response of TSH to TRH. He had a partial Prl deficiency with a normal basal Prl (5.4 μg/L), but no response to TRH stimulation. Insulin-induced hypoglycemia and glucagon stimulation resulted in deficient GH release. An A-to-G mutation in codon 216 was identified in one allele resulting in an amino acid change from a lysine to a glutamic acid (Cohen et al., 1994). Codon 216 encodes the third amino acid in a phosphorylation consensus sequence of the Pit-1 gene (Kapiloff et al., 1991). K216E mutant Pit-1 bind to the GH-1 and Prl-1P sites and has superactivation of both the GH and Prl promoters. However, the K216E mutant has defective retinoic acid signaling on the Pit-1 gene enhancer. Thus, the ability to selectively impair interaction with the superfamily of nuclear hormone receptors is also a mechanism responsible for CPHD (Cohen et al., 1996).

Other Pit-1 gene mutations have been identified but not characterized. A patient with CPHD was noted to have a C-to-T transition in exon 4 inherited in an autosomal recessive manner. This resulted in an alteration of the arginine (R) at codon 172 to a stop codon. Hypothetically, the mutant Pit-1 should lose transcriptional activation activity, as well as DNA binding (Tatsumi et al., 1992). Another patient had an A-to-G transition resulting in an arginine (R) to glutamine (Q) in codon 143, 5′ to helix A in the POU-S, and also transmitted in an autosomal recessive manner (Ohta et al., 1992b). A third patient had a T-to C-transition resulting in a proline (P) to a leucine (L) in codon 24 of one allele, which has been postulated to disrupt transactivation (Ohta et al., 1992b). A final patient carried an autosomal recessive nonsense mutation in codon 250, altering a glutamate (E) to a stop codon, with the result of complete loss of helix 3 of the POU-HD and presumably loss of DNA binding (Irie et al., 1995).

Since Pit-1 has different functions in the somatotroph, lactotroph, and thyrotroph, the phenotypic variability seen in patients with Pit-1 mutations is consistent with the different locations and types of Pit-1 gene mutations. Mutant Pit-1 may not be able to bind to target genes. If both mutant and wild type Pit-1 are present, mutant Pit-1 may interfere with binding to target genes by forming homodimers that compete for DNA binding or by forming heterodimers with wild type Pit-1 on DNA and inhibiting its action. Mutant Pit-1 may also interact with and inhibit the function of other nuclear factors or hormone receptors, such as the RAR.

SUMMARY

Pit-1 functions in the development of the GH-secreting somatotrophs, the Prl-secreting lactotrophs, and a population of the TSHβ-secreting thyrotrophs. Pit-1 activates GH and Prl gene expression and regulates Prl and TSHβ gene expression

through binding to sites on these target genes and interacting with a wide number of additional factors. Pit-1 also regulates its own gene expression, although it is not responsible for initial activation of its gene. Naturally occurring murine and human Pit-1 gene mutations have been crucial in elucidating Pit-1 structure-function relationships.

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Chapter 3

Regulation of Growth Hormone Gene Expression

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Advances in Molecular and Cellular Endocrinology
Volume 1, pages 51-82.

ISBN: 0-7623-0158-9

INTRODUCTION

As with many genes expressed in a tissue-specific manner, the growth hormone (GH) gene has been found to be regulated by a variety of mechanisms including 5′ flanking *cis* elements with their corresponding *trans*-factors, precursor mRNA splicing machinery, and the 3′ flanking poly(A) addition apparatus. Although the GH gene may be judged as a rather "small" segment of DNA, these multiple modes of regulation make it very attractive in terms of defining the molecular mechanisms responsible for GH synthesis. In this review, we have addressed regulation of GH synthesis at the transcription and messenger RNA processing levels, and at the level of GH release from somatotrophs.

GH is a peptide hormone which is synthesized and released by somatotroph cell types in the anterior lobe of the pituitary gland (Andrews, 1966; Miller et al., 1980). In addition to its role in promoting linear growth (Raben, 1958; Martin, 1978; Palmiter et al., 1982; Chen et al., 1990), GH is responsible for a variety of metabolic processes which include nitrogen, lipid, mineral, and carbohydrate metabolism (Martin, 1978). GH's effects on growth and metabolism are exerted directly or mediated indirectly via the actions of somatomedins released from the liver and other tissues in response to GH stimulation. Somatomedin C, also known as insulin-like growth factor I (IGF-I), a peptide hormone which is structurally similar to insulin, appears to be the primary regulator of linear growth in animals (Darnell et al., 1990).

GH is synthesized as a 217 amino acid precursor protein including a 26 amino terminal signal peptide which is removed by proteolytic cleavage during secretion (Miller et al., 1980; Leung et al., 1986). The mature secreted form of the protein is approximately 191 amino acids in length and has a molecular mass of about 22 kDa (Dellacha et al., 1996). In bovine GH (bGH), we have observed microheterogeneity in the length of the mature protein due to variable cleavage of the signal peptide at predominately the first or second amino acids, alanine or phenylalanine, respectively, during the secretion process (Leung et al., 1986).

The three-dimensional structure of the porcine and human GH (hGH) molecules has been determined by X-ray crystallography (Adbel-Meguid et al., 1987; de Vos et al., 1992). The major structural features common to both hormones is the presence of four α-helices and two intrachain disulfide linkages. While the three-dimensional structure of bGH has not been determined, the greater than 90% amino acid sequence identity between the two suggests a similar three-dimensional structure (Chen et al., 1990).

The binding of GH to its receptor (GHR) has been reported to involve a sequential two-step process in which the first binding site of GH interacts with one GHR molecule and then the second binding site interacts with a second GHR molecule (Cunningham et al., 1991; de Vos et al., 1992; Fuh et al., 1992). GH binding site one was determined by traditional receptor binding assays and includes amino acids within α-helix 1 and α-helix 4 and the loop between α-helices 1 and

2 (Cunningham and Wells, 1989; Cunningham et al., 1989). The second binding site has been shown to involve amino acids at the amino terminus and within the third α-helix of GH (Cunningham et al., 1991; Chen et al., 1990).

Work in our laboratory has found that the third α-helix of GH is important for its growth promoting activity (Chen et al., 1990, 1991a, b, 1994). Mutational analyses of the third α-helix has revealed that glycine-119 (Gly-119) of bGH and glycine-120 (Gly-120) of hGH are critical for this activity (Chen et al., 1991b). Substitution or deletion of the codon for the bovine Gly-119 (Chen et al., 1991b, c; Okada et al., 1992) or human Gly-120 (Chen et al., 1994) results in GH analogues which function as GH antagonists both *in vitro* and *in vivo*. Interestingly, a comparison among GH molecules of other species has shown that this glycine residue is invariant (Watahiki et al., 1989; Chen et al., 1994). We have postulated that the minimal side chain size of the invariant glycine creates a "cleft" which interacts with a "second target" involved in GH mediated signal transduction (Chen et al., 1990, 1991a, c, 1994).

GH belongs to a family of hormones that includes prolactin (PRL) and placental lactogens (PL). This family of genes share extensive sequence homology (>90%) and is, therefore, believed to have evolved by duplication of a single precursor gene over more than 350 million years (Miller and Eberhardt, 1983; Slater et al., 1986; Chen et al., 1989). Common among all the GH-related genes that have been studied is that the structural portions are composed of five exons separated by four intervening sequences, also known as introns. The 5′ flanking regions also share a high degree of sequence conservation. Despite the extensive sequence similarity, all the hormones are expressed in a specialized tissue-specific manner.

The GH gene is approximately 3,000 base pairs in length and includes a 5′ flanking region, five exons, four intervening sequences and a 3′ flanking region (Figure 1). The first 300 base pairs in the 5′ flanking region comprise the promoter/enhancer region which is responsible for tissue-specific expression of the gene and for its responsiveness to hormonal control. As with most genes expressed in a tissue-specific manner, the promoter/enhancer contains *cis* elements for transcriptional regulatory factors.

In humans, the GH gene exists as part of a gene cluster comprised of five GH-related genes located within a 47 kilobase (kb) segment on chromosome 17 (George et al., 1981; Chen et al., 1989). The human PRL gene is located on

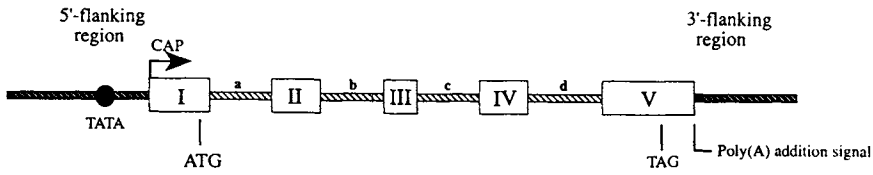

Figure 1. Typical growth hormone-related gene.

chromosome 6 (Owerbach et al, 1981) and is expressed in the lactotrophs of the anterior pituitary, human chorion-decidual tissue (Golander et al., 1978; Riddick et al., 1978), and decidualized endometrium during the menstrual cycle (Maslar and Riddick, 1979). The GH-related genes in the cluster are linked, in the same transcriptional orientation, in a tandem array in which the 5′-most gene is the normal GH gene (hGH-N). Located just 3′ of hGH-N is a chorionic somatomammotropin pseudogene (hCS-L) which, although it is presently not known to have any specific function, has weak GH-like activities (Slater et al., 1986). It has been suggested that the hCS-L may function during periods of natural starvation during pregnancy to provide maternal glucose to the fetus (Simpson and MacDonald, 1981; Slater et al., 1986). The three remaining genes include a functional human chorionic somatomammotropin-like gene (hCS-A), a GH variant gene (hGH-V) and a second functional human chorionic somatomammotropin-like gene (hCS-B), respectively (Seeburg, 1982; Cooke et al., 1988a). The hCS-A and hCS-B genes are expressed in the syncytiotrophoblastic villous epithelium of the placenta (McWilliams and Boime, 1980; Cooke et al., 1988a). In 1987 Frankenne et al. detected hGH-V-specific mRNA in both human placenta and in a single human pituitary tumor. In 1988, Cooke et al. cloned and characterized the hGH-V mRNA from human placenta and found that the hGH-V gene encodes two mRNA species generated by alternative splicing of the fourth intron. The fully spliced mRNA encodes a 22 kDa protein (hGH-V) while the other variant-related mRNA, retaining the fourth intron, is predicted to encode a 26 kDa protein (hGH-V2) (Cooke et al., 1988a; Liebhaber et al., 1989).

ALTERNATIVE SPLICING OF GH PRE-mRNA

GH cytoplasmic mRNA containing the last intron, intron **d**, has also been detected in cells which express the bGH gene (Hampson and Rottman, 1987; Hampson et al., 1989; Dirksen et al., 1994, 1995). In 1987, Hampson et al. reported their observation of a larger than usual bGH specific mRNA in bovine pituitary tissue as well as in Chinese hamster ovary (CHO) cells stably transfected with the bGH gene. Upon examination of these transcripts, they discovered that intron **d** was retained in a small fraction (approximately 0.1%) of the GH mRNA. Inspection of the intron **d** sequence revealed an open reading frame through the entire intron, terminating 50 nucleotides into the last GH exon, i.e., exon V. If this bGH variant mRNA is translated, it would be 42 amino acids longer than the wild-type polypeptide and have a molecular mass of approximately 27 kDa. Although the variant polypeptide has not been observed, the presence of the variant mRNA on polysomes suggests that it is translated (Hampson and Rottman, 1987). bGH mRNA species containing any of the other three introns have not been detected (Hampson and Rottman, 1987).

Sequences located within bGH exon V are important for efficient splicing of intron **d** (Hampson et al., 1989). In an experiment designed to study the importance

of bGH 3′ flanking sequences for efficient and accurate poly(A) addition, a series of bGH minigene deletion mutants were generated in which many of them had portions of exon V removed by Bal31 nuclease digestion. Following transfection into mammalian cells, they discovered that deletion mutants, lacking the region between the exon V FspI-PvuII restriction endonuclease cleavage sites, expressed bGH-specific mRNAs of which the majority (approximately 95%) retained intron **d**.

As mentioned previously, mRNA transcribed from the wild-type gene retains intron **d** in about 0.1% of the cytoplasmic bGH specific mRNA. When the FspI-PvuII fragment (FP) from bGH exon V is removed, the ratio of spliced to unspliced is reversed, resulting in cytoplasmic bGH mRNA in which intron **d** is almost completely retained (Hampson et al., 1989). Visual analysis of the FP fragment revealed the existence of a 10-bp inverted repeat sequence (CTTCCGGAAG) which they found to be sufficient for partially rescuing the splicing efficiency of intron **d** to about 50% of the wild-type level. They later found, however, that the purine-rich portion of the inverted repeat (GGAAG) alone was sufficient for restoring partial splicing efficiency and that insertion of multiple copies of the purine-rich element resulted in near wild- type levels (Dirksen et al., 1994). To examine whether this "exonic" effect was specific for the bGH intron **d**, the researchers attached the bGH exon V sequences to the corresponding position on the bovine prolactin (bPRL) gene. Upon removal of the FP element from the bGH exon V, no effect was observed upon splicing of the bPRL intron **d** (Hampson et al., 1989).

Following further examination of bGH intron **d** sequence, Dirksen et al. (1994) reasoned that, because the 5′ splice donor sequence (CCG:GUGGGG) matches the mammalian consensus splice donor sequence ((C/A)AG:GU(A/G)AGU) at only six out of nine positions, and the bPRL intron **d** 5′ splice donor site (CAG:GUAAGC) matches at eight out of nine positions, the bGH 5' splice donor site may be suboptimal or "weak" and, therefore, may require positive elements located in the downstream FP fragment which function as an exonic splice enhancer (ESE). To test this hypothesis, three mutations were introduced into the bGH intron **d** 5′ splice donor site to generate a sequence identical to the mammalian consensus. This resulted in the complete removal of intron **d** independent of the downstream ESE.

In an *in vitro* study, Sun et al. (1993) demonstrated that a 35-kDa protein(s) specifically cross-links to the FP ESE located in bGH exon V and is necessary for efficient *in vitro* splicing of bGH intron **d**. Dirksen et al. (1994) showed that cross-linking of a protein-doublet is dependent on the GGAAG repeat and that cross-linking is greatly diminished in S100 fractions which would implicate serine/arginine-rich splicing factor.

It has been reported that hGH-N mRNA is also alternatively processed (DeNoto et al., 1981; Lewis, 1984; Cooke et al., 1988b). Removal of hGH-N intron **b** by splicing sometimes involves the use of an alternative splice acceptor site 45 nucleotides into exon III resulting in a hGH-N mRNA variant which encodes a polypeptide with a molecular mass of approximately 20 kDa, lacking 15 internal

amino acids. Although an analogous alternative 3′ splice acceptor site is present 42 nucleotides into exon III of the bGH gene, there has not been any evidence for the use of this site in bGH mRNA processing (Hampson and Rottman, 1987).

REGULATION OF GH GENE TRANSCRIPTION

Advances in molecular biology techniques over the past 15 years have greatly facilitated the study of GH gene transcription. We now know that GH gene transcription is essentially regulated at two levels. The first level is the hormonal regulation, while the second level involves the tissue-specific regulation whereby specific and ubiquitous transcription factors interact with the evolutionarily conserved 5′ flanking sequence to effect tissue-specific expression of the gene.

Interestingly, more than one transcriptional initiation site, also known as CAP sites, has been identified in the hGH gene 5' flanking sequence. *In vitro* transcription of chimeric plasmid DNA minigenes driven by the hGH promoter/enhancer in non-pituitary nuclear extracts indicated that three transcriptional start sites could be observed by primer extension and RNase protection analyses (Lemaigre et al., 1989; Courtois et al., 1992). In addition to the +1 position, which is used to generate hGH mRNA, two other CAP sites were found to be located at positions –54 and –197. A putative TATA box, a *cis*-acting target sequence for binding transcription factor IID (TFIID), was identified approximately 30 base pairs upstream of each CAP site (Figure 2). When *in vitro* transcription was performed using pituitary cell extracts, transcription from the +1 position was stimulated while no initiation could be detected from CAP 2. Transcription driven from sequences 5′ position –197 was found to be dependent on the upstream stimulatory factor (USF) *cis*-element located at position –253 to –266 (Courtois et al., 1992). Lemaigre et al. (1989a) suggest that transcription from position -54 is blocked in pituitary nuclear extracts by GHF-1/Pit-1 because of competition with TFIID for binding to overlapping *cis*-sites.

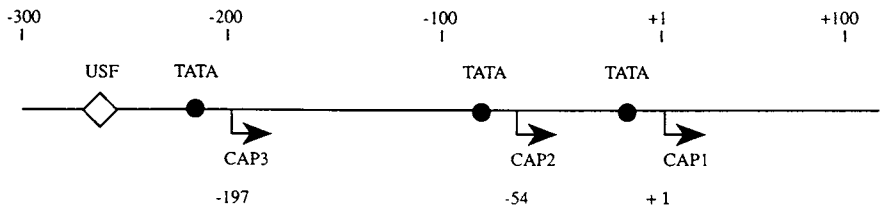

Figure 2. Diagram showing three hGH CAP sites. Transcriptional start sites are indicated by the black arrows labeled CAP 1, 2, and 3. The numbers below each arrow refer to CAP positions relative to the +1 position. TATA boxes are indicated as solid black circles and the upstream stimulatory factor (USF) is indicated as a white diamond. Figure adapted from Lemaigre et al., 1989a.

HORMONAL REGULATION

GHRF

GH releasing factor (GHRF) was first isolated in 1982 from human pancreatic tumors which overexpressed GH and caused acromegaly (Guillemin et al., 1982; Rivier et al., 1982). It was later isolated from the hypothalamus (Spiess et al., 1983; Ling et al., 1984). GHRF is synthesized as a precursor polypeptide of approximately 103 to 108 amino acids in length and includes an amino terminal signal peptide and a carboxy terminal peptide (Mayo et al., 1995). Following proteolytic cleavage of the precursor, the mature secreted protein is approximately 44 amino acids in length. With the exception of the rodent peptide (Spiess et al., 1983), the mature GHRF undergoes carboxy terminal amidation following proteolytic removal of the carboxy terminal peptide (Mayo et al., 1995).

While GHRF-secreting cells have been predominantly found in the arcuate nuclei of the hypothalamus (Merchenthaler et al., 1984; Sawchenko et al., 1985), GHRF, like many neuropeptides, is expressed in other areas outside the brain and, therefore, may have biological roles in addition to its role in controlling GH synthesis and secretion (Mayo et al., 1995). GHRF exerts its effects by first binding to its receptor (GHRFR) on the cell surface. A family of GHRFRs has recently been cloned from rat, human and mouse pituitary (Lin et al., 1992; Mayo, 1992; Gaylinn et al., 1993). All of the receptors contain 423 amino acids including a putative amino terminal signal peptide and the structural feature characteristic of other G- protein coupled receptors, i.e., seven hydrophobic domains which form seven transmembrane-helices (Mayo et al., 1995). GHRFR belongs to a family of gut-brain receptors which include the secretin receptor (Ishihara et al., 1991), the vasoactive-intestinal peptide receptor (Ishihara et al., 1992), the glucagon receptor (Jelineck et al., 1993), the glucagon-like peptide receptor (Thorens, 1992), the pituitary adenylate cyclase-activating peptide receptor (Pisegna and Wank, 1993), and the gastric inhibitory peptide receptor (Usdin et al., 1993).

GHRF stimulates transcription of the GH gene via a cyclic-adenosine 3′, 5′-monophosphate (cAMP)-mediated action involving protein kinase A (Barinaga et al., 1983). It also stimulates GH secretion by way of a calcium-mediated mechanism. Treatment of pituitary cells with agents that block voltage-dependent calcium channels such as verapamil, or Ca^{2+} chelators such as ethylene glycol-bis[α-amino ethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), block GHRF-stimulated GH release (Barinaga et al., 1985).

Somatostatin

Another hypothalamic neuropeptide, somatostatin, (SST) also called somatotropin release-inhibiting factor, functions by inhibiting GH secretion. Somatostatin,

a cyclic 14 amino acid peptide hormone/neurotransmitter, was first isolated by Brazeau et al. (1973) from the ovine hypothalamus. It was initially shown to inhibit adenylate cyclase activity (Jakobs et al., 1983) and to partially attenuate the levels of cAMP induced by GHRF binding to its receptor (Bilezikjian and Vale, 1983). Barinaga et al. (1985) reported that somatostatin inhibits GH secretion without altering basal or GHRF-induced GH gene transcription. However, other *in vitro* studies, involving longer periods of exposure, have shown that somatostatin reduces the steady-state levels of GH mRNA and GHRF-induced GH mRNA production (Wood et al., 1987; Tanner et al., 1990).

Like GHRF, somatostatin is expressed in a number of tissues outside the brain and mediates its affects by binding to high-affinity membrane-bound receptors (SSTR), most of which are coupled to G-proteins (Bell and Reisine, 1993). In addition to its role in the central nervous system (Rezek et al., 1977; Haroutunian et al., 1987), somatostatin has been found to inhibit Ca^{2+} conductance (Wang et al., 1990) and to potentiate K^+ currents (Jacquin et al., 1988; Wang et al., 1989).

The cloning and characterization of five different subclasses of somatostatin receptors from human, mouse and rat has been reported (Bell and Reisine, 1993; Raynor et al., 1993). Among the five subclasses of somatostatin receptors is the pituitary specific SSTR4 cloned from rat (O'Carroll et al., 1992). In cells which express this receptor, it has been shown to mediate the inhibition of forskolin-induced cAMP accumulation following treatment with somatostatin or somatostatin analogues (O'Carroll et al., 1992; Raynor et al., 1993). Forskolin is a pharmacological agent which activates adenylate cyclase. When these cells are pretreated with pertussis toxin, a substance known to block the inhibition of adenylate cyclase by ADP- ribosylation of the inhibitory G protein (G_i) (Stryer, 1988), somatostatin or somatostatin analogues are no longer able to inhibit forskolin-induced cAMP accumulation (Raynor et al., 1993).

Insulin

In addition to its regulation by neuropeptides such as somatostatin and GHRF, GH secretion is also modulated by other peptide hormones such as insulin, IGF-I, and even GH itself. Insulin exerts its cellular effects by binding specific receptors on the cell surface. The binding of insulin to its receptor results in the entry of metabolizable substrates into the cell and the regulation of specific cellular genes. *In vitro* analyses involving the use of rat and human pituitary cells has shown that insulin suppresses GH expression (Ivarie et al., 1981; Ceda et al., 1985). In 1988, Prager and Melmed showed that the hGH gene 5′ flanking region conferred insulin responsiveness to the hGH gene as well as to the bacterially derived chloramphenicol acetyltransferase (CAT) reporter gene following transfection into cultured HeLa and rat anterior pituitary GC cells. Although specific *cis*-acting regulatory sequences have not yet been identified, the 5′ flanking region of the hGH gene appears to be necessary for insulin responsiveness.

IGF-I

IGF-I functions in a long loop negative feedback system to inhibit GH secretion at the level of both the hypothalamus (Berelowitz et al., 1982; Abe et al., 1983; Tannenbaum et al., 1983) and the pituitary (Berelowitz et al., 1982; Yamashita and Melmed, 1986). IGF-I, like insulin, initiates its cellular action by binding membrane bound receptors. Specific receptors for IGF-I have been found in rat and human pituitary (Goodyer et al., 1984; Rosenfeld et al., 1984; Ceda et al., 1985). IGF-I has been shown to suppress basal and GHRF-stimulated GH secretion and GH-specific mRNA levels in human pituitary adenoma cells (Yamashita et al., 1986). Experiments involving human choriocarcinoma cells transfected with the hGH gene showed that the hGH gene was expressed and negatively regulated by IGF-I through, apparently, non-tissue-specific *cis*-acting regulatory elements located within either the 5′ flanking region or perhaps even within an intron as shown for glucocorticoids (Slater et al., 1985; Yamashita et al., 1987). Using nuclear "run-on" transcription assays, Yamashita and Melmed (1987) showed that GH gene transcription was inhibited following treatment of rat pituitary cells with IGF-I. The specific *cis*-acting element(s) responsible for IGF-I responsiveness has not yet been identified.

While IGF-I inhibition of GH secretion at the level of the pituitary is via transcriptional regulation of the GH gene (Yamashita et al., 1986, 1987), it is likely that IGF-I regulates GH secretion at the level of the hypothalamus by stimulating release of somatostatin. Berelowitz et al. (1981) showed that a 20 minute incubation of rat hypothalamus with IGF-I was sufficient to stimulate release of somatostatin at a level almost four times the basal level.

GH

Evidence has been reported which suggests that GH may also participate in a short loop negative feedback mechanism to inhibit GH secretion. Results have been presented which suggest that GH may inhibit its own secretion by stimulating somatostatin and/or suppressing GHRF. The possible role of somatostatin was first suggested by the observation that somatostatin levels declined in hypox rats (Patel, 1979). Also, *in vitro* and *in vivo* studies have shown that GH stimulates the release of somatostatin (Berelowitz et al., 1981; Chihara et al., 1981). Chomczynski et al. (1988) found that GHRF mRNA increased approximately sixfold in hypox rats. Administration of GH to the hypox rats results in a reduction in GHRF mRNA. In another study, male rats treated for seven days with injections of either rat GH (rGH) or GH- secreting rat pituitary tumor homogenate resulted in a drastic reduction in GHRF levels and an increase in the levels of somatostatin (Miki et al., 1989). Together with the data presented by Sugihara et al. (1993), suggesting that somatostatin has an inhibitory affect on GHRF, it is likely that the role of somatostatin in mediating the negative feedback by GH is to inhibit GHRF. The direct effect(s) of GH on the pituitary is still unclear.

Glucocorticoid Hormones

Glucocorticoids are produced by the adrenal cortex and are known to have diverse affects on metabolism. These hormones are steroidal in nature and, therefore, can readily enter the cell where they complex with a cytoplasmic receptor. The receptor-hormone complex traverses the nuclear membrane and activates responsive genes in the nucleus. Glucocorticoids have been shown to stimulate transcription of the GH gene through response elements (GREs) located on the promoter/enhancer (Brent et al., 1988; Dana and Karin, 1989) and in the first intron (Slater et al., 1985) of the human gene, and also in the 5′ flanking sequence (Treacy et al., 1991) and structural portion of the rat gene (Birnbaum and Baxter, 1986). Upon examination of the bGH structural gene sequences, we have identified three putative sequence elements within the first and second introns which, in addition to possessing the highly conserved hexanucleotide TGT(C/T)CT at the 3′ end, very closely match the consensus sequence QGGTRCAVRNTGTQCT (Slater et al., 1985) at 11 or 12 positions out of 16 (Woodley and Kopchick, 1996a). Other sequences sharing less homology with the consensus have been found to act as functional glucocorticoid receptor binding sites (Figure 3). Paek and Axel (1987) reported that glucocorticoids also enhance the stability of hGH mRNA. The

Source		*Sequence*	*~% Match*	*References*
Consensus		**QGGTRCAVRNTGTQCT**	16/16 (100)	Slater et al., 1985
hGH-a	+95	GGGCACAA**TGTGTCCT***+107	14/16 (87.5)	Slater et al., 1985
d-rGH	−250	− CACCCAAT**GTGTCCT***−265	11/15 (73.3)	Treacy et al., 1991
p-rGH	−111	AGCACAAGC**TGTCAGT***−96	11/15 (73.3)	Treacy et al., 1991
mMTV	−185	GGTTACAAAC**TGTTCT***−170	14/16 (87.5)	Pfahl et al., 1983
hMT-II$_A$	−263	CGGTACACTG**TGTCCT***−248	16/16 (100)	Karin et al., 1984
mMTV-II$_A$	−128	TGGTATCAAA**TGTTCT***−113	14/16 (87.5)	Karin et al., 1984
hGH	−224	GCTGACACTC**TGTGCA***−209	10/15 (66.7)	Eliard et al., 1985
hGH(as)	−234	CCACCCAACT**TGTCCT***−249	11.15 (73.3)	Lemaigre et al., 1989a
hCS	−225	GCTGACACTC**TGTGCA***−240	12/16 (75.0)	Eliard et al., 1985
hCS	+96	GGC-ACAACG**TGTCCT***+109	12/15 (80.0)	Eliard et al., 1985
bGH-1a	+392	TACCATTCGCG**TGTCCT**+407	11/16 (68.8)	Woodley and Kopchick, 1996a
bGH-2a	+509	AAGTTTGAAA**TGTTCT**+524	12/16 (75.0)	Woodley and Kopchick, 1996a
bGH-b	+984	TGGGTGGGTG**TGTTCT**+999	14/16 (87.5)	Woodley and Kopchick, 1996a

Figure 3. Comparison of glucocorticoid receptor binding sites. Sixteen nucleotides of the glucocorticoid receptor *cis-* element from various sources are aligned for comparison. The numbers located on either side of the sequence refer to the distance from the transciptional start site to the first nucleotide of the binding element and the sixteenth nucleotide. (*) designates those elements shown to be bound by glucocorticoid receptor. (as) refers to the antisense strand. (d-, p-) refers to distal and proximal positions with respect to the (+1) transcriptional start site. (a and b) refer to GH introns. In the consensus sequence R = A or T, Q = T or C, and V = A or C.

increase in hGH mRNA stability was associated with an increase in the length of the poly(A) tail (Paek and Axel, 1987).

Thyroid Hormone

Thyroid hormone, another steroid-like hormone, also has been reported to regulate GH gene expression. The action of this hormone is mediated by a chromatin-associated nuclear receptor known as the c-*erb*-A protein (Glass et al., 1987). In transfection experiments, hGH gene transcription was inhibited, however, the stability of hGH mRNA was found to be stabilized by thyroid hormone (Morin et al., 1990). Also detected following transfection into rat pituitary cells was 5′ flanking region-dependent inhibition of the hGH gene (Cattini et al., 1986). In the 5′ flanking region of the rGH, Glass et al. (1987) identified a sequence, CAGGGACGTGACCGCA, which they found to be bound with high affinity by the rat thyroid hormone receptor. A similar sequence, CAGGGGGCATGATCCC, is located at position −159 to −144 in the hGH gene promoter/enhancer (Lemaigre et al., 1989a).

Thyroid and glucocorticoid hormones have been shown to function synergistically. Recently, Nagomi et al. (1995) reported that thyroid hormone exerted a stimulatory effect on fetal GH gene expression, however, only in the presence of glucocorticoid. Other evidence for synergism with respect to the action of thyroid hormone comes from work reported by Ye et al. (1988). They found that thyroid hormone regulation of the rGH required cell- specific elements. The mechanism underlying the synergistic effects of glucocorticoid and thyroid hormones is as yet unclear (Nagomi et al., 1995).

Retinoic Acid

Retinoic acid, a metabolic form of vitamin A, has been shown to be an important signaling molecule during vertebrate development (Cunningham et al., 1994). It transduces its signal via cytoplasmic retinoid binding proteins (CRBP) and several receptors localized in the nucleus (Cunningham et al., 1994). Retinoic acid, when bound to its receptor, can interact with the hGH promoter/enhancer and transactivate CAT gene expression in pituitary GH1 cells propagated *in vitro* (Bedo et al., 1989). Like thyroid and glucocorticoid hormones, retinoic acid and thyroid hormone have been shown to function synergistically to upregulate GH (Bedo et al., 1989).

TRANS-ACTING ELEMENTS

Cloning of the GH promoter/enhancer regions has facilitated the search for the various *trans*-acting regulatory factors which regulate its expression. DNase-1 footprinting has been commonly used to identify specific *cis*-acting DNA se-

quences which are bound by specific proteins and thereby protected from enzymatic degradation. This technique has been used to identify a region –253 to –289 base pairs upstream of the transcriptional start site on the hGH promoter/enhancer which was protected by a protein(s) present in a pituitary nuclear extract (Lefevre et al., 1987; Lemaigre et al., 1989). Upon sequencing of the protected region, a particular motif (CCACGTGACC) was identified which had been previously shown in other genes to be bound by a USF. Inclusion of a saturating amount of USF-binding DNA in a subsequent footprinting reaction caused the USF to be "competed off." However, only the proximal portion of the protected region, –253 to –266, disappeared in competition suggesting that the remaining portion, –267 to –289, was protected by one or more other factors. Courtois et al. (1990) identified nuclear factor 1 (NF-1) as the other factor involved in protection of the distal portion of the –253 to –289 region. Also, activation protein-2 (AP-2) has been shown to compete with NF-1 for binding to overlapping sites (Courtois et al., 1990).

Similar results were observed with HeLa cell nuclear extracts indicating that these factors were not involved in the tissue-specific regulation of the gene (Lemaigre et al., 1989b; Courtois et al., 1990). Several ubiquitous transcription factors including NF-1, AP-2, USF, and Sp1 (Briggs et al., 1986) have been shown to bind the GH promoter/enhancer and are believed to be responsible for basal activity of the gene (Lafontaine et al., 1987; Lefevre et al., 1987). These ubiquitous factors, even when purified from nonpituitary cell extracts, are capable of stimulating transcription from the GH gene promoter/enhancer (Lafontaine et al., 1987; Lefevre et al., 1987). Interestingly, however, intact Hela cells cannot express minigene constructs whose transcription is driven by the hGH 5′ flanking region (Cattini and Eberhardt, 1987). It was concluded that Hela cells possess a repressor mechanism that is somehow destroyed during the preparation of the cell extract (Lafontaine et al., 1987). Repression in Hela cells is eliminated when the region –450 to –291 is deleted from the hGH 5′ flanking region (Peritz and Eberhardt, 1987).

When pituitary-derived nuclear factors are added to nonpituitary cell extracts, the transcription rate by the GH promoter/enhancer can be proportionally increased until, eventually, a maximum rate of transcription is achieved (Lafontaine et al., 1987; Lefevre et al., 1987). This was some of the first evidence implicatiang the presence of a pituitary-specific factor(s) capable of positively regulating transcription of the GH gene beyond the basal rate achieved with nonpituitary- derived cell extracts.

Subsequent footprinting analyses of the GH promoter enhancer identified a single Sp1 footprint at region –115 to –139 using HeLa nuclear extracts and a double footprint at –65 to –92 and –105 to –130 using pituitary nuclear extracts (Lefevre et al., 1987; Lemaigre et al., 1989c, 1990). Sequence analysis of the double footprint fragments revealed that the nucleotide sequence, TTATCCAT, was common to both. When this sequence was used in competition analyses, the double footprint disappeared and the single Sp1 footprint, seen with the HeLa cell nuclear extract, replaced it (Figure 4) (Lemaigre et al., 1990).

-130	GGAGCTTCTAAA**TTATCCAT**TAGCAC CCTCGAAGATTT**AATAGGTA**TTCGTG	-105	Site 1 (Distal)
-92	GGCCCC**ATGCATAA**ATGTACACAGAAAC CCGGGG**TACGTATT**TAGATGTGTCTTTC	-65	Site 2 (Proximal)

Figure 4. GHF-1/Pit-1 binding sites in hGH promoter/enhancer. The figure above illustrates the two GHF-1/Pit-1 binding sites in the 5′ flanking region of the hGH gene. Distal and proximal refer to their positions with respect to the +1 position.

The pituitary-specific factor capable of binding the TTATCCAT *cis*-element was named GH factor 1 (GHF-1) and found to be capable of stimulating GH gene transcription *in vitro* (Bodner and Karin, 1987; Lefevre et al., 1987). In 1988 it was cloned and expressed by both Ingraham et al. (1988) and Bodner et al. (1988). Ingraham et al. (1988) named it Pit-1. GHF-1/Pit-1 is comprised of 291 amino acids (~33 kDa) having a region of approximately 60 amino acids near its carboxy terminal end which resembles a homeobox consensus sequence, also known as a homeodomain (Theill et al., 1989; Karin et al., 1990). Homeodomains, although considerably divergent, are DNA binding domains that share sequence homology and characteristic α-helical structures. They were originally identified in developmentally important homeotic transcription factors that regulate position-specific determination in *Drosophila* (Awgulewitch et al., 1986; Gehring, 1987).

The homeodomain of GHF-1/Pit-1 is very similar to the one found in three other nuclear proteins known to function as transcriptional regulators. They are the widely expressed octamer binding protein (Oct1), the B lymphocyte-specific octamer binding protein (Oct2) which regulates immunoglobulin gene transcription, and Unc-86, a regulatory gene product in *Caenorhabditis elegans* that controls development of the nervous system (Finney et al., 1988). Additionally, these four proteins share another region of near identical sequence homology which is also involved in DNA binding (Theill et al., 1989; Karin et al., 1990). This region, approximately 75 amino acids in length, is located toward the amino terminal end of the homeodomain and appears to be unique to a family of transcription factors known to be involved in the transcriptional activation of specific genes. Because these two regions, separated by a putative "hinge" region (Ingraham et al., 1990), are common to GHF-1/Pit-1, the octomer binding proteins and Unc-86, they have collectively been termed the POU domain (Figure 5). The two regions comprising this motif are known individually as the POU-specific domain and the POU homeodomain (Herr et al., 1988). Although both parts of the POU domain are involved in DNA binding, only the POU homeodomain interacts directly with the DNA. Transactivation is mediated via a separate domain located near the amino terminal end and is comprised of 72 hydroxy-rich amino acids (Theill et al., 1989).

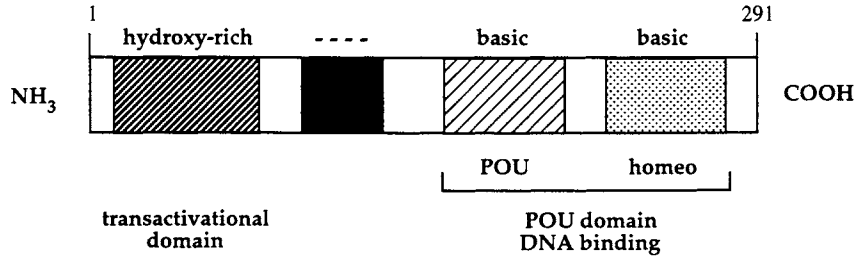

Figure 5. Structural architecture of GHF-1/Pit-1. Located nearest its carboxy-terminal end is the homeodomain (dotted box). Upstream of the homeodomain is the POU-specific domain (hatched box). Nearest of amino-terminus is the *trans*-activational domain (densely hatched box) followed by a region rich in negatively charged (——) amino acids. Figure was adapted from Karin et al., 1990 and Rousseau, 1992.

Cyclic-AMP has been shown to be an important second messenger in the regulation of GH gene transcription. As previously mentioned, Barinaga et al. (1983, 1985) demonstrated the GHRF stimulates rat GH gene transcription in anterior somatotrophs via a cAMP-mediated pathway involving protein kinase A. Because the hGH gene shares approximately 75% identity with the rat homologue, Barinaga et al. (1985) suggested that it too may be regulated by cAMP. It was later shown that, indeed, the hGH gene was also activated by GHRF through a cAMP-mechanism that required the GH gene promoter enhancer (Brent et al., 1988; Dana and Karin, 1989).

Transcriptional regulation of a number of genes by cAMP is mediated by either AP-2 (Imagawa et al., 1987) or the cAMP response element binding protein/activating transcription factor (CREB/ATF-1) (Roesler et al., 1988; Montminy et al., 1990; Meyer and Habener, 1993). However, AP-2 binding sites in the hGH gene promoter enhancer have been shown to be unimportant for the action of GHRF. Also, phorbol esters, which are known to activate AP-2 responsive genes, did not stimulate expression of the intact rGH gene whose 5′ flanking region also contain putative AP-2 elements (Copp and Samuels, 1989).

In the 5′-flanking region of the rat, bovine, and/or human GH genes, several attempts have been made to identify cAMP response elements (CREs) (Brent et al., 1988; Copp and Samuels, 1989; Dana and Karin, 1989; Shepard et al., 1994). In transient transfection studies of rat pituitary tumor cells, rGH, hGH, and bGH promoter enhancers are all induced by the cAMP analogue, 8-Bromo-cAMP (8-Br-cAMP) (Brent et al., 1988). The sequences required for cAMP-mediated induction of hGH and rGH lie within 183 bps of the transcriptional start site, a region highly conserved among the three GH promoter enhancers (Brent et al., 1988). In a similar experiment, using forskolin-induced rat pituitary tumor cells

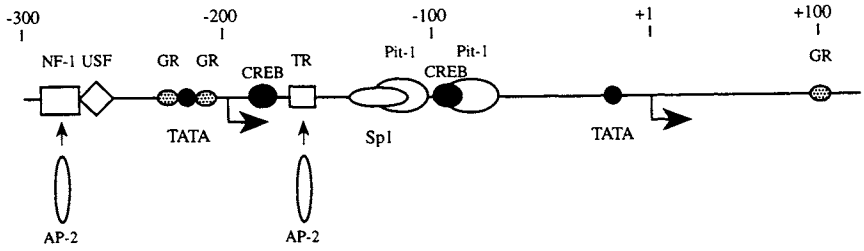

Figure 6. *trans*-factors on the hGH 5′ flanking region. TATA boxes are indicated by small black circles. GHF-1/Pit-1 factors (Pit-1) are indicated by large white ovals. Sp1 is indicated by a long horizontal oval. The nuclear factor-1 (NSF) is represented by a large square. The upstream regulatory factor (USF) is represented by a white diamond. Glucocorticoid receptors (GR) are represented by small dotted ovals. cAMP response element binding proteins (CREB) are represented by medium sized grey shaded ovals. The thyroid hormone receptor (TR) is represented by a small square. Activator protein-2 *trans*-factors are represented by long vertical ovals. Figure was adapted from Lemaigre et al., 1989a.

transiently transfected with an hGH-CAT minigene, a CRE consensus binding element was not found, although the 5′ flanking region of hGH could confer cAMP inducibility to the CAT reporter gene (Dana and Karin, 1989). The cAMP responsive region was, however, localized to within 82 base pairs 5′ of the hGH transcriptional start site. While the GHF-1/Pit-1 *cis*-element was not required for cAMP-mediated induction of CAT activity, in at least one experiment, evidence was found to suggest that cAMP-mediated induction may be potentiated it (Dana and Karin, 1989).

In a later study, two core CRE motifs (CGTCA) (at positions –187 to –183 and –99 to – 95) (Figure 6) and a GHF-1/Pit-1-specific *cis*-element (at position –123 to –112) were identified using mutational and gel shift analyses (Shepard et al., 1994). All three of these sights were found to be important for cAMP responsiveness. The nuclear factor(s) binding the CGTCA motif was identified as being CREB/ATF-1-related by using an unlabeled CREB consensus oligonucleotide in competition analyses (Shepard et al., 1994).

CREB is a nuclear DNA binding protein which belongs to the "bZIP" class of transcription factors (Vinson et al., 1989). It has a negatively charged transactivational domain (glutamate residues), a leucine zipper dimerization domain, and a stretch of basic amino acids involved in DNA binding. It is known to mediate the actions of cAMP on a number of genes by binding to a CRE (Montminy et al., 1990; Shepard et al., 1994). When the levels of cAMP rise within the cell in response to hormonal stimulation, the catalytic subunit of protein kinase A (pKA) (C-subunit) is released from the inactive enzyme and traverses the nuclear membrane. Once inside the nucleus, the C-subunit phosphorylates CREB at serine 133 which

induces a conformational change in the previously inactive CREB. The change in conformation allows the basic domain of CREB to interact with the acidic domain of an RNA polymerase II-associated protein factor (Montminy et al., 1990).

The gene encoding GHF-1/Pit-1 contains two functional CREs (McCormick et al., 1990). Stimulation of GH gene transcription by GHRF is believed to be accomplished, in part, via the induction of GHF-1/Pit-1 gene expression (McCormick et al., 1990). Also, GHF-1/Pit-1 gene transcription itself is autoregulated. It has been suggested that a GHF-1/Pit-1 positive feedback mechanism may be important during pituitary development (McCormick et al., 1990).

DEVELOPMENTAL IMPORTANCE OF GHF-1/PIT-1

Development of the anterior pituitary gland arises from a plate-like thickening of somatic ectoderm in Rathke's pouch and involves very dynamic processes by which distinct cell types develop within the organ (Gilbert, 1995). Within the developing pituitary, five distinct cell types arise that are defined by the specific trophic hormones they produce. These cell types arise in a specific order and include the corticotrophs (synthesize adrenocorticotropic hormone [ATCH]), gonadotrophs (synthesize gonadotropins), thyrotrophs (synthesize thyroid stimulating hormone), somatotrophs (synthesize GH), and lactotrophs, the latter of which synthesizes PRL, an activator of milk production during pregnancy (Simmons et al., 1990).

In the mouse fetus, mRNA specific to the GHF-1/Pit-1 enhancer-binding protein is first detectable at day 16, approximately 1 day following formation of a definitive Rathke's pouch (Simmons et al., 1990; Dollé et al., 1990; Gilbert, 1995). However, translation of this mRNA into detectable levels of protein does not occur until approximately day 18 or 19 of gestation. Although GHF-1/Pit-1 mRNA is found at high levels in the mature pituitary gland, translation only appears to occur in the thyrotrophs, somatotrophs, and lactotrophs (Simmons et al., 1990). GHF-1/Pit-1 mRNA remains untranslated in the corticotrophs and gonadotrophs. The mechanism for this is unknown.

In addition to its role of mediating the transcription of specific genes such as GH, PRL, and even the GHRFR (Mayo et al., 1995), GHF-1/Pit-1 also appears to play an important role for specifying the phenotype of distinct cell types within the anterior pituitary. In the Snell dwarf mouse (*dw*), a point mutation (a G-to-T transversion) affects a critical residue (Trp to Cys) within the POU-homeodomain of the GHF-1/Pit-1 protein and a structural alteration of the GHF-1/Pit-1 gene occurs in the Jackson dwarf mouse (dw^J) (Li et al., 1990). Both mutations result in a dwarf phenotype, similar to that seen with the nonallelic Ames dwarf (*df*) (Cheng et al., 1983), in which the anterior pituitary is devoid of thyrotrophs, somatotrophs, and lactotrophs in homozygous mice. Using immunohistochemistry and *in situ* hybridization, Li et al. (1990) detected no GHF-1/Pit-1 mRNA or protein in the Jackson dwarf pituitary and only low levels of GHF-1/Pit-1 gene expression in the

Snell dwarf. Similar results were obtained with the phenotypically similar but nonallelic Ames dwarf suggesting to this group that the Ames mutation is epistatic to expression of the GHF-1/Pit-1 gene (Li et al., 1990).

INTRON EFFECTS ON GH GENE REGULATION

In addition to 5′ flanking *cis*-elements used to control transcription of the GH gene, introns have also been found to be involved in regulation of GH synthesis. We have observed some variability in bGH expression directed by different bGH minigenes possessing different combinations of bGH introns (Figures 7 and 8; Woodley et al., 1992). Using a transcription run-on assay to compare the rate of transcription of a representative low expressing construct (pbGH-9) with that of a high expressing construct (pbGH-17), we have detected no significant difference (Figure 9; Woodley and Kopchick, 1996b). Transient expression data from bGH minigenes containing different combinations of bGH introns show that higher expression of mRNA and protein can be achieved when three or more introns are present. Transient expression of bGH minigenes containing one or two introns results in reduced levels of expression when compared with the cDNA containing minigene, pbGH-7. One explanation for the observed differences in bGH gene expression between and among various bGH minigenes possessing different bGH introns might be that some precursor mRNA transcripts are less stable. The results of the nuclear run-on assay preclude the possibility of intron-borne *cis*-elements. Whether bGH introns produce a similar effect *in vivo* remains to be determined.

Also, in an unrelated experiment designed to determine whether bGH introns can be removed by splicing when "precisely" repositioned to another intron position within the bGH gene, bGH intron **a** was precisely repositioned to intron **d**'s position. Northern blot analysis indicates no detectable retention of intron **a** from this position (Figure 10; Woodley and Kopchick, 1996c).

POLY(A) SIGNALS IN THE 3′ FLANKING SEQUENCE

The GH gene 3′ flanking sequence has been well characterized for bGH. In 1984, Woychik et al. reported the results of a study in which they examined the 3′ flanking sequence of the bGH gene to determine whether there exists any signal(s) that, in addition to the evolutionarily conserved hexanucleotide AATAAA, may function to regulate polyadenylation. They reasoned that the AATAAA alone, although widely shown to be essential for the production of polyadenylated mRNAs, could not be sufficient for directing efficient and accurate poly(A) addition because some genes contain additional copies of the hexanucleotide that have no apparent function in polyadenylation. In an attempt to identify possible regulatory signals, a series of bGH minigenes were generated each containing the SV40 late region promoter enhancer and a copy of the genomic bGH structural gene possessing

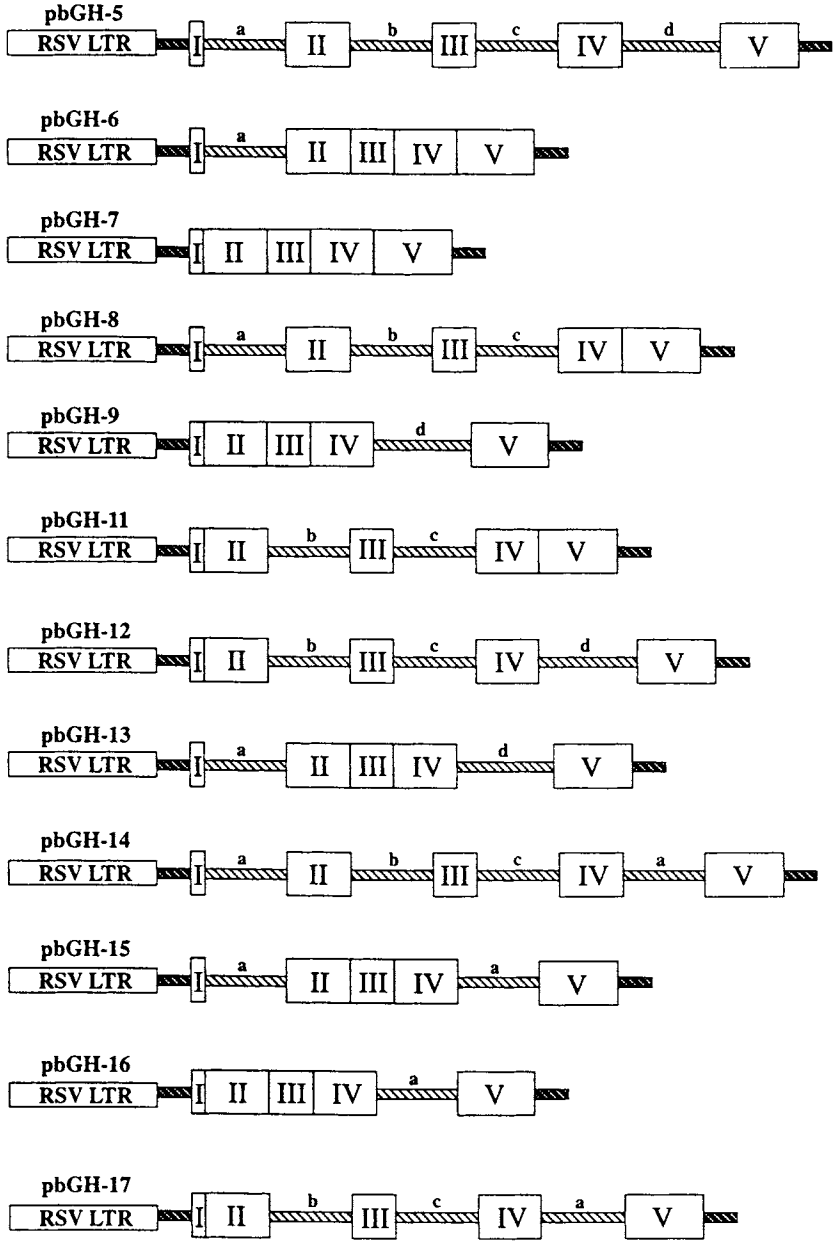

Figure 7. bGH-Containing minigenes with different intron compositions. All bGH minigenes contained idential 5′ (RSV-LTR) and 3′ transcriptional regulatory elements. RSV LTR refers to the Rous sarcoma long terminal repeat sequence.

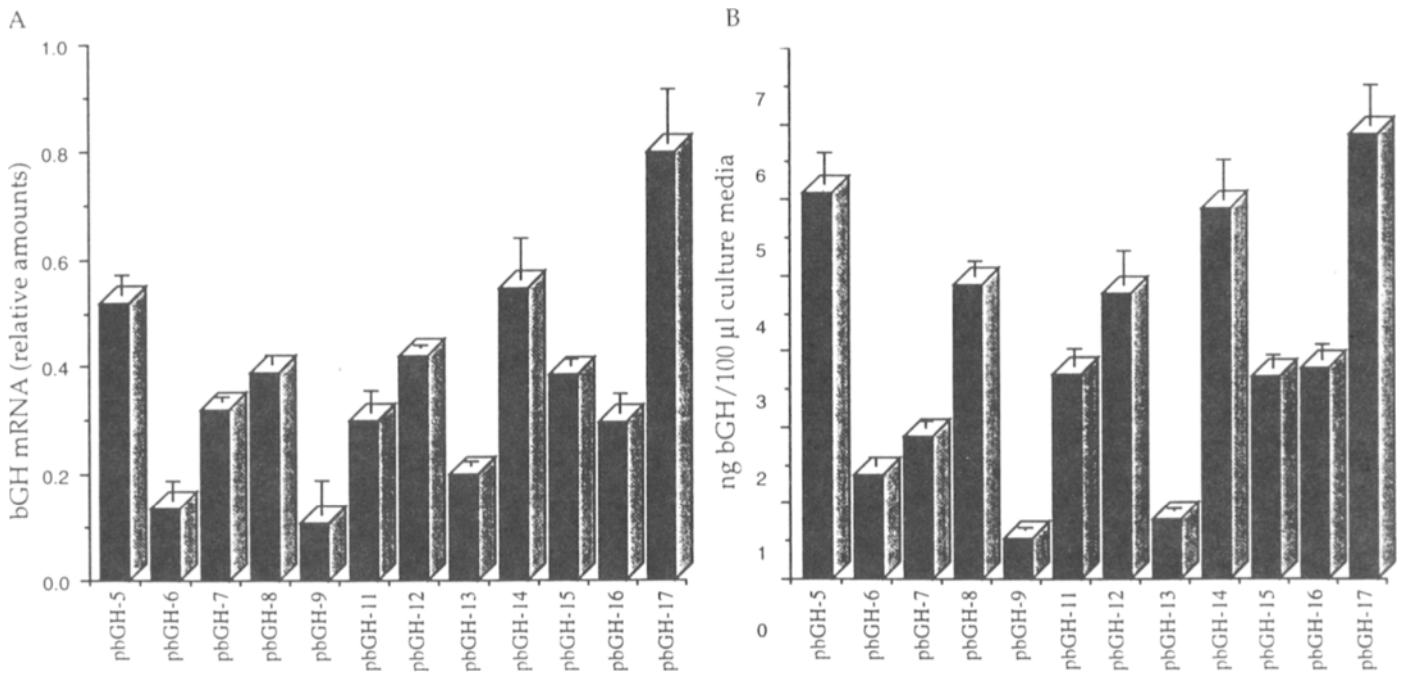

Figure 8. Expression data from bGH minigenes having different intron compositions. Panel (**A**) shows the relative amounts of mRNA, determined by RNA slot blot analysis, generated from the different bGH minigenes used in this study (see Figure 7). Relative mRNA levels were normalized using actin as an internal standard. Panel (**B**) shows corresponding protein levels determined using radioimmunoassay.

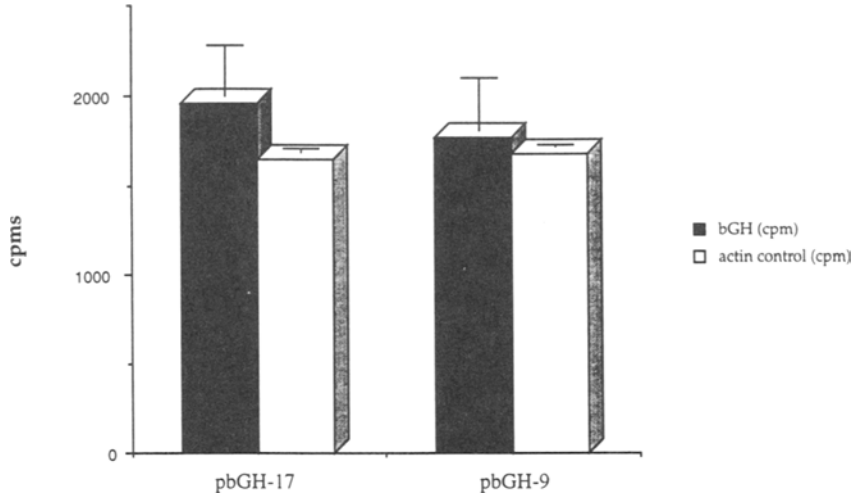

Figure 9. Comparison of transcription rates between a "higher" and a "lower" expressing bGH minigene. The rates of transcription driven by bGH minigenes pbGH-17 and pBGH-9 were compared using a nuclear run-on assay. The data above is expressed in counts per minute (cpms).

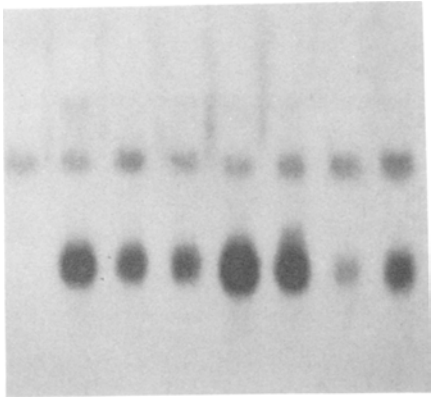

Figure 10. Splicing of intron **a** from intron **d**'s position. Northern blot analysis of mRNA transribed (in mouse L cells) from bGH minigenes containing intron **a** in intron **d's** position. Lane (1) pbGH-14, (2) pbGH-15, (3) pbGH-16, (4) pbGH-17, (5) pbGH-5, (6) pbGH-6, (7) pbGH-7 (see Figure 7).

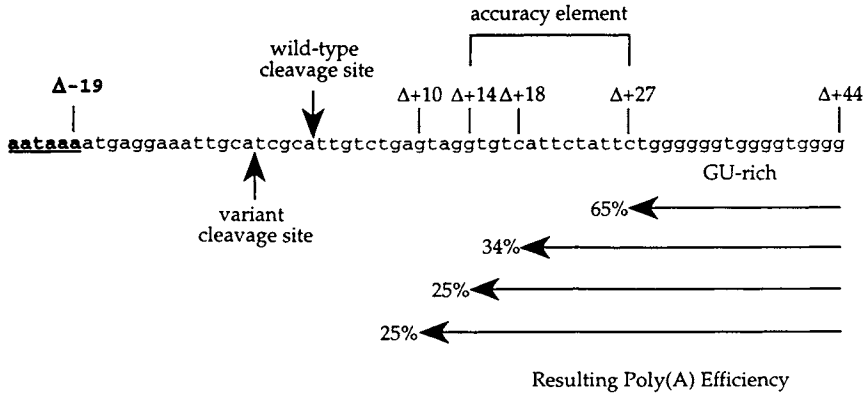

Figure 11. Diagram illustrating the bGH 3′ flanking region poly (A) addition machinery. **aataaa** represents the poly (A) addition signal. The wild-type and variant clevage sites are indicated by verticle arrows. The horizontal arrows indicate 1) the sequences that are removed by Bal31 digestion, and 2) the resulting poly(A) efficiencies. The cleavage accuracy element and GU-rich region are also indicated.

different lengths of the bGH-specific 3′ flanking sequence. bGH minigenes were transiently transfected into green monkey kidney cells (Cos1).

The data showed that bGH minigenes containing 84 base pairs of bGH-specific 3′ flanking sequence (Δ+84) was capable of expressing mRNA that was polyadenylated at the wild-type poly(A) addition site in greater than 90% of the transcripts (Woychik et al., 1984). Minigenes possessing only 1, 10, or 13 nucleotides of bGH-specific 3′ flanking sequence beyond the poly(A) addition site (Δ+1, Δ+3, and Δ+10, respectively), however, expressed mRNA that were predominately polyadenylated at sites 5′ or 3′ of the wild-type position but did not appear to have any detectable decrease in efficient polyadenylation. The wild-type poly(A) addition site is located 19 nucleotides 3′ of the AATAAA hexanucleotide after the nucleotides C-A (Figure 11). In this study, the estimation of poly(A) addition efficiency was determined using a simple transfection and an S1 nuclease protection assay and, therefore, was limited in its ability to detect changes in steady-state bGH mRNA levels less than a two-fold decline (Woychik et al., 1984). From the data, it was concluded that the bGH 3′ flanking sequence contains, within its first 84 nucleotides, a signal which influences the site of GH poly(A) addition (Woychik et al., 1984). The 3′ flanking sequence of the bGH gene, however, does not possess a uridine-rich region and only a portion of the general GU-rich consensus sequence common to many poly(A) addition signals (McDevitt et al., 1986).

In another study, Goodwin and Rottman (1992) used intronless bGH minigenes containing varying lengths of bGH 3′ flanking sequence whose deletion points fell within the Δ+14 to Δ+84 region, i.e., Δ+18, Δ+27, Δ+44, Δ+53, Δ, and Δ-19 (Figure 11).

The deletion (Δ-19) removed all of the bGH sequence 3' of the hexanucleotide element. Removal of 3' flanking sequence to Δ+69, Δ+53, and Δ+44 did not influence the efficiency of the poly(A) addition signal while further deletion to Δ+27 (a GU-rich region) caused a decrease in efficiency to 65% when compared to the wild-type. Gradual removal of 3´ flanking sequence to Δ+10 resulted in a further declines in efficiency to 25% (Figure 11). Because poly(A) addition efficiency declined gradually with the increasing removal of 3´ flanking sequence, it was concluded that poly(A) addition elements are scattered throughout the entire region between Δ+10 and Δ+44 and, therefore, are not consistent with a requirement for a "discrete efficiency element" proposed to exist in other mammalian poly(A) addition signals (Proudfoot, 1991; Goodwin and Rottman, 1992).

To more accurately identify and then characterize individual elements scattered throughout this region, a number of block and point mutations were generated using site-directed mutagenesis (Goodwin and Rottman, 1992). The block mutations generated were m(19-27), m(15-17), and m(10-14) in which the particular spans contained introduced nucleotide transversions relative to the wild-type cleavage site and the bGH 3´ flanking sequence. Three point mutations were also generated (m(15), m(16), and m(17)) which targeted the UGU located at the core of a poly(A) addition efficiency element consensus sequence proposed by McLauchlan et al. (1985). A double mutant, m(19, 24), was also generated which disturbed a tandem repeat of the consensus element CAYUG proposed by Berget (1984). Each block mutation produced an approximate 25-45% decline in poly(A) addition efficiency, supporting the deletion data which originally implicated each of these segments as part of an efficiency signal. The point mutations, however, had no effect on the efficiency of poly(A) addition. It was concluded that the bGH poly(A) addition efficiency signal does not require a discrete GU-rich element like that proposed to exist in other mammalian systems (Goodwin and Rottman, 1992).

To precisely identify the accuracy element located within the bGH 3´ flanking sequence (Figure 11), the deletion and block mutants were examined for accurate cleavage using an S1 nuclease mapping assay. Deletion of the bGH 3´ flanking sequence to Δ+27 had no effect on wild-type cleavage site selection while deletion to Δ+18 caused a partial shift to the variant cleavage site. Further deletion to Δ+14 shifted the preferred cleavage site to favor the variant site. This suggests that an element responsible for accurate cleavage of the bGH mRNA was contained within the region between Δ+14 and Δ+27 (Goodwin and Rottman, 1992).

SUMMARY AND PERSPECTIVE

In addition to its regulation by mechanisms which include the precursor mRNA processing machinery, GH regulation is under the control of a variety of different factors which act, essentially, on two tissues, i.e., the hypothalamus and somatotrophs in the pituitary. The hypothalamus produces two neuropeptides which function to either stimulate (GHRF) or inhibit (somatostatin) the synthesis and release of GH (Figure 12). The model illustrated in Figure 12 is an adaptation of a

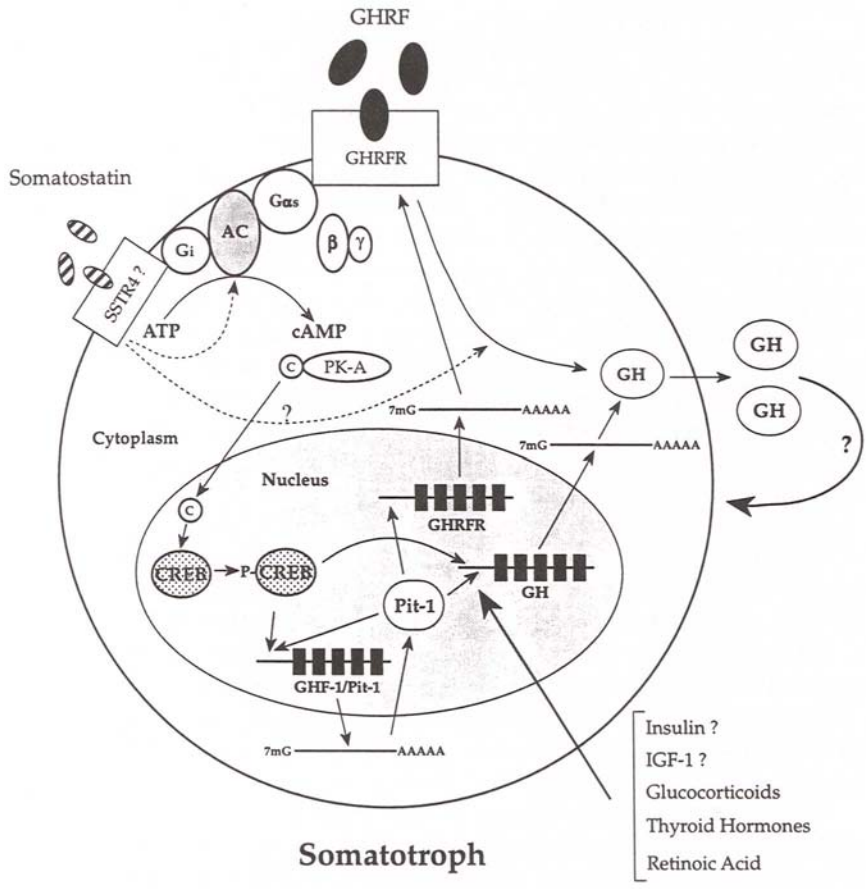

Figure 12. GH regulation at the level of the somatotroph. The figure was adapted and modified from Mayo et al., 1995. See text for description.

model proposed by Mayo et al. (1995) for the action GHRF. We have modified their model to include the effect of somatostatin and other hormones on the somatotroph.

GHRF binds to a G-protein-coupled receptor on the surface of somatotrophs in the anterior pituitary. Binding triggers the activation of adenylate cyclase which causes an increase in intracellular cAMP and the subsequent activation of PK-A. Upon activation, the catalytic subunit of PK-A dissociates from the holoenzyme and enters the nucleus where it then activates CREB by phosphorylating it at serine 133. When phosphorylated, CREB undergoes a conformational change which enables it to interact with CREs in the 5′ flanking regions of various target genes,

which include GH, GHF-1, and Pit-1 to activate their transcription. The binding of somatostatin to its receptor on the somatotroph is believed to produce an opposite effect. A pituitary-specific somatostatin receptor, SSTR4, has been cloned and characterized. Pharmacological analyses using pertussin toxin have indicated that this receptor is coupled to an inhibitory G protein (Gi) and is capable of inhibiting adenylate cyclase when somatostatin or one of its analogues is bound. Although this receptor has not been shown to be the "authentic" receptor responsible for mediating the effects of somatostatin on the somatotroph, it certainly is a plausible candidate.

Experiments performed *in vitro* have demonstrated that somatostatin can block GHRF- induced GH release but only partially attenuate the levels of cAMP. Also, there are conflicting reports with respect to somatostatin's effect on GH gene transcription. Some have reported a reduction in GHRF-induced GH mRNA levels while others report no change in basal or GHRF- stimulated GH mRNA levels in response to somatostatin. One consistency in the data, however, is that somatostatin does inhibit GH release from the pituitary, perhaps by interfering with events related to the secretory process.

GH gene transcription is negatively regulated by insulin and IGF-1 via a mechanism(s) which involves membrane bound receptors and requires the GH 5′ flanking region. Steroid hormones such as glucocorticoids and thyroid hormone also effect changes in GH gene transcription. Also, via an unknown mechanism(s), glucocorticoid and thyroid hormones act in a synergistic manner to upregulate transcription of the GH gene. Retinoic acid, when coupled to a CRBP and one of its receptors, is also capable of transactivating GH gene transcription. And, like glucocorticoids and thyroid hormone, retinoic acid can function synergistically with thyroid hormone to upregulate GH gene transcription.

GH is also regulated by negative feedback mechanisms at the level of the hypothalamus by both IGF-I and GH itself. Studies have shown that GH and IGF-I both stimulate the release of somatostatin. And, in hypox rat where the levels of GH are low or nonexistent, the levels of somatostatin are low while the levels of GHRF are high. However, it is still not clear as to whether GH negatively regulates GHRF directly or whether somatostatin functions to inhibit GHRF in response to GH.

Clearly, much progress has been made toward elucidating the mechanisms involved in the regulation of GH synthesis and release. Because GH affects a number of different tissues and metabolic processes, an understanding of its regulation is critical to our ability to develop therapeutic regimens for the treatment of diseases related to aberrant GH synthesis, release and/or signaling. Further research is expected to clarify the picture of how this multitude of regulatory factors interrelate to modulate the synthesis and release of GH.

ACKNOWLEDGMENTS

J.J. Kopchick and F.W. Woodley are supported in part by the State of Ohio's Eminent Scholar Program which includes a grant from Milton and Lawrence Goll.

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Chapter 4

Molecular Aspects of the Insulin-like Growth Factor (IGF) Genes

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Advances in Molecular and Cellular Endocrinology
Volume 1, pages 83-121.

ISBN: 0-7623-0158-9

INTRODUCTION

Insulin-like growth factors (IGFs) were discovered during a search for factors that mediate the growth stimulating effect of growth hormone (Salmon and Daughaday, 1957). Purification and characterization of these factors revealed that two types of IGFs exist, IGF-I (70 amino acids long) and IGF-II (67 amino acids long) (Rinderknecht and Humbel, 1978a, b). The amino acid sequences and predicted tertiary structures of IGF-I and IGF-II show a striking resemblance with those of proinsulin (Dafgard et al., 1985; Sato et al., 1993; Torres et al., 1995). Besides the canonical types of IGFs some variants have been described (Jansen et al., 1985; Zumstein et al., 1985; Sara et al., 1986; Van den Brande et al., 1990). In serum both of the IGFs are associated with a series of binding proteins (BPs), each with its specific abundance and binding affinities for IGF-I and -II (LeRoith et al., 1992; Baxter, 1993). The roles of the BPs are still obscure. Functions in storage or delivery of IGFs as well as in regulation of the biological activity of IGFs have been proposed. In order to exert their action it is necessary that IGFs bind to membrane-associated receptors. The two main types of IGF receptors are the Type I receptor that binds both IGF-I and IGF-II, although IGF-I binding is stronger than that of IGF-II, and the Type II receptor that preferentially binds IGF-II. Most of the physiological effects of the IGFs are mediated by the Type I receptor (LeRoith et al., 1995).

IGF-I and -II play important roles in growth and development. IGF-II is an essential factor during fetal development, although in humans (in contrast to rodents) the level of circulating IGF-II remains high postnatally. The levels of IGF-I are rather low during fetal development and rise gradually after birth, which has led to the notion that this growth factor is mainly involved in postnatal growth. However, in detailed localization studies it could be demonstrated that both IGFs are expressed in most embryonic tissues.

The physiological importance of IGFs is most clearly demonstrated by *in vivo* experiments, in which null mutations were introduced into the IGF-I, -II and/or Type I receptor genes (DeChiara et al., 1990; Liu et al., 1993). Homozygous elimination of the IGF-I gene leads to a reduction in body weight at birth of 40% in otherwise normally proportioned mice. Depending on the genetic background some of the IGF-I knock-outs die shortly after birth, while others survive and reach adulthood. A small phenotype was also observed in mice with a targeted disruption of the IGF-II gene. The importance of the IGF-I/Type I receptor combination was clearly demonstrated by the fact that null mutants for the Type I receptor gene die at birth of respiratory failure and exhibit severe growth deficiency (45% of normal size). These studies support the notion that, in addition to IGF-II, IGF-I and the Type I receptor have essential functions in embryogenesis.

In all stages of life, a clear pattern of tissue specificity in the expression of the IGF genes is observed. Hepatocytes are the main source of circulating IGFs, but in addition numerous non-hepatic tissues are capable of IGF synthesis. In these tissues, the IGFs probably function in para- or autocrine ways. The regulation of

expression in consecutive developmental stages and in different tissues has become a major topic of IGF research. After the detailed description of the IGF-I and -II genes in various species, research is now focused on the analysis of their promoter regions and on the factors involved in the regulation of their activity. In this report, we will review the present knowledge of the structures of the human IGF genes and their expression. The results of recent studies on the molecular aspects of IGF promoter activation and posttranscriptional mRNA processing will be summarized.

THE IGF-I GENE AND ITS REGULATION OF EXPRESSION

The structural analysis of IGF-I encoding mRNAs started in 1983 with the isolation of a human IGF-I cDNA clone (Jansen et al., 1983). The nucleotide sequence of the cDNA revealed that IGF-I is synthesized as a larger precursor molecule carrying a signal peptide at its N-terminus and a C-terminal extension of 35 (IGF-Ia) amino acids. Since then, nucleotide sequences have been reported for numerous IGF-I cDNAs of a variety of species including sheep (Wong et al., 1989), cow (Fotsis et al., 1990), pig (Tavakkol et al., 1988), goat (Mikawa et al., 1995), dog (Delafontaine et al., 1993), guinea pig (Bell et al., 1990), rat (Murphy et al., 1987a), mouse (Bell et al., 1986), chicken (Kajimoto and Rotwein, 1989), frog (Kajimoto and Rotwein, 1990), trout (Shamblot and Chen, 1992), salmon (Cao et al., 1989), and catfish (McRory and Sherwood, 1994). Comparison of these data has revealed much of the phylogeny of IGF-I and its evolutionary conservation, and has provided insight into the essential regions of the peptide for its biological functions (LeRoith et al., 1993). The amino acid sequence of mature, bioactive IGF-I is highly conserved in evolution, being identical for species as diverse as human, cow, dog, pig, and guinea pig. When more distant species like *Xenopus*, chicken, and salmon are included, 54 out of 70 amino acids are still invariant in all of the IGF-I sequences. Nonetheless, the organization and structure of the corresponding genes and mRNAs have diverged considerably among the above species. Their homologies and differences are discussed in more detail in the following paragraphs.

IGF-I Gene Structure

Detailed structural information on IGF-I genes has been obtained for a limited number of species, e.g., human (reviewed in Sussenbach et al. 1992), rat (Shimatsu and Rotwein, 1987), sheep (Dickson et al., 1991), chicken (Kajimoto and Rotwein, 1991), and salmon (Wallis and Devlin, 1993; Kavsan et al., 1994). Some aspects of their structures are summarized in Figure 1. Invariably, mature IGF-I in all of these genes is encoded by two relatively small exons. The size of the intron separating these exons varies considerably, and predominantly determines the overall length of the gene. The salmon gene is the smallest IGF-I gene described to date; in fact the salmon contains two IGF-I genes that are virtually identical in

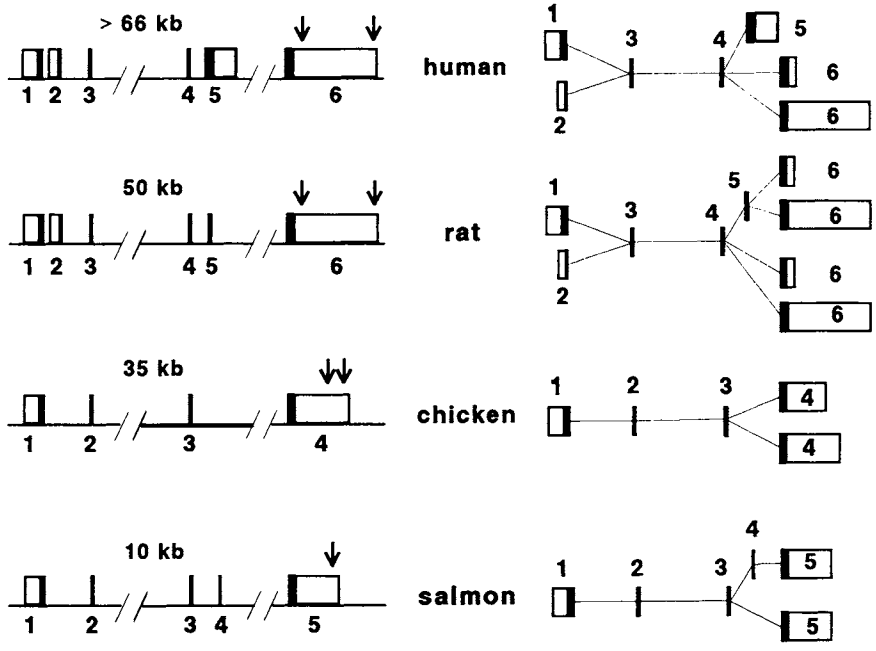


Figure 1. Structures of the human, rat, chicken, and salmon IGF-I genes and mRNAs. Exons are depicted by boxes. Coding regions are black. The length of the largest intron in each of the genes is indicated. The arrows show the positions of poly(A) addition signals. See text for references on gene structures.

structure. The intron between the IGF-I encoding exons is 10 kb in length and the entire gene is comprised within 20 kb of chromosomal DNA. In the over 90 kb long human gene, the corresponding intron has a size of over 65 kb. The number of mRNA species with different structures derived from the same IGF-I gene can be considerable as a consequence of alternative polyadenylation and the presence of alternatively used leader- and 3′-terminal exons.

Heterogeneity at the 3′-end of human, rat, and chicken IGF-I transcripts is generated by alternative polyadenylation. Downstream poly(A) addition signals in the 3′-terminal exons of the rat and human gene result in long transcripts of about 7.6 kb (Lund et al., 1989). The primary structure of the entire 7.6 kb human IGF-I mRNA has been determined (Steenbergh et al., 1991). The function of the long 3′-untranslated regions (UTRs) in these mRNA species is not clear, but effects on translatability (Thissen and Underwood, 1992) and stability (Hepler et al., 1990) have been reported. The upstream poly(A) addition signals yield shorter mRNAs of somewhat over 1 kb. They encode preproIGF-I precursor proteins identical to those encoded by the long transcripts (see Figure 1).

The number of different transcripts derived from the same gene is further increased by alternative splicing. The structure of the chicken gene seems the least complicated in this respect. It contains four exons that are represented in all of its transcripts. The salmon gene has five exons, of which exon 4 is a cassette exon that may or may not be represented in mature transcripts. In the rat IGF-I gene, exon 5 is a cassette exon that can be spliced in or out yielding mRNAs of different lengths (Shimatsu and Rotwein, 1987). In human IGF-I mRNA, the presence of exon 5 or 6 derived sequences at the 3′-end has been reported to be mutually exclusive, since cDNAs containing either exon 5 or 6 sequences were found, but not both (Jansen et al., 1983; Rotwein, 1986). IGF-I precursors with divergent C-terminal domains, designated preproIGF-Ia and -b, result from the two alternate splicing routes. Very recently a minor human IGF-I mRNA species containing both exon 5 and 6 derived sequences has been described (Chew et al., 1995). This mRNA, detected by reverse transcriptase-polymerase chain reaction, seems to be liver-specific. Alternative splicing causing 3′-terminal heterogeneity of transcripts has also been observed in transcripts from the salmon genes, but here no frameshift resulting in different C-terminal sequences of the encoded precursors occurs.

Finally, mRNA species with heterogeneous 5′-terminal sequences are produced in mammals, since their IGF-I genes contain more than one leader exon. Two are present in the human (Jansen et al., 1992), pig (Weller et al., 1993), rat (Roberts et al., 1987), and mouse (Bell et al., 1986) genes, and the ovine (Ohlsen et al., 1993) IGF-I gene has been reported to contain three different leader exons. Again, the sequence of preproIGF-I is affected by the choice of exon represented in the transcript. Further divergence in 5′-terminal mRNA results from the presence of multiple transcription initiation sites in each of the leader exons. The single leader exons found in the chicken and fish IGF-I genes are homologous to the mammalian exon 1 sequence. Exon 2 seems to be a more recent evolutionary addition to the IGF-I gene.

Transcription of the IGF-I Gene

Initiation of Transcription

The transcription start sites (tss) have been determined in the human (Jansen et al., 1991), rat (Adamo et al., 1991), sheep (Ohlsen et al., 1993), chicken (Kajimoto and Rotwein, 1991), and salmon (Koval et al., 1994) IGF-I genes. Figure 2 summarizes the data schematically. Without exception, dispersed initiation sites were found, giving rise to mRNA products of different lengths. The relative frequencies of transcription initiation at these sites are not identical and preferred or so-called major tss are clearly present. In human, the majority of the exon 1 containing transcripts is derived from the tss located about 245 nucleotides (nt) upstream of the 3′-terminus of the exon. Even here, the exact start sites seem somewhat scattered over a small region with the sequence 5′-CTCTTCTGTTT-3′. Analysis employing reverse transcriptase-polymerase chain reactions has revealed that longer exon 1 derived sequences, up to 900 nt upstream of the major tss, are

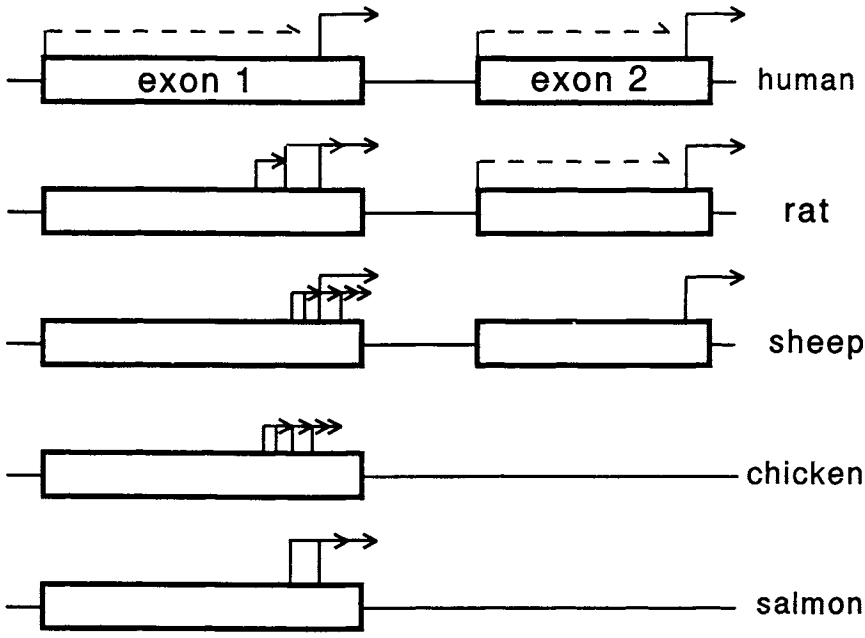


Figure 2. Transcription initiation sites in the leader exons of the human, ovine, chicken, and salmon IGF-I gene. Major tss are indicated by larger arrows. The broken lines signify the presence of additional minor tss at undefined positions and indicate the most upstream tss detected. See text for references on tss in the different species.

contained in some of the mRNAs. A major tss located at the same position as in the human gene has been identified in the rat, sheep, and salmon IGF-I genes, and mRNAs with 245 nt long exon 1 derived leaders are present in these species. In the chicken gene, however, all of the tss have been mapped to positions located 40 to 120 nt upstream of this major tss in the other species, although its sequence is also conserved in the chicken gene.

Transcription initiation in the alternative leader exon, exon 2 in the human, rat, and sheep genes, is much less frequent than in exon 1. The majority of the exon 2 transcripts is initiated at a short stretch of nucleotides between positions 65 to 75 upstream of the 3′-end of the exon (Adamo et al., 1991; Jansen et al., 1991). However, minor mRNA species with considerably longer exon 2 derived sequences have been detected (Jansen et al., 1992).

IGF-I Promoters

DNA fragments encompassing the previously identified tss of the IGF-I genes have been tested for intrinsic promoter activity. To this end, plasmids were con-

structed containing these fragments linked to the luciferase reporter gene. With the fusion constructs, gene transfer experiments have been performed in a variety of cell lines. The 5′-flanking regions of the leader exons in the human (Jansen et al., 1992), rat (Hall et al., 1992; Lowe and Teasdale, 1992; Adamo et al., 1993), chicken (Kajimoto et al., 1993), and salmon (Koval et al., 1994) gene were shown to possess promoter activity by this approach. In all instances, however, the activity is rather moderate. This is generally regarded as consistent with the absence of specific elements in the flanking regions that are usually found in stronger promoters, e.g., TATA and CAAT boxes. Commonly, a maximal activity of 10-fold higher than that of an identical construct lacking a promoter was measured. However, since the activity of such a control construct should be zero by definition, any signal detected must be regarded as variable background noise. Therefore, we preferred to use the basal promoter region of the thymidine kinase (TK) gene as comparative standard (Jansen et al., 1992). The promoter activities of the human IGF-I gene derived fragments depend on the cell type used in the transient transfection assays. In cells not expressing the IGF-I gene endogenously at significant levels (e.g., HepG2, Hep3B), their activities are only a few percent of that of the TK promoter. In IGF-I expressing cells, however, the activity may rise to 30% (exon 1 flanking sequences in SK-N-MC cells) and 40% (exon 2 flanking region in OVCAR-3 cells) of the activity of the TK promoter (Jansen et al., 1992). In the latter—ovarian carcinoma derived cell line—a clear preference for the usage of tss in the flanking region of exon 2 was observed. This is consistent with the observation that exon 2 containing IGF-I mRNAs are almost exclusively produced by these cells (Steenbergh et al., 1993). In other cell lines and tissues, exon 1 is the predominant leader exon. Over 80% of IGF-I gene transcripts in human adult liver are derived from exon 1.

In conclusion, weak promoters have been shown to be present upstream of the IGF-I leader exons. The promoter regions flanking exons 1 and 2 in the human and rat genes have been designated P1 and P2, respectively. The promoter regions of the IGF-I genes with a single leader exon, e.g. the salmon and chicken genes, are homologous to P1.

Regulation of Promoter Activity

The observed developmental stage and tissue-specific expression pattern of IGF-I is a consequence of the interaction of specific transcription factors with the promoter(s) of the IGF-I gene. Many hormones and other signaling molecules have been implicated in the regulation of IGF-I bioactivity by endocrinological studies (Table 1). With a few exceptions (e.g., growth hormone (GH), prostaglandin-E2), it is not known whether they affect the transcription rate of the IGF-I gene or interfere with posttranscriptional processes. Observed differences in steady-state IGF-I mRNA levels may be due to effects on the transcription rate as well as to effects on the stability (half-life) of the transcripts. The molecular mechanisms by which factors influencing the transcription rate exert their regulatory functions are

Table 1. Compilation of Factors Stimulating (+) or Inhibiting (–) IGF-I Expression

Factor	*Tissue / Cells*	*Effect On*	*+ / –*	*Reference*
ACTH	Adrenal fasciculata cells	protein	+	Penhoat et al. (1991)
Angiotensin II	Adrenal fasciculata cells	protein	+	Penhoat et al. (1991)
	Vascular smooth muscle cells	transcription	+	Delafontaine and Lou (1993)
Calcitonin	MC3T3-E1 osteoblast cells	mRNA	+	Kobayashi et al. (1994)
Cortisol	(pre)Osteoblast cells	mRNA	–	McCarthy et al. (1990)
Dexamethasone	Osteoblast-like cells	protein	–	Chen et al. (1991)
EGF	Renal collecting duct cells	mRNA	+	Rogers et al. (1991)
Estrogen	Osteoblast cells	transcription	+	Ernst and Rodan (1991)
	Uterus (*in vivo*)	mRNA	+	Kapur et al. (1992)
	HepG2 hepatoma cells	transcription	+	Umayahara et al. (1994)
FGF	Aortic smooth muscle cells	mRNA	–	Bornfeldt et al. (1990)
	Osteoblast cells	mRNA	–	Canalis et al. (1993)
FSH	Granulosa cells	protein	+	Hsu and Hammond (1987)
Glucagon	Primary hepatocytes	mRNA	+	Kachra et al. (1991)
Glucose	C6 glioma cells	mRNA	+	Straus and Burke (1995)
Growth Hormone	Primary hepatocytes	mRNA	+	Norstedt and Moeller (1987)
	Ob1771 adipocytes	mRNA	+	Doglio et al. (1987)
	Liver (*in vivo*)	transcription	+	Bichell et al. (1992)
IGF-I	Aortic smooth muscle cells	mRNA	+	Bornfeldt et al. (1990)
IGF-II	MC3T3-E1 osteoblast cells	protein	+	Tremollieres et al. (1991)
Insulin	Aortic smooth muscle cells	mRNA	+	Bornfeldt et al. (1990)
	Primary hepatocytes	mRNA	+	Phillips et al. (1991)
	Liver (*in vivo*)	transcription	+	Pao et al. (1992)
Interferon-γ	Macrophages	mRNA	–	Arkins et al. (1995)
Interleukin-1β	Leydig cells	mRNA	–	Lin et al. (1992)
LH	Testis (*in vivo*)	mRNA	+	Closset et al. (1989)
PDGF	Aortic smooth muscle cells	mRNA	–	Bornfeldt et al. (1990)
	Vascular smooth muscle cells	mRNA	–	Giannella-Neto et al. (1992)
	Osteoblast cells	mRNA	–	Canalis et al. (1993)
Progesterone	Uterus (*in vivo*)	mRNA	+	Kapur et al. (1992)
Prostaglandin E2	Osteoblast cells	transcription	+	Pash et al. (1995)
PTH	(pre)Osteoblast cells	mRNA	+	McCarthy et al. (1989)
	Osteoblast cells (*in vivo*)	mRNA	+	Watson et al. (1995)
Somatostatin	Serum (*in vivo*)	protein	–	Pollak et al. (1989)
Testosterone	Uterus (*in vivo*)	mRNA	+	Sahlin et al. (1994)
T3	GH3 pituitary cells	mRNA	+	Fagin et al. (1989)
	Liver (*in vivo*)	mRNA	+	Wolf et al. (1989)
	Primary hepatocytes	mRNA	+	Tollet et al. (1990)
	Heart (*in vivo*)	mRNA	+	Kupfer and Rubin (1992)
TGF-β	MC3T3-E1 osteoblast cells	protein	–	Tremollieres et al. (1991)

continued

Table 1. Continued

Factor	*Tissue / Cells*	*Effect On*	*+ / –*	*Reference*
	Thyroid follicular cells	protein	–	Beere et al. (1991)
	Osteoblast cells	mRNA	–	Canalis et al. (1993)
Thyrotropine	Thyroid follicles (ex *vivo*)	mRNA	+	Hofbauer et al. (1995)
Vitamin D3	Osteoblast-like cells	protein	+	Chen et al. (1991)

Note: If experimental proof (e.g., run-on assay) has been obtained for a direct effect on the transcription rate of the IGF-I gene, "transcription" is indicated. Effects on steady-state levels of IGF-I mRNA are indicated by "mRNA," and "protein" stands for all techniques showing altered IGF-I levels after administration of the factor. More general factors known to affect IGF-I levels (e.g., nutritional status, injury, mechanical strain) have not been included in this table. Abbreviations: ACTH, adrenocorticotrophic hormone; EGF, epidermal growth factor; FGF, fibroblast growth factor; FSH, follical stimulating hormone; LH, luteinizing hormone; PDGF, platelet derived growth factor; PTH, parathyroid hormone; T3, triiodothyronine; TGF-β, transforming growth factor-β.

still largely obscure. Their responsive elements in the promoters of the IGF-I gene have not yet been established.

Transcription Factors Regulating IGF-I Expression

In recent years, it has become clear that predominant expression of a gene in the liver is caused by the concerted action of liver-specific transcription factors. In the promoter of such a gene, e.g., the albumin gene (Herbommel et al., 1989), multiple *cis*-acting elements are present to which members of various liver-specific transcription family members can bind. This does not imply that the expression of the gene is totally restricted to liver tissue. Firstly, additional elements responsive to factors that are not restricted to hepatic tissue may be present in the promoter. Secondly, all of the so-called liver-specific transcription factors have been shown to be present in certain extra-hepatic tissues and cell types as well (De Simone and Cortese, 1992; Aran et al., 1995). Although IGF-I gene expression is not confined to hepatocytes, liver clearly is the major site of IGF-I production, suggesting a role for liver-specific factors in this process. Binding sites for liver-specific transcription factors have now indeed been identified in the IGF-I gene. Transient transfection experiments have revealed the potent stimulatory effect of these factors on IGF-I promoter activity. Since P1 is by far the more active promoter of the IGF-I gene in liver tissue, yielding over 80% of the transcripts, studies have concentrated on this promoter

C/EBP

Members of the CAAT/enhancer binding protein (C/EBP) family have been shown to transactivate P1 of the human IGF-I gene (Nolten et al., 1994). Both C/EBPα and C/EBPβ (also known as liver activator protein, LAP) are able to stimulate the basal activity of P1 in transient transfections using P1-luciferase

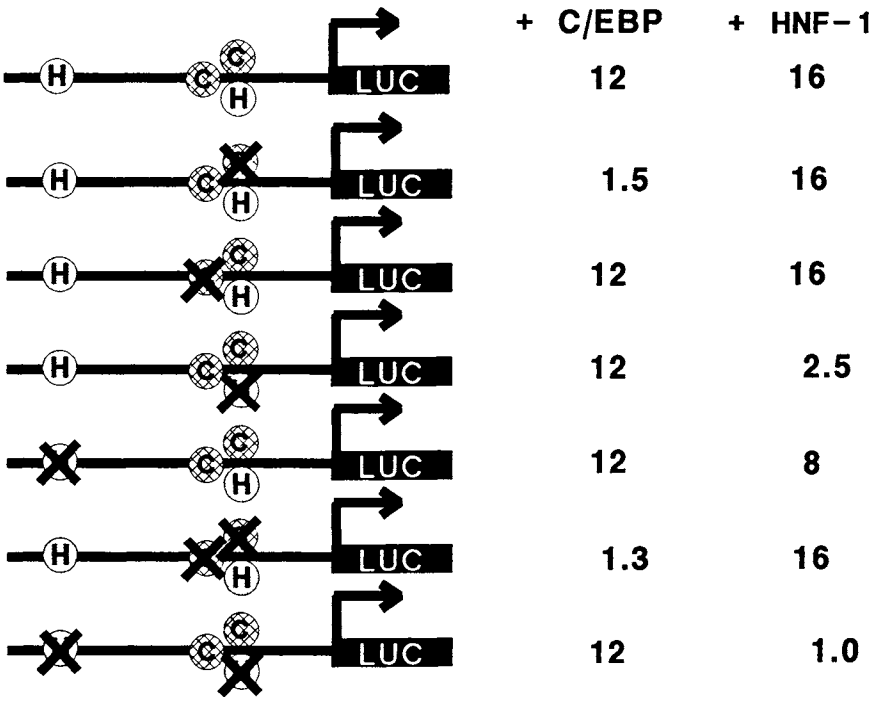
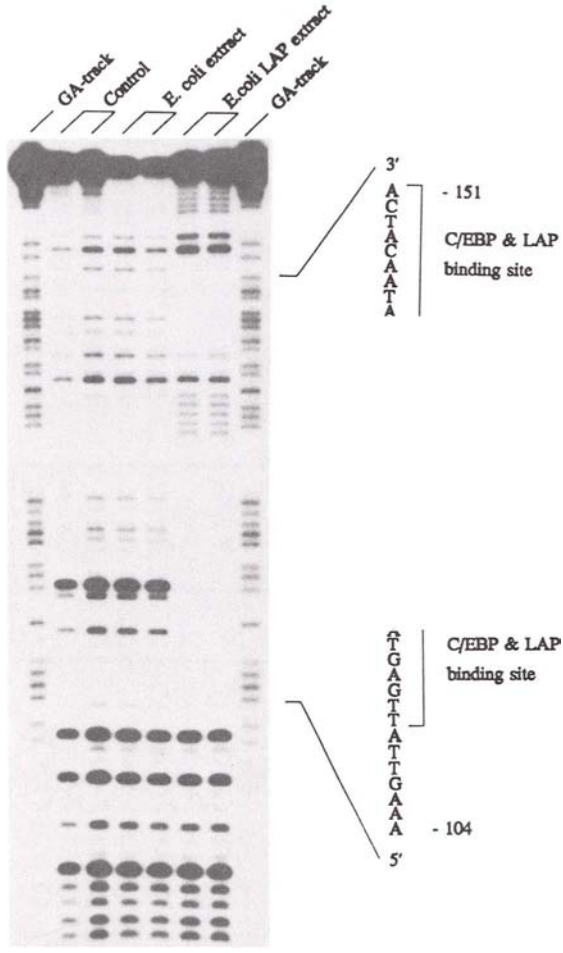


Figure 3. Induction of P1 (-733 to +55)-luciferase constructs by C/EBPβ and HNF-1α in transient transfection experiments in Hep3B cells. The figure indicates the number of times a given construct is activated in comparison with the same construct when no expression vector for C/EBPβ or HNF-1α was added to the transfection mixture. The wild-type construct is shown at the top, the various mutants are indicated by crossing out the binding site(s). Data are from Nolten et al., 1994, 1995.

constructs and expression vectors encoding the transcription factors. C/EBPβ was found to have a stronger effect than C/EBPα.

C/EBPα and -β belong to a family of b-zip (basic zipper) transcription factors comprising at least seven members (Wedel and Loems Ziegler-Heitbrock, 1995). Some of them, e.g., liver inhibitor protein (LIP) lack the N-terminal activating sequence present in C/EBPα and -β, and function as inhibitors of gene expression, probably by inactively blocking responsive elements. LIP has been shown to counteract the stimulatory effects of C/EBPα and -β on P1 of the human IGF-I gene.

The artificially constructed optimal binding site for the C/EBPs has the palindromic sequence 5′-GATTGCGCAATC-3′. Functional C/EBP responsive elements may, however, differ significantly from this optimal binding sequence and


GA-track
Control
E. coli extract
E.coli LAP extract
GA-track
3'
ACTACAATA
- 151
C/EBP & LAP
binding site
TGAGTTATTGAAA
C/EBP & LAP
binding site
- 104
5'

site extends from −111 to −119, and the other site is located shortly upstream between residues −143 and −151. Subsequent studies employing promoter-luciferase constructs with mutations disrupting either of the two binding sites have indicated that the stimulatory effect on promoter activity is dependent on C/EBP binding to the more downstream site, with hardly any contribution of the upstream element (Figure 3). The latter binds C/EPB with lower affinity than the more downstream site and the position of the downstream site at a distance of 115 nt from the tss may also be more favorable for its role in transcriptional regulation. The nucleotide sequence of the C/EBP responsive element has been highly conserved in the IGF-I genes of different species and thus C/EBP may well function as a regulator of IGF-I expression in all of them (see Figure 5).

High levels of C/EBP mRNAs and proteins have only been detected in a limited number of specific cells, i.e., hepatocytes, adipocytes, myeloid cells, and B cells. The expression of these regulating factors is itself regulated in a developmental stage-dependent fashion. In general, the expression of C/EBPs increases during ontogenesis and is high only in terminally differentiated cells (Birkenmeier et al., 1989). In this respect there is a suggestive parallel with IGF-I gene expression,

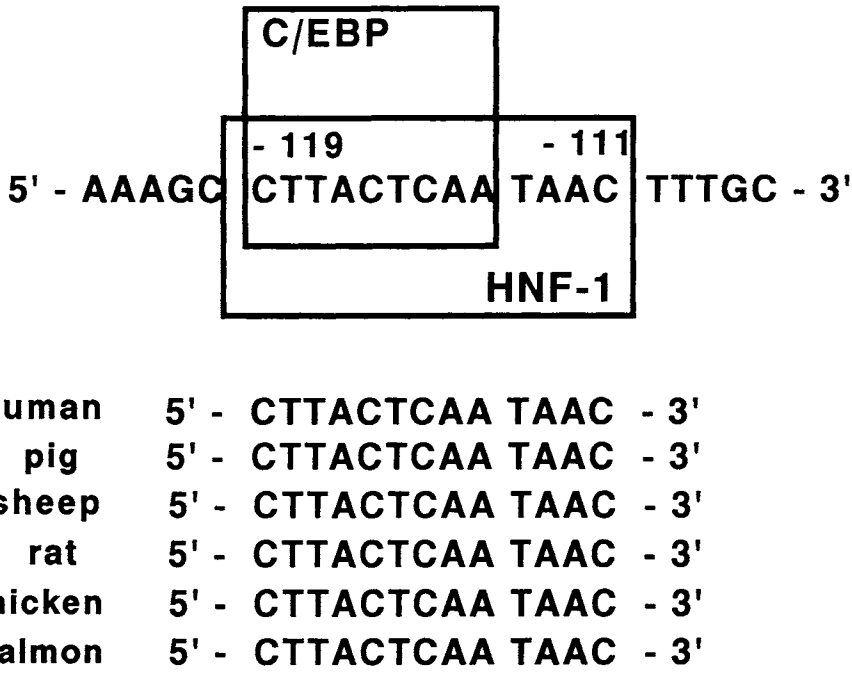


Figure 5. Conservation of the nucleotide sequence of the overlapping C/EBP and HNF-1 binding elements in the promoter P1 of the human IGF-I gene. The major tss is at position +1.

which also seems to be restricted to terminally differentiated cells, e.g., hepatocytes (Norstedt and Moeller, 1987) and adipocytes (Doglio et al., 1987), while absent in their proliferating stages.

HNF-1

A second class of transcription factors capable of enhancing the activity of the IGF-I P1 promoter is constituted by the hepatocyte nuclear factor-1 (HNF-1) family. Strong stimulatory effects of HNF-1α on the activity of both the human P1 promoter (Nolten et al., 1995) and on the homologous (and only) promoter of the salmon IGF-I gene have recently been described (Kulik et al., 1995). Like the C/EBPs, HNF-1 plays a prominent role in regulating the promoter activity of many genes that are predominantly expressed in the liver. The members of this transcription factor family are distantly related to the POU-homeodomain containing transcription factors (Frain et al., 1989). They recognize the palindromic sequence 5′-GTTAAT(N)ATTAAC-3′, but again it has now become clear that elements with sequences deviating considerably from the perfect palindrome, although usually retaining one intact half-site, can bind HNF-1 with high affinity (Mendel and Crabtree, 1991). Two elements interacting with HNF-1 have been identified in P1 of the human IGF-I gene by EMSA. One of them is located between positions –282 and –270 (major tss is at +1). This site contains one perfect half-site, GTTAAT, but three of the residues in the other half-site deviate from the perfect palindrome. Nevertheless, HNF-1α binds with high affinity to this site. The second HNF-1 binding element is formed by residues -119 to -107. Although neither half site completely adheres to the ideal binding sequence, HNF-1α binds with high affinity to this element, albeit with about twofold lower affinity than to the upstream element. Transient transfection experiments with wild type and mutant promoter-luciferase constructs have shown that both of the HNF-1 binding elements contribute to the stimulatory effect of HNF-1α on the activity of human promoter P1. However, in spite of its weaker binding the contribution of the downstream element to the overall effect was found to be more important than that of the upstream higher affinity binding site. Mutations abolishing HNF-1α binding to the upstream element reduced the level of activation by HNF-1α observed with the wild type promoter to about 50%, mutations to the downstream element to 15%. No residual stimulation was observed when both of the binding sites were mutated to sequences incapable of HNF-1 binding (Figure 3).

As can be inferred from its position numbers, the downstream HNF-1 binding site co-localizes with and completely overlaps the previously described downstream C/EBP binding site (Figure 5). This suggests that the -110 to -120 region can bind members of different families of transcription factors and thus plays a very prominent role in the regulation of IGF-I gene expression. In binding assays using extracts of cells in which both C/EBPβ and HNF-1α were overexpressed, or using extracts from adult rat liver, no evidence has been recorded for simultaneous

binding of the two classes of transcription factors to the same DNA molecule. In view of their sizes, it is well conceivable that steric hindrance prevents a second protein molecule from binding to the already occupied recognition sequence. Even so, slight variations in the concentrations of stimulating (e.g., C/EBP, HNF-1) and inhibiting (e.g., LIP) factors capable of binding to this regulatory hotspot close to the transcription start site may well allow for intricate regulation of transcriptional activity of the IGF-I gene.

Like the C/EBP binding element, the downstream HNF-1 binding sequence has been well conserved during evolution (see Figure 5). Although interspecies homology between the human and salmon sequences is lower than that observed with other species, the salmon element has been shown to bind (mouse) HNF-1α. Moreover, this binding results in strong activation of the salmon IGF-I gene promoter (Kulik et al., 1995). Both the *trans*-acting factor, HNF-1, and its binding element are well conserved principles, as was recently confirmed by the isolation of salmon HNF-1. This protein interacts in the same way with both the salmon and rat albumin promoters (Deryckere et al., 1995).

HNF-1α protein levels in hepatocytes are unmatched by any other cell type, but lower levels have also been found in kidney, intestine, stomach and pancreas. Here, HNF-1 may well contribute to the observed IGF-I gene expression in these tissues.

AP-1

In addition to binding sites for liver-enriched transcription factors, only one functional *cis*-acting element has been localized in IGF-I gene promoters so far. The chicken IGF-I promoter homologous to P1 of the human gene has been shown to be regulated by AP-1 transcription family members. Upon addition of tetradecanoyl (TPA) to cells transiently transfected with chicken IGF-I promoter constructs, a five-fold higher promoter activity was observed (Kajimoto et al., 1993). The responsive element is located 420 bp upstream of the transcription start site. Subsequently, this AP-1 site has been implicated in the mechanism by which estrogen enhances IGF-I gene expression (Umayahara et al., 1994).

The sequence constituting the AP-1 site in the chicken promoter, however, is not conserved in the mammalian and fish promoters, and functional AP-1 motifs have not been identified in other IGF-I genes to date.

Growth Hormone and IGF-I Expression

The important role of GH in IGF-I expression was recognized almost 40 years ago (Salmon and Daughaday, 1957). The concept formulated at that time, known as the somatomedin hypothesis, has been confirmed in numerous experiments. The strong stimulatory effect of GH on IGF-I gene expression has been well documented *in vivo*. A single *intraperitoneal* injection of GH rapidly stimulates hepatic IGF-I gene transcription in hypophysectomized rats through activation of the P1

promoter (Bichell et al., 1992). In contrast, IGF-I expressing cells in tissue culture in general do not show this response upon addition of GH, with the exception of primary hepatocyte cultures (Norstedt and Moeller, 1987) and Ob1771 mouse adipocytes in their differentiated state (Doglio et al., 1987). There are no reports on artificial constructs containing the IGF-I promoters that are activated by the addition of GH to transfected cells harboring the constructs.

Enormous progress has recently been made in the unraveling of the signal transduction pathway leading from the activation of the ligand bound GH-receptor to transcriptional effects on GH responsive genes. This pathway is now known to involve activation of the Janus Kinase 2 (JAK2) the GH-receptor associated tyrosine kinase (Argetsinger et al., 1993). JAK2 subsequently diversifies the signal by activating both the mitoagen-activated protein (MAP) kinase and phosphatidylinositol 3-Kinase (PI-3 Kinase) pathways, as well as by the direct activation of transcription factors binding to the promoters of GH regulated genes, such as the signal transducers and activators of transcription (STATs) and others (Winston and Bertics, 1992; Meyer et al., 1994; Argetsinger et al., 1995).

These new insights have resulted in the identification of specific GH responsive elements in a number of genes, e.g., the serine protease inhibitor gene Spi2.1 (Le Cam et al., 1994). Similar elements have not been identified in the IGF-I promoters to date, in spite of elaborate research. Conceivably, the IGF-I promoter-luciferase constructs used in these studies, harboring several kilobases of promoter sequence, do not contain the responsive element. The element may reside elsewhere in the exonic or intronic regions of the IGF-I gene. Based on this assumption, DNaseI hypersensitive sites in the rat IGF-I gene have been extensively studied. This study has revealed the presence of a GH induced change in chromatin structure in the second intron (Bichell et al., 1992). However, upon stimulation with GH no alterations in the pattern of proteins binding to this region have been detected, and the significance of the GH-responsive DNaseI hypersensitive site remains to be established (Thomas et al., 1995).

In our view a plausible alternative mechanism linking GH to enhanced IGF-I expression would involve the transcription factors previously described. Mediators of transcriptional activation like the C/EBPs and HNFs may well be stimulated in their synthesis or be activated by posttranslational modifications as a result of GH action. Convincing evidence for GH control over synthesis and activity of C/EBPs has recently been reported. GH was shown to transcriptionally activate C/EBPδ and an even more rapid activation of C/EBPβ was observed to result in significantly elevated levels of C/EBPβ binding to its recognition site (Clarkson et al., 1995). The same principle may apply to hormones other than GH. Moreover, one might well envisage nutritional status, an important parameter for IGF-I synthesis, to be directly reflected by the activity of liver-specific transcription factors.

In summary, a limited number of transcription factors has now been identified as potential regulators of IGF-I gene expression. The developmental expression pattern of these factors is consistent with IGF-I levels observed in different stages of development. During adult life, the levels of these, and possibly additional

factors, may be regulated in specific tissues by the numerous hormonal and metabolic agents known to influence IGF-I levels (see Table 1). Further investigations, including studies on the regulation of transcription factor activity, are necessary to resolve the mechanisms that control IGF-I gene expression.

THE IGF-II GENE AND ITS REGULATION OF EXPRESSION

The IGF-II gene is a complex transcription unit with many interesting regulatory aspects. Expression of IGF-II is controlled at multiple levels ranging from tissue-specific and developmental stage-dependent transcription initiation, alternative splicing, and multiple polyadenylation sites to endonucleolytic cleavage of IGF-II mRNAs. The structure of the IGF-II gene with the corresponding mRNAs will be discussed and a summary of the factors that regulate the four promoters of the IGF-II gene will be presented in detail in the following paragraphs.

IGF-II Gene Structure

The human IGF-II gene consists of nine exons numbered 1 to 9, of which exons 7, 8, and the first part of exon 9 code for the IGF-II precursor protein. Exons 1 to 6 are 5′-untranslated exons of which exons 1, 4, 5, and 6 are each preceded by a separate promoter (P1-P4), resulting in the production of a family of mRNAs that contain four different leader sequences (Figure 6). All transcripts share the IGF-II coding region, but have different 5′- and 3′-UTRs. The four IGF-II promoters are differentially active in a tissue-specific and development-stage dependent manner and give rise to mRNAs of 5.3 kb (P1), 5.0 kb (P2), 6.0 kb (P3), and 4.8 kb (P4). Further diversity in mRNA species is created at the 3′ end where two functional polyadenylation signals are present in exon 9, resulting in, e.g., P3 transcripts of 6.0 kb and 2.2 kb (Holthuizen et al., 1991; Sussenbach et al., 1992).

Since the isolation of IGF-II cDNA from human liver in 1984 (Bell et al., 1984; Dull et al., 1984), many IGF-II cDNAs have been characterized in other species from which the corresponding amino acid sequence of IGF-II was deduced. The protein sequence of IGF-II is highly conserved showing only four variable residues out of 67 amino acids and thus an overall homology of 94%. IGF-II cDNAs were isolated from a number of vertebrates, and the (partial) nucleotide sequences were determined for horse (Otte and Engström, 1994), cow (Brown et al., 1990), sheep (O'Mahoney and Adams, 1989; Brown et al., 1990; Demmer et al., 1993), pig (Catchpole and Engström, 1990), guinea pig (Levinovitz et al., 1992), mink (Ekström et al., 1993), rat (Dull et al. 1984; Soares et al., 1985), and mouse (Stempien et al., 1986). The complete genomic structure has been determined for the human (summarized in Sussenbach et al., 1992), ovine (Ohlsen et al., 1994), rat (Frunzio et al., 1986; Soares et al., 1986), and mouse (Rotwein and Hall, 1990) IGF-II genes. The overall structure of the IGF-II genes is extremely well conserved (Figure 6).

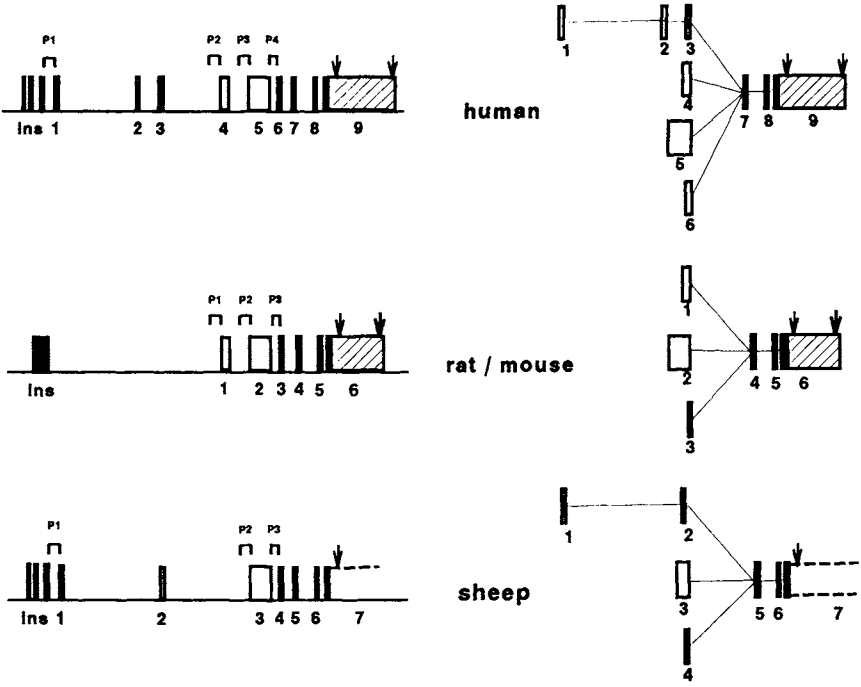


Figure 6. Structures of the human, rodent, and ovine IGF-II genes and mRNAs. Exons are numbered according to Holthuizen et al., 1991 for the human, rat, and mouse genes and are represented by boxes. For the ovine gene only those exons are indicated that have been identified in cDNAs; the numbering deviates from that in Ohlsen et al., 1994. The coding regions for the IGF-II precursor-peptides are indicated by filled boxes, the leader exons by open boxes, and the 3'-UTRs are striped. Arrows show the positions of poly(A) addition signals. The promoters for the human (P1-P4) and rodent (P1-P3) genes have been identified. Based on the structure and sequence of the isolated sheep cDNAs, putative sheep promoters are also indicated. See text for references on gene structures.

The human and the ovine IGF-II genes contain a promoter (P1), that is activated exclusively in adult liver tissue. The presence and activation of promoter P1 are directly correlated with the presence of circulating IGF-II after birth, which is exclusively produced in the liver. In rat and mouse, which lack the adult liver promoter, expression rapidly declines after birth, whereas in human and in sheep IGF-II expression is maintained in the liver throughout life (Daughaday and Rotwein, 1989).

For all species examined, IGF-II mRNAs are abundantly expressed in many tissues during fetal growth and development (Scott et al., 1985; Han et al., 1988).

Increased expression is found during growth and tissue repair as well as in a number of malignancies where it acts as the major autocrine growth factor (Haselbacher et al., 1987; El-Badry et al., 1989). In all non-neuronal tissues of the rat, IGF-II expression is greatly reduced after birth, but expression persists in adult rat brain (Brown et al., 1986; Lund et al., 1986; Murphy et al., 1987b; Beck et al., 1988; Chiariotti et al., 1988; Rotwein et al., 1988; Stylianopoulou et al., 1988). However, in several other species, i.e., human (Schofield and Tate, 1987; Gray et al., 1987; Irminger et al., 1987), horse (Otte and Engström, 1994), cow (Boulle et al., 1993), and sheep (O'Mahoney et al., 1991), the IGF-II gene is expressed, albeit at a lower level, in a number of adult tissues such as heart, brain, kidney, liver, muscle, skin, the reproductive organs, and the nervous system.

With the elucidation of the complete IGF-II gene structure, attention is now directed towards interpreting the complex regulation of transcription and to characterize the nature of transcription factors involved in activation of the multiple IGF-II promoters.

Transcription of the IGF-II Gene

Factors Involved in the Regulation of the Human Promoter P1

A distinct example of developmental regulation of IGF-II transcription is found in the human liver. Before birth, IGF-II transcription is directed by the promoters P2, P3, and P4, of which P3, yielding an mRNA transcript of 6.0 kb, is the most active (De Pagter-Holthuizen et al., 1987). After birth, these promoters are down-regulated and within the first postnatal year they are completely switched off (Davies, 1994). Simultaneously, promoter P1 is activated and subsequently the 5.3 kb IGF-II mRNA becomes the only IGF-II encoding transcript in the adult-liver. The adult liver-specific IGF-II promoter P1, located immediately downstream of the insulin gene, comprises 1 kb (Figure 7). Based on the transcriptional activity of various P1 reporter gene constructs, the promoter can be subdivided into two regions. The first 200 nt upstream of the transcription start site contain several regions to which nuclear proteins can bind and thus regulate transcription. The distal promoter region -900 to -175 contains two 67 nt long inverted repeat (IR) elements that act as negative regulatory elements (van Dijk et al., 1991).

Several elements of promoter P1 were initially identified by DNase I footprinting analyses using protein extracts from various sources (van Dijk et al., 1992b). Element PE1-1 located from -74 to -47 contains a perfect consensus binding site for the ubiquitous transcription factor Sp1. Mutational analysis of PE1-1 further showed that it is absolutely required for basal P1 activity, suggesting that it may be involved in the recruitment of the transcriptional machinery to this TATA-less promoter.

Obviously, liver-enriched transcription factors may play a role in the adult liver-specific expression of P1. Around position -100, a functional binding site was

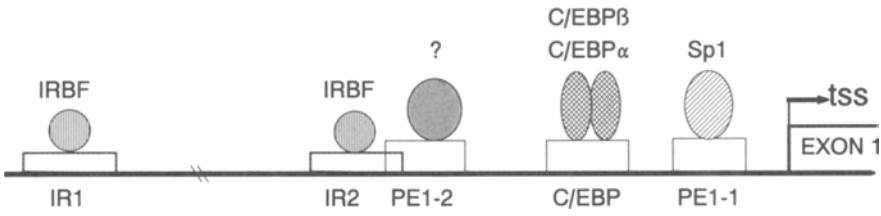


Figure 7. *Cis*-elements and *trans*-acting factors that participate in activation or repression of the human IGF-II promoter P1. The human IGF-II P1 region is 1 kb long and is flanked at the 5'-end by the insulin gene. The transcription start site (tss) (+1) is indicated by an arrow. The positions of the various *cis*-elements are indicated by boxes and the *trans*-acting factors are represented by ellipses. Footprinting analysis has shown protection of proximal element PE1-1 (-47/-74), of the C/EBP binding element (-91/-112), and of PE1-2 (-154/-192) to which an unknown factor binds. IR1 (-812/-887) and IR2 (-176/-243) represent the 67 nucleotides inverted repeat elements. Positions of the elements are not drawn to scale.

identified to which proteins of the C/EBP transcription factor family bind (van Dijk et al., 1992b). Using nuclear extracts enriched for C/EBPα, a footprint was detected between positions -112 and -91. Since the members of the C/EBP family play an important role in the course of hepatic differentiation, the effect of the C/EBP proteins on P1 transcription was examined in detail. The C/EBPs are themselves expressed in a tightly controlled manner, suggesting their potential importance for modulating gene expression in the liver during development. The concentrations of C/EBPα and C/EBPβ in fetal liver are minimal and start to increase around birth. After a short perinatal peak in expression, the levels of C/EBPα and C/EBPβ slowly increase, and maximum levels are reached in adult liver (Birkenmeier et al., 1989; Descombes et al., 1990).

Two of the C/EBP family members are able to activate P1: C/EBPα six-fold and C/EBPβ 15-fold. D-element binding protein (DBP), another member of the C/EBP family, does not activate P1 and mutational analysis indicated that DBP binding is prevented by a specific G-C basepair in the P1 element (Rodenburg et al., 1995). LIP, the inhibiting variant of C/EBPβ (Descombes and Schibler, 1991), suppresses the C/EBPα and C/EBPβ activation of P1 by forming C/EBP-LIP heterodimers that can bind to the P1 element but do not transactivate. *In vivo*, the expression patterns of C/EBPα and C/EBPβ in adult liver correlate with the observed P1 expression pattern, indicating that these transcription factors are major contributors to the postnatal liver-specific activation of the human IGF-II promoter P1.

The effects of other liver-enriched transcription factors involved in human P1 regulation have been screened but not fully characterized. Several different HNFs, were tested predominantly by transient co-transfection assays. HNF-1α, a nucleoprotein related to the homeodomain proteins, does not influence P1 activity. Of the

three HNF-3 (forkhead protein related) family members, HNF-3α, HNF-3β, and HNF-3γ, only HNF-3β strongly activates P1 (Rodenburg, unpublished results). Since this factor is highly expressed in liver it is thought to play an important role in the tissue-specific IGF-II expression directed by P1.

Furthermore, using deletion constructs it was found that the distal region of P1 exerts a negative effect on P1 promoter activity indicating that in addition to stimulatory factors such as HNF3-β and C/EBP, inhibition of P1 activity also takes place (van Dijk et al., 1991). It has been shown that the IGF-II promoter P1 contains two homologous inverted repeat elements (IR1 and IR2) of 67 nucleotides long that act as suppressors of P1 activity. In transient transfection experiments using P1 deletion constructs that lack one or both of the elements, it was shown that these two IR elements act as cell type-dependent suppressors of P1 activity. Similar to previously identified silencer elements, the IR elements affect promoter activity in an orientation- and position-independent manner. Using EMSA it could be shown that the IR elements are specifically bound by a novel protein, called inverted repeat binding factor (IRBF). Interestingly, the amounts of IRBF found to be present in different cell lines correlate directly with the levels of suppression of P1 activity in those cell lines (R.J.T. Rodenburg et al., 1996).

Sheep is the only species other than human of which the nucleotide sequence of a region upstream of the first exon has been determined (Ohlsen et al., 1994). Although promoter activity of this region of the sheep gene still needs to be established, it shows considerable sequence homology with the human promoter P1. In contrast to the observation of the authors, the sheep IGF-II gene also contains a perfect Sp1 consensus sequence homologous to the putative Sp1 site in human element PE1-1. In addition, one of the two IR elements is conserved among the human and ovine IGF-II genes.

Factors Involved in Activating Promoter P3

The human IGF-II promoter P3 is active in many fetal and non-hepatic adult tissues and in most IGF-II expressing cell lines, giving rise to an abundant 6.0 kb and a minor 2.2 kb transcript, depending on the polyadenylation signal used. P3 contains canonical TATA- and CCAAT-boxes and the promoter region is highly GC-rich (Figure 8). Transient transfection experiments using P3-reporter gene constructs indicate that P3 consists of a proximal region that supports transcription in most cell types, and an upstream region that enhances P3 activity in cell types that endogenously express IGF-II, suggesting that these cells contain additional factors which stimulate the P3 promoter (van Dijk et al., 1992a; Raizis et al., 1993). In EMSA experiments it was shown that the upstream P3 region (-1231/-1063) can form cell type-specific DNA-protein complexes (Schneid et al., 1993).

DNaseI footprint analysis, EMSA, and *in vitro* transcription have revealed that the proximal promoter region contains a number of elements that are recognized by nuclear proteins. Promoter P3 can be bound and activated by the general

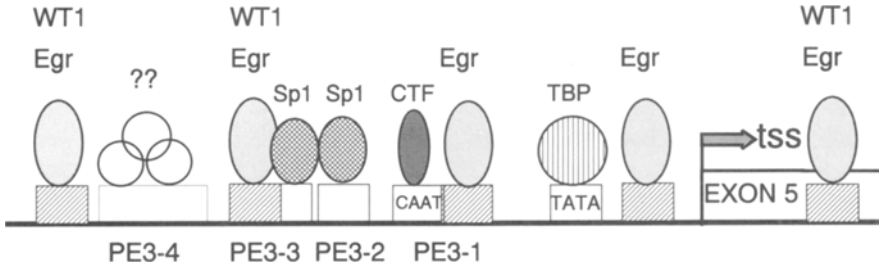


Figure 8. *Cis*-elements and *trans*-acting factors that participate in activation or repression of the human IGF-II promoter P3. The human IGF-II P3 region is 1,350 nt long and is flanked at the 5′-end by exon 4 of the IGF-II gene. Only the proximal 250 nt are shown. The transcription start site (tss) (+1) is indicated by an arrow. The positions of the various *cis*-elements are indicated by boxes and the *trans*-acting factors are represented by ellipses. TBP represents the TATA-box binding protein. Footprinting analysis using protein extracts from HeLa cells showed protection of proximal elements PE3-1 (-64/-90), PE3-2, and PE3-3 (-103/-146), and element PE3-4 (-172/-192), that is protected by a multiprotein complex (Rietveld, unpublished results). Binding of Egr-1 and WT1 was confirmed by footprint analysis and EMSA using partially purified Egr-1 and WT1 protein. Binding of Sp1 at -109/-117 and -120/-128 prevents binding of Egr-1. The binding site for Egr/WT located within exon 5 (+63/+74) is bound by two different WT1 isoforms, WT[-KTS] and WT[+KTS]. This figure is a compilation of data from van Dijk et al., 1992a, Drummond et al., (1992, 1994), and Raizis et al., 1993.

transcription factors NF1 nuclear factor 1 (NF1) and Sp1, that bind to proximal elements PE3-1 and PE3-2, respectively (Figure 8). Elements PE3-3 and PE3-4 are bound by still unidentified transcription factors. It was shown that PE3-4 is quite important since truncation of P3 at position -180 leads to a severe loss of promoter activity both in transient transfection and *in vitro* transcription assays, suggesting that the factors binding to PE3-4 are major contributors to P3 activity (van Dijk et al., 1992a). Some additional binding sites for regulators of promoter activity have been identified by transfection experiments and DNaseI footprinting, but the specific transcription factors that bind to them have yet to be identified.

Interestingly, the early growth response proteins Egr-1 and Egr-2 have multiple recognition sites in promoter P3. These transcription factors contain zinc-finger domains in which three zinc fingers are present that recognize the target consensus sequence 5′-CGCCCCCGC-3′. The activation of P3 by the Egr zinc-finger transcription factors has received a lot of attention, since the specific DNA motif that can be bound by the zinc-fingers of Egr-1 and Egr-2 is also recognized by the Wilms' tumor WT1 gene product (Rauscher, 1993). Binding studies further revealed that partially purified Egr-1, Egr-2, and WT1 proteins are indeed able to recognize and to bind to these sites, albeit with different affinities. The WT1 protein is present *in*

vivo in four different isoforms due to alternative splicing. The WT1(–KTS) which is a minor component *in vivo*, was shown to bind to all Egr/WT1 sites of P3 (Drummond et al., 1992). WT1(+KTS), however, could only bind with high affinity to the site located within exon 5 at position +63/+71 (Figure 8). In addition it was shown in transient transfection experiments that Egr-1 has a strong stimulatory effect on expression of IGF-II promoter P3 (Madden and Rauscher, 1993). It was further demonstrated in co-transfection experiments that a high level of WT1 expression of the –KTS type represses IGF-II P3 activity. From these and other results it has been postulated that the Egr proteins may play a role in stimulating expression of the IGF-II gene resulting in autocrine growth stimulation of specific tumors, whereas WT1 may act as a repressor of P3 activity. The results obtained so far indicate that promoter P3 is a complex promoter and activation involves binding of several *trans*-acting factors that act in concert in an intricate mechanism of regulation. It is not known whether these factors compete for the same binding site. Some will bind with higher affinity than others and, moreover, not all factors are present in each cell type.

Factors Involved in Activating Promoter P4

The human promoter P4, preceding exon 6, contains a TATA-box and transcription results in an mRNA of 4.8 kb. P4 is moderately active and is expressed in most fetal and non-hepatic adult human tissues examined. The general transcription factor Sp1 is the major regulator of P4 (Figure 9). Four recognition sequences for nuclear factor Sp1 are located in the first 125 nucleotides upstream of the transcription start site (van Dijk et al., 1992a; Hyun et al., 1993). However, two sites fit the

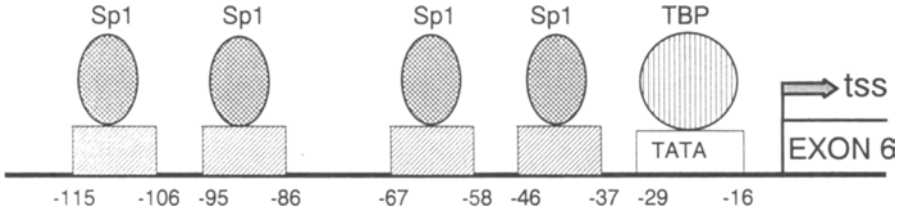


Figure 9. *Cis*-elements and *trans*-acting factors that participate in activation of the human IGF-II promoter P4. The human IGF-II P4 region is 1,000 nt long and is flanked at the 5'-end by exon 5 of the IGF-II gene. Only the proximal region of the promoter is shown. The transcription start site (tss) (+1) is indicated by an arrow. The positions of the various *cis*-elements are indicated by boxes and the *trans*-acting factors are represented by ellipses. TBP represents the TATA-box binding protein. Additional elements that were identified are RCE elements (-56/-61) and (-68/-73) flanking the second Sp1 site, and an Egr-1 binding site (-90/-98) overlapping with the third Sp1 site. The numbering is based on the sequence as presented in Holthuizen et al., 1993, and differs from that used by Kim et al., 1992, and Hyun et al., 1994.

consensus sequence of Sp1 completely and two sites contain some mismatches, suggesting that the contribution of the latter two to transcriptional activation is less significant (Holthuizen et al., 1993). When transient transfection experiments with a P4 promoter-CAT construct were performed in insect cells that do not produce any endogenous Sp1, no residual P4 activity could be detected (Kim et al., 1992). Two retinoblastoma control elements (RCE) were identified flanking the Sp1 site at -67 to -58 and it was shown that the retinoblastoma gene product can act as a positive regulator on the Sp1-mediated transcription of P4 (Kim et al., 1992). The human P4 promoter also contains one Egr-1 consensus sequence which can be bound by bacterially expressed Egr-1 protein. Furthermore, it was shown that P4-reporter gene constructs could be activated by Egr-1 (Hyun et al., 1994).

The homologous promoters in rat and mice were shown to be the most active promoters in these species. Transcripts of 3.6 kb derived from this promoter are in general 10-fold more abundant in rat and mouse tissues than the 4.6 kb transcripts from the promoter homologous to the prominent human P3 promoter. Interestingly, in the rat (Evans et al., 1988; Matsuguchi et al., 1990) and the mouse (Rotwein and Hall, 1990) promoters the Sp1 binding sites are also present, and in these species all four sites were shown to bind Sp1 (compiled in Holthuizen et al., 1993). Furthermore, it was shown that the mouse IGF-II promoter contains two non-consensus AP-1 sites which can be bound by recombinant c-jun protein. In a transient expression system using CP-1 embryonic stem cells it was demonstrated that this promoter is activated by AP-1 (Caricasole and Ward, 1993).

Factors Involved in Activating Promoter P2

Human IGF-II promoter P2 is a very weak promoter that lacks TATA- and CAAT-boxes, the transcription start sites are heterologous, and no enhancer elements have been described (Holthuizen et al., 1990). Transcription derived from P2, resulting in a 5.0 kb mRNA, has only been detected at low levels. Some human tumor tissues, however, exhibit elevated IGF-II expression derived from P2 (Ikejiri et al., 1991). In leiomyosarcomas, it was shown that expression of P2 derived mRNAs is enhanced 20-fold compared to that of normal smooth muscle tissue (Gloudemans et al., 1990). Promoters analogous to the human P2 were detected and characterized in sheep, rat, and mouse. For these promoters, features similar to the human promoter have been described. Although transcripts derived from this promoter have been detected in mouse and rat tissues by Northern blotting, this IGF-II mRNA also represents a minor transcript in these species.

Imprinting

A new level of complexity in the control of IGF-II expression has been added by the fact that the IGF-II gene is imprinted. It was demonstrated first in the mouse (DeChiara et al., 1990) and later in human (Giannoukakis et al., 1993) and rat

(Pedone et al., 1994) tissues that the paternally inherited allele is expressed and the maternally inherited allele is silent. This was clearly demonstrated by the phenotype of mice carrying a paternally inherited mutant allele of the IGF-II gene which resulted in a greatly reduced body size (DeChiara et al., 1990). Conversely, it was shown that fetal mice artificially overexpressing IGF-II have an increased growth phenotype (Ward et al., 1994). In a subset of human Wilms' tumors and Beckwith-Wiedemann syndrome patients it was further shown that loss of heterozygosity and duplication of the paternal allele can result in increased expression of IGF-II in tumors because in these instances both alleles are transcriptionally active (Ohlsson et al., 1993; Weksberg et al., 1993).

Recent publications have addressed the question whether there is a correlation between IGF-II promoter usage and monoallelic or biallelic expression of the human IGF-II gene. Interestingly, it could be shown that the P2, P3, and P4 IGF-II promoters are activated only on the paternal allele during prenatal and early postnatal development, while in human adult liver biallelic expression directed by P1 is observed (Davies, 1994; Vu and Hoffman, 1994). Biallelic expression of the IGF-II gene was further detected in two cell regions of the central nervous system, the choroid plexus and leptomeninges (Ohlsson et al., 1994). In the rat and the mouse IGF-II transcripts are derived from the three promoters on the paternal allele in all tissues examined with the exception of some regions of the central nervous system both in rat (Pedone et al., 1994) and in mouse (DeChiara et al., 1991; Hu et al., 1995).

Site-Specific Endonucleolytic Processing of IGF-II mRNAs

Expression of the IGF-II gene is not only regulated at the level of initiation of transcription, but also at the level of posttranscriptional mRNA processing. As an initial observation, a non-IGF-II encoding RNA species of 1.8 kb derived from the IGF-II gene was detected on Northern blots when a DNA fragment corresponding to the 3′-UTR of IGF-II mRNA was used as a probe. Further examination revealed that high levels of this 1.8 kb RNA are present in IGF-II expressing fetal and postnatal tissues (de Pagter-Holthuizen et al., 1988). Extensive analysis of these RNA fragments revealed that they are the products of a site-specific endonucleolytic cleavage reaction in the 3′-UTR of full length IGF-II mRNAs (Meinsma et al., 1991; Nielsen and Christiansen, 1992). Endonucleolytic cleavage of IGF-II mRNAs was shown to occur in all types of full length IGF-II transcripts, irrespective of the promoter from which the transcripts are derived. Although the 3′-terminal 1.8 kb cleavage product lacks a cap structure it is an extremely stable RNA. The 5′-cleavage product containing the coding region does possess a cap structure but lacks a poly(A) tail and is rapidly degraded (Figure 10). In the human IGF-II mRNAs the cleavage site has been mapped to single nucleotide resolution. Employing mutant IGF-II genes in an *in vivo* system, it was demonstrated that two widely separated sequence elements (designated I and II) in the 3′-UTR of IGF-II

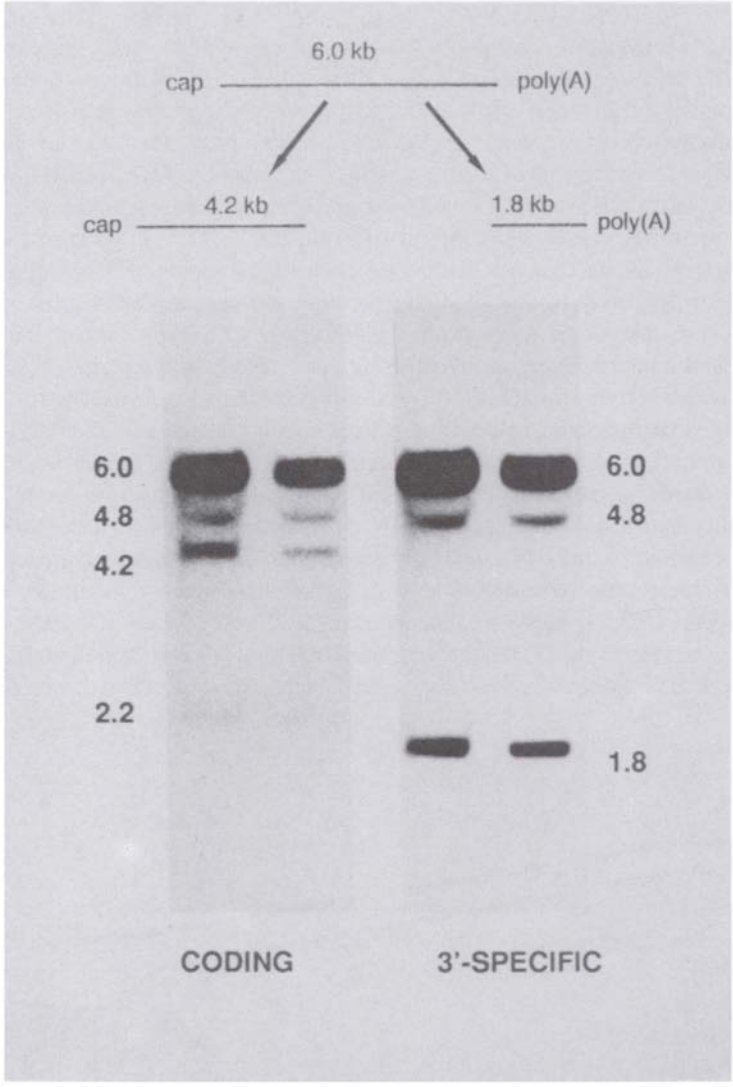


Figure 10. Site-specific endonucleolytic cleavage of IGF-II mRNA. **Top**. Schematic representation of the cleavage reaction. The 6.0 kb transcript is cleaved in its 3'-UTR. Two cleavage products are formed, a capped 5'-terminal 4.2 kb RNA and a 3'-terminal polyadenylated 1.8 kb RNA. **Bottom**. Northern blot of total RNA (5μ and 10μg) isolated from the human neuroblastoma cell line SHSY-5Y, that predominantly expresses 6.0 kb transcripts derived from P3, and as a minor component the 4.8 kb mRNA derived from P4. Blots were hybridized with a cDNA probe specific for the IGF-II encoding region (left), and a DNA fragment specific for the 3'-UTR (right).

mRNAs are essential for the site-specific endonucleolytic cleavage reaction to occur (Meinsma et al., 1992). It was shown that a 350 nt region (element II) surrounding the cleavage site is necessary but not sufficient for cleavage, and that an additional 150 nt element (element I) located 2 kb upstream of element II is also required to confer cleavage. The presence of both elements in the 3′-UTR of mRNA is sufficient for cleavage, as was demonstrated when these elements were transferred to a heterologous gene (Meinsma et al., 1992). This suggests that the two structural elements that are distant in the primary sequence are brought into proximity by RNA folding, thereby forming the recognition determinant for cleavage. Structural analysis using RNA folding algorithms demonstrates that element II contains a region immediately upstream of the cleavage site that can form two stem-loop structures. The region downstream of the cleavage site is very G-rich. Furthermore, a stable 80 nucleotides long double-stranded (ds) RNA stem structure ($\Delta G = -100$ kcal/mol) has been identified that can be formed between the G-rich region in element II and the far upstream element I (Figure 11). Subsequently, using *in vitro* synthesized RNA it was shown that such a stable ds RNA structure could indeed be formed between the two elements and that the presence of this stable stem structure as well as its sequence are important for proper processing of IGF-II mRNAs (Scheper et al., 1995).

Christiansen et al., 1994, have proposed that the G-rich stretch directly downstream of the cleavage site adopts an intramolecular quadruplex structure. Support

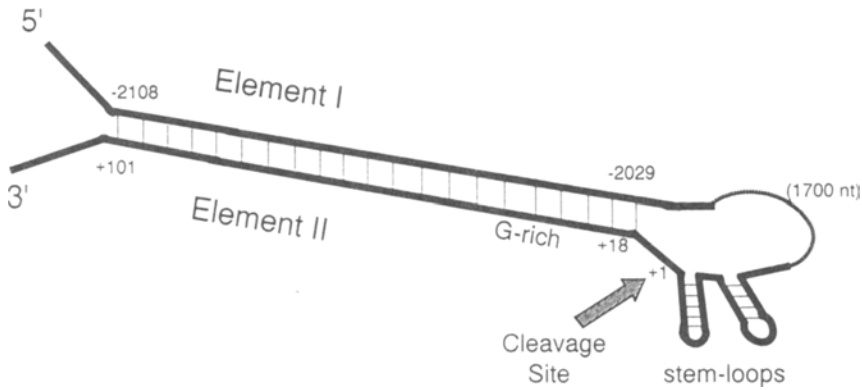


Figure 11. Secondary structure of the minimal cleavage unit required for endonucleolytic cleavage of the 3′-UTR of IGF-II mRNAs. Elements I and II harboring the regions in exon 9 that are essential for the site-specific endonucleolytic cleavage are indicated. An 80 nt long ds RNA stem-structure is formed between element I (-2108/-2029) and the downstream region of element II (+18/+101). The IGF-II cleavage unit binding protein (ICU-BP) binds to this stem structure. Element II further contains two small but stable stem-loops (-139/-3). The cleavage site (+1) is located in a ssRNA region, indicated by an arrow. All structural elements are extremely well conserved in human, rat, and mouse IGF-II mRNAs.

for this model was obtained by structural probing of synthetic RNA with RNase T1 and reverse transcriptase. However, formation of the ds RNA structure between elements I and II as shown in Figure 11, and the formation of the quadruplex structure are mutually exclusive because the same G-residues are involved either in the formation of the stem structure or in the quadruplex structure, but not in both. Given the requirement for both elements I and II in the cleavage reaction to occur, we favor the possibility that the stem structure is the predominant structure in full-length IGF-II mRNA. The quadruplex structure may form at the 5′ end of the 1.8 kb RNA after cleavage has taken place. This structure then may protect the 1.8 kb RNA from 5′-exonucleolytic degradation which would account for the unusual stability of the 3′-terminal cleavage product.

Since both the secondary structure and the sequence of the stem-structure are important for cleavage, it is probable that elements I and II cooperate in the binding of *trans*-acting factors involved in cleavage of IGF-II mRNAs. The identification of proteins binding to the cis-acting elements required for cleavage may provide insight into the mechanism underlying the specific endonucleolytic cleavage.

The mechanism of site-specific endonucleolytic cleavage of IGF-II mRNAs is unique because of the large distance separating the two elements as well as the lengths of the interacting regions. IGF-II mRNA cleavage has also been detected in other species such as rat and mouse (Holthuizen et al., 1993). The biological function of the cleavage reaction is still elusive. It may be a first step in the degradation of IGF-II mRNAs, as has been suggested for other endonucleolytic cleavage reactions of mRNA molecules (Binder et al., 1994; Beelman and Parker, 1995). Alternatively, the 3′-terminal cleavage product may have an intrinsic biological activity. Effects of 3′-UTRs on differentiation and tumorigenesis in mammalian cells have been reported (Rastinejad and Blau, 1993; Rastinejad et al., 1993).

Translational Regulation of IGF-II Expression

In recent years, a number of mechanisms by which translation of specific mRNAs is regulated have been elucidated. One of these, termed leader mediated control of translation, now seems highly relevant when discussing regulation of IGF-II expression. In IGF-II mRNA species, four different leaders or 5′-UTRs have been identified. The leaders are designated leader 1 to 4, corresponding to promoters P1 to P4 (Figure 6). Three of the leaders are much longer than the average 5′-UTR (leader 1, exons 1-3, 586 nt; leader 2, exon 4, 408 nt; leader 3, exon 5, 1171 nt) and can be expected to be translated less efficiently than leader 4 (exon 6), which has a length of 109 nt. Studies on the polysomal distribution of the four types of mRNA in different cell lines and in fetal liver have revealed that the most abundant human IGF-II mRNA carrying leader 3 is hardly found in the polyribosome fraction indicating that this mRNA species is poorly translated (Nielsen et

al., 1990; de Moor et al., 1994a, b). On the other hand, mRNAs with leader 2 or 4 are almost exclusively found in the polyribosome fraction and are efficiently translated. Analysis of the proteins binding to leader 3 has revealed that a 16 nt region in the 3′-end of the leader specifically binds four proteins with apparent molecular weights of 57, 43, 37, and 36 kDa (de Moor et al., 1995). The 57 kDa protein is indistinguishable from the polypyrimidine tract binding protein by size, binding properties, and immunoprecipitation characteristics (Hellen et al., 1993).

Recently, it has been demonstrated that translation of IGF-II mRNA in the rhabdomyosarcoma cell line RD is considerably affected by the growing state of the cells (Nielsen et al., 1995). P3 derived 6.0 kb mRNA (leader 3) is not translated in quiescent cells, but is selectively mobilized and translated in exponentially growing cells. Activation of translation is inhibited by rapamycin and mimicked by anisomycin, suggesting that translation of the 6.0 kb mRNA species is regulated by the S6 kinase signaling pathway. In contrast, the translation of P4 derived 4.8 kb mRNA is not dependent on the growth state of the cells. Therefore, the minor 4.8 kb mRNA generates constitutive production of IGF-II, whereas translation of the major 6.0 kb mRNA is posttranscriptionally regulated. It is known that the activation of the S6 kinase is controlled by growth factors. Consequently, administration of IGF-I or -II to IGF-II producing cells may lead to enhanced translation of the 6.0 kb IGF-II mRNA species via stimulation of the phosphatidyl inositol 3 kinase (PI-3 kinase) pathway and the activation of S6 kinase.

PERSPECTIVE

As described in this overview, extensive insight has been obtained in the regulatory mechanisms involved in expression of the IGF genes in the last few years. The IGF gene structures have been elucidated for different species and is has become clear that regulation of expression takes place at the transcriptional as well as the posttranscriptional level. With the identification of a number of tissue-enriched transcription factors the first steps have been made towards a better understanding of the tissue-specific and developmental stage-dependent expression patterns of IGF-I and -II. Future studies will focus on the complex interplay between these and other ubiquitous and cell type-specific transcription factors and their concerted action in the regulation of IGF gene expression during development of the vertebrate body. Such studies will yield more insight into the regulation of expression of locally synthesized IGFs in different developmental stages and will contribute to a better understanding of the roles of IGFs in cell proliferation and differentiation during the development from embryo to adult organism.

ACKNOWLEDGMENTS

The authors thank their co-workers Drs. L.A. Nolten, W. Scheper, R.J.T. Rodenburg, and L.E.G. Rietveld for communicating unpublished results and for critical reading of the manuscript. They are grateful to Mrs. A.C.M.B. Koonen-Reemst and F.M.A. van Schaik for technical assistance to research performed in the laboratory of the authors. This research is supported by several grants from the Netherlands Organization for The Advancement of Research (NWO).

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Chapter 5

Molecular Aspects of Insulin-Like Growth Factor Binding Proteins

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Advances in Molecular and Cellular Endocrinology
Volume 1, pages 123-159.

ISBN: 0-7623-0158-9

INTRODUCTION—THE IGF BINDING PROTEIN FAMILY

The biological activities of many peptide hormones and growth factors may be modulated by their interaction with larger soluble proteins, which may serve to restrict or facilitate their transit between different body compartments, inhibit or enhance their access to cell-surface receptors, or affect their susceptibility to proteolytic degradation. In some cases—for example, soluble binding proteins for growth hormone (Martha et al., 1992) and tumor necrosis factor-α (Van Zee et al., 1992)—these regulatory proteins are structurally related to a membrane-bound receptor for the hormone or growth factor, and may be proteolytically cleaved forms of the receptor, lacking the membrane-anchoring domain. Other peptides have specific binding proteins which bear no structural resemblance to their receptor. Among these binding proteins are the family of insulin-like growth factor binding proteins (IGFBP), six proteins that have been numbered IGFBP-1 to IGFBP-6 (Ballard et al., 1989; Drop et al., 1992).

The existence of one or more proteins that could convert IGF-I and IGF-II to high molecular weight forms was established two decades ago (Hintz and Liu, 1977; Kaufmann et al., 1977). In humans and rats, IGFs were recognized to circulate predominantly in growth hormone-dependent complexes of approximately 150 kDa, with a smaller proportion in 30 to 50 kDa forms, and very little as the free peptide (~7.5 kDa) (Moses et al., 1976; White et al., 1981). The 150 kDa complex was observed to be irreversibly converted by acidification to smaller forms (Hintz and Liu, 1977; Furlanetto, 1980). In time, the proteins responsible for binding IGFs were purified from serum or from cell culture media, and six structurally distinct species were identified (Baxter and Martin, 1989a; Shimasaki et al., 1991c; Drop et al., 1992); the 150 kDa complex was found to involve one of the IGFBPs (IGFBP-3) and another, non-IGF-binding protein (Furlanetto, 1980; Baxter and Martin, 1989b). Denaturation of this protein, known as the acid-labile subunit or ALS, at low pH was responsible for the irreversible loss of the large complex (Baxter, 1988).

The number of laboratories studying the IGFBP family has increased greatly in recent years, as pure proteins, antibodies, and molecular probes became more widely distributed. As a consequence, a wealth of new data has been published in a relatively short time. It is beyond the scope of this chapter to review the extensive literature on the physiology of IGFBP regulation in isolated cells, animals, and humans. Several comprehensive reviews of these topics have appeared recently (Baxter, 1995; Jones and Clemmons, 1995; Zapf, 1995). Instead, this review will concentrate on structural aspects of the proteins, their mRNAs and their genes, and will emphasize in particular the recent advances in knowledge of posttranslational modifications to the proteins, which are increasingly recognized as playing important roles in their regulation.

THE IGFBP AND ACID-LABILE SUBUNIT GENES

Consistent with the very strong structural relationships among the mRNAs and proteins for all members of the IGFBP family, the organization of the genes show marked similarities. Based on analyses of human and rodent genes, the genes encoding all six proteins have four coding exons, although the intron sizes show considerable variability. Table 1 summarizes the known structures.

The structure and regulation of the IGFBP-1 gene have been studied extensively in recent years. The human gene, located in the 7p12-p13 region of chromosome 7 (Alitalo et al., 1989), contains four exons over a length of 5.2 kb (Brinkman et al., 1988; Cubbage et al., 1989). It encodes a single mRNA species of approximately 1.55 kb, including 0.78 kb of coding sequence, which is expressed predominantly in the liver and in pregnancy decidua (Lee et al., 1993). A similarly-sized transcript is seen in the rat, where it shows tissue-specific (predominantly hepatic) expression, with marked age dependence, falling rapidly after birth (Ooi et al., 1990).

The IGFBP-1 gene promoter has TATA and CCAAT elements in the region from 28 to 72 bp 5′ to the cap site. Further upstream are regulatory elements in common with the gene for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK); both genes show potent suppression by insulin and stimulation by glucocorticoids. Identical insulin response sequences have been identified in the two genes, although only a single copy is found in the PEPCK promoter region, whereas the IGFBP-1 promoter has two copies (Suwanichkul et al., 1994; O'Brien et al., 1995). For both genes, hepatic nuclear factor-3 (HNF-3) appears to act as an accessory factor, the binding of which, to a site overlapping the insulin response sequence region, is required for the full induction of gene expression by glucocor-

Table 1. Organization of the IGF Binding Protein Genes

Gene	*Exon 1*	*Intron 1*	*Exon 2*	*Intron 2*	*Exon 3*	*Intron 3*	*Exon 4*	*Intron 4*
hIGFBP-1	0.51	1.5	0.17	1.2	0.13	0.9	0.70	
hIGFBP-2	0.60	27.0	0.23	1.0	0.17	1.9	0.50	
hIGFBP-3	0.54	3.3	0.23	0.54	0.12	1.6	0.14	0.94
rIGFBP-4		6.4		0.6		2.7		
hIGFBP-5		~25		0.6		1.3		
rIGFBP-5		10		0.6		0.7		
rIGFBP-6		2.4		0.2		1.2		

Notes: Exon/intron sizes in kb.
Human and rat genes designated by prefix h and r, respectively. Data from Rechler and Brown (1992) and original sources cited in the text.

ticoids. Liver-specific expression may also depend on the liver-enriched factor HNF-1 (Babajko et al., 1993). In the rat, the IGFBP-1 gene promoter similarly has corticosteroid, cyclic AMP and phorbol ester responsive elements, the stimulatory activity of which can be abolished by insulin (Goswami et al., 1994; Robertson et al., 1994; Suh et al., 1994)

The gene for human IGFBP-2 is found as a single copy on chromosome 2q33-q34 (Ehrenborg et al., 1991; Binkert et al., 1992). Over 32 kb in length, its three introns are 27.0, 1.0, and 1.9 kb. Similarly, the first intron of the rat and mouse genes are very long, 32 and 23 kb, respectively (Kutoh et al., 1993; Landwehr et al., 1993). A suggested promoter region, between nucleotides –635 and –2 of the human gene, lacks TATA or CAAT motifs, which are similarly absent in the putative promoter regions of the rat and mouse genes. There are, however, several GC boxes, binding sites for the transcription factor Sp1, which appear to be important in IGFBP-2 gene regulation (Kutoh et al., 1993; Landwehr et al., 1993). A human IGFBP-2 cDNA, isolated from a fetal liver library, was reported to be 1.43 kb, of which almost 1 kb is coding region (Binkert et al., 1989). It detected an mRNA of approximately 1.5 kb in Northern blots of liver, brain, and other tissues. In the rat, the mRNA is described as approximately 2 kb. It is detectable in liver, kidney, intestine, lung, and other tissues, and similar to IGFBP-1, its expression shows a strong inverse age dependence (Brown et al., 1989; Ooi et al., 1990).

The IGFBP-3 gene differs from those of the other IGFBPs in that it contains an additional intron, located in the 3′-untranslated region (Cubbage et al., 1990). It spans 8.9 kb on human chromosome 7p14-p12, where it exists as a single copy, 20 kb from the gene for IGFBP-1, and oriented tail-to-tail with it (Ehrenborg et al., 1992). Similarly, in the mouse genome, the IGFBP-1 and IGFBP-3 genes are found together on chromosome 1 (Kou et al., 1994a). Recently, the rat IGFBP-3 gene promoter region has been characterized. Like the human gene promoter, a TATA box, but no CAAT box, was identified, together with a number of putative response elements for AP-2, insulin, and growth hormone (Albiston et al., 1995). The full-length human IGFBP-3 cDNA is 2.5 kb, of which almost 0.8 kb encodes the protein (Wood et al., 1988); in the rat, the cDNA is 2.6 kb (Shimasaki et al., 1989). IGFBP-3 expression, first detected in the liver, is widespread among the tissues, including fibroblasts, spleen, ovary, heart, muscle, etc. (Naya et al., 1991), and the distribution is similarly broad in the rat (Shimasaki et al., 1989; Albiston and Herington, 1992). Hepatic expression shows a striking positive age-dependence in the mouse (Schuller et al., 1994), though in the rat it is not as marked as the age dependence of IGF-I mRNA expression (Albiston and Herington, 1992). Interestingly, in contrast to IGFBP-1 expression, the hepatic site of IGFBP-3 gene expression is not hepatocytes, but non-parenchymal cells, in particular sinusoidal endothelium (Takenaka et al., 1991; Chin et al., 1994).

The human IGFBP-4 gene, on chromosome 17q12-21.1 (Bajalica et al., 1992), is located very close to the hereditary breast cancer gene BRCA1 (Tonin et al., 1993). The gene organization has been reported for rat IGFBP-4 (Gao et al., 1993).

Its three introns span approximately 6.4, 0.6, and 2.7 kb of the 12 kb gene. In the promoter region are found TATA and CAAT boxes, as well as a variety of regulatory elements including cyclic AMP response elements, AP-1 binding sites, and a progesterone receptor binding site. The mRNA for IGFBP-4 is approximately 2.6 kb (Shimasaki et al., 1990). Its expression has been described in every rat tissue examined, including spleen, heart, lung, kidney, and brain, with liver the most predominant. In mouse liver, relatively little age dependence of expression is seen (Schuller et al., 1994).

IGFBP-5 gene has been localized to human chromosome 2q33-34, linked with the IGFBP-2 gene, but in the opposite orientation. Similarly to IGFBP-2, it has a very long first intron of some 25 kb, for a total length of about 33 kb (Allander et al., 1994). The rat and mouse IGFBP-5 genes are considerably shorter, at 17 kb (Zhu et al., 1993a; Kou et al., 1994b). In both man and rodent, there appears to be a tight linkage with the fibronectin gene. A TATA box is found 33 bp from the cap site in the human gene. Additional regulatory elements have been described in the rat, including a CAAT box, AP-1 and AP-2 sites and, similar to the IGFBP-4 gene, a progesterone receptor binding site (Zhu et al., 1993a). The rat IGFBP-5 mRNA is approximately 6 kb, and its expression is widespread, including kidney, lung, heart, and stomach; the liver, however, is a rather poor site of expression, in contrast to other IGFBPs (Shimasaki et al., 1991b).

The gene structure for rat IGFBP-6 has been described (Zhu et al., 1993b). Spanning 5.1 kb, it has a similar 4-exon structure to the other IGFBP genes. Like IGFBP-1, its promoter region lacks TATA or CAAT elements, but contains a putative Sp1 site, and estrogen and retinoic acid response elements. A 1.3 kb IGFBP-6 mRNA has wide tissue distribution, including liver, lung, kidney, intestine, etc. (Shimasaki et al., 1991a).

Very recently, the organization of the genes for rat and mouse ALS have been reported (Delhanty and Baxter, 1995; Ooi et al., 1995). Southern analysis suggests a single copy in the rat genome. A single intron of 1.1 kb interrupts the coding sequence, 16 bp 3′ of the translation initiation codon. The mature peptide is fully encoded within exon 2. Analysis of a putative promoter region, up to 2.3 kb upstream of the translation start site, reveals putative response elements for the growth hormone- and cytokine-dependent factors of the STAT (signal transducer and activator of transcription) family, and sites for AP1, AP2 and nuclear factor κB, possibly involved in the cyclic AMP and glucorticoid regulation of ALS. The ALS mRNA is 2.0-2.2 kb in human and rat (Leong et al., 1992; Dai and Baxter, 1994). Its expression appears exclusively hepatic by Northern hybridization, although *in situ* hybridization reveals some expression in kidney. In both tissues there is marked positive age dependence (Chin et al., 1994; Dai and Baxter, 1994).

Expression of a gene encoding a novel member of the IGFBP family has recently been identified in senescent human mammary epithelial cells (Swisshelm et al., 1995). It had previously been reported in meningiomas, where it had been postulated to have a growth-regulatory function (Murphy et al., 1993). Termed mac25, it is transcribed to a 1.1 kb mRNA. The gene has been localized to chromosomal

region 4q12-q13. Although there is sufficient similarity with the other IGFBP structures to classify mac25 within the family, no IGF-binding function for the protein has been described.

STRUCTURAL ASPECTS OF THE IGFBPS

Common Features of the IGFBP Family

The structures of the six IGFBPs share marked overall similarities. The human proteins have core molecular masses, estimated from their cDNA sequences, ranging from 22.8 kDa for IGFBP-6 to 31.3 kDa for IGFBP-2 (Drop et al., 1992). Their structures may be seen as consisting of three domains of approximately equal size. The N-terminal domains contain 12 corresponding Cys residues in IGFBPs -1 to -5, and 10 in IGFBP-6, whereas six corresponding Cys residues are found in the C-terminal domains of all of the proteins. It seems likely that none of these residues exists in a free sulfhydryl form, and that they disulfide bond within their own domain (Brinkman et al., 1991; Sommer et al., 1991). Other marked sequence similarities among the six proteins are also found in these domains; in contrast, the central domains of the proteins contain no Cys residues, except for two in IGFBP-4, and share no sequence similarities. A phylogenetic tree for the human IGFBPs indicates that IGFBP-3 and IGFBP-5 diverged first from a common origin, followed by IGFBP-6, which in turn diverged earlier than IGFBP-1, -2, and -4. IGFBP-1 and -4 appear to have diverged most recently from this cluster (Lee et al., 1993).

IGFBP-Binding Determinants on IGF-I and IGF-II

The use of natural and synthetic variant forms of IGF-I and IGF-II has greatly enhanced understanding of the structural determinants responsible for interaction with the IGFBPs. As recently reviewed, the first was the naturally occuring, amino-terminally truncated form of IGF-I, des(1-3)IGF-I (Francis et al., 1994), which has a significantly reduced affinity for IGFBP-3 and greatly reduced affinity for other IGFBPs (Forbes et al., 1988; Oh et al., 1993). Studies with this analogue established Glu^3 of IGF-I as an important residue in IGFBP interaction, as confirmed by the use of variants [Arg^3]IGF-I and [Gln^3, Ala^4] IGF-I (Bayne et al., 1988; Francis et al., 1994). Other residues of the B-domain of IGF-I are clearly also important, since [Gln^3, Ala^4, Tyr^{15}, Leu^{16}]IGF-I binds much more poorly to IGFBP-1 and IGFBP-3 than variants of either residues 3 and 4, or 15 and 16, alone (Clemmons et al., 1990; Baxter et al., 1992).

Residues 49-51 of the A-domain (Thr-Ser-Ile), which are located on a surface of IGF-I near to important B-domain residues, also appear to be involved in IGFBP-3 interaction, and their substitution abolishes binding to IGFBP-1 (Clemmons et al., 1990; Baxter et al., 1992). Tyr^{60} in the A-domain has also been

implicated in IGFBP-2 binding (Moss et al., 1991), but has little involvement in binding to intact IGFBP-3 although, as discussed below, it is essential for binding to proteolyzed IGFBP-3 (Bayne et al., 1990; Baxter and Skriver, 1993). In contrast, the IGF-I C-domain, which is important in type I receptor binding, and the D-domain, appear to have little involvement in IGFBP interaction; indeed, deletion of the C-domain, or truncation of the D-domain, slightly increase the apparent affinity of IGF-I for IGFBP-3 (Bayne et al., 1989; Baxter et al., 1992).

Analogous to the important role of Glu^3 and other B-domain residues in IGF-I, Glu^6 and nearby residues are essential in IGF-II binding to IGFBPs; for example, substitution of Leu^8 of IGF-II abolishes binding to IGFBPs 1-6 (Francis et al., 1994; Perdue et al., 1994). A-domain residues 48-50, Thr-Ser-Ile, are also required, most notably to retain affinity for IGFBPs -1 and 4-6, whereas, as seen for IGF-I, the C- and D-domains of IGF-II have little involvement in IGFBP interactions (Bach et al., 1993).

IGFBP-1 and -2

Human IGFBP-1 is a protein of 234 amino acids, with a calculated molecular mass of 25.3 kDa, first identified in amniotic fluid (Chochinov et al., 1977) and purified from placenta, where it was known as placental protein 12 (Bohn and Kraus, 1980). The details of its isolation by several independent groups, the characterization and sequencing of its cDNA by four laboratories in 1988-1989, and its regulation and functions, have been reviewed comprehensively (Lee et al., 1993). IGFBP-1 has no *N*-linked glycosylation sites, but a 4% carbohydrate content, presumably *O*-linked, was reported by Bohn and Kraus (1980), although it is unclear whether this exists on the protein from all sources. Binding studies of IGFBP-1 isolated from human amniotic fluid indicate similar affinities for IGF-I and IGF-II, of 6.6×10^9 and 3.2×10^9 L/mol, respectively (Baxter et al., 1987). However, as detailed in the following section, IGFBP-1 exists in at least four phosphorylated forms, with phosphorylation apparently increasing its IGF-binding affinity. Important IGF-binding determinants reside towards the amino-terminus of IGFBP-1, since a naturally occurring amino-terminal fragment of 21 kDa is able to bind IGF-I (Huhtala et al., 1986). This is supported by mutagenesis experiments showing that a 60-residue amino-terminal deletion abolished binding, although point mutations did not, except when disulfide bonding was disrupted (Brinkman et al., 1991; Lee et al., 1993). A region in the central domain rich in Pro, Glu, Ser, and Thr residues, common to many rapidly degraded proteins, might be involved in the very fast turnover of IGFBP-1 that is observed *in vivo* (Lee et al., 1993).

IGFBP-2 was first characterized from medium conditioned by rat BRL-3A cells, a liver-derived line (Knauer et al., 1981), and human and rat cDNA sequences were reported in 1989 (Binkert et al., 1989; Brown et al., 1989). The calculated size of the protein is 31.3 kDa in human, and 29.5 kDa in rat, although it typically appears

approximately 34 kDa on SDS-PAGE. Initial binding studies on the protein isolated from bovine kidney cells showed a slight preferential affinity for IGF-II when [^{125}I]IGF-I tracer was used, but a very strong preference for IGF-II in competition for [^{125}I]IGF-II binding (Forbes et al., 1988); however, this distinction is not so clear for human IGFBP-2 (Oh et al., 1993). There are no *N*-glycosylation sites, and *O*-linked glycosylation has not been described. Although the components of its IGF-binding domain have not been delineated, carboxy-terminal fragments of 12-14 kDa from the rat and human proteins retain some IGF binding activity (Wang et al., 1988; Ho and Baxter, 1994).

Among the six IGFBPs, only IGFBP-1 and -2 contain an Arg-Gly-Asp motif, located a few residues amino-terminal to the eighteenth (i.e., carboxy-terminal) conserved Cys. This sequence is characteristic of proteins, such as fibronectin, that interact with cell membranes via receptors of the integrin class (Ruoslahti and Piersbacher, 1987). In IGFBP-1 this motif appears to be involved in the IGF-independent regulation of cell motility, since an Arg→Trp mutation inhibited the migration of cells expressing the mutated IGFBP-1 compared to those expressing wild-type protein (Jones et al., 1993c). The membrane component interacting with Arg-Gly-Asp in IGFBP-1 was shown to be $\alpha 5\beta 1$ integrin, the fibronectin receptor. A different function has been demonstrated for Arg-Gly-Asp in IGFBP-2. The binding protein, secreted by transfected Chinese hamster ovary (CHO) cells, was able to potentiate IGF-II-stimulated DNA synthesis in sheep choroid plexus cells. An Asp→Glu mutation abolished the potentiating activity, instead causing the IGFBP-2 to inhibit IGF-II action (Delhanty and Han, 1993). The authors concluded that the Arg-Gly-Asp domain was important for the potentiating effect of IGFBP-2, which required cell association of the protein.

IGFBP-3

IGFBP-3 was purified from human plasma in 1986 (Martin and Baxter, 1986); the rat serum protein and human and rat cDNAs were characterized within the following few years (Baxter and Martin, 1987; Wood et al., 1988; Shimasaki et al., 1989). The mature human protein has 264 amino acids, and a molecular mass of 28.7 kDa. However, the core nonglycosylated protein, though extensively studied in recent years, does not occur naturally; rather, its size is increased to 40-45 kDa by extensive *N*-linked glycosylation. Human IGFBP-3 in virtually all biological fluids and cell culture media examined exists as a doublet in this size range. In human serum the larger form predominates, whereas in many culture media the smaller form is more abundant. IGFBP-3 has three potential *N*-glycosylation sites, all in the central domain, and recent mutagenesis studies indicate that all three may be used (Firth and Baxter, 1995). Asn^{89} and Asn^{109} always appear glycosylated, carrying some 4 and 5 kDa of carbohydrate, repectively; in contrast, Asn^{172} optionally carries approximately 6 kDa of carbohydrate, accounting for the observed doublet. Interestingly, rat IGFBP-3, which has four potential *N*-glycosyla-

tion sites in its central domain (Shimasaki et al., 1989), appears as a diffuse triplet on SDS-PAGE (Baxter and Martin, 1987). The role of the carbohydrate in IGFBP-3 is not clearly understood, since neither ligand binding, cell association, or the ability to modulate IGF activity, appear to depend strongly on the glycosylation state of the protein (Conover, 1991b; Sommer et al., 1993; Firth and Baxter, 1995).

IGF-I and IGF-II bind to a single binding site on human IGFBP-3, with IGF-II binding having a slightly higher affinity than IGF-I. Determined by equilibrium binding in solution, the association constant (K_a) for IGF-I was originally reported as 2 x 10^{10} L/mol and for IGF-II, 3 x 10^{10} L/mol (Martin and Baxter, 1986). The IGF-I estimate has been confirmed by a solid-phase biosensor method (Sommer et al., 1993), which shows essentially no difference between glycosylated and nonglycosylated IGFBP-3. However, IGF binding to IGFBP-3 is remarkably pH-dependent (Figure 1), having a broad optimum at pH 4-5.5, and greatly reduced activity at pH 7.4 (Holman and Baxter, 1996). It is stimulated several-fold at near-physiological, compared to low, ionic strength such that under near-physiological conditions, K_a values of 7.4 x 10^{10} L/mol for IGF-I and 10.8 x 10^{10} L/mol for IGF-II, have recently been reported (Holman and Baxter, 1996). The recent observation that IGFBP-3 contains a sequence [12-16] resembling the active site of protein disulfide isomerase, and displays isomerase activity, also raises the

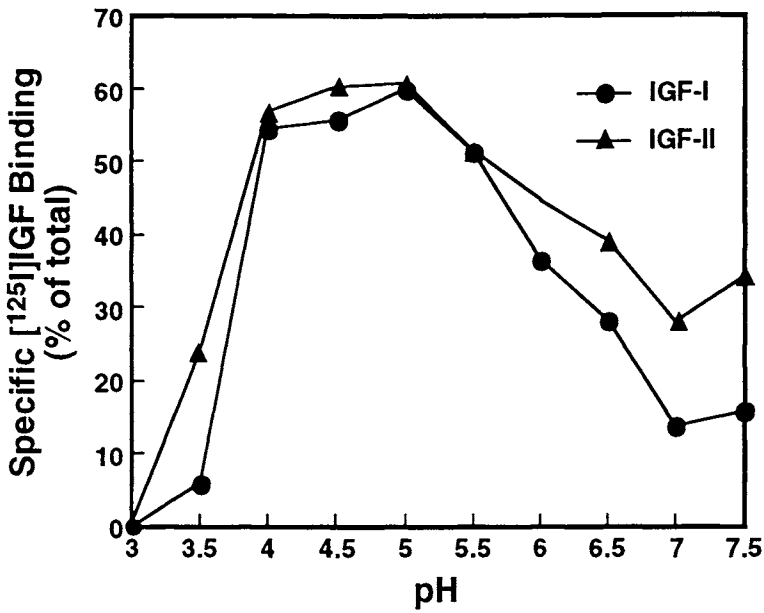

Figure 1. pH dependence of IGF-I and IGF-II binding to IGFBP-3. Natural human IGFBP-3 (1 ng) was incubated at various pH values with radiolabeled IGF-I or IGF-II, and the complexes precipitated with IGFBP-3 antiserum.

interesting possibility that IGF binding affinity may be directly influenced by the activity of the binding protein itself (Koedam and Van den Brande, 1994).

Deletion mutants of human IGFBP-3 expressed in CHO cells, in which either the central, nonconserved domain or the carboxy-terminal conserved domain, or both, are deleted (IGFBP-3[1-88], IGFBP-3[1-184], and IGFBP-3[Δ89-184]), fail to bind IGF-I by ligand blotting (Baxter and Firth, 1995), although binding to IGFBP-3[1-88] and other major deletion mutants, detected by affinity-labeling, has been reported (Sommer et al., 1991). These domains therefore either contain important IGF-binding determinants and/or are needed to maintain the conformation of the binding site. As expected from their lack of IGF binding, they also fail to bind ALS (see below). Interestingly, whereas the mutant forms lacking the carboxy-terminal domain cannot bind to the CHO cells that secrete them, the central domain deletion mutant does cell-associate. Cell-bound IGFBP-3 has been shown to be displaceable by IGF-I, but not by IGF-I analogues which do not bind to the binding protein (Martin et al., 1992), and consistent with this observation, the binding of IGFBP-3[Δ89-184], which does not bind IGF-I, to CHO cells is not displaced by IGF-I (Baxter and Firth, 1995).

The region of the carboxy-terminal domain of IGFBP-3 postulated to interact with cells is an 18-residue sequence [215-232] containing 10 Lys or Arg residues (Baxter, 1993). Only IGFBP-5 has a corresponding basic region (see below). These basic residues are likely to interact with polysulfated glycosaminoglycans (Cardin and Weintraub, 1989), and IGFBP-3 has been shown to be displaced from fibroblasts by heparin (Martin et al., 1992). A charge-reversal mutation of residues 228-232 of IGFBP-3, Lys-Gly-Arg-Lys-Arg, to the corresponding residues of IGFBP-1, Met-Asp-Gly-Glu-Ala, eliminated IGFBP-3 binding to CHO cells, emphasizing the importance of this basic region (Baxter and Firth, 1995). It is, however, unlikely that heparan sulfate proteoglycans constitute the IGFBP-3 cell binding sites, since heparinase or chlorate treatment of various cell types, both of which greatly reduce these proteoglycans on the cell surface, fail to eliminate IGFBP-3 binding (Booth et al., 1995; Yang et al., 1995).

The Acid-Labile Subunit and the Ternary Complex

IGFBP-3 differs from the other IGFBPs in its ability to form a complex with a liver-derived glycoprotein, ALS. ALS is a member of a diverse family of proteins which have an exceedingly high proportion of Leu residues—22% of total amino acids in the case of ALS. Approximately 80% of the 578-residue sequence of ALS is comprised of 18-20 repeating units of 24 amino acids, each containing six Leu residues, with some 50 additional amino acids at the amino-terminus and 70 at the carboxy-terminus (Leong et al., 1992). Similar leucine-rich repeats are found in a diverse range of other proteins, such as the cartilage proteoglycan core proteins decorin and biglycan, and ribonuclease inhibitor (Lee and Vallee, 1990). Proteins

with this structure tend to be involved in protein-protein interactions, either in solution or within membranes.

Within their estimated mature protein sizes of 63.3 kDa and 64.1 kDa, respectively, human and rat ALS each have seven potential *N*-glycosylation sites, six of which are conserved between the two proteins (Leong et al., 1992; Dai and Baxter, 1992). Analyzed by SDS-PAGE, ALS appears as a doublet band of approximately 85 kDa, more obvious in the human than the rat protein, which can be reduced to a single species of 60-65 kDa by extended enzymatic deglycosylation (Baxter et al., 1989; Baxter and Dai, 1994). More limited deglycosylation of human ALS reveals at least four additional bands of intermediate size, suggesting that most of the seven sites can probably carry carbohydrate (Liu et al., 1994). The function of this carbohydrate has not yet been elucidated. Of 13 Cys residues in human ALS, 12 are conserved in the rat protein. Four of these are clustered in the non-repeating amino-terminal region, and six in the nonrepeating carboxy-terminal region.

ALS appears to have no binding affinity for IGF-I or IGF-II alone, and its influence on the affinity of the IGFs for IGFBP-3 is variable. Binding studies with pure plasma IGFBP-3 have failed to show any effect of ALS on the binding of either IGF-I or IGF-II (Baxter et al., 1989). However, as discussed below, ALS increases the affinity of IGF binding to IGFBP-3 which has been subjected to limited proteolysis by enzymes in pregnancy serum (Baxter and Skriver, 1993). Similarly, IGF-I binding to nonglycosylated IGFBP-3 expressed in *Escherichia coli* is increased by ALS (Barreca et al., 1995). Whether this is due to partial proteolysis of the recombinant IGFBP-3, or to a difference between natural glycosylated IGFBP-3 and the recombinant nonglycosylated form, remains to be established.

The affinity of ALS binding to IGFBP-3, measured at 37 °C, pH 7.4, and near-physiological ionic strength, is much weaker than the binding of the IGFs. ALS binding is dependent upon whether IGF-I or IGF-II is bound to the IGFBP-3, with a mean K_a for binding to IGF-I-IGFBP-3 of $2.5 \times 10_8$ L/mol, and to IGF-II-IGFBP-3, of $5.8 \times 10_7$ L/mol (Holman and Baxter, 1996). These estimates are lower than those previously observed at lower temperature, pH, and ionic strength, since each of these factors influences the affinity (Baxter et al., 1989). A variety of studies using human IGFBP-3 and ALS preparations have concluded that ALS only binds to IGFBP-3 weakly, if at all, unless either IGF-I or IGF-II is present. This has been demonstrated by competition studies using gel permeation chromatography (Baxter, 1988), affinity-labeling and SDS-PAGE (Baxter and Martin, 1989b), and solution binding of radiolabeled ALS followed by immunoprecipitation with anti-IGFBP-3 antibodies (Baxter et al., 1993). Only by affinity-labeling can a weak IGFBP-3-ALS complex be demonstrated (Baxter and Martin, 1989b), a result that needs to be interpreted cautiously since this technique stabilizes even very low-affinity interactions. Figure 2 illustrates the same requirement for occupancy of IGFBP-3, using radiolabeled IGFBP-3 and immunoprecipitation with anti-ALS antibodies. These studies all point to the conclusion that the affinity of ALS for unoccupied IGFBP-3 must be exceedingly weak compared to that for the occupied protein.

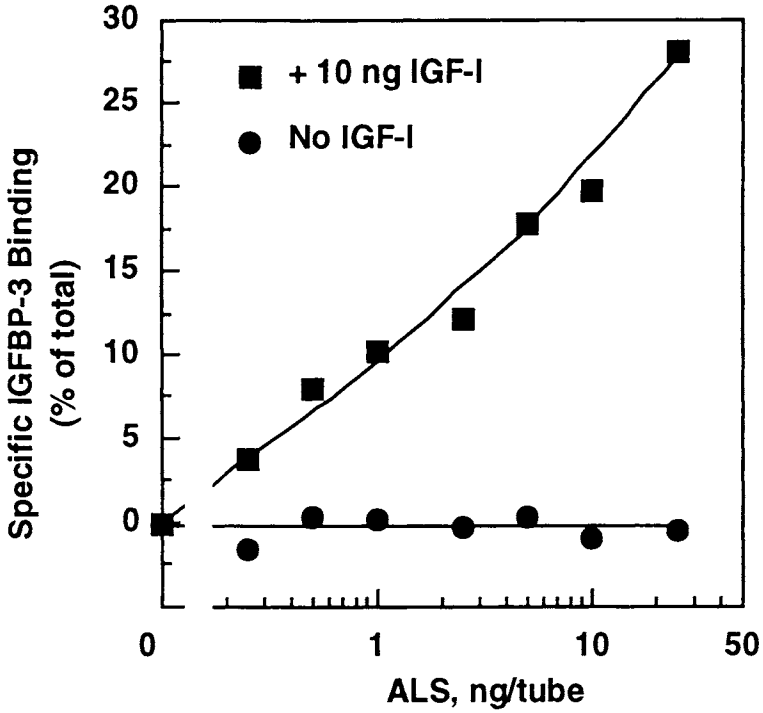

Figure 2. Binding of radiolabeled natural human IGFBP-3 to increasing concentrations of pure human ALS. Binding was carried out in the presence or absence of 10 ng/tube of IGF-I, as indicated. Reaction mixtures, in 0.3 mL, were essentially as previously described (Baxter et al., 1992), except that complexes were precipitated with a specific antiserum against human ALS. No specific binding of IGFBP-3 to ALS was seen in the absence of IGF-I.

This concept is supported by studies using IGF-I analogues with site-specific mutations. As described above, residues in the amino-terminal B-domain of the IGFs are crucial for IGFBP interaction. Thus, the variants [$Tyr^{15}Leu^{16}$]IGF-I and [$Gln^{3}Ala^{4}Tyr^{15}Leu^{16}$]IGF-I show reductions in their affinity for IGFBP-3 of fivefold and > 100-fold, respectively (Bayne et al., 1988). Scatchard plots of ALS binding to IGFBP-3 in the presence of these IGF-I variants show relatively little change in ALS affinity, but a marked reduction in the estimated binding site concentration, which is lowest in the presence of the IGF analogue with the lowest affinity for IGFBP-3. This indicates that it is the binary IGF-IGFBP-3 complex, not IGFBP-3 alone, that provides the ALS binding site (Baxter et al., 1992). ALS binding studies at different pH values lead to the same conclusion. IGF-I or IGF-II

binding to IGFBP-3 is lower by at least 50% at pH 6.5 compared to pH 5.5, and ALS binding is also decreased. When ALS binding kinetics are determined under the two conditions, the affinity of binding is unchanged by increasing the pH from 5.5 to 6.5, but the estimated binding site concentration is decreased by about 50%, again implicating the binary IGF-IGFBP-3 complex, rather than free IGFBP-3, as the site of ALS binding (Holman and Baxter, 1996).

However, two recent studies have concluded that under certain conditions there may be an interaction between ALS and IGFBP-3 in the absence of IGFs. In one such study, Lee and Rechler (1995) analyzed IGFBP-3 in adult rat serum, and found that a portion of it was partially proteolyzed, such that its affinity for IGF-I was selectively decreased. This IGFBP-3 form could be extracted from the 150 kDa complex of rat serum on an IGF-I affinity column, and therefore it was concluded that it circulated with its IGF binding site unoccupied, although still bound to ALS. However, the possibility of exchange between IGF-I bound with low affinity to this IGFBP-3 form, and the huge excess of IGF-I immobilized on the affinity column, would need to be excluded before this conclusion was justified. It also needs to be emphasized that even IGFBP-3 and IGF forms with a reduced affinity of interaction can still form a ternary complex with ALS under conditions (such as those found in serum) where their concentrations are sufficiently high to force the binding equilibrium substantially in the direction of the binary IGF-IGFBP-3 complex (Baxter et al., 1992).

Covalent cross-linking studies with recombinant human IGFBP-3 (nonglycosylated, produced in *E. coli*) have also been interpreted as indicating that IGFBP-3 and ALS can form a complex in the absence of IGFs, since a cross-linked ALS-IGFBP-3 complex could bind radiolabeled IGF-I by ligand blotting after SDS-PAGE; IGFBP-3 cross-linked to ALS without IGFs could be detected immunologically at high molecular weight; and radiolabeled IGFBP-3 could be cross-linked to ALS without IGFs (Barreca et al., 1995). All of these results point to an interaction between *E. coli* IGFBP-3 and ALS, but, as noted above, the question of physiological relevance remains, since (i) the IGFBP-3 used was the unnatural, nonglycosylated form, and (ii) the covalent cross-linking technique stabilizes even interactions of extremely low affinity, leading to artifactual results. As an example of this artifact, radiolabeled IGF-II can be covalently cross-linked to albumin, even though equilibrium binding studies fail to show any interaction (Martin and Baxter, 1988).

IGFBP-5

IGFBP-5 was isolated and characterized from human and rat serum in 1991 (Kiefer et al., 1991; Shimasaki et al., 1991b). Due to its susceptibility to limited proteolysis (see below), it may appear smaller in some biological fluids—around 22-24 kDa (Roghani et al., 1991; Camacho-Hubner et al, 1992)—than its estimated size of approximately 28 kDa. Although it has no potential *N*-glycosylation sites,

its ususal appearance on SDS-PAGE as an approximately 30-32 kDa doublet or triplet (Martin and Baxter, 1990; Camacho-Hubner et al., 1992) suggests a degree of *O*-glycosylation. IGFBP-5 has an approximately fivefold preferential affinity for IGF-II over IGF-I (Clemmons et al., 1992), but IGF-binding domains on the protein have not been investigated. However, it has in its carboxy-terminal domain an 18-residue basic sequence almost identical to that described above for IGFBP-3 and, in addition, another highly basic sequence in its central domain. Both of these regions might be involved in its association with acidic elements on the cell surface or matrix (Jones et al., 1993b; Booth et al., 1995).

In bone, IGFBP-5 binds to hydroxyapatite, where it may serve to concentrate a pool of IGFs, particularly IGF-II for which it has a preferential affinity (Bautista et al, 1991; Andress and Birnbaum, 1991; Mohan, 1993). In skin fibroblasts, matrix components with which IGFBP-5 can associate include types III and IV collagen, laminin, and fibronectin. These interactions appear to be largely ionic, since the binding is readily reversible by increasing the ionic strength. Binding to matrix components decreases the K_a for IGF-I from 1.4×10^{10} L/mol for IGFBP-5 in solution, to $1\text{-}2 \times 10^9$ L/mol for matrix- or collagen-bound IGFBP-5, and may lead to the binding protein potentiating IGF-I-stimulated cell growth (Jones et al., 1993b). In contrast, binding of IGFBP-5 to membranes of SV-40-transformed fibroblasts is thought to be inhibitory to IGF-I-mediated cell growth (Reeve et al., 1995).

The carboxy-terminal basic region of IGFBP-5, like that of IGFBP-3, specifically suggests the potential to interact with heparinlike molecules (Cardin and Weintraub, 1989), and the 30 kDa IGFBP-5 doublet can be released from skin fibroblasts into the culture medium by heparin (Martin et al., 1992). Booth et al. (1995) have demonstrated direct binding of radiolabeled IGFBP-5, as well as IGFBP-3, to endothelial cell membranes and matrix. IGFBP-5 binding is particularly susceptible to displacement by heparin, heparan sulfate, and dermatan sulfate, as well as the polycation protamine. Similarly to IGFBP-3 binding, however, heparinase treatment of cells did not decrease IGFBP-5 binding. In contrast, binding could be displaced by a synthetic peptide equivalent to the 18-residue basic region of IGFBP-3[215-232], although not by the IGFBP-5 central-domain basic sequence, IGFBP-5[130-143] (Booth et al., 1995). These experiments indicate that the carboxy-terminal basic region of IGFBP-5, like that of IGFBP-3, is important in its cell-association, but leave some question as to the nature of the cell elements to which these proteins bind.

IGFBP-4 and -6

Rat and human IGFBP-4 were initially identified and characterized from serum and bone cell medium (LaTour et al., 1990; Shimasaki et al., 1990). Both have two additional Cys residues in their central domain, in addition to the 18 conserved Cys residues common to IGFBP-1 to -5. A potential *N*-glycosylation site in the central

domain appears to be utilized, as the protein is found both in a form of 24-25 kDa, similar to its predicted size of approximately 26 kDa, and a larger form of 28-30 kDa, which can be converted to the small form by endoglycosidase-F (Ceda et al., 1991). IGF-I and IGF-II appear to bind to IGFBP-4 with essentially identical affinities (Clemmons et al., 1992).

IGFBP-6 differs from the other binding proteins in its extremely high affinity for IGF-II relative to IGF-I. It was initially identified in human cerebrospinal fluid for its preferential IGF-II binding (Hossenlopp et al., 1986), and later isolated from that source, as well as human fibroblast lines (Roghani et al., 1989; Forbes et al., 1990; Martin et al., 1990). Characterization of its cDNA was reported by Shimasaki et al. (1991a); the human protein has a predicted size of 22.8 kDa and the rat, 21.4 kDa. The affinity difference between the two IGFs is reported to be between 10- and 100-fold, with an estimated K_a as high as 3×10^{11} L/mol for IGF-II and 3×10^9 L/mol for IGF-I (Martin et al., 1990). Human IGFBP-6 lacks an adjacent pair of Cys residues corresponding to the sixth and seventh from the amino-terminus in the other IGFBPs, and rat IGFBP-6 in addition lacks the third and fourth Cys residues (Shimasaki et al., 1991c). Originally observed to bind to wheat germ lectin but not concanavalin A (Martin et al., 1990), IGFBP-6 has no consensus *N*-glycosylation site, but is always found in forms larger than its calculated size of approximately 22 kDa, due to the presence of *O*-linked glycosylation (Bach et al., 1992). Complete deglycosylation with neuraminidase, fucosidase, and *O*-glycanase has no effect on the affinity for either IGF-I or IGF-II (Bach et al., 1992). The extent of IGFBP-6 glycosylation is variable, as the form found in human cerebrospinal fluid appears smaller than that secreted by fibroblasts (Martin et al., 1990), but larger than that in human serum (Baxter and Saunders, 1992).

There is no evidence to date that the activities of either IGFBP-4 or IGFBP-6 are modified by association with cell surfaces.

IGFBP PHOSPHORYLATION

Several of the IGFBPs are secreted as phosphoproteins. Most extensively studied is IGFBP-1, which is exclusively serine-phosphorylated, principally at residues 101, 169, and 119, all potential casein kinase II sites (Frost and Tseng, 1991; Jones et al., 1991, 1993a). The existence of different phosphorylation states of IGFBP-1 was first suggested by the experiments of Busby et al. (1988), who showed that anion-exchange chromatography separated the protein, isolated from amniotic fluid, into fractions of different charge, which had different effects on IGF-I-stimulated DNA synthesis in porcine smooth muscle cells. At least four phospho-forms are identifiable on non-denaturing polyacrylamide gel electrophoresis. The phosphorylation state of IGFBP-1 has a major influence on the activity of the protein, since the phosphorylated forms bind IGFs with high affinity and inhibit their biological activities, whereas the dephospho form has a four- to sixfold lower

binding affinity and appears able to potentiate IGF activity (Jones et al., 1991). The importance of phosphorylation in regulating IGFBP-1 affinity is emphasized by the demonstration that the mutation ^{101}Ser→Ala lowered its affinity, and dephosphorylation of ^{119}Ser and ^{169}Ser caused a further loss of affinity (Jones et al., 1993a). However, the precise way in which altered affinity for IGF-I is reflected in such a profound switch in the regulation of IGF-I activity is not understood.

Recent studies have investigated the regulation of IGFBP-1 phosphorylation in different body fluids and physiological states. Separating different phospho-forms by anion-exchange and analyzing them electrophoretically, Koistinen et al. (1993) showed that both in decidua and in amniotic fluid, the extent of phosphorylation of IGFBP-1 increased through pregnancy. IGF-binding affinity increased with the degree of phosphorylation. In serum, IGFBP-1 phosphorylation may also vary under different conditions. Although a relatively high proportion of the nonphosphorylated form was observed in fetal human serum, compared to decidua or HepG2 hepatoma cells (Jones et al., 1991), adult serum has been described as having a single, highly phosphorylated species, with some lower phosphorylation states evident in pregnancy (Westwood et al., 1994). In patients with severe trauma, phosphorylated IGFBP-1 forms increase dramatically, and elevated levels are also seen in diabetic subjects (Frost et al., 1994).

Since changes in binding affinity accompany the changes in the phosphorylation state of IGFBP-1 in different states of health and disease, these observations have been interpreted as indicating possible modulation of the inhibitory potential of IGFBP-1 in different circumstances. While these interpretations may in time prove to be correct, the current uncertainty about the roles of IGFBP-1—whether as a glucoregulator or an regulator of IGF-mediated mitogenic function (Lee et al., 1993; Baxter, 1995)—leave the full significance of IGFBP-1 phosphorylation to be determined.

IGFBP-3 phosphorylation was first described by Mukku et al. (1991), who noted that in mammalian cells labeled with ^{32}P-phosphate, the radioactivity in IGFBP-3 was located on serine residues. Subsequently, two major phosphorylation sites, Ser^{111} and Ser^{113}, have been identified, both consensus sites for casein kinase II (Hoeck and Mukku, 1994). Phosphorylation/dephosphorylation does not appear to have any influence on the binding affinity for IGF-I, but the affinity of IGFBP-3 for ALS is slightly increased by dephosphorylation (Baxter, 1993; Hoeck and Mukku, 1994). IGFBP-3 phosphorylation has recently been shown to be IGF-I-stimulated (Coverley and Baxter, 1995). By using IGF-I analogues with selective affinity for either the type I receptor or the IGFBPs, stimulation of phospho-IGFBP-3 was shown to require type I receptor interaction, whereas an increase in total IGFBP-3 occurred even with a receptor-inactive analogue that retained IGFBP-binding. This is illustrated in Figure 3. Since receptor-independent stimulation of IGFBP-3 is believed to involve release of the protein from cell-surface or matrix binding sites (Martin et al., 1992), and a receptor-inactive IGF-I analogue increased total IGFBP-3 but not phospho-IGFBP-3, this implies that cell-associated IGFBP-3

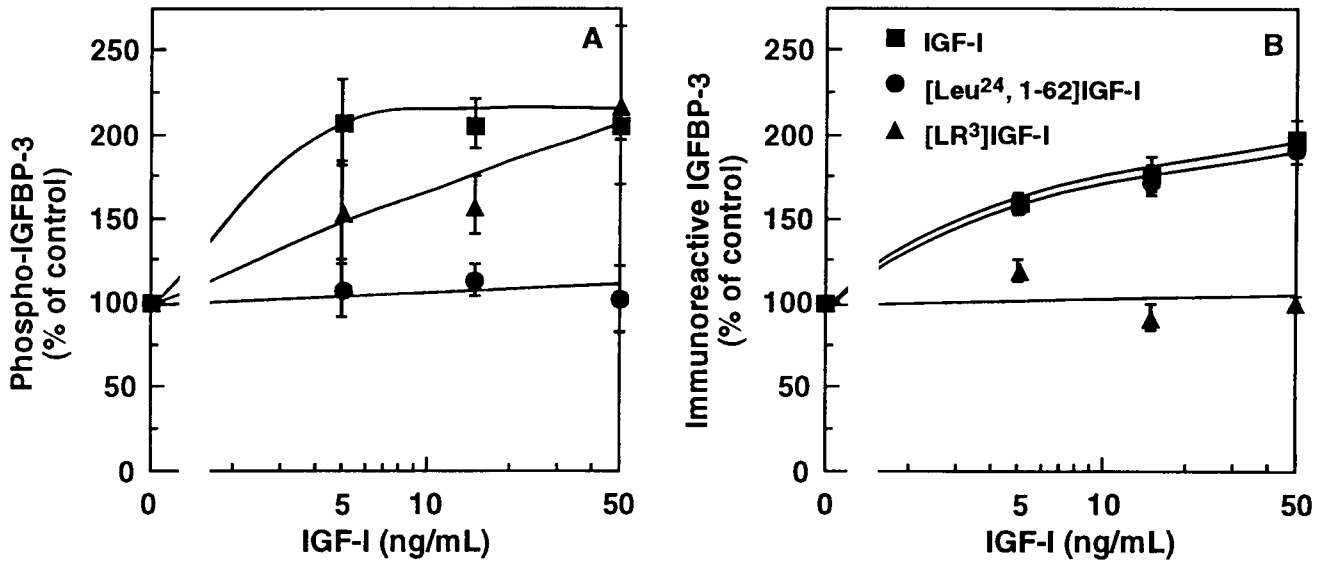

Figure 3. Stimulation of phosphorylated IGFBP-3 production by IGF-I. Human skin fibroblasts were exposed to 50 ng/mL of IGF-I, [Leu^{24}, 1-62]IGF-I which binds poorly to the type I IGF receptor, or long [Arg^3]IGF-I (LR^3) which binds poorly to IGFBPs. Long [Arg^3]IGF-I increased the amount of phospho-IGFBP-3 without any increase in total immunoreactive IGFBP-3, indicating type I-receptor mediated stimulation of phosphorylation. [Leu^{24}, 1-62]IGF-I increased total IGFBP-3, but not phospho-IGFBP-3. Since this analogue is believed to act by releasing IGFBP-3 from the cell surface (Martin et al., 1992), this suggests that cell-associated IGFBP-3 is not phosphorylated, or is dephosphorylated on release. Phospho-IGFBP-3 was detected by ^{32}P incorporation and immunoprecipitation with an IGFBP-3 antiserum. Data from Coverley and Baxter (1995), with permission from The Endocrine Society.

may not be phosphorylated, or is dephosphorylated on release, whereas IGFBP-3 in the extracellular medium is phosphorylated.

Finally, a preliminary report suggests that IGFBP-5 is also a phosphoprotein (Jones et al., 1992), but the regulation of its phosphorylation is as yet unexplored. However, Hoeck and Mukku (1994) point out that both IGFBP-5 and IGFBP-6 contain serine residues in an acidic environment that might be sites for phosphorylation.

IGFBP PROTEOLYSIS

The possibility that IGFBPs might be subject to limited degradation to stable, smaller forms has been recognized for many years. Morris and Schalch (1982) described the appearence of a 30 kDa IGFBP form after exposure of a 60 kDa serum IGF-I-IGFBP complex (presumably IGFBP-3) to alkaline pH, and Clemmons et al. (1983) reported that serum proteases could improved the access of IGF-I bound in serum complexes to an antiserum. Subsequently, a purified preparation of human IGFBP-3 was shown to generate a major breakdown product of near 30 kDa on prolonged storage (Martin and Baxter, 1986). The idea that specific proteases might be involved in the processing of IGFBPs to stable products became a well-established principle with demonstration that human pregnancy serum could degrade IGFBP-3 to a 30 kDa form, unable to bind IGF tracers when analyzed by ligand blotting (Giudice et al., 1990; Hossenlopp et al., 1990). Subsequently, numerous studies have described IGFBP-proteolytic activity in the circulation, where it has been proposed to regulate IGF access to the tissues, and in the extracellular environment, where it is thought both to liberate sequestered IGFs, allowing them access to their receptors, and to generate IGFBP forms that might have IGF-potentiating activity.

Circulating IGFBP Proteases

The discovery of pregnancy-related serum IGFBP proteases rapidly led to many studies examining their regulation and significance. Inhibition studies with aprotinin and EDTA established the involvement of an enzyme (or enzymes) of the serine-protease class, with cation-dependence (Hossenlopp et al., 1990). The failure to detect IGFBP-3 by ligand blotting was initially interpreted as evidence for a marked decline in serum IGFBP-3 during pregnancy (Giudice et al., 1990), a conclusion inconsistent with the earlier demonstration that immunoreactive IGFBP-3 increases significantly (Baxter and Martin, 1986). Indeed, both IGFBP-3 and ALS increase with increasing gestational duration (Suikkari and Baxter, 1992), and IGFBP-3 levels at 15 weeks are significantly higher in multiple than singleton pregnancies, despite a correspondingly greater level of IGFBP-3 protease activity (Langford et al., 1995).

The discrepancy between radioimmunoassay and ligand blot results was clarified with the demonstration that all of the protein was present in a 30 kDa, non-IGF-binding form when analyzed by immunoblotting after SDS-polyacrylamide gel electrophoresis (Hossenlopp et al., 1990). However, the concept that all of the IGFBP-3 in pregnancy circulates in a low-affinity 30 kDa form does not account for the observations that serum IGF levels increase in pregnancy (Wilson et al., 1982), that they are found predominantly in a 150 kDa complex indistinguishable from that in nonpregnancy serum (Gargosky et al., 1991), that IGFBP-3 itself circulates predominantly in the usual 150 kDa complex (Suikkari and Baxter, 1992), and that, even after acidification of serum to destroy the ALS, the IGFBP-3, analyzed by high-resolution gel chromatography which separates proteins of 30 kDa and 50 kDa, is identical in size (approximately 50 kDa) to that in nonpregnant controls (Suikkari and Baxter, 1992).

Binding studies *in vitro* have helped to clarify the enigma of why the ternary complex appears normal in human pregnancy, yet IGFBP-3 isolated from its other components may have reduced IGF affinity. Since all of the IGF-binding studies used iodinated ligands, the possibility existed that iodination changed the affinity of IGF-I for proteolyzed IGFBP-3. This proved to be the case, since in the presence of nonradioactive iodo-IGF-I, ALS binding occurred normally to nonpregnancy IGFBP-3, but not to pregnancy IGFBP-3, whereas in the presence of natural IGF-I, both forms of IGFBP-3 bound ALS (Suikkari and Baxter, 1991). Studies with site-specific IGF-I variants indicated that Tyr^{60}, and possibly Tyr^{24}, were likely to be the IGF-I residues affected by iodination, which then inhibited binding to proteolysed IGFBP-3. Analogues in which these residues were altered by mutagenesis had greatly reduced affinity for proteolyzed IGFBP-3, and did not support ternary complex formation, whereas with intact IGFBP-3 they had normal binding affinity and supported ALS binding (Baxter and Skriver, 1993; Baxter et al., 1993). In contrast, substitution of Tyr^{31} of IGF-I, or deletion of the C-domain in which this residue occurs, did not selectively affect IGF-I or ALS binding to proteolyzed IGFBP-3.

Notwithstanding the severely inhibitory effect of IGF-I iodination on its binding to proteolyzed IGFBP-3, several studies clearly showed that the affinity for unsubstituted IGF-I was also reduced (Baxter and Skriver, 1993; Lassarre and Binoux, 1994), raising the question why ALS binding to proteolyzed IGFBP-3 *in vitro*, and the pregnancy serum ternary complex *in vivo*, appear normal. As discussed above, several independent methods have shown that normal IGF binding is an essential prerequisite for normal ALS binding, and that IGF-I analogues with reduced affinity for IGFBP-3 form reduced amounts of ternary complex in the presence of ALS. Therefore IGFBP-3 forms with reduced affinity for IGF-I should not allow normal ALS binding, as seen with IGFBP-3 isolated from pregnancy serum under mild conditions (Baxter et al., 1993). The answer appears to lie with ALS itself. Although the presence of ALS was shown previously to have no effect on the binding of IGF-I or IGF-II to natural human IGFBP-3, it caused a severalfold

increase in the affinity of IGF-I for proteolyzed IGFBP-3 (Baxter et al., 1989; Baxter and Skriver, 1993). Thus it may be concluded that the circulating IGF complex in pregnancy serum appears normal, despite limited proteolysis of IGFBP-3, because the presence of ALS serves to increase the affinity of ALS for IGF-I. Indeed, since IGFBP-3 levels are disproportionately increased throughout pregnancy, so that the ratio of immunoreactive ALS:IGFBP-3 rises with increasing gestation (Suikkari and Baxter, 1992), ALS may have an increasingly protective effect on the circulating IGF complex as pregnancy develops.

A similar decline in serum IGFBP-3 detectable by ligand blot has been reported in rodents, attributable to a cation-requiring serine protease (Davenport et al., 1990; Fielder et al., 1990; Gargosky et al., 1990). In contrast to humans, however, this is accompanied by a marked decrease in circulating IGF-I, at least after day 10 of pregnancy (Donovan et al., 1991). The fall in IGF-I does not appear to be a consequence of IGFBP-3 proteolysis, since steady-state IGF-I mRNA levels are decreased by 60% in pregnant rat liver (Davenport et al., 1990). Further investigation of the enzymes involved in IGFBP-3 proteolysis has implicated the matrix metalloproteinases (MMPs), since TIMP-1, a specific inhibitor of all MMPs, or antibodies against MMP-1 and MMP-3, inhibited the IGFBP-3 proteolytic activity in rat pregnancy serum (Fowlkes et al., 1994b). As discussed by Fowlkes et al., the MMPs are cation-dependent, and their precursors may be activated by serine proteases, thus accounting for the inhibition of pregnancy serum protease activity by serine protease inhibitors. Furthermore, at least three enzymes of the MMP class increase during human pregnancy, while the inhibitor TIMP-1 decreases, consistent with the human pregnancy serum IGFBP-3 proteolysis (Fowlkes et al., 1994b).

In contrast to the findings in humans and rodents, an increase, rather than a decline, in serum IGFBP-3, as detected by ligand blotting, is seen in pregnant nonhuman primates (Giudice et al., 1993). Measured by radioimmunoassay, the increase is over twofold in the rhesus monkey and 12-fold in the baboon. This remarkable difference between humans and other primates has been attributed to their different placental strucures, since only in species with an invasive trophoblast does serum MMP activity, and IGFBP-3 proteolysis, increase during pregnancy. This would support the concept that the enzymes involved are of placental origin (Giudice et al., 1993; Fowlkes et al., 1994b).

IGFBP-3 proteolysis, as detected by reduced activity by ligand blotting, is also present in human serum in various catabolic states. This is seen in severe illness (Davies et al., 1991), following surgery (Davenport et al., 1992a), in non-insulin dependent diabetes mellitus (Bang et al., 1994), and in patients with malignancies (Muller et al., 1994). Nutritional state appears to be one factor affecting serum protease levels, which were higher in severely ill patients when fasting than when undergoing parenteral feeding (Davies et al., 1991). Figure 4 illustrates a study from the author's laboratory, comparing the degradation of iodinated natural human IGFBP-3 by serum from healthy nonpregnant, pregnant, and critically ill subjects, after incubation at 37 °C, fractionation by SDS-PAGE, and detection by phos-

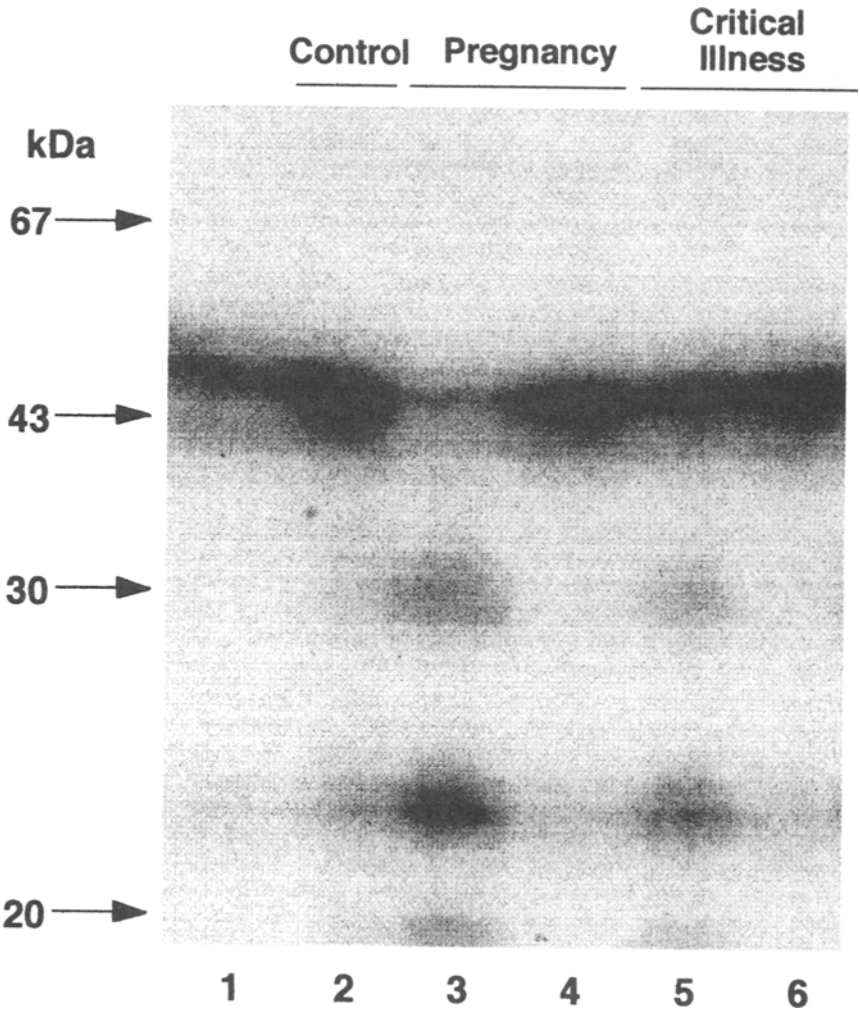

Figure 4. Serum proteolysis of IGFBP-3 in pregnancy and critical illness. Serum samples were incubated at 37 °C with radiolabeled natural human IGFBP-3 tracer, and the reaction mixtures were fractionated by non-reducing SDS-PAGE and detected by phosphorimaging. Lane 1: ^{125}I-IGFBP-3 tracer alone, showing the characteristic glycosylated doublet; lane 2: tracer incubated with normal serum; lanes 3 and 4: tracer incubated with third-trimester pregnancy serum, in the absence or presence, respectively, of 10 mM EDTA; lanes 5 and 6: tracer incubated with serum from a critically ill patient, in the absence or presence, respectively, of 10 mM EDTA. Degradation of IGFBP-3 to products of 30, 24, and 20 kDa in pregnancy and critical illness is substantially inhibited by EDTA, indicating the action of a cation-dependent protease.

phorimager. The pattern of degradation products is identical in pregnancy and critical illness, with major bands seen at 30, 24, and 20 kDa and, in both cases, the presence of EDTA inhibits degradation.

Most authors studying circulating IGFBP-3 proteolysis have interpreted it in terms of altering IGF bioavailability, increasing the access of IGFs to tissues to overcome catabolism, or to enhance uterine or fetal growth. Although this is an attractive hypothesis, it has been difficult to provide direct experimental support for it. In pregnancy, for example, the increased circulating concentrations of IGF-I and IGF-II argue against a lower binding affinity within the complex, or enhanced release of complexed IGFs to the tissues. To quantitate IGF bioavailability *in vitro*, Blat et al. (1994) compared the ability of pregnancy and nonpregnancy serum samples with identical IGF-I and IGF-II content to stimulate DNA synthesis in chick embryo fibroblasts. The stimulation, which was largely inhibitable by anti-IGF antibodies, was twice as high in pregnancy than nonpregnancy serum, suggesting greater IGF bioavailability in pregnancy. However, the contributions of other IGFBPs, and other IGF-modulating factors which change in pregnancy, and the effect of altered binding equilibria caused by serum dilution in the *in vitro* assay system, are impossible to assess, leaving the consequence of IGFBP-3 proteolysis on IGF bioavailability *in vivo* an open question.

IGFBPs apart from IGFBP-3 may also be affected by circulating proteases. Ligand blotting of pregnancy human serum has revealed a loss of binding proteins believed to correspond to IGFBP-2 and IGFBP-4, after 8 weeks gestation (Giudice et al., 1993). *In vitro* studies with iodinated pure IGFBPs have shown that IGFBP-4 and IGFBP-5, but not IGFBP-1 or IGFBP-6, are degraded by incubation with pregnancy serum, the effect being cation-dependent (Claussen et al., 1994). Whether the enzymes involved are the same as those which proteolyze IGFBP-3 is unknown, and the significance of such findings will remain obscure until the functions of these IGFBPs in the circulation are elucidated.

Cell and Tissue IGFBP Proteases

In parallel with the observation of serum IGFBP-3 proteolysis in pregnancy, a variety of tissues from pregnant rats were shown to be able to degrade IGFBP-3 (Davenport et al., 1992b). While highest levels of proteolysis were seen in decidua and other reproductive tissues, brain, liver, and spleen also had measurable activity. Schmid et al. (1991) found that carboxy-terminally truncated IGFBP-3 had lost the ability to inhibit IGF-I-stimulated osteoblast growth, leading to the idea that IGFBP proteolysis might be an important regulatory mechanism operating at the cellular level. Since then, cell culture studies have revealed proteolytic activity for IGFBP-2, -3, -4, and -5 in many different cell types.

IGFBP-2

In porcine smooth muscle cells, a serine protease has been shown to degrade IGFBP-2. After cation depletion, activity could be restored by calcium but not zinc.

This proteolytic activity was found to be enhanced by IGF-II, and to a smaller extent, IGF-I (Gockerman and Clemmons, 1995). Although in this respect the enzyme involved resembles an IGFBP-4 protease produced by smooth muscle cells, zymographic analysis suggests that the IGFBP-2 protease is 36 kDa, smaller than the 48-kDa IGFBP-4-degrading enzyme (Parker et al., 1995). The products of proteolysis by smooth muscle cells appear to be fragments of 22 and 14 kDa (Cohick et al., 1995). Two IGFBP-2 proteolysis products have also been identified in human milk, although the source and type of the protease responsible is unknown (Ho and Baxter, 1994). Amino-terminal sequencing indicated that these fragments were generated by cleavage of IGFBP-2 after Lys^{168} and Lys^{180}, yielding peptides of apparent size 12-14 kDa which retain weak IGF binding activity, as determined by ligand blotting.

IGFBP-3

In human skin fibroblasts, IGF-I has been shown to increase the concentration of IGFBP-3 in the cell medium by a mechanism that does not require interaction with the type I receptor (Conover, 1991a; Neely and Rosenfeld, 1992). While there is evidence that this may be due in part to release of IGFBP-3 from cell surface or matrix stores (Martin et al., 1992), Camacho-Hubner et al. (1992) reported that IGF-I was acting to inhibit IGFBP-3 proteolysis. Conover (1992) also observed that cell-associated IGFBP-3 in bovine fibroblasts was subject to degradation to smaller forms, a process that has been implicated in the ability of IGFBP-3 to potentiate IGF action after preincubation with cells. A subsequent analysis of fibroblast culture medium by immunoblotting specifically identified precursor forms of the proteases MMP-1, -2, and -3, corresponding in size to bands of IGFBP-3 proteolytic activity seen by zymography (Fowlkes et al., 1994a). The major IGFBP-3 cleavage site for these MMPs was shown to be after Tyr^{99} in the central domain of the protein, yielding fragments of 10.8 and 19.0 kDa; MMP-3 additionally cleaved after Asn^{109} and Glu^{176}. Another, acid-activated IGFBP-3 protease in fibroblast medium was identified by its low pH optimum, inhibition by pepstatin, and immunoreactivity, as cathepsin D (Conover and De Leon, 1994). Interestingly, in that study, no activity corresponding to the MMPs was detected, possibly due to the presence of MMP inhibitors. Cathepsin D also acted on IGFBP-3 in breast cancer and osteoblastic cell lines. Although it may be postulated that estrogen-stimulated proteolysis of IGFBP-3 by cathepsin D could in part be involved in the growth regulation of estrogen-dependent breast cancer cells, the very acidic pH optimum of this enzyme leaves the exact mechanism unclear.

In human osteosarcoma cells, a different IGFBP-3 proteolysis system has been described. Lalou et al. (1994) reported the presence of a plasminogen activator/plasminogen activator inhibitor system, regulated by IGF-I, and capable of leading to IGFBP-3 proteolysis, suggesting that IGFBP-3 processing by plasmin may be important in these cells. Finally, another cancer where IGFBP-3 proteolysis may

be implicated in growth regulation is carcinoma of the prostate. In 1992 the interesting observation was made that prostate-specific antigen (PSA) can proteolyze IGFBP-3 (Cohen et al., 1992); proteolytic activity for IGFBP-4 and -5 has now also been demonstrated (Lee et al., 1994). PSA-cleaved IGFBP-3 has only a slightly reduced affinity for IGF-II, but a 10-fold reduced affinity for IGF-I (Cohen et al., 1994). Since the inhibition by IGFBP-3 of IGF-stimulated prostate epithelial cell growth was reversible by PSA, the enzyme has the potential to be growth-stimulatory to prostate cancer cells. The sites of IGFBP-3 cleavage by PSA have recently been identified (Fielder et al., 1994). At least seven fragments are generated, several of which retain some IGF-binding activity. A major cleavage site was identified after Tyr^{159}, with others after Arg^{97} and Phe^{173}. While some of the identified sites were described as "kallikrein-like," consistent with the classification of PSA as one of the kallikrein family, two sites were more typical of hydrolysis by chymotrypsin (Fielder et al., 1994).

IGFBP-4

IGF-I, which increases the concentrations of IGFBP-3 and IGFBP-5 in human skin fibroblast culture medium, was independently observed in several laboratories to lead to the disappearance of the 24 kDa binding protein, IGFBP-4 (Conover, 1991a; Camacho-Hubner et al., 1992; Fowlkes and Freemark, 1992; Martin et al., 1992; Neely and Rosenfeld, 1992). This phenomenon has now been extensively investigated in several cell types. In fibroblasts, IGF-II was found to be more potent than IGF-I in stimulating IGFBP-4 proteolysis, and the IGF stimulation was shown to be blocked by phorbol esters (Conover et al., 1993a,b). Although it has not been clear whether the IGF effect was due to occupancy of IGFBP-4 or direct stimulation of the protease, the observation that an IGF-II:IGFBP-4 ratio of 1:4 induced complete hydrolysis was more consistent with the latter explanation (Conover et al., 1993b). However, since IGF analogues with little or no affinity for IGFBP-4 fail to induce proteolysis (Kanzaki et al., 1994), the IGF specificity of the enzyme, if it is directly activated by IGFs, would have to be very similar to that of the IGFBP-4 itself.

IGF-dependent IGFBP-4 proteolysis is also active in bone-derived cell cultures, where products of 14 and 18 kDa have been identified (Kanzaki et al., 1994; Durham et al., 1994). The observation that IGF-II treatment of osteoblast cultures (leading to IGFBP-4 proteolysis) is able to enhance IGF-I-stimulated DNA synthesis provides evidence that IGFBP-4 plays a regulatory role in these cells.The biological importance of IGFBP-4 protease has been further investigated using protease-resistant IGFBP-4 variants (Conover et al., 1995). After analysis of degradation products indicated a site of cleavage after Met^{135} of IGFBP-4, this residue was substituted to inhibit proteolysis, although, surprisingly, extended incubation with cell-conditioned medium still resulted in degradation to 14 and 18 kDa forms. Over shorter incubation times, the variant showed resistance to prote-

olysis, and was able to block the enhancement by IGF-II of IGF-I-stimulated DNA synthesis (Conover et al., 1995), further implicating IGFBP-4 proteolysis in cell growth regulation.

Proteolysis of IGFBP-4 in different cell types is not necessarily due to the activity of the same enzyme, or enzyme class, as there are discrepancies between fragment sizes and cleavage sites reported in different studies. For example, whereas cleavage after Met^{135} was observed in fibroblasts, a 16 kDa IGFBP-4 fragment generated by a neuronal cell protease terminated at Lys^{120}, with another fragment starting at residue 132 (Chernausek et al., 1995). In cultured bone cells, the enzyme was described as being between 67 and 160 kDa (Kanzaki et al., 1994), whereas the protease in smooth muscle cells was 48 kDa (Parker et al., 1995). However, all of the enzymes appear to act on sites in the central, nonconserved domain of IGFBP-4, which may account for their specificity for a unique IGFBP.

IGFBP-5

Human skin fibroblasts secrete proteolytic activity capable of reducing IGFBP-5 to a nonbinding form of 23 kDa (Camacho-Hubner et al., 1992). The activity is partly inhibited by IGF-I or IGF-II, but not insulin, which may contribute to the remarkable increase in 31 kDa IGFBP-5 elicited by the IGFs (Martin and Baxter, 1990; Camacho-Hubner et al, 1992). Purification of the enzyme involved revealed a calcium-dependent serine protease that could generate 22, 20, and 17 kDa fragments. Heparin was found to inhibit the proteolysis (Nam et al., 1994), consistent with a previous demonstration that heparin treatment increased the concentration of IGFBP-5 (identified as a 30-32 kDa IGFBP) in fibroblast medium (Martin et al., 1992). Osteoblasts have also been shown to produce IGFBP-5-proteolytic activity, partly inhibitable by IGF-II when added to cell cultures, but not when added to conditioned, protease-containing medium (Kanzaki et al., 1994).

Studies in rat granulosa cells suggest an important role for IGFBP proteolysis in ovarian follicle development. IGFBP-4 and -5, detected in primary granulosa cell culture medium, were absent when the cells were incubated with follicle-stimulating hormone (FSH), with only IGFBP-4 fragments of 21.5 and 17.5 kDa, and an IGFBP-5 band of 21 kDa, detectable (Liu et al., 1993). The loss of the intact IGFBPs in the presence of FSH was shown to be due in part to a cessation of new synthesis, complemented by the proteolysis of existing IGFBPs. Together, these mechanisms were postulated to contribute to IGF-I-stimulated, FSH-dependent follicle development. In keeping with their action in other cell types, IGFs were shown to inhibit the FSH-induced IGFBP-5 proteolysis, suggesting a complex regulatory interplay between FSH and the growth factors (Fielder et al., 1993).

Taken together, the numerous studies described above indicate that the controlled hydrolysis of IGFBPs to smaller forms of reduced affinity is a widespread mechanism by which cells modulate IGF-dependent growth and other functions. A broad range of enzymes, including members of the the kallikrein, MMP, and

cysteine protease classes, has been implicated, and even for a single IGFBP, there may be a variety of cleavage sites, depending on the cell type involved. In all cases, however, cleavage occurs in the nonconserved central domain of the IGFBPs, a region thought to be less structurally constrained than the highly disulfide-bonded amino- and carboxy-terminal domains. IGFBP proteolysis at the cellular level appears important in the regulation both of normal physiological processes, such as ovarian follicle selection, and pathological process such as the growth of malignant mammary and prostatic epithelial cells. The physiological significance of circulating IGFBP proteolysis in pregnancy and catabolic states is somewhat less obvious, but the widespread view that it leads to increased IGF availability to the tissues appears to be a very plausible working hypothesis on which to base future experimentation.

SUMMARY

IGF actions are modulated by a family of six proteins, the IGFBPs. This review discussed structural aspects of the proteins and their genes, emphasizing in particular the posttranslational modifications—glycosylation, phosphorylation and proteolysis—which are believed to be important modifiers of IGFBP activity. The IGFBP genes all contain four coding exons, with only the IGFBP-3 gene having a fifth, non-coding exon. Consistent with a relatively conserved gene structure, the proteins themselves, all approximately 20-30 kDa in size, are highly conserved in primary structure, each consisting of three domains: cysteine-rich amino-terminal and carboxy-terminal domains with marked sequence similarity among all of the proteins, and central domains showing no conservation. Putative sites of *N*-linked glycosylation (three in IGFBP-3, and one in IGFBP-4 and -6) occur in this region, and appear occupied by carbohydrate in IGFBP-3 and -4, whereas IGFBP-1, -5, and -6 have varying amounts of *O*-linked carbohydrate. All of the IGFBPs bind both IGF-I and IGF-II, with IGFBP-6 having an outstanding preferential affinity for IGF-II. IGFBP-3, alone among the IGFBPs, binds to another ligand, the 85 kDa, leucine-rich acid-labile subunit, with which it forms a ternary complex, together with IGF-I or IGF-II, in the circulation.

Certain of the IGFBPs can associate with cell surfaces or matrix, in the case of IGFBP-1 and -2, via an Arg-Gly-Asp motif which interacts with receptors of the integrin class, and in the case of IGFBP-3 and -5 via a highly basic motif in their carboxy-terminal domain. IGFBP-1, -3, and -5 can be serine-phosphorylated, resulting in changes in their cell-binding and/or IGF affinity. Finally, limited proteolysis by enzymes of a variety of classes can convert IGFBPs to stable forms of lower molecular weight with reduced IGF-binding affinity. These proteases are postulated to be of major importance in modulating the ways in which IGFBPs affect IGF activity in the cellular environment, and may also be important in determining the bioavailability of circulating IGFs.

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Chapter 6

Molecular Basis of Insulin Action

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INTRODUCTION

Insulin is a small protein (Mr 5,600) that is initially synthesized in the β cells of the pancreatic islet as a preprohormone (Hutton, 1990; Steiner et al., 1993; Bennett and Hutton, 1994). Cotranslational cleavage of the signal sequence during translo-

Advances in Molecular and Cellular Endocrinology
Volume 1, pages 161-202.

ISBN: 0-7623-0158-9

cation across the endoplasmic reticulum results in the formation of proinsulin. Sequestration of proinsulin into an endosomal compartment occurs in concert with proteolytic processing of the internal C peptide (Hutton, 1990; Steiner and James, 1992; Steiner et al., 1992). This maturation process results in the formation of an active form of insulin which is composed of an A and B chain in which there is one intrachain and two interchain disulfide bonds. Insulin is stored in the secretory vesicles of the islet β cells and is released in response to various stimuli (Li et al., 1994a). This occurs primarily following a meal in response to ingestion and absorption of nutrients, most notably glucose. In addition to the regulation of glucose homeostasis, insulin is also essential in stimulating the synthesis of glycogen, triglyceride, protein, RNA, and DNA as well as participating in cell growth and development. Thus, the release of insulin into the circulation has pleiotropic effects on whole body metabolism and is the most potent anabolic hormone known.

In mammals the three major insulin responsive tissues are adipocytes, liver, and muscle. In the liver, insulin functions to increase glycogen synthesis and inhibit gluconeogenesis. In muscle, insulin enhances glycogen synthesis and stimulates glucose uptake, whereas in adipocytes, insulin promotes triglyceride synthesis in addition to stimulating glucose transport. Thus, these different tissues display overlapping but distinct responses to insulin. The sum total of these and other tissue-specific biological responses are ultimately responsible for the maintenance of normal homeostasis, growth, and development.

All of the biological effects of insulin require the presence of a plasma membrane localized insulin receptor, which binds circulating insulin with a high degree of specificity and affinity. The insulin receptor functions to transduce extracellular binding of the ligand into the activation of intracellular signaling pathways. It is the activation of these pathways which are responsible for mediating the appropriate cellular response. However, the molecular basis for insulin signaling in responsive cells is currently unknown. Defining the precise pathways involved in insulin receptor signaling will undoubtedly be of enormous value not only in understanding the basic molecular signaling machinery but also in the defects associated with various insulin resistant states such as non-insulin dependent diabetes mellitus (NIDDM). This disease affects approximately 5% of the population and is predominantly characterized by insulin resistance in peripheral target tissues (DeFronzo et al., 1992; Granner and O'Brien, 1992). Thus, numerous laboratories continue to invest a substantial effort towards understanding the signaling pathways utilized by insulin and their dysregulation in the pathology associated with diabetes. In this chapter, we will review the current molecular concepts regarding insulin receptor signaling specificity and its relationship to other tyrosine kinase and non-tyrosine kinase receptor signaling pathways.

INSULIN RECEPTOR STRUCTURE

The mature insulin receptor (Ebina et al., 1985 Ullrich et al., 1985), present at the cell surface, is classified as a Type I receptor (Ullrich and Schlessinger, 1990; van der Geer et al., 1994) being composed of two α subunits and two β subunits which are disulfide-linked into an $\alpha_2\beta_2$ heterotetrameric complex (Czech, 1985; Kahn, 1985; Rechler and Nissley, 1985; Goldfine, 1987)(Figure 1). The receptor subunits are initially synthesized as an αβ polypeptide fusion precursor which undergoes extensive co- and posttranslational modifications (Jacobs and Cuatrecasas, 1983; Ullrich et al., 1985; Knutson, 1991; Taylor et al., 1992). Initially, the Mr 155,000 αβ receptor precursor undergoes cotranslational acylation and Asn-linked glyco-

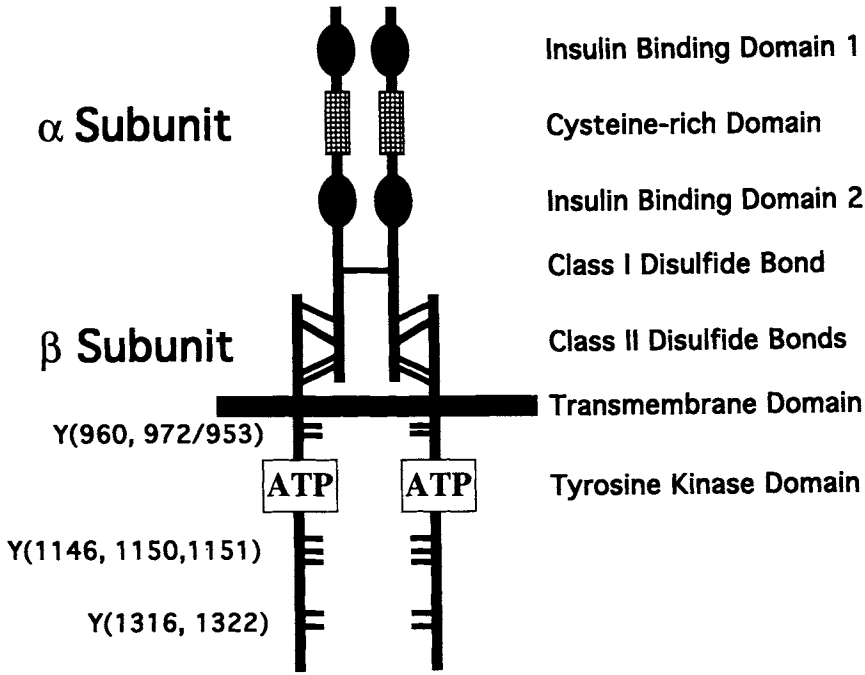

Figure 1. Schematic representation of the human insulin receptor. The mature insulin receptor (Ex11-) is composed of two α subunits (723 amino acids) and two transmembrane β subunits (620 amino acids). The α subunits are composed of two regions responsible for high affinity insulin binding separated by a cysteine rich domain. The two α subunits are covalently attached to each other through a disulfide linkage and anchored to the plasma membrane via disulfide linkages with the transmembrane β subunits. The intracellular domain of the β subunits contains a consensus tyrosine kinase domain and three clustered regions of tyrosine autophosphorylation acceptor sites numbered according to Ullrich et al. 1985 for the Ex11- isoform.

sylation. As the precursor is processed from the endoplasmic reticulum through the trans-Golgi network, intramolecular disulfide bonds are formed linking the a subunit to the b subunit. Concurrently, two of the αβ fusion protein precursors non-covalently dimerize and are proteolyzed at a tetrabasic sequence (RKRR) separating the a subunit from the b subunit. The dimerized αβ half-receptors are then covalently linked through the formation of a disulfide bond between the two a subunits. Coincident with this is the addition of terminal sialic acid residues and emergence of the native $\alpha_2\beta_2$ heterotetrameric receptor at the cell surface.

Although the insulin receptor is derived from a single gene copy (Seino et al., 1989), there are several mechanisms which result in insulin receptor heterogeneity. Splicing variation in the primary human insulin receptor transcript results in two different mRNAs that differ only by the inclusion in one of a small 36 bp exon (Exon 11) (Seino and Bell, 1989). This exon encodes for a 12 amino acid insert at the carboxyl-terminal end (Arg 723) of the α subunit, which is just amino-terminal to the tetrabasic cleavage site separating the α subunit from the β subunit. Translation of these transcripts results in two naturally occurring forms of the human insulin receptor (HIR-A and HIR-B). The liver predominantly expresses Ex11+ or the HIR-B isoform, whereas muscle primarily expresses the Ex11- or HIR-A isoform (Benecke et al., 1992). In contrast, adipocytes and placenta express both isoforms at approximately equal levels (Benecke et al., 1992). The different tissue distribution of these two isoforms may underlie a mechanism by which insulin responsiveness can be fine tuned. Several studies have observed that the Ex11+ insulin receptor has an approximately twofold reduction in binding affinity relative to the Ex11- form and a concomitant reduction in the rate of receptor internalization stimulated by insulin (McClain, 1991; Vogt et al., 1991; Yamaguchi et al., 1991, 1993; Kellerer et al., 1992). Since newly secreted insulin initially passes through the liver, the lower binding and internalization rate of hepatocytes may be necessary to allow for and perhaps regulate the amount of insulin entering the general circulation (McClain, 1991).

A second level of insulin receptor heterogeneity results from the ability of the αβ half-receptor precursors to dimerize with non-identical partners. For example, many cells express the related insulin-like growth factor 1 (IGF1) receptor, which is also synthesized as an αβ precursor and undergoes a similar maturation process resulting in an $\alpha_2\beta_2$ heterotetrameric IGF1 receptor (Ullrich et al., 1986). Several studies have documented that in cells expressing both the insulin and IGF1 receptors, insulin and IGF1 αβ half-receptors dimerize and are processed into mature insulin/IGF1 hybrid receptors. Each hybrid receptor is composed of an αβ insulin half-receptor and an αβ IGF1 half-receptor (Jonas et al., 1986; Moxham et al., 1989; Soos et al., 1990; Chin et al., 1991; Garofalo and Barenton, 1992; Kasuya et al., 1993; Grako et al., 1994; Langlois et al., 1995; Seely et al., 1995b). Functional studies have demonstrated that these heterotypic hybrid receptors have distinct binding affinities compared to the homotypic insulin and IGF1 receptors (Frattali

and Pessin, 1993; Soos et al., 1993). In addition to their potential role in cellular regulation, the isolation of insulin/IGF1 hybrid receptors provided an important experimental tool in our understanding of the mechanism by which extracellular insulin binding stimulates activation of the intracellular protein kinase domain (Treadway et al., 1989a, 1991, 1992; Frattali and Pessin, 1993).

As indicated above, the insulin receptor α subunits encompass the insulin binding site(s) and are exclusively located on the exofacial side of the plasma membrane. Both molecular and biochemical approaches have identified various α subunit domains responsible for its specificity and high affinity ligand binding. The primary determinants of the insulin binding domain appear to be very complex, with domains located within the α subunit amino terminus, the cysteine-rich domain as well as in regions carboxyl-terminal to the cysteine-rich domain (Yip et al., 1988, 1991; Gustafson and Rutter, 1990; Kjeldsen et al., 1991; Schumacher et al., 1991; Zhang and Roth, 1991) (Figure 1).

It appears that each α subunit contains both a high affinity and low affinity insulin binding domain such that insulin occupancy results in the physical bridging of the two adjacent α subunits. This model is consistent with the kinetics of insulin binding and is analogous to that established for the binding of growth hormone to the growth hormone receptor.

Insulin binding to the α subunits results in an intramolecular signal that activates the tyrosine-specific protein kinase domain of the β subunit located within the cell. Like all growth factor receptor tyrosine kinases so far tested, the insulin receptor undergoes a series of intramolecular trans-autophosphorylation reactions in which one β subunit phosphorylates the adjacent β subunit on specific tyrosine residues (Shia et al., 1983; Sweet et al., 1985; Boni-Schnetzler et al., 1988; Treadway et al., 1989a; Shoelson et al., 1991; Frattali et al., 1992; Lee et al., 1993b). Each β subunit contains eight tyrosine phosphorylation acceptor sites located at amino acid positions 953, 960, 972, 1146, 1150, 1151, 1316, and 1322 (White et al., 1984; Tornqvist et al., 1987; Tavare and Denton, 1988; Feener et al., 1993; Kohanski, 1993). Autophosphorylation of these tyrosine residues underlies several distinct functional properties of the insulin receptor kinase. Several studies have demonstrated that tyrosine phosphorylation of the carboxyl terminal 1316 and 1322 residues regulates the mitogenic actions of insulin (Thies et al., 1989; Myers et al., 1991; Ando et al., 1992). Interestingly, these residues appear to direct the association of the insulin receptor with the p85 regulatory subunit of phosphatidylinositol 3-kinase (Backer et al., 1992b; Yamamoto et al., 1992; Seely et al., 1995a). Similarly, tyrosine phosphorylation of the juxtamembrane residues 960 and/or 953/972 appears to enhance the ability of the insulin receptor to associate and tyrosine phosphorylate two important substrates (Shc and IRS1) which are important intermediates in several downstream signaling pathways (White et al., 1988a; Kaburagi et al., 1995). In addition, tyrosine residues 953 and 960 are located within consensus sites for clathrin-coated pit mediated internalization (Rajagopalan et al., 1991).

Tyrosines 1146, 1150 and 1151 are found within the kinase domain and phosphorylation of these residues markedly stimulates the substrate tyrosine kinase activity of the insulin receptor β subunit (Klein et al., 1986; Kohanski and Lane, 1986; Yu and Czech, 1986; White et al., 1988b; Flores-Riveros et al., 1989). In fact, following phosphorylation of two or three of these sites, insulin is no longer required for the substrate kinase activity of the insulin receptor. Recent analysis of the crystals prepared from a truncated insulin receptor β subunit demonstrated that tyrosine 1150 is located within the catalytic pocket functioning as an autoinhibitory substrate (Hubbard et al., 1994). Once this tyrosine is phosphorylated, the β subunit undergoes a conformational change in which this residue swings out of the catalytic site making it accessible to exogenous substrates. These data are not only consistent with an intramolecular trans-autophosphorylation mechanism, but also indicate that dephosphorylation of this residue may be necessary for inactivation of the insulin receptor substrate kinase.

Contrary to the majority of experimental data indicating that the insulin receptor kinase activity is essential for insulin action, several early structure-function studies suggested that not all of the actions of insulin require either receptor autophosphorylation or the tyrosine phosphorylation of endogenous receptor substrates. For example, a point mutant replacing the catalytic lysine residue in the active site with alanine was reported to mediate insulin stimulation of pyruvate dehydrogenase activity as effectively as the wild-type insulin receptor (Gottschalk, 1991). Another report indicated that ATP binding alone, and not its hydrolysis, was sufficient to produce the conformational change in the insulin receptor β subunit necessary for activating the downstream biological actions of insulin (Maddux and Goldfine, 1991; Baron et al., 1992). Other studies utilizing certain insulinomimetic antibodies demonstrated insulin-like responses in cells in the absence of any measurable receptor tyrosine phosphorylation (Hawley et al., 1989). However, these data are difficult to interpret since cellular responses to insulin require the activation of only a very small percentage of the insulin receptors, which could be below the level of detection. Nevertheless, based upon the recent identification and molecular analysis of several key intermediates, it is generally accepted that the activity of the insulin receptor tyrosine kinase is absolutely essential for activation of the signaling pathways linked to these effectors.

Insulin activation of the insulin receptor also results in serine/threonine phosphorylation of the β subunit, which occurs subsequent to autophosphorylation on tyrosine residues (Pang et al., 1985). It has been suggested that these phosphorylations result in receptor desensitization since the insulin receptor substrate kinase activity appears to decrease following serine/threonine phosphorylation (Treadway et al., 1989b). Several kinases potentially involved in the serine/threonine phosphorylation of the insulin receptor have been proposed, including the cAMP-dependent protein kinase and protein kinase C (Bollag et al., 1986; Jacobs and Cuatrecasas, 1986; Stadtmauer and Rosen, 1986; Takayama et al., 1988; Lewis et al., 1990; Chin et al., 1993). However, since the effect of counter-regulatory

hormones are very rapid and generally occur before increases in insulin receptor serine/threonine phosphorylation are discernible, the physiological significance of these findings remains to be determined.

INSULIN RECEPTOR KINASE SUBSTRATES

The first evidence for an insulin receptor specific substrate came from early studies by White and colleagues who demonstrated the insulin-stimulated tyrosine phosphorylation of a 185 kDa cytosolic protein using phosphotyrosine immunoblotting (White et al., 1985). Subsequently, tyrosine phosphorylation of this protein was detected in several insulin-responsive cell types and was originally referred to as pp185 based on its migration in SDS-polyacrylamide gels. The eventual purification of pp185 from rat liver and 3T3L1 adipocytes led to the cloning of its cDNA and its designation as IRS1 for insulin receptor substrate 1 (Keller et al., 1991 Rothenberg et al., 1991; Sun et al., 1991). Human IRS1 has been cloned from a hepatic carcinoma cell line and from skeletal muscle (Nishiyama and Wands, 1992; Araki et al., 1993). More recently, the cDNA for a protein highly related to IRS1, termed IRS2, has been cloned and found to have a similar function and tissue distribution (Sun et al., 1995).

Another insulin receptor substrate termed Shc, for Src homology 2/α collagen related, was identified and its cognate cDNA cloned based upon its homology with the human *c-fes* SH2 domain (Pelicci et al., 1992; Kovacina and Roth, 1993; Pronk et al., 1993; Skolnik et al., 1993). The Shc proteins are actually composed of three distinct species (46, 52, and 66 kDa) which are derived from a single gene copy. The 46 and 52 isoforms result from a single transcript containing two different translation initiation sites, whereas the 66 kDa species results from an alternative spliced transcript containing additional 5′ sequences (Pelicci et al., 1992).

Although IRS1 and Shc are currently the best characterized insulin receptor substrates, evidence exists for several other substrate proteins. Insulin stimulation increases the tyrosine phosphorylation of the liver p120 ecto-ATPase, the adipocyte fatty acid binding protein 422/aP2, and a RasGAP-associated 62 kDa protein, which has sequence homology with the small nuclear RNA binding proteins that may be involved in mRNA processing (Laio et al., 1991; Porras et al., 1992; Najjar et al., 1993; Sung et al., 1994). However, it is not known if these proteins are direct substrates of the insulin receptor kinase or if these tyrosine phosphorylations have any physiological significance. In primary isolated rat adipocytes, insulin also stimulates the rapid tyrosine phosphorylation of a 60 kDa protein which is unrelated to the RasGAP-associated 62 kDa protein (Momomura et al., 1988; Mooney et al., 1989). Interestingly, tyrosine phosphorylation of pp60 is only detected in primary rat adipocytes and not in rat liver or muscle, perhaps indicating it may be an adipocyte specific protein (Rothenberg et al., 1991). Recently, a tyrosine phosphorylated 55 kDa protein, termed $p55^{PIK}$, which has strong sequence similarity to the

p85 regulatory subunit of phosphatidylinositol 3-kinase, has been identified and its cDNA cloned (Pons et al., 1995). The smaller size of $p55^{PIK}$ relative to p85 results from a deletion of the amino terminal SH3 and bcr domain (see below). Similar to p85, $p55^{PIK}$ is constitutively associated with the catalytic p110 phosphatidylinositol 3-kinase subunit and binds tyrosine phosphorylated IRS1 through the $p55^{PIK}$ SH2 domains resulting in a small activation of phosphatidylinositol 3-kinase activity. Although $p55^{PIK}$ itself appears to be tyrosine phosphorylated in insulin-stimulated cells, this event does not have a significant effect on its association with IRS1 or on phosphatidylinositol 3-kinase activity. Thus, the physiological function of this protein remains to be determined.

A major breakthrough in our understanding of receptor tyrosine kinase signaling was made by Pawson and colleagues who identified the src homology 2 (SH2) binding motif within the protein kinase src (Pawson and Gish, 1992; Pawson, 1995). Parenthetically the src homology 1 (SH1) domain is the catalytic tyrosine kinase domain in src. SH2 domains are found in numerous intracellular proteins and are composed of approximately 100 amino acids which bind to phosphotyrosine residues embedded in specific sequence contexts. For example, the SH2 domains of the p85 regulatory subunit of phosphatidylinositol 3-kinase preferentially bind phosphorylated tyrosine within a YMXM or YXXM motif, whereas the SH2 domain of Grb2 has specificity for YINS or YVNS sequences (Zhou et al., 1993; Songyang et al., 1994). The binding of SH2 domains to tyrosine phosphorylated proteins can have several functions including enhancing the physical association of two proteins, affecting the subcellular location of a protein, or regulating enzyme activity by allosteric mechanisms (Myers et al., 1992; Skolnik et al., 1993; Sabe et al., 1994).

The deduced amino acid sequence of IRS1 indicated that this protein contained more than 20 potential tyrosine phosphorylation sites which might interact with various SH2 domain containing effectors. To date, IRS1 has been directly demonstrated to associate with the SH2 domains of phosphatidylinositol 3-kinase (Y608>Y939>Y460 and Y987), the small adapter protein Grb2 (Y895), and the protein tyrosine-specific phosphatase SHPTP2 (Y1172 and Y1222) (Sun et al., 1993; Sugimoto et al., 1994). The small adapter protein Nck has also been reported to associate with tyrosine phosphorylated IRS1 (Lee et al., 1993a), but the specific site on IRS1 involved in this interaction has not been determined. Co-immunoprecipitation experiments have also demonstrated that a single IRS1 molecule can simultaneously complex with several different SH2-domain containing proteins, suggesting that the formation of a multi-factor signaling complex is likely (Keller and Lienhard, 1994). Although the specific effectors interacting with IRS2 have not been reported, we expect a similar cadre of associations based on the sequence similarity of the IRS2 tyrosine phosphorylated docking sites with those of IRS1 (Figure 2).

In contrast to the multi-site phosphorylation of IRS1 and IRS2, Shc contains only one major tyrosine phosphorylation site (Y317) (Skolnik et al., 1993; Salcini

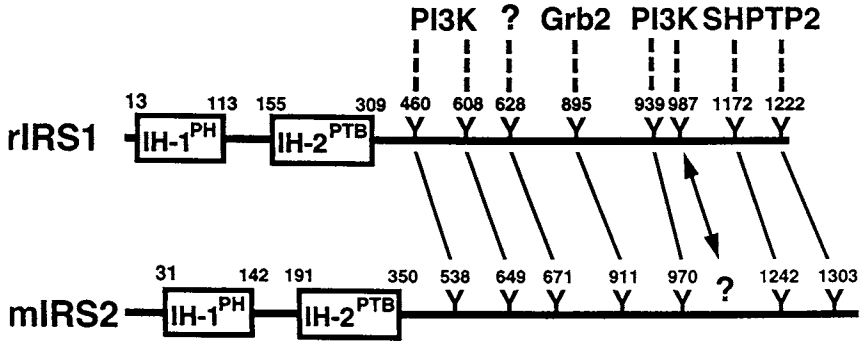

Figure 2. Structural features of rat insulin receptor substrate 1 (rIRS1) and mouse insulin receptor substrate 2 (mIRS2). The insulin receptor substrate homology 1 (IH-1) domain and the insulin receptor substrate homology 2 (IH-2) domain correspond to the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, respectively. The number of amino acid residues from the amino terminus defining the beginning and end of these domains is shown. *In vitro* phosphorylation experiments using purified insulin receptor and rIRS1 identified eight sites of tyrosine phosphorylation on rIRS1. The same sites were also identified in *in vivo* experiments and are indicated in the figure. The amino-terminal src homology 2 (SH2) domain of phosphatidylinositol 3-kinase (PI3K) binds to rIRS1 with the relative affinity order: Y608>Y939>Y460 and Y987. The equivalent IRS1 tyrosine phosphorylation site Y987 does not appear to be present in IRS2 as indicated by the double arrow. The SH2 domain of Grb2 binds phosphotyrosine 895 of rIRS1 and the SH2 domains of the tyrosine-specific phosphatase SHPTP2 binds phosphotyrosines 1172 and 1222.

et al., 1994). This site directs its association with the small adapter protein Grb2. Shc, IRS1, and IRS2 also contain newly described motifs termed protein tyrosine binding (PTB) domain, protein interaction domain (PID), or SAIN (Shc and IRS1 NPXY binding) domain (Blaikie et al., 1994; Bork and Margolis, 1995; Gustafson et al., 1995; Kavanaugh and Williams, 1995; Kavanaugh et al., 1995). This domain directs the interaction of both IRS1 and Shc with tyrosine 960, an autophosphory-

lation site in the juxtamembrane region of the insulin receptor. Thus, mutations in either the PTB domain or that of the insulin receptor tyrosine 960 greatly reduces the ability of the insulin receptor to tyrosine phosphorylate IRS1 or Shc (Chen et al., 1995; Kaburagi et al., 1995). Interestingly, IRS1 and IRS2 contain another interaction domain termed the pleckstrin homology (PH) domain (Mayer et al., 1993; Musacchio et al., 1993). The precise role of the PH domain has not yet been fully elucidated but may mediate the interaction with both the βγ subunits of trimeric G proteins and/or phosphatidylinositides (Touhara et al., 1994). Although the PH domain of IRS1 is not essential for insulin signaling, deleting it does reduce the tyrosine phosphorylation of IRS1 by the insulin receptor (Myers et al., 1995). This suggests that the PH domain of IRS1 may have a role in mediating the interaction of IRS1 with the insulin receptor.

In addition to SH2 domains, many proteins involved in intracellular signaling events also contain src homology 3 (SH3) domains that are responsible for the binding to various proline-rich motifs within a general consensus sequence of XPXXPPP¥XP, where ¥ is any hydrophobic amino acid (Ren et al., 1993). In contrast to SH2 domains which require recognition of a tyrosine phosphorylated residue, SH3 domains are associated with effector proteins in unstimulated cells. For example, Grb2 was identified as a 23 kDa growth factor receptor binding protein which contains a single SH2 domain flanked by two SH3 domains (Margolis et al., 1992). The SH3 domains of Grb2 direct its association with the proline-rich motifs of several proteins including the microtubule-associated protein dynamin (Scaife et al., 1994; Seedorf et al., 1994) and a 150 kDa guanylnucleotide exchange factor specific for Ras, termed SOS (Chardin et al., 1993; Li et al., 1993). SOS was originally cloned in *Drosophilia* and is the product of the Son of Sevenless gene (Simon et al., 1991). Association of the Grb2-SOS complex with tyrosine phosphorylated receptors, IRS1 and Shc have all been directly implicated in the activation of the Ras signaling pathway (described below).

The current identification of the various proximal substrates in receptor tyrosine kinase signaling is probably just the tip of a very large iceberg. Nevertheless, the elucidation of the SH2, SH3, PH and PTB domains that mediate specific types of protein interactions has provided us with an important conceptual framework by which we can address the critical issue of signaling specificity at the molecular level. Currently, IRS1, IRS2, and Shc are the best characterized proximal substrates of the insulin receptor; however, as other critical substrate molecules are identified, the specificity of tyrosine kinase receptor signaling will undoubtedly undergo substantial refinement.

ROLE OF IRS1 AND IRS2 IN BIOLOGICAL RESPONSIVENESS

Following the initial cloning of IRS1, it was observed that its expression in Chinese hamster ovary (CHO) cells transfected with the human insulin receptor (Sun et al.,

1992), or in unprimed Xenopus oocytes, resulted in a significant enhancement in insulin-stimulated DNA synthesis (Chuang et al., 1993). These data indicate that IRS1 functions as a mediator of insulin-stimulated mitogenesis. This hypothesis is consistent with other experiments utilizing anti-sense IRS1 RNA expression or injection of IRS1 blocking antibodies, which reduced cell growth and insulin-stimulated DNA synthesis (Waters et al., 1993 Rose et al., 1994). Recently, a more definitive role for IRS1 in mitogenesis has been established by the use of two myeloid progenitor cell lines. In the myeloid-derived FDC-P2 cell line, interleukin 4 (IL4) induces cell division and the tyrosine phosphorylation of a 190 kDa protein (termed 4PS). In contrast, mitogenesis in the 32D myeloid cell line is insensitive to IL4 (Wang et al., 1993a, b). Further examination of the 32D cells demonstrated the presence of functional IL4 receptors, but no detectable 4PS expression. Thus it was hypothesized that the IL4 insensitivity of the 32D cells was due to a lack of 4PS. Surprisingly, expression of IRS1 in the 32D cells not only restored IL4 responsiveness but, following coexpression with the insulin receptor, causes a remarkable stimulation of the mitogenic pathway in response to insulin (Wang et al., 1993b). Together, these data provided compelling evidence for an important role of IRS1/4PS in mediating insulin-stimulated mitogenesis in tissue culture cell lines.

To assess the signaling role of IRS1 *in vivo*, two groups independently produced IRS1 gene knockout mice using homologous recombination (Araki et al., 1994; Tamemoto et al., 1994). Homozygous mice lacking any functional IRS1 protein had reduced intrauterine growth rates and were approximately 50% the size of heterozygous or wild-type litter mates. This reduction in weight which was accompanied by normal development, supports a role for IRS1 in regulating growth. Interestingly, these animals also displayed a mild insulin-resistance characterized by normal basal glucose levels, fasting hyperinsulinemia and decreased glucose tolerance. This relatively small change in the phenotype of IRS1 knockout mice was surprising considering the dramatic developmental and cellular signaling defects that occurred in a patient which lacked the insulin receptor gene or in some patients which produce a truncated form of the insulin receptor (Wertheimer et al., 1993; Krook et al., 1994). Further analysis has suggested that the mild phenotype associated with these mice results from the presence of a second IRS1 isoform apparently identical to the 4PS protein found in the myeloid cell lines (Patti et al., 1995; Tobe et al., 1995). This protein, now termed IRS2 is approximately 190 kDa, which is slightly larger than the 185 kDa estimate for IRS1. In the liver, IRS2 is tyrosine phosphorylated by the insulin receptor, which directs its association with at least Grb2 and phosphatidylinositol 3-kinase (Patti et al., 1995). These data are also consistent with an earlier study in Fao hepatoma cells indicating the presence of a distinct, but IRS1 related, protein that is tyrosine phosphorylated in response to insulin (Miralpeix et al., 1992).

Based on the initial identification, characterization, and cloning of the IRS1 cDNA, this protein was thought to be a relatively specific substrate for the insulin and the related IGF1 receptor tyrosine kinases (Shemer et al., 1987; Lamphere and Lienhard, 1992). This concept was supported further by the fact that IRS1 is not tyrosine phosphorylated by the epidermal growth factor receptor or the platelet-derived growth factor receptor tyrosine kinase, (Kadowaki et al., 1987; Madoff et al., 1988). However, the subsequent identification of IRS1 and IRS2 as substrates for the IL4 receptor suggested that other receptors may also utilize IRS1 (Morla et al., 1988; Wang et al., 1992, 1993b). Recently it has been reported that IRS1 and IRS2 are substrates for the growth hormone, IL9, IL13, interferon a, interferon γ, and leukemia inhibitory factor receptors (Souza et al., 1994; Argetsinger et al., 1995; Uddin et al., 1995; Welham et al., 1995; Yin et al., 1995). These cytokine receptors do not contain any intrinsic tyrosine kinase activity, but instead associate with various members of the Janus family of protein tyrosine kinases (JAK and Tyk) (Ihle et al., 1994). Activation of these kinases then function to tyrosine phosphorylate IRS1 and IRS2. For example, the IL4 and IL9 receptors utilize both JAK1 and JAK3 to tyrosine phosphorylate IRS1 and IRS2, whereas the growth hormone receptor activates JAK2 to phosphorylate IRS1 (Argetsinger et al., 1993; Yin et al., 1994, 1995).

Since multiple receptors can couple to the same substrates within the same cell type, the question of signaling specificity can not be solely dependent upon any given effector protein. Instead, the specificity of cellular responsiveness must be dependent upon multiple factors, including site-specific phosphorylation of substrates, unique activation of specific substrate combinations, and perhaps temporal differences in these phosphorylation events. At one level this is controlled by the specific expression of defined receptor populations in target cells. For example, rat adipocytes express relatively high levels of the insulin receptor with significantly lower levels of the epidermal growth factor (EGF) and IGF1 receptors. In contrast, liver expresses relatively high levels of the EGF receptor and muscle expresses both the insulin and IGF1 receptors. At another level, different receptors can lead to different patterns of substrate utilization. For example, both IL4 and insulin enhance IRS1 phosphorylation, however, insulin also induces Shc phosphorylation whereas IL4 does not (Pruett et al., 1995). Similarly, insulin does not induce JAK activation and therefore tyrosine phosphorylation of the signal transducers and activators of transcription (STAT) family of transcription factors. In contrast, cytokines are potent activators of JAK family kinases and the STAT proteins (Figure 3).

METABOLIC ACTIONS OF INSULIN

Insulin shares many of the properties of growth factors in that activation of the insulin receptor can function as an important activator of mitogenesis, growth,

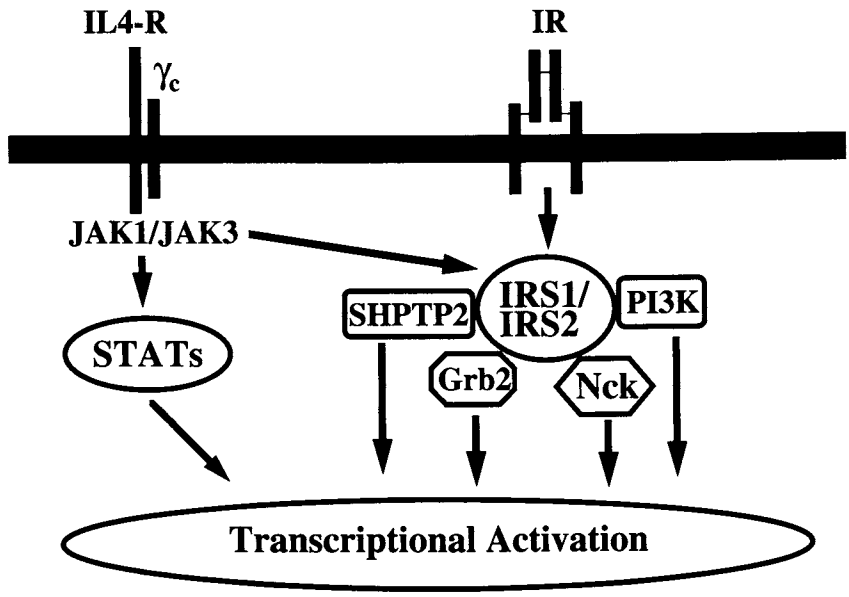

Figure 3. Activation of the interleukin 4 (IL4) receptor and the insulin receptor result in the tyrosine phosphorylation of the insulin receptor substrates 1 and 2 (IRS1/IRS2). The insulin receptor (IR) tyrosine kinase phosphorylates IRS1 and IRS2 directly whereas activation of the cytokine receptor (IL4-R) results in both the tyrosine phosphorylation of IRS1/IRS2 and the STAT (signal transducers and activators of transcription) proteins by the Janus family of tyrosine kinases (JAK). Tyrosine phosphorylation of IRS1 and IRS2 trigger their association with various effector molecules. It is not currently known whether the effectors associated with IRS1 and IRS2 differ. Other abbreviations used in the figure are SHPTP2 (also referred to as Syp and phosphotyrosine phosphatase 1D), PI3K (phosphatidylinositol 3-kinase), Grb2 (growth factor receptor bound protein 2), Nck (adapter protein consisting primarily of three src homology 3 (SH3) domains and one src homology 2 (SH2) domain).

development, and cell fate determination. However, in the adult the primary role of insulin is to regulate glucose homeostasis and intermediary metabolism. One of the most intensively studied and enigmatic areas of insulin action has been, and continues to be, the mechanism by which insulin activates glucose transport activity in adipocytes and muscle. Understanding the molecular mechanism for this physiological response may provide a basis to explain the most common defect in patients with NIDDM, in which there is a marked decrease in the sensitivity and/or responsiveness of insulin to stimulate glucose uptake into muscle and adipose tissues. This pathophysiological state is commonly referred to as insulin resistance.

A major advance in understanding insulin-stimulated glucose transport was independently obtained by the groups of Cushman and Kono. These two

laboratories demonstrated that in primary isolated adipocytes, the acute effect of insulin was to recruit or translocate an intracellular pool of glucose transporters to the plasma membrane in an energy-dependent but protein synthesis-independent manner (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Kono et al., 1981). Subsequently, this phenomenon was also found to occur in cardiac and skeletal muscle and to be impaired in various states of insulin resistance (Watanabe et al., 1984; Hirshman et al., 1990; Friedman et al., 1991; Marette et al., 1992a,b; Rodnick et al., 1992; Guma et al., 1995). During the past 15 years, this general mechanism has been somewhat refined and the essential features have been confirmed by numerous laboratories.

We now know that the facilitative glucose transporters are members of a multi-gene family with distinct but overlapping tissue distributions (Kahn, 1992; Pessin and Bell, 1992; Mueckler, 1994). The most prevalent glucose transporter expressed in adipose and muscle is the GLUT4 isoform with lower levels of the GLUT1 isoform. In the basal state, GLUT1 predominantly exists on the plasma membrane and accounts for the majority of non-insulin stimulated glucose transport (Holman et al., 1990; Clark et al., 1991; Piper et al., 1991; Satoh et al., 1993; Wilson and Cushman, 1994; Lund et al., 1995). In contrast, GLUT4 is primarily stored in an intracellular vesicle compartment with similarities to secretory granules of neural or endocrine origin. Several reports have suggested that the GLUT4 protein contains specific intracellular vesicle retention motifs at either the amino- or carboxyl-terminal ends which direct the localization of GLUT4 to an insulin-regulated vesicle population (Haney et al., 1991; Asano et al., 1992; Piper et al., 1992; 1993; Czech et al., 1993; Marshall et al., 1993; Verhey et al., 1993; Haney et al., 1995).

Recent studies have demonstrated that these GLUT4 vesicles are continually recycling between their intracellular location and the plasma membrane (Figure 4) (Jhun et al., 1992; Satoh et al., 1993; Yang and Holman, 1993). The net combination of the endocytosis and exocytosis rates, under basal conditions, results in the majority of the GLUT4 vesicles spending a significantly longer time in the intracellular compartment relative to the time spent at the cell surface. However, following insulin stimulation, approximately 50% of the intracellular GLUT4 compartment fuses with the plasma membrane due to a large increase in the rate of exocytosis and a small reduction in the rate of internalization (Czech and Buxton, 1993; Kanai et al., 1993). This causes a large increase in the total number of GLUT4 transporters at the plasma membrane which is substantially greater than the amount of GLUT1. In addition, GLUT4 is a significantly more efficient carrier of glucose than GLUT1 (Keller et al., 1989; Nishimura et al., 1993). Thus, the combination of an increase in the number of cell surface GLUT4 transporters and increased carrier efficiency accounts for most, if not all, of the insulin-stimulated enhancement of glucose transport. It should also be noted that several studies have indicated that insulin can cause a small increase the specific activity of GLUT4. However,

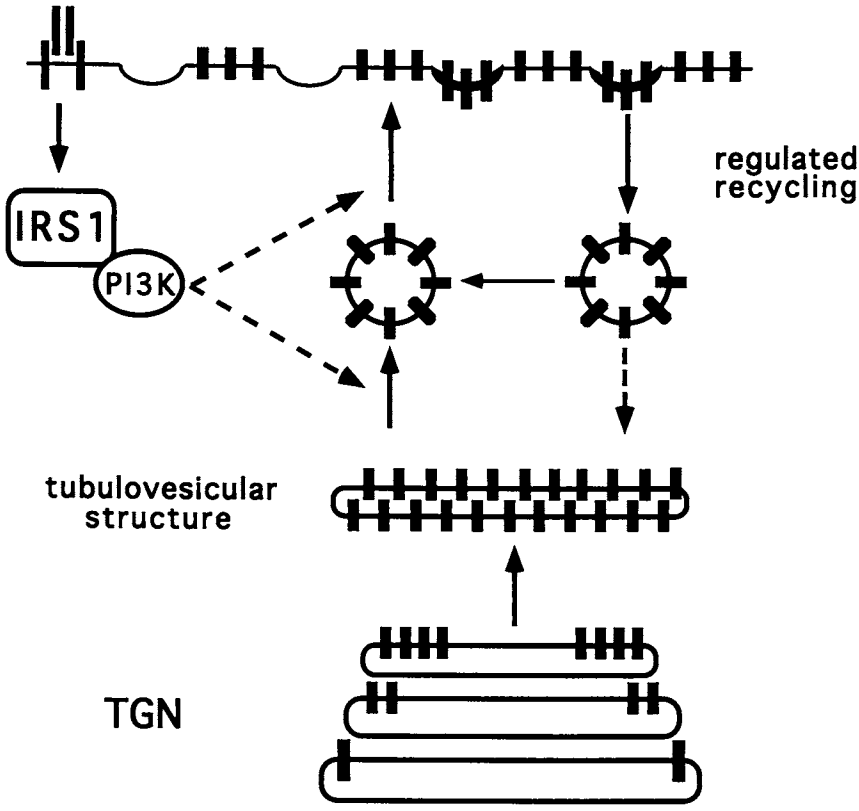

Figure 4. Regulation of GLUT4 glucose transporter trafficking in adipose and muscle cells. The GLUT4 protein is synthesized and transported from the endoplasmic reticulum to the trans-Golgi network (TGN). In contrast to other glucose transporter isoforms, GLUT4 appears to be retained in intracellular storage sites composed of tubulovesicular structures. In the basal state, the majority of the GLUT4 protein is sequestered into this intracellular pool with only a small fraction found at the plasma membrane. However, the GLUT4 protein appears to continually recycle between these locations in the absence of insulin. In one model, insulin stimulation through the activation of the phosphatidylinositol 3-kinase (PI3K) results in an increased movement of GLUT4 from the retention pool into the regulated recycling pathway. Following translocation to the plasma membrane, the GLUT4 proteins are clustered into clathrin coated pits and internalized through an endosomal compartment. In the presence of insulin, the capture of internalized GLUT4 proteins by the retention pool is inhibited allowing for the recycling of GLUT4 back to the plasma membrane.

the physiological relevance of this increase in specific activity is currently uncertain since it is small relative to the increase in the number of cell surface GLUT4 molecules.

In certain pathological states, the GLUT4 gene is expressed at reduced levels with a concomitant decrease in GLUT4 protein. This occurs in adipose tissue and several different muscle fiber types in states of prolonged insulin deficiency (Berger et al., 1989; Garvey et al., 1989; Kahn et al., 1989; Sivitz et al., 1989; 1990; Charron and Kahn, 1990; Kahn and Flier, 1990; Strout et al., 1990; Richardson et al., 1991; Garvey, 1992; Kahn, 1992; Kahn and Pedersen, 1993). In addition, there is a marked decrease in GLUT4 expression in adipose tissue of obese NIDDM patients (Sinha et al., 1991). However, since GLUT4 levels in skeletal muscle are not affected (Pedersen et al., 1990; Dohm et al., 1991; Eriksson et al., 1992; Garvey et al., 1992; Kahn et al., 1992) and muscle is the major site for postprandial glucose disposal, the underlying defect in NIDDM is due to a defect in GLUT4 translocation and not due to a decrease in the level of GLUT4 expression.

To examine the specific molecular pathways linking the insulin receptor kinase to the activation of GLUT4 translocation, various approaches have been taken to enhance or ablate specific intracellular functions followed by determination of GLUT4 translocation and glucose transport activity. For example, expression of an IRS1 ribozyme to reduce IRS1 expression levels was observed to inhibit the translocation of an expressed epitope tagged GLUT4 protein in adipocytes (Quon et al., 1994). This suggests that insulin stimulation of IRS1 phosphorylation is an important component in the pathway leading to GLUT4 translocation in adipose tissue. This is also consistent with data obtained from the IRS1 knockout mice in which there is approximately a 50% decrease in insulin-stimulated glucose transport in isolated adipocytes (Araki et al., 1994; Tamemoto et al., 1994). However, since the IRS1 knockout mice also have increased phosphorylation of IRS2, the relative contribution of IRS1 and the potential compensatory role of IRS2 can not be readily assessed until IRS1 and IRS2 knockout mice can be studied. Further experimentation is clearly needed to assess the potential role of both IRS1 and IRS2 in this pathway.

PHOSPHATIDYLINOSITOL 3-KINASE

The mammalian heterodimeric phosphatidylinositol 3-kinases (PI3K) are composed of tightly associated p85 regulatory and p110 catalytic subunits (Soltoff et al., 1993 Kapeller and Cantley, 1994). Currently, three isoforms of the p85 regulatory subunit have been cloned. These include the p85α and p85β isoforms and the $p55^{PIK}$ isoform (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991; Pons et al., 1995). In addition, three isoforms of the p110 catalytic subunit have been identified, including p110α, p110β and p110γ (Hiles et al., 1992; Hu et al., 1993; Stoyanov et al., 1995). PI3K is activated by numerous receptor tyrosine kinases and functions to phosphorylate the D3 position of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate *in vivo* as well as phosphatidyli-

nositol *in vitro* Auger et al., 1989). In addition to its lipid kinase activity, PI3K also displays a low level of serine/threonine protein kinase activity (Dhand et al., 1994b; Lam et al., 1994). Although the PI3K activity appears to be essential for several signaling pathways, a specific role for the lipid products of PI3K remains poorly defined. To date the only signaling function ascribed to the lipid products PI-3,4-P or PI-3,4,5-P is in the stimulation of the protein kinase C isozymes δ, ε, η and ζ (Nakanishi et al., 1993; Toker et al., 1994).

The p85 subunit contains an amino terminal SH3 domain followed by a proline rich region with sequence homology to the carboxyl terminus of the breakpoint cluster region (bcr) gene product (Heisterkamp et al., 1985). Carboxyl to the bcr domain of p85 are two SH2 domains separated by an intervening sequence termed the inter-SH2 (IS) domain. The IS domain directs the high affinity interaction of p85 with the amino terminus of the 110 kDa catalytic subunit and is also present in the recently identified $p55^{PIK}$ regulatory subunit (Klippel et al., 1993; Dhand et al., 1994a; Holt et al., 1994; Pons et al., 1995). This interaction between the IS domain and p110 is necessary for PI3K activity and, in fact, a constitutively active p110 subunit was generated by fusing the p85 IS domain to the amino terminus of p110 (Hu et al., 1995). Although a functional role for the p85 SH3 or bcr domains has not been established, both SH2 domains direct the binding to tyrosine phosphorylated residues in a characteristic YMXM or YXXM motif (Yonezawa et al., 1992). Engagement of either the amino- or carboxyl-terminal SH2 domain not only serves to target the PI3K to specific effectors, but it also results in stimulation of its PI3K activity (Myers et al., 1992; Carpenter et al., 1993).

Several important roles for PI3K have been determined. Early studies using site-directed mutations of the EGF and platelet-derived growth factor (PDGF) receptors demonstrated that the association of these receptors with PI3K is necessary for inducing maximal EGF- or PDGF-stimulated mitogenesis and internalization of the PDGF receptor (Seedorf et al., 1992; Valius and Kazlauskas, 1993; Joly et al., 1994, 1995; Roche et al., 1994; Carraway et al., 1995). In addition, PI3K has been implicated in the regulation of actin stress fibers and membrane ruffling (Kotani et al., 1994, 1995; Wennstrom et al., 1994; Wymann and Arcaro, 1994). Other cellular functions for PI3K have been determined following the discovery that the microbial metabolite wortmannin is a potent inhibitor of PI3K activity (Arcaro and Wymann, 1993; Okada et al., 1994). These data indicate that PI3K is involved in the fusion and release of secretory granules. Treatment with wortmannin causes cellular effects which are similar to the vesicular trafficking defects occurring in yeast mutants expressing defective Vps34, a PI3K homologue.

In terms of insulin action, PI3K has been reported to associate with the carboxyl terminus of the autophosphorylated β subunit of the insulin receptor (Backer et al., 1992b; Yonezawa et al., 1992; Liu and Livingston, 1994). However, the predominant interaction of PI3K in insulin responsive cells is with tyrosine phosphorylated

IRS1 and perhaps IRS2 (Backer et al., 1992a; Tobe et al., 1995). The importance of the IRS1-PI3K interaction could account for the inhibition of GLUT4 translocation in the adipocytes transfected with the IRS1 ribozyme (Quon et al., 1994). Consistent with the role of PI3K in vesicular trafficking, treatment of adipocytes with PI3K inhibitors such as wortmannin, ML-9, and LY294002 were found to completely inhibit insulin-stimulated GLUT4 translocation (Inoue et al., 1993; Cheatham et al., 1994; Clarke et al., 1994; Barros et al., 1995). Although these compounds have a relatively high affinity for PI3K, the question of inhibitor specificity is always a concern and makes interpretations tenuous without alternative verification. For example, the PI3K inhibitor ML-9 was originally identified as an inhibitor of the myosin light chain kinase (Hashimoto et al., 1994). Even assuming that these inhibitors are specific for PI3K, the interpretation of these data are at best limited since PI3K appears to be necessary for general vesicular movement. Thus, PI3K activity probably functions in a permissive fashion for insulin-stimulated GLUT4 translocation, but may not necessarily be specific for insulin-dependent vesicular trafficking. Obviously, a more detailed analysis of the function of PI3K isoforms, their intracellular localization, mechanisms of activation, and its interaction with IRS1 and IRS2 are important issues which should be clarified within the next several years.

RAS-DEPENDENT DOWNSTREAM SIGNALING EVENTS

Recently, a direct pathway linking receptor tyrosine kinase activation to transcriptional activation by the mitogen-activated protein (MAP) kinases has been established (Schlessinger and Bar-Sagi, 1994). In the case of the insulin receptor, tyrosine phosphorylation of IRS1 and the Shc proteins results in their association with Grb2-SOS complexes via the SH2 domain of Grb2 (Baltensperger et al., 1993 Skolnik et al., 1993). This interaction between Grb2 and SOS is mediated by the two SH3 domains of Grb2 and the proline rich carboxyl terminus of SOS (Chardin et al., 1993; Egan et al., 1993; Sastry et al., 1995). Several studies have demonstrated that the association of the Grb2-SOS complex with tyrosine phosphorylated Shc and/or IRS1 results in the interaction of SOS with plasma membrane bound p21Ras, thereby stimulating exchange of GDP for GTP (Simon et al., 1991; Bowtell et al., 1992; Lowenstein et al., 1992; Rozakis-Adcock et al., 1992, 1993; Baltensperger et al., 1993; Chardin et al., 1993; Draznin et al., 1993; Egan et al., 1993; Li et al., 1993; Skolnik et al., 1993; Aronheim et al., 1994; Pronk et al., 1994; Quilliam et al., 1994; Yonezawa et al., 1994). Thus, the conversion of inactive GDP-bound Ras to active GTP-bound Ras results from the specific targeting and/or activation of the guanine nucleotide exchange activity of SOS through a Grb2-mediated association with tyrosine phosphorylated docking proteins (Figure 5).

Elucidation of the pathway leading to Ras activation is an important milestone in our understanding of intracellular signaling. In the early 1980s mutations which resulted in constitutive activation of Ras were originally identified as the primary cause of a majority of colon and pancreatic cancers in humans (Barbacid, 1987). In addition, activation of Ras has been shown to be an important event in differentiation and cell fate determination. Thus, the functional signaling events emanating from Ras have also received considerable attention. It has now been established that once in the GTP-bound state, Ras associates with the Raf family of MAP kinase kinase kinases resulting in activation of the serine/threonine kinase activity of Raf (Koide et al., 1993; Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). In this manner, Ras functions as a molecular

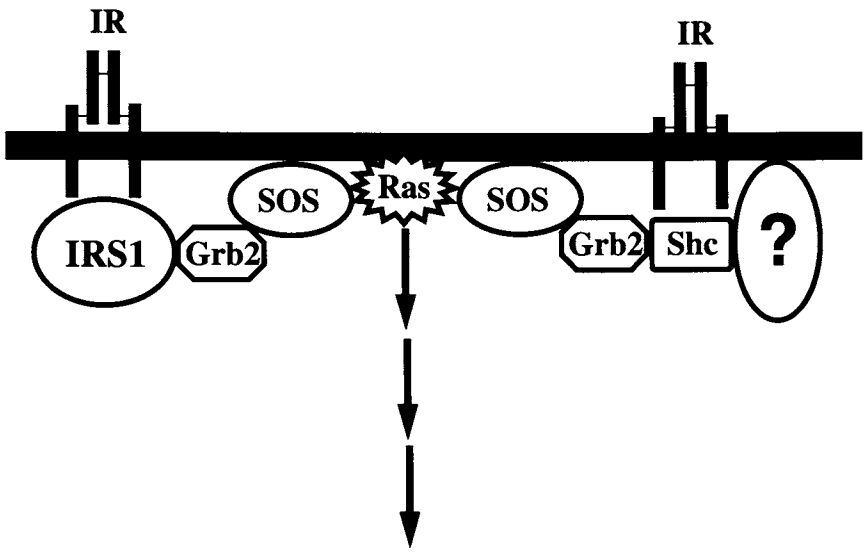

Figure 5. Insulin activation of p21 Ras can occur by at least two signaling pathways. In one pathway, activation of the insulin receptor results in the tyrosine phosphorylation of IRS1 and its association with the adaptor protein, Grb2 and the guanylnucleotide exchange factor, SOS. This complex is then trageted to Ras at the plasma membrane through a weak association of IRS1 with the insulin receptor or an unknown membrane component. Alternatively, membrane targeting may be due to specific domains in SOS. Similarly, insulin receptor phosphorylation of Shc also results in the rapid formation of a Shc-Grb2-SOS complex and the targeting of SOS to Ras at the plasma membrane. At the plasma membrane, the GDP/GTP exchange factor SOS can then promote the conversion of GDP-bound Ras into active GTP-bound Ras. Activated Ras can then stimulate various downstream signaling events.

switch converting upstream receptor tyrosine kinase activity into serine/threonine phosphorylation events. The activation of Raf results in this kinase specifically phosphorylating MAP kinase kinase family members, also known as MEKs (Crews et al., 1992; Huang et al., 1993; Zheng and Guan, 1993). The MEKs are unusual kinases in that they phosphorylate both tyrosine and threonine residues and are therefore classified as dual function kinases. Activated MEK specifically phosphorylates the 42 and 44 kDa ERKs on threonine and tyrosine residues (T183 and Y185, respectively for ERK2), which is necessary for the full activation of ERK (Anderson et al., 1990; Boulton et al., 1990; Boulton et al., 1991) (Figure 6). ERK provides an important juncture for the control of various biological processes since ERK activation results in the divergence of the insulin signal into several nuclear and cytoplasmic events (Davis, 1993). For example, the ERK pathway has been shown to phosphorylate PHAS1, which results in derepression of protein translation (Haystead et al., 1994; Lin et al., 1994). ERK activation also results in translocation of ERK to the nucleus and the subsequent phosphorylation and activation of several transcription factors including TCF/Elk1 and NF-IL6 (Gille et al., 1992; Janknecht et al., 1993; Nakajima et al., 1993; Chuang and Ng, 1994; Gille et al., 1995; Whitmarsh et al., 1995). These data have therefore established a direct link between tyrosine kinase receptor activation and activation of intracellular serine/threonine kinase regulated

Several studies have examined the potential role of Ras and the ERK pathway in the metabolic actions of insulin. Stimulation of the ERK pathway appears to phosphorylate and inactivate glycogen synthase kinase 3 (GSK-3) (Sutherland et al., 1993; Welsh and Proud, 1993). Similarly, insulin activation of p70 S6 protein kinase (which occurs through a poorly defined pathway) also phosphorylates GSK-3 (Sutherland et al., 1993). In addition, an ERK sensitive kinase has been reported to phosphorylate and activate $pp90^{Rsk}$, which has been implicated in the regulation of protein phosphatase 1 (Dent et al., 1990). The combination of GSK-3 inactivation and stimulation of protein phosphatase 1 has been hypothesized to account for increases in insulin-stimulated glycogen synthase activity. However, recent experments in 3T3-L1 adipocytes and L6 myotubes demonstrate that the MAP kinase pathway does not appear to be the signaling mechanism by which protein phosphatase 1 activity is regulated in response to insulin. Using the MEK-specific inhibitor PD98059, it is possible to block ERK activation in response to insulin without affecting increases in glycogen synthase activity or glycogen synthesis (Dudley et al., 1995; Lazar et al., 1995). These experiments demonstrate that ERK activity is not required for insulin stimulation of glycogen synthesis. Furthermore, stimulation of 3T3-L1 adipocytes with PDGF activates ERK to the same extent as insulin, however, only insulin stimulation significantly increases glycogen synthesis, indicating that ERK activation alone is not sufficient for activation of glycogen synthesis (Weise et al., 1995).

Two reports have also suggested that Ras activation may be involved in stimulating glucose transport. Microinjection of Ras antibodies inhibit insulin-

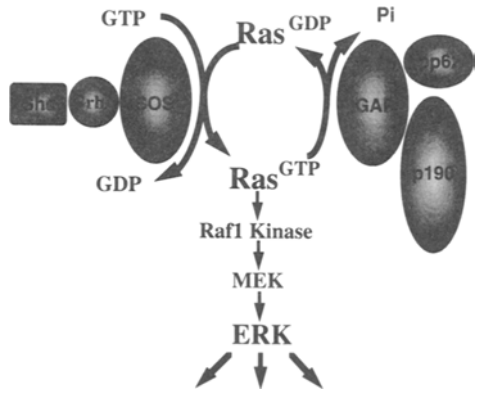

Figure 6. The Ras GTP/GDP cycle is regulated by both the guanylnucleotide exchange factor, SOS and the Ras GTPase activating protein, GAP. Currently, the predominant route for insulin stimulation of Ras activation is thought to occur by the formation of the Shc-Grb2-SOS ternary complex. Ras is also inactivated by the action of GAP proteins which function to hydrolyze the bound GTP into GDP and free phosphate, P_i. The 120 kDa form of GAP is found in a complex composed of a pp62 protein having homology with RNA-binding proteins and a 190 kDa protein which has GAP activity for the small GTP-binding protein Rho (Settleman et al., 1992; Wong et al., 1992; Foster et al., 1994; Taylor and Shalloway, 1994). In the activated state, Ras associates with the Raf family of serine/threonine kinases resulting in Raf kinase activation. The Raf kinase then phosphorylates and activates the dual functional protein kinase MEK. In turn, MEK phosphorylates the mitogen-activated protein kinase ERK on both a threonine and tyrosine residue resulting in the activation of ERK protein kinase activity and the phosphorylation of several transcription factors.

stimulated glucose transport in cardiac myocytes to a small extent. In addition, expression of a constitutively active form of Ras in both cardiac myocytes and adipocytes increases the amount of both GLUT4 and GLUT1 at the plasma membrane (Kozma et al., 1993; Manchester et al., 1994). However, other studies found no effect of oncogenic Ras in 3T3-L1 adipocytes and expression of dominant-interfering Ras did not inhibit insulin-stimulated GLUT4 translocation (Hausdorff et al., 1994; Quon et al., 1995). One interpretation of these data is that increased expression of Ras is sufficient for activating glucose transport, but it is not necessary. Thus, the ability of Ras to stimulate glucose transport may simply reflect promiscuous coupling to the normal insulin regulated pathway utilized by insulin responsive cells. As support for this hypothesis, the EGF and PDGF receptors present in muscle and adipocytes can activate both Ras and the ERK pathway (Robinson et al., 1993; Fingar and Birnbaum, 1994; Gould et al., 1994; van den Berghe et al., 1994). However, stimulation of the EGF or PDGF receptor does not cause GLUT4 translocation or increase glucose transport activity despite activation of both Ras and ERK. In contrast, expression of high levels of the EGF

receptor in 3T3-L1 adipocytes can result in an EGF-stimulation of GLUT4 translocation and glucose transport activity (Hardy et al., 1995). Furthermore, treatment of cells with a MHC class I peptide plus EGF, a combination that enhances EGF receptor signaling, can stimulate the glucose transport to a similar extent as insulin (Stagsted et al., 1993). Together, these data indicate that increased signals generated by multiple factors can somehow couple to glucose transport activation, but whether this activation intersects with the normal insulin signaling pathways at some level distal to the insulin receptor is unknown.

Recently, a very interesting finding has been made which may reconcile the apparent discrepancies between the various studies examining the relationship of Ras with glucose transport. As described above, the best characterized pathway leading to Ras activation involves the plasma membrane targeting and interaction of the guanylnucleotide exchange factor SOS with Ras. An alternative route for Ras activation has been demonstrated via the direct interaction of the p110 catalytic subunit of PI3K with the effector domain of Ras (Rodriguez-Viciana et al., 1994; Hu et al., 1995). Assuming that the activation and/or subcellular localization of PI3K can initiate GLUT4 translocation, then expression of constitutively active Ras may stimulate a sufficient PI3K-mediated signal. Since the most likely pathway for an insulin-stimulated PI3K signal is through IRS1, this would account for the necessity of high levels of Ras expression and the observation that dominant-interfering Ras mutants were unable to prevent glucose transporter translocation. Whether or not this speculation actually accounts for the observed data will undoubtedly be addressed in the near future.

SH2 DOMAIN CONTAINING PROTEIN TYROSINE PHOSPHATASE, SHPTP2

The SHPTP2 phosphatase (also termed, PTP1D, SHPTP3, PTP2C, PTPL1 or Syp) is one member of a family of cytosolic protein tyrosine-specific phosphatases that contain two amino terminal SH2 domains and a carboxyl terminal catalytic domain (Freeman et al., 1992 Ahmad et al., 1993). These SH2 domains target SHPTP2 to the tyrosine phosphorylated EGF receptor, PDGF receptor, and IRS1 (Feng et al., 1993; Kuhne et al., 1993; Li et al., 1994b). In addition to mediating the targeting of SHPTP2, the SH2 domains also appear to regulate SHPTP2 catalytic activity. Several studies have demonstrated that the unoccupied SH2 domains maintain the SHPTP2 phosphatase activity in a repressed state whereas the binding of tyrosine phosphorylated proteins or peptides results in a marked activation of protein tyrosine phosphatase activity (Kazlauskas et al., 1993; Kuhne et al., 1993; Lechleider et al., 1993a,b). Furthermore, the phosphorylation of SHPTP2 on tyrosine 542 in response to PDGF may also contribute to the stimulation of its phosphatase activity (Bennett et al., 1994; Li et al., 1994b).

In general, tyrosine phosphatases function as the inactivating arms of kinase activation pathways. For example, the highly related hematopoietic protein tyrosine-specific phosphatase, SHPTP1C, is responsible for the inactivation of erythropoietin receptor mediated signaling (Klingmuller et al., 1995). However, protein tyrosine phosphatases may also function as positive effectors for downstream signaling. In this regard SHPTP2 is the mammalian homologue of the *Drosophilia* SH2 domain-containing protein tyrosine phosphatase, termed *corkscrew* (Freeman et al., 1992; Perkins et al., 1992). Genetic epistasis experiments have demonstrated that *corkscrew* is an essential gene for the appropriate development of anterior/posterior structures during embryogenesis, functioning downstream of the *torso* tyrosine kinase, a homologue of the mammalian PDGF receptor, and upstream of *polehole*, a homologue of the mammalian raf serine/threonine kinase (Ambrosio et al., 1989).

Recently, several studies in mammalian cells have also suggested that SHPTP2 plays an important positive role in insulin signaling. Although increased levels of tyrosine phosphatase activity would be expected to inhibit signaling events, expression of the wild-type catalytically active SHPTP2 had no significant effect on insulin-stimulated biological responsiveness (Yamauchi et al., 1995a). Furthermore, microinjection of SHPTP2 specific antibodies completely blocked insulin-stimulated DNA synthesis in fibroblasts expressing high levels of the insulin receptor (Xiao et al., 1994). In addition, expression of the SHPTP2 SH2 domains or a full length catalytically inactive SHPTP2 mutant blocked activation of the MAP kinase pathway, as well as c-fos transcription and DNA synthesis, without significant effect on the tyrosine phosphorylation state of IRS1 or Shc (Milarski and Saltiel, 1994; Noguchi et al., 1994; Yamauchi et al., 1995a). These effects appear to occur through an inhibition of insulin-stimulated Ras GTP binding suggesting that SHPTP2 functions in a proximal event necessary for Ras activation.

The apparent coupling of SHPTP2 function with the Ras pathway is consistent with its role in the mitogenic and developmental pathways in both *Drosophilia* and in mammalian cells. In this regard, homozygous SHPTP2 knockouts result in an embryonic lethal phenotype demonstrating the essential role of SHPTP2 in mouse development (Livingston, personal communication). Furthermore, microinjection of SHPTP2 blocking antibodies in adipocytes had no effect on insulin-stimulated GLUT4 translocation (an acute metabolic action) but did prevent the transcriptional increase in GLUT1 expression (a mitogenic response) (Hausdorff et al., 1995). Currently, several potential SHPTP2 associated binding proteins and substrates have been suggested including proteins of 115 and 40 kDa (Milarski and Saltiel, 1994; Yamauchi and Pessin, 1995; Yamauchi et al., 1995b; Zhao et al., 1995). However, until a molecular target for SHPTP2 function has been identified the elucidation of the signaling pathway utilized by SHPTP2 remains undefined.

SUMMARY

During the past several years, we have made enormous progress in our understanding of the cellular mechanisms involved in insulin receptor signaling. These findings have come about due to the coordinate use of physical, molecular, and cellular biological approaches to the complex issues of intracellular protein-protein interactions, subcellular localization and activation of enzyme activities. Although we have come a long way, we still have a lot to learn before the entire scheme of insulin signaling is established at the molecular level. Presently, there are several pressing issues that need to be resolved in order to determine the basis for the mitogenic and metabolic actions of insulin.

For example, one important issue is the molecular basis of receptor signaling specificity. Does this result from the regulation of the signal amplitude (receptor number and/or hormone dose) or from the intrinsic cellular context of a particular receptor. If cell context-dependent, is this due to receptor substrate specificity, site specific phosphorylation, and/or combinational associations within a defined group of effectors. Although some progress is being made examining the potential importance of effector compartmentalization, are the temporal patterns of activation events important? Finally, what are the unidentified factors which may be necessary in the pleiotropic actions of insulin? These issues will only be resolved once each of the biochemical pathways leading to a particular biological response have been defined. We are looking forward to a very exciting future for the elucidation of the molecular basis of insulin action.

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Chapter 7

Molecular Aspects of the Glucagon Gene

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INTRODUCTION

In vertebrates, glucose homeostasis is mainly regulated by two peptide hormones, insulin and glucagon, produced in the islets of Langerhans. About 40 years ago,

Advances in Molecular and Cellular Endocrinology
Volume 1, pages 203-228.

ISBN: 0-7623-0158-9

glucagon was shown to be synthesized in the α-cells of the endocrine pancreas and the peptide was purified, crystallized, and sequenced (Sutherland and de Duve, 1948; Staub et al., 1953; Bromer et al., 1956). Since then, basic studies on its physiological role have been conducted revealing its importance in the activation of glycolysis and gluconeogenesis in response to low plasma glucose concentrations as well as hormonal and neural stimuli.

A new era in the study of glucagon has been opened by the availability of recombinant DNA technology and the cloning of the glucagon gene. It was previously established that glucagon is part of a large family of peptide hormones produced in the endocrine pancreas, the gut, as well as in the central and peripheral nervous systems. Like all other members of the glucagon superfamily, glucagon is now known to be derived from a large precursor by proteolytic processing. Surprisingly, two related peptides, glucagon like peptides-1 and -2 (GLP-1 and GLP-2) were found to be encoded by the same gene. Since then, considerable information has accumulated on where and how proglucagon is produced and processed, the physiological roles of GLP-1, and the pancreatic α-cell-specific expression and regulation of the glucagon gene.

This review will focus on the molecular aspects of glucagon physiology: the structure of the glucagon and the glucagon superfamily genes and the cell-specific expression and regulation of the glucagon gene. Since gene expression in eukaryotes is mainly controlled at the level of transcription initiation, experiments were first performed to characterize the promoter and enhancers of the glucagon gene. *Cis*-acting DNA control elements were localized within the 5′ flanking region which in interaction with *trans*-acting factors promote cell specificity of glucagon gene expression and response to physiological changes. In addition to the level of transcriptional control, glucagon synthesis is in some species regulated posttranscriptionally by differential splicing of the primary transcript (Irwin and Wong, 1995) and, in mammals, posttranslationally by tissue-specific proteolytic processing (Habener 1990).

Studies on the molecular biology of the α-cell should also have an impact on our knowledge of islet physiology. Recent studies on glucagon and insulin gene transcription have taught us that both peptide hormones share common control elements and *trans*-acting factors (Cordier-Bussat et al., 1995; Diedrich and Knepel, 1995). The transcriptional level of gene expression in the endocrine pancreas is therefore regulated in two steps by islet- and cell specific mechanisms. In addition, transcription factors involved in cell-specificity may have a much wider role than previously expected in processes such as cell proliferation, differentiation, and maintenance of the differentiated state (Ohlsson et al., 1993; Peers et al., 1994; Jonsson et al., 1994; Slack, 1995).

Identification of the *cis*- and *trans*-acting factors responsible for the cell-specific expression of the glucagon gene will thus be important to understand transcriptional regulation in response to extracellular stimuli and the development of the α-cell.

Studies on the physiological control of the glucagon gene should also bring some light on the dysregulation which occurs in diabetes.

STRUCTURE AND EVOLUTION OF THE GLUCAGON GENE

The Glucagon Gene

The structure and sequence of the human and rat glucagon genes has been determined and found to be strongly conserved (Bell et al., 1983a,b; Heinrich et al., 1984a; White and Saunders, 1986). The human gene is composed of six exons and five introns spanning 10 kb (White and Saunders, 1986) and is located on chromosome 2 (Tricoli et al., 1984). Nucleotide sequence analyses have indicated that glucagon is synthesized as part of a large biosynthetic precursor, preproglucagon. In agreement with the evolutionary theory of exon-shuffling, each functional domain of preproglucagon is encoded by a distinct exon, separated by introns which are located in connecting peptides (Bell et al., 1983a; Heinrich et al., 1984a,b, White and Saunders, 1986). As illustrated in Figure 1, the 5′- and 3′-untranslated regions of glucagon mRNA are encoded by exons 1 and 6, respectively; exon 2 codes for the signal peptide and part of the amino-terminal end of glucagon-related pancreatic peptide (GRPP), while exons 3, 4, and 5 contain the coding information for glucagon, and the glucagon like peptides GLP-1 and GLP-2, respectively.

The cDNA sequences of anglerfish, rainbow trout, chicken, hamster, rat, guinea pig, degu, bovine, mouse, and human glucagon have been determined (Lund et al., 1982, 1983; Bell et al. 1983a,b; Lopez et al., 1983; Heinrich et al., 1984a; White and Saunders, 1986; Bell 1986; Seino et al., 1986; Nishi and Steiner, 1990; Hasegawa et al., 1990; Irwin and Wong, 1995; Rothenberg et al., 1995). Additionally, the glucagon peptides from three other classes of vertebrates (amphibia,

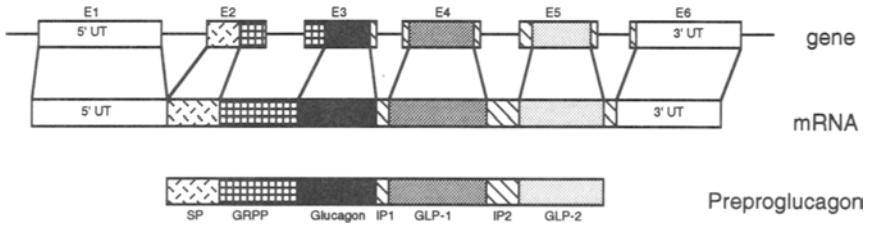
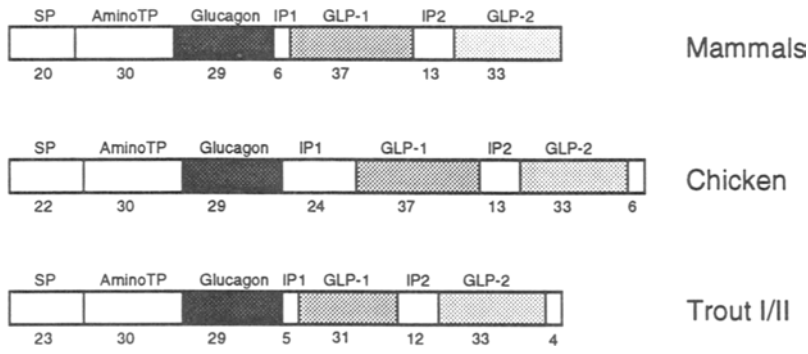


Figure 1. Schematic diagram illustrating the structure of the human glucagon gene, its mRNA and the encoded precursor protein, preproglucagon. Exons are represented by boxes designated by E1 to E6 and introns by solid lines. White boxes and boxes with specific patterns indicate 5′ and 3′-untranslated sequences (UT); the signal peptide (SP), glucagon-related pancreatic peptide (GRPP), intervening peptides 1 and 2 (IP1 and IP2), glucagon-like peptide 1 and 2 (GLP-1 and GLP-2), and glucagon, respectively.

cartilaginous fish, and jawless fish) have been purified and sequenced (Gail Pollock et al., 1988; Conlon et al., 1989, 1993). Whereas mammals, birds, and amphibia most probably possess a single glucagon gene, most fishes synthesize two glucagon mRNAs from two different genes and, in the rainbow trout, even four proglucagon genes have been identified (Lund et al., 1983; Conlon et al., 1988; Irwin and Wong, 1995). In all species analyzed, glucagon is part of a biosynthetic precursor, preproglucagon. Although fish and bird preproglucagon genes were for a long time thought not to encode a GLP-2 sequence, recent analyses of genomic DNA or cDNA have revealed the presence of sequences encoding this peptide in chicken and rainbow trout (Irwin and Wong, 1995). Thus, preproglucagon genes from mammals, and at least chicken and trout, possess a similar structure coding for a characteristic hydrophobic signal peptide (SP), an aminoterminal peptide (AminoTP), glucagon, as well as the glucagon like peptides GLP-1 and GLP-2 (Figure 2a,b). The functional domains are separated from each other and from the intervening peptides by basic amino acids allowing posttranslational cleveage of the precursor (Figure 2b).

Mammalian preproglucagons are 180 aa long and have a molecular weight of 18 kDa. Human GLP-1 and GLP-2 share 35% identity and 68% homology and display 48% and 38% identity with glucagon, respectively. Phylogenetic analyses of glucagon and the GLPs suggest that an ancestral glucagon-encoding exon duplicated about 1.2 billion years ago to give rise to GLP-1 and then triplicated (either from the glucagon or the GLP-1 encoding exon) about 160 million years ago to result in two different GLPs. Thus, a preproglucagon gene comprising glucagon and two glucagon like peptides existed long before the separation of fish and mammals; this ancestral gene was then duplicated in fish (Lopez et al., 1984). Although GLP-2 is much less conserved than GLP-1 (86% and 57% identity of chicken and human GLP-1 and GLP-2, respectively), the presence of GLP-2 in all vertebrate classes suggests that it plays a functional, but yet unknown, role in these species (Irwin and Wong, 1995).

A



B

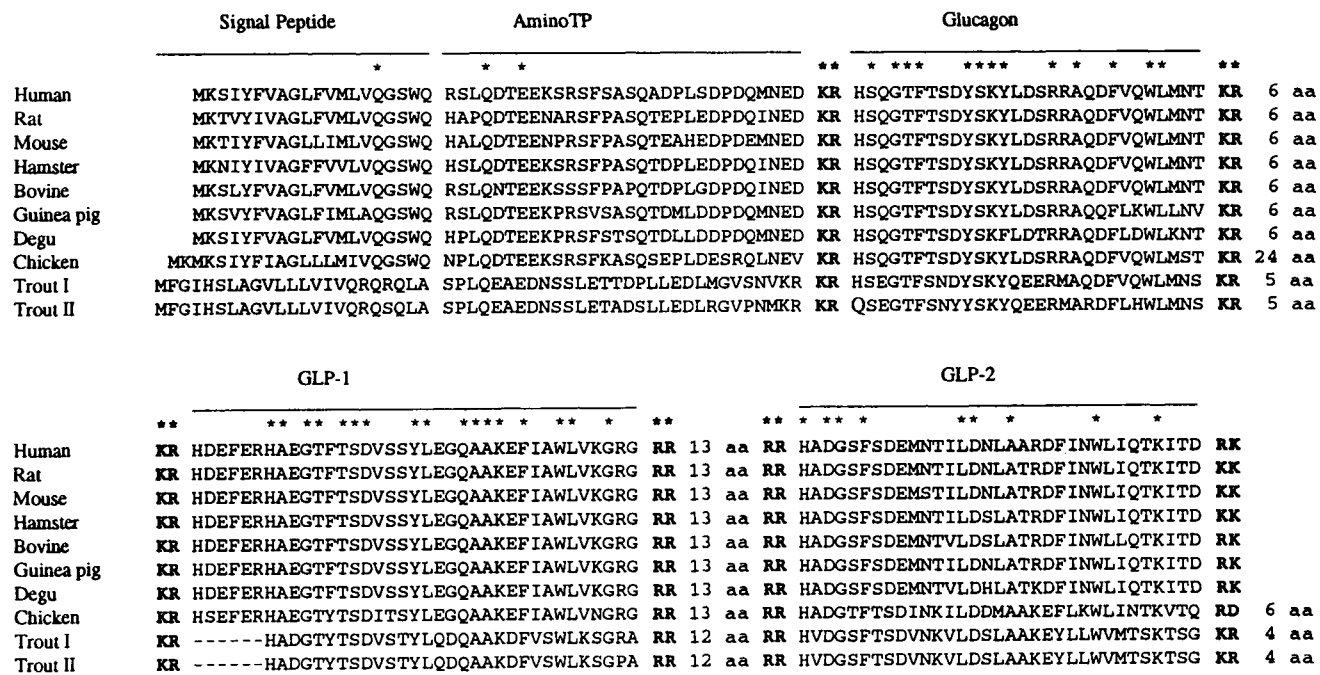


Figure 2. (**A**) Schematic diagram illustrating the glucagon precursor protein, preproglucagon from mammals, chicken, and trout. In the trout, two nonallelic glucagon genes are present encoding two glucagon precursors. Numbers indicate the length of the respective peptides in amino acids. SP, signal peptide; Amino TP, amino-terminal peptide; IP, intervening peptide, and GLP, glucagon-like peptide. (**B**) Sequence alignment of the preproglucagon proteins from human, rat, mouse, hamster, beef, guinea pig, degu, chicken, and trout. Asterisks indicate amino acids which are conserved in all species analyzed.

The 29 aa glucagon peptide is identical in human, rat, mouse, hamster, and beef; the sequence shares 97%, 86%, 86%, 72%, and 55% identity with the chicken, guinea pig, degu, trout I and II equivalents, respectively (Figure 2b). Despite the high degree of conservation of the glucagon aa sequence throughout evolution, it is interesting to note that within mammals the histricomorph rodents degu and guinea pig represent exceptions. The glucagons of both New World rodents differ from other mammalian glucagons mainly in their C-terminal regions by five amino acids (Seino et al., 1986; Nishi and Steiner, 1990). As the C-terminus of glucagon seems to be important for receptor-binding affinity and biological activity, the changes in primary sequence result in decreased biological potency. Interestingly, the insulin gene from these rodents has also undergone multiple mutations resulting in insulin molecules which have only about 60% identity with human insulin. The mutations in the insulin gene have led to peptides exhibiting more growth stimulating, but less metabolic, activity than other mammalian insulins (King and Kahn, 1981). Therefore, changes observed in the glucagon sequence of histricomorph rodents might serve to counterbalance the metabolically less active insulins found in these species (Nishi and Steiner, 1990).

The Glucagon Superfamily

Glucagon is structurally related to several other regulatory peptides including vasoactive intestinal peptide (VIP), gastric inhibitory polypeptide (GIP), growth hormone releasing factor (GHRF), secretin, and pituitary adenylate cyclase activating protein (PACAP). Comparison of their primary amino acid sequences reveals similarities at the N-terminus of each hormone. Despite size variations of their precurser molecules, each prepropeptide comprises a signal sequence, an N-terminal peptide and one, two, or three peptides related to glucagon (Figure 3). The peptides belonging to the glucagon superfamily are found in the gut, pancreas, as well as in the central and peripheral nervous systems where they may function as hormones and/or neurotransmitters.

VIP is a 28 aa peptide, present in the intestine and both the central and peripheral nervous system. As deduced from the cloned rat and human cDNAs (Nishizawa et al., 1985; Tsukada et al., 1985), preproVIP comprises 170 aa. The precursor molecule is composed of a signal peptide (21 aa), a 58 aa N-terminal peptide, PHM-27 (a 27 aa peptide), a 12-residue spacer peptide, VIP itself, and a 15 aa carboxy-terminal peptide. The exon-intron organization of the VIP gene is similar to the human glucagon gene; it contains 6 exons and 5 introns spanning about 9 kb (Tsukada et al., 1985). Exons 1 and 6 encode the 5′- and 3′-untranslated sequences, exon 2 the signal peptide, and exons 3, 4 and 5 the spacer peptide, PHM-27 and VIP, respectively.

Human GHRF is a 44 aa prepeptide (43 aa in the rat) produced in the hypothalamus that stimulates biosynthesis and secretion of growth hormone in the anterior pituitary. Human preproGHRF is 108 aa long (104 aa for the rat preprotein) and

contains a signal peptide, an amino-terminal peptide, GHRF, and a carboxyterminal peptide. The human and rat single copy genes are about 10 kb long and contain five exons encoding each functionally distinct domain of preproGHRF (Mayo et al., 1983, 1985). By contrast to glucagon and VIP, GHRH is encoded by two exons and is less conserved in primary sequence; rat GHRF exhibits only 66% identity with its human homologue (Gubler et al., 1983; Mayo et al., 1983).

GIP is a 42 aa hormone synthesized in the duodenum. PreproGIP is 153 aa long and organized similarly to precurser molecules of other members of the glucagon superfamily. The human gene spans approximately 10 kb and contains 6 exons; like GHRF, the GIP coding sequence is disrupted by an intron (Inagaki et al., 1989).

Secretin is a 27 aa peptide produced by the S cells of the intestine and the brain. Preprosecretin is 134 aa long and contains a signal peptide, a short N-terminal peptide, secretin, and a long 72 aa carboxy-terminal extension (Kopin et al., 1991). The rat gene spans 0.8 kb including four exons. Like glucagon and VIP—but unlike GHRF and GIP—the entire secretin sequence is encoded by a single exon. In contrast to other members of the glucagon gene superfamily, the secretin gene does not have an intron separating the transcriptional and translational start sites.

PACAP was originally isolated from the hypothalamus and was shown to stimulate adenylate cyclase in rat anterior pituitary cell cultures. Human preproPACAP is 172 aa long and contains a signal peptide, a 55 aa N-terminal peptide, a 29 aa PACAP-related peptide (PRP), a linker peptide, and PACAP. The gene is composed of five exons and four introns (Hosoya et al., 1992). PRP and PACAP are each encoded by a single exon arranged in tandem in the PACAP gene, suggesting exon duplication. Two isoforms of this peptide have been detected, PACAP38 and PACAP27, the latter corresponding to the 27 amino-terminal

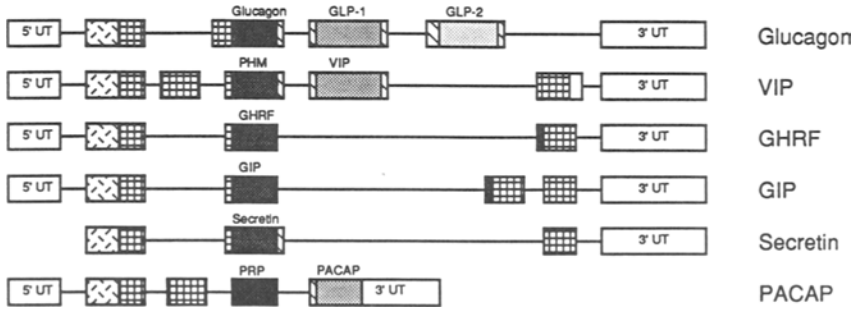


Figure 3. Structural organization of the genes encoding peptides of the glucagon superfamily: glucagon, vasoactive intestinal peptide (VIP), growth hormone-releasing factor (GHRF), gastric inhibitory polypeptide (GIP), secretin, and pituitary adenylate cyclase activating protein (PACAP). Exons are represented by boxes and introns by solid lines. Functional domains and intervening peptides of the preprohormones are indicated by specific patterns, white boxes represent untranslated sequences. GLP, glucagon-like peptides; PHM, peptide histidine-methionine; PRP, PACAP-related peptide.

residues of PACAP38. Although all three preproPACAP-derived peptides are present in the hypothalamus, PACAP38 is the predominant peptide being 60-fold and 10-fold more abundant compared to PACAP27 and PRP, respectively (Hannibal et al., 1995).

Comparison of the genes encoding peptides of the glucagon superfamily reveals organizational similarities but also significant differences (Figure 3). Whereas proGHRF, proGIP, and prosecretin contain a single biologically active peptide, proVIP, proPACAP, and proglucagon are composed of two or three distinct functional peptides. Furthermore, exon boundaries relative to the biologically active peptides have not been highly conserved within these two groups. If the genes encoding the glucagon superfamily of peptides are derived from a common ancestral gene, they have since then diverged extensively. The only exception is represented by the VIP and PACAP genes which show both similar structure and high sequence homology and seem to have separated more recently than the other members of this superfamily (Hosoya et al., 1992; Parker et al., 1993).

EXPRESSION OF THE GLUCAGON GENE

Posttranscriptional Processing of the Glucagon Transcript

Expression of the glucagon gene is restricted to the endocrine pancreas, intestine, and brain. In these tissues, transcription is initiated at identical sites suggesting that a common promoter region regulates transcriptional activity. In addition, preproglucagon cDNAs isolated from pancreas, intestine, and brain of mammals are identical in sequence indicating that in all glucagon expressing cells a single and unique glucagon mRNA is generated (Drucker and Asa, 1988; Novak et al., 1987). However, proteins derived from the preproglucagon precursor differ between the pancreas and the intestine. For mammals it was established that differential proteolytic processing of proglucagon gives rise to glucagon and precursor forms of the amino- and carboxy-terminal peptides in the pancreas, whereas in the intestine, glucagon remains in incompletely processed forms and the glucagon-like peptides GLP-1 and GLP-2 are liberated (Mojsov et al., 1986; Orskov et al., 1986).

Until recently, the posttranslational mechanism of tissue-specific differential expression of the glucagon-related peptides was also assumed for other vertebrates. Surprisingly, proglucagon cDNA sequences isolated from bird or fish pancreas contain glucagon and GLP-1, but lack GLP-2 (Lund et al., 1982, 1983; Hasegawa et al., 1990). Since GLP-2 peptide could not be detected by immunological analyses (Martínez et al., 1991), it was suggested that GLP-2 is not encoded by the preproglucagon gene of these species. However, recent cDNA analyses from trout and chicken intestine have revealed that in contrast to the pancreas, the intestinal preproglucagon comprises glucagon as well as both GLP-1 and GLP-2 (Irwin and Wong, 1995). Sequence comparison of a partial genomic and complete cDNA

clones isolated from these tissues indicated that generation of different proglucagon transcripts is due to alternative splicing of the pre-mRNA. As illustrated in Figure 4, a stop codon is located immediately downstream of the GLP-1 sequence in the trout preproglucagon gene. In the pancreas, the proglucagon reading frame terminates six codons 3′ to GLP-1 and a polyA site is located about 0.2 kb further downstream. In contrast, the genomic stop codon is contained within sequences of an alternative intron in the corresponding intestinal transcript. Splicing of this intron generates a continuous proglucagon reading frame coding for glucagon and the two glucagon-like peptides (Irwin and Wong, 1995). A similar situation has been found for the chicken proglucagon gene; tissue-specific expression of the derived peptides is equally regulated at the level of mRNA splicing. Interestingly, the genomic sequence of mammalian proglucagon genes contains a possible stop-codon at the same position as in the trout and chicken homologues which is however, located within intron 4 and not present in the mature mRNA. Thus, within vertebrates two different mechanisms have been developed for tissue-specific expression of the proglucagon-derived peptides. It will be of interest to analyze if alternative splicing

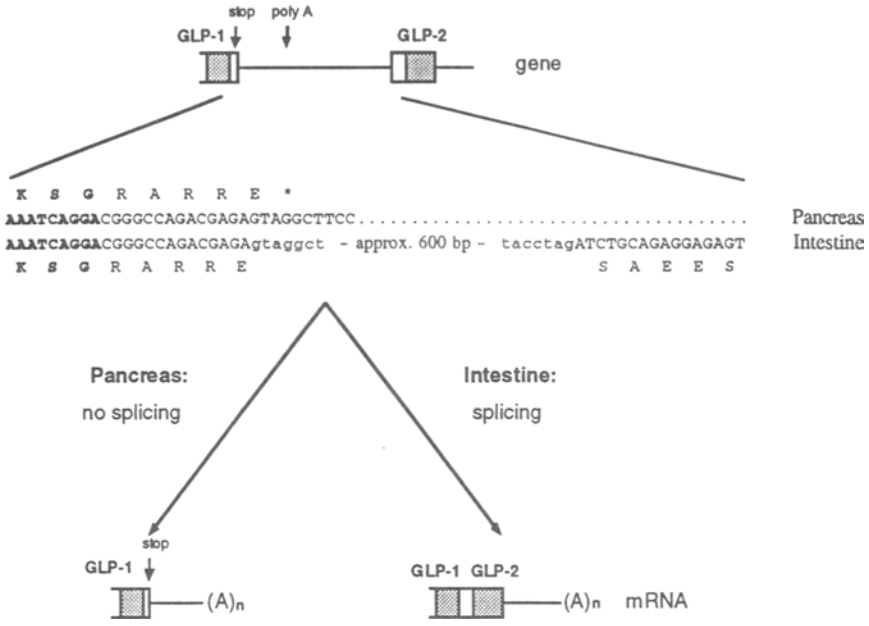


Figure 4. Schematic diagram illustrating the tissue-specific alternative splicing of the glucagon pre-mRNA in the trout. Dots in the pancreatic preproglucagon gene sequence indicate continuation of the gene, bold and small characters represent sequences coding for GLP-1, and sequences that are recognized as intronic in the intestine, respectively. GLP, glucagon-like peptides; (A)n, poly A of the mRNA.

of the proglucagon transcript in trout and chicken is typical for these vertebrate classes and at which level proglucagon gene expression is regulated in amphibians and reptiles.

Among the glucagon superfamily, alternative splicing of the respective transcript has been demonstrated for the secretin gene. In porcine intestine, two different secretin mRNAs are generated; the longer encodes a full-length preprosecretin, whereas in the shorter and less abundant transcript, exon three has been deleted by alternative splicing (Kopin et al., 1991). The physiological importance of the processing remains unclear as the third exon encodes an amino acid sequence located within the C-terminal extension peptide of secretin which has no known biological function and no tissue-specific regulation of intron splicing has been reported. Similarly, in two salmon species, different transcripts encoding peptides related to GHRH and PACAP are present (Parker et al., 1993). Whereas a longer mRNA contains a large reading frame including both peptides, an exon encoding the amino-terminal part of the GHRF-like peptide is excised in the shorter form. It is unclear, however, if these transcripts are derived from different genes or generated by alternative splicing. In mammals, both corresponding peptides are encoded by separate genes (Figure 3) and the possibility of independent expression of each peptide has been conserved in salmon irrespective of the mechanism applied.

Posttranslational Processing of Proglucagon

In mammals, proglucagon gene expression gives rise to an identical transcript in the pancreatic islets, intestine, and brain (Novak et al., 1987; Drucker and Asa, 1988). Proteolytic processing of the derived prohormone is enabled by pairs of basic amino acids flanking six potential peptides: an amino-terminal peptide (GRPP), glucagon, GLP-1, GLP-2, and the intervening peptides IP1 and IP2 (Figure 2a,b). Analysis of the processed products in the proglucagon-expressing tissues showed that processing is cell-specific and generates different peptides (Mojsov et al., 1986; Orskov et al., 1986). In pancreatic α-cells, the first proteolytic cleavage produces glicentin and the major proglucagon fragment (MPF). From the amino-terminal peptide, two different glucagon precursor molecules, 9K-glucagon (GRPP + glucagon) and oxyntomodulin (glucagon + intervening peptide, 1), are generated which finally lead to glucagon. MPF is besides glucagon the most abundant final processing product in the α-cells and is cleaved only to a small extent liberating some GLP-1 (Figure 5) (Orskov, 1992; Rothenberg et al., 1995). In contrast, the main proglucagon-derived peptides in the intestine are GLP-1 and GLP-2, whereas glucagon remains in the form of precursor molecules such as glicentin and oxyntomodulin (Orskov et al., 1986). GLP-1 is produced in three different forms, GLP-1 (aa 1-37), GLP-1 (aa 7-37), and the amidated form GLP-1 (aa 7-36); its physiological function is the stimulation of insulin biosynthesis and secretion in response to nutrient ingestion (Mojsov et al., 1986; Drucker et al., 1987c; Holz et al., 1993). A biological role for the second major proglucagon-de-

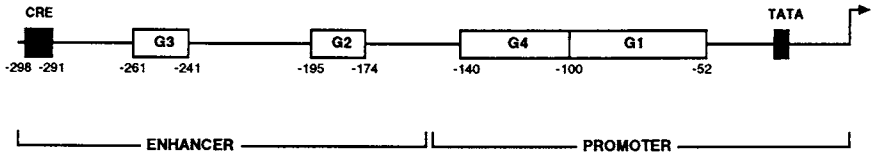


Figure 5. Schematic diagram illustrating the DNA control elements present in the first 300 bp of the rat glucagon gene upstream sequence. The 5′-flanking sequence can be divided into an α cell-specific promoter and an islet cell-specific enhancer. Numbers indicate the position relative to the transcriptional start site which is represented by an arrow. CRE, cyclic AMP-response element.

rived peptide of the intestine, GLP-2, remains to be elucidated. In the third proglucagon-expressing tissue, the brain, proteolytic processing is similar to the intestine, however, glucagon is also released (Lui et al., 1990).

Most of the peptidases which process proglucagon in the endocrine pancreas have been identified; they belong to a family of mammalian precursor processing endopeptidases specific for cleavage at dibasic amino acids (e.g., Lys-Arg) which are designated prohormone convertases (PCs). The most abundant PC in α-cells is PC2 (Marcinkiewicz et al., 1994; Rouillé et al., 1994) which is colocalized with glucagon in secretory granules (Rothenberg et al., 1995). The importance of this convertase for glucagon release has also been demonstrated by transfection of a PC2 antisense cDNA-construct in a mouse glucagon-producing cell line which inhibited proglucagon processing (Rouillé, et al., 1994). However, in *in vitro* studies, PC2 was not able to generate a completely processed form of glucagon (Rothenberg et al., 1995). In addition to PC2, at least one other prohormone convertase, PC1/PC3 is expressed in the pancreatic α-cells and PC1/PC3 can *in vitro* perform the same reactions as PC2 leading to MPF, glicentin, and oxyntomodulin, but not to free glucagon (Neerman-Arbez et al., 1994; Rothenberg et al., 1995). It will be of interest to determine which prohormone convertase is responsible for liberating glucagon from its precursor molecules in the pancreas and which enzymes mediate proglucagon processing in the brain and the L-cells of the intestine. Cell-specific posttranslational processing of proglucagon in mammals may thus be achieved by differential expression of the responsible endopeptidases.

REGULATION OF GLUCAGON GENE EXPRESSION

Cell-Specific Expression of the Glucagon Gene

The glucagon gene is expressed in three different tissues, the brain, intestine, and endocrine pancreas. In the brain, expression of the glucagon gene is mainly

localized to the brainstem and hypothalamus (Drucker and Asa, 1988) with an additional transient expression in the cortex during embryogenesis (Lee et al., 1993). The physiological role of the proglucagon-derived peptides in the brain is, however, still unknown. Expression of the glucagon gene is also found in the endocrine L-cells of the stomach, and the small and large intestine (Mojsov et al., 1986; Novak et al., 1987); developmental studies indicate that glucagon mRNA is first synthesized in the intestine and later, during the first 10 days postnatal, in the stomach (Lee et al., 1992). Most studies on glucagon gene expression however, have been focused on the endocrine pancreas and the *cis*-acting sequences directing glucagon gene transcription to the α-cells. *In vivo* analyses in transgenic mice containing the SV40 large T antigen linked to different fragments of the rat glucagon 5′-flanking sequences revealed that control elements for glucagon gene expression in the gastrointestinal tract present between position –1300 and –2000 are necessary, whereas the first 1300 bp upstream sequences are sufficient to restrict glucagon gene expression to the brain and pancreatic α-cells (Efrat et al., 1988; Lee et al., 1992).

More detailed information on the *cis*-acting elements controlling islet and α cell-specific expression has been obtained from gene transfer experiments using cultured cell lines. Progressively shortened 5′ and 3′, and internally deleted sequences of the 5′- flanking region of the rat glucagon gene fused to the coding region of the bacterial reporter gene chloramphenicol acetyl transferase (CAT) were transfected into glucagon- and nonglucagon-producing cells and analyzed for transcriptional activity (Drucker et al., 1987a,b; Philippe et al., 1988; Knepel et al., 1990a; Philippe and Rochat, 1991; Morel et al., 1995). Plasmid constructs containing 1300 and 300 bp of upstream sequences showed no difference in transcriptional activation indicating that α cell-specific control elements are contained within the first 300 bp of the glucagon 5′ flanking region.

The upstream sequences responsible for cell-specific expression of the glucagon gene in the endocrine pancreas are composed of a proximal promoter from bp -1 to -150 and an enhancer region from bp -150 to -300. Within the two functionally different regions several *cis*-acting control elements have been identified by binding assays and functional analyses: the proximal promoter contains the TATA box, G1 and G4 whereas two enhancerlike elements and a cAMP-response element (CRE) are located in the distal enhancer (Figure 5).

G2 and G3 are capable of independently increasing transcription when directly upstream of the proximal glucagon promoter or a heterologous promoter in islet cell lines. Transcriptional activation conferred by both control elements is strongly distance-dependent; in heterologous promoters they need to be located within 100 bp from the TATA box to be active (Philippe and Rochat, 1991). G2, but not G3, functions in both orientations relative to the transcriptional start. The major enhancer element is represented by G2, its deletion leading to an 80% decrease in activity. In contrast, 5′-deletions of the two most distal elements, the CRE and G3, do not result in any significant loss of basal transcription (Philippe et al., 1988).

G2 is located at position –174 to –195 and binds two complexes in gel retardation assays. One of these complexes (A2) is islet cell-specific and acts as a positive regulator of glucagon gene expression. The second complex represents the liver-enriched factor, hepatocyte nuclear factor 3 β1 (HNF-3 β1) (Figure 6). Both complexes bind to overlapping and probably mutually exclusive sites in G2. Interestingly, overexpression of HNF-3 β1 leads to a decrease of glucagon gene transcription whereas mutations that affect HNF-3 β1 and not A2 binding result in increased transcriptional activity. Therefore, HNF-3 β1 acts as a repressor of glucagon gene expression in contrast to A2 which functions as an activator (Philippe et al., 1994).

Recent studies indicate the presence of at least three different HNF-3 β isoform proteins in α-cells which are generated by alternative splicing from the HNF-3 β pre mRNA (Philippe, 1995). The three isoforms are distinguished by their amino-terminal ends; whereas HNF-3 β1 is identical to the liver transcription factor, HNF-3 β2 possesses a N-terminal extension of 5 aa and HNF-3 β3 represents a truncated protein lacking the first 30 aa. Interestingly, a transcriptional activation domain of HNF-3 β1 is located in the amino-terminal end (Pani et al., 1992; Qian and Costa, 1995) and the three HNF-3 β proteins indeed exhibit different transcriptional properties. Although all 3 HNF-3 β isoforms bind G2, two of them, HNF-3 β2 and β3 do not affect glucagon gene transcription but compete with the negatively acting HNF-3 β1 isoform to decrease its repressing effects. Further studies will elucidate the functional role of both positive and negative regulatory factors interacting with the major enhancer G2 and its impact on glucagon gene transcription.

The upstream enhancer element G3 is located at position -241 to -261 and composed of two separate domains, A and B (Knepel et al., 1990a; Philippe, 1991a;

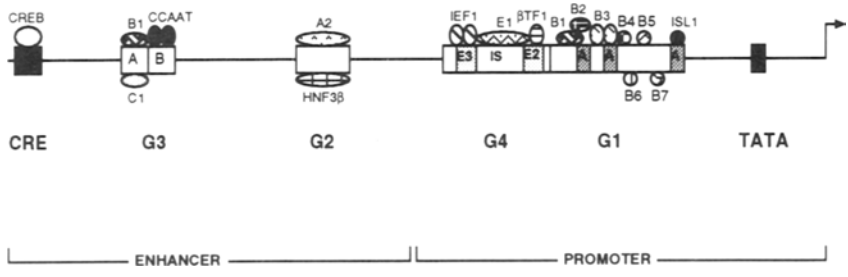


Figure 6. Schematic representation of the *cis*-acting DNA elements and *trans*-acting factors assumed to be implicated in the transcriptional regulation of the glucagon gene. Boxes indicate DNA control elements and circles or ovals, protein complexes interacting with them. The transcriptional start site is represented by an arrow. CCAAT, CCAAT-binding protein; HNF3β, hepatocyte nuclear factor-3 β; IEF1, insulin enhancer factor; βTF1, β cell transcription factor 1; CRE and CREB, cyclic AMP responsive element and CRE-binding protein, respectively.

Philippe et al., 1995). The A domain between nt –249 and –261 acts both as an insulin response element (IRE) mediating repression of glucagon gene expression and as a positive enhancer. Two islet-specific complexes, C1 and B1, bind to the A domain (Figure 6). B1 also interacts with the proximal upstream promoter element G1, implicated in α-cell specific expression. In contrast, the B domain (bp –241 to –247) binds a ubiquitous DNA-binding protein that probably belongs to the family of CCAAT-box proteins. The functional relevance of the B domain, however, is unclear as mutations of the B domain do not significantly affect the transcriptional regulation conferred by G3 (Philippe, 1989, 1991a; Chen et al., 1989a; Philippe et al., 1995).

The proximal promoter of the rat glucagon gene is composed of at least three regulatory sequences: the general TATA box and the specific *cis*-acting elements G1 and G4. G4 is a complex DNA element localized immediately upstream of G1 between nt -100 and -140; it contains two E box motifs (E2 and E3) which are separated by an intervening sequence (IS). E2 and E3 do not activate transcription as isolated elements but rather necessitate their IS to form a functional unit (Cordier-Bussat et al., 1995). E box motifs are characterized by the palindromic sequence CANNTG and interact with transcription factors of the basic helix-loop-helix (bHLH) protein family. The distal E box motif, E3, is located between nt -130 to -135 and is identical to the E1 and E2 boxes (formally: NIR and FAR) of the rat insulin I gene and to the E1 element (formally: ICE/RIPE 3a) of the insulin II gene (for review see Philippe, 1991b; German et al., 1995). The glucagon promoter sequence E3 interacts with a protein complex similar or identical to the insulin enhancer factor 1 (IEF1; see Figure 6), an islet cell-specific activator involved in the regulation of insulin, gastrin, and secretin gene expression (Wang and Brand, 1990; Ohlson et al., 1991; Wheeler et al., 1992; Cordier-Bussat et al., 1995). The second E box motif, E2, is present downstream of E3 and IS at position –103 to –108 and does not function as a typical E-box. E2 is homologous to DNA motifs (known as B-elements) which are present in the rat insulin I, somatostatin, and elastase I genes. Interestingly, two complexes interact with E2 which are highly similar to the B-element binding complex αTF1 and an unidentified ubiquitous factor, respectively (Kruse et al., 1993; Cordier-Bussat et al., 1995).

The enhancer elements G2 and G3, as well as the the distal promoter element G4, function as islet cell-specific *cis*-acting sequences but they are not able to confer α cell-specific expression in transformed islet cell lines. The major determinant that restricts glucagon gene expression to α as opposed to other islet cells is represented by the most proximal promotor element, G1, which confers only low transcriptional activation (Philippe et al., 1988; Morel et al., 1995).

G1 is present downstream of G4 from nt –52 to –100 and binds at least seven protein complexes. Four of them (B1, B2, B3, and B6) are detected by gel retardation assays using the whole G1 element and three additional complexes interact with fractions of G1 (Morel et al., 1995; Morel, unpublished data). B1, B2, and B3 bind to overlapping sequences and generate similar methylation interfer-

ence patterns; they may represent related complexes. Mutational analyses within G1 indicate that complex B1—which interacts with both G1 and G3—is critical for transcriptional activity (Morel et al., 1995). G1 contains three AT-rich sequences which are possible binding sites for homeodomain transcription factors. Recently, a LIM-homeodomain (LIM for: *lin-11 isl-1 mec-2)* factor *isl-1* was found to interact with the most proximal motif (Wang and Drucker, 1995). The two distal AT-rich sequences form an imperfect direct repeat binding a homeodomain protein (*cdx-2/3*) related to the *Drosophila* gene *caudal. Cdx-2/3* acts as a homo- or heterodimer forming a subunit of complex B3 (Laser et al., 1996). Most of G1-binding complexes observed in gel retardation assays are islet-specific, but some of them represent ubiquitous proteins. None of the presently identified complexes, however, has been found to be specific for glucagon-producing cells. Additional factors yet to be identified must interact with G1 to confer α-cell specificity. Identification of the proteins forming complexes with G1 will thus be nescessary to unravel the molecular mechanisms of alpha cell-specific expression.

Homologous Mechanisms Involved in the Control of Glucagon and Insulin Gene Expression

The glucagon-producing α-cells are colocalized with β, δ, and PP cells synthezising insulin, somatostatin, and pancreatic polypeptide, respectively, in the islets of Langerhans which are embedded in the exocrine pancreas. Both tissues are of endodermal origin arising from two buds which are formed at the dorsal and ventral sides of the duodenum (for review see Slack, 1995). Primitive islet cells differentiate in the epithelium of the foregut prior to morphogenesis of the exocrine pancreas and developmental studies indicate that the four islet hormone genes are expressed roughly at the same time rather than sequentially (Alpert et al., 1988; Herrera et al., 1991; Gittes and Rutter, 1992; Teitelman et al., 1993). Recent studies indicate that the homeobox transcription factor *IPF1* (also known as *Idx-1* or *STF1*) may be a major determinant to initiate pancreatic development because in knockout mice the pancreas is completely absent. *IPF1* is expressed in the whole pancreas in early embryogenesis but later restricted to the β and δ islet cells where it acts as a transcriptional activator of the insulin and somatostatin genes, respectively (Ohlsson et al., 1993; Jonsson et al., 1994; Miller et al., 1994; Guz et al., 1995). Glucagon mRNA is first detected in the mouse embryo at E 8.5 to 9 at the same time as insulin mRNA.

In agreement with their origin from common precursor cell lineages, glucagon-producing α and insulin-producing β-cells share *cis*-acting control elements and *trans*-acting factors determining an islet-specific level of gene regulation. These include a cAMP response element binding (CREB), AT-rich elements (A-boxes) interacting with homeodomain proteins, E-boxes which bind bHLH proteins, and yet unclassified elements (Figure 7).

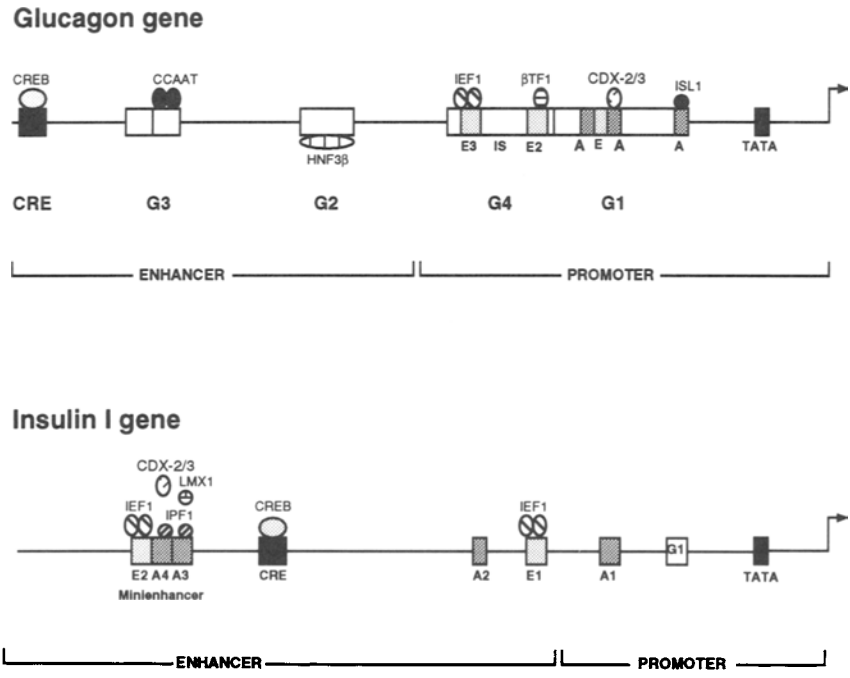


Figure 7. Schematic comparison of characterized *cis*-acting DNA elements and *trans*-acting factors assumed to be implicated in the transcriptional regulation of the rat glucagon and insulin I genes. CCAAT, CCAAT-binding protein; HNF3β, hepatocyte nuclear factor-3 β; IEF1, insulin enhancer factor 1; βTF1, β cell transcription factor 1; IPF1, insulin promotor factor 1; cdx-2/3, caudal-type homeobox 2/3; lmx-1, LIM-homeo box 1; CRE and CREB, cyclic AMP responsive element and CRE-binding protein, respectively.

One major transcription factor of the insulin gene is likely to be the heterodimeric complex IEF1 that is composed of the ubiquitous bHLH protein E47 and a recently identified bHLH factor (BETA2) present in α and β cells mediating islet cell specificity (Naya et al., 1995). IEF1 binds to both E-boxes of the insulin gene and transactivates its promoter. β cell-specific expression, however, seems to be conferred in a second step by the homeodomain protein *IPF1* that interacts with both A boxes of the insulin minienhancer (Ohlsson et al., 1993; Miller et al., 1994). Interestingly, two other homeodomain transcription factors, *cdx-2/3* and *lmx-1* are also able to bind and transactivate the minienhancer (German et al., 1992). A possible competition of several transcription factors of the homeodomain family and their relative impact on insulin gene regulation remains to be elucidated.

In glucagon-producing cells, the islet-specific complex IEF1 is a transcriptional activator that binds to the E-box E3 within the promotor element G4; E3 is identical to the E1 and E2 elements of the insulin gene (Figure 7). The functional relevance of IEF1 in glucagon gene expression is further enhanced by the observation that overexpression of the negative bHLH regulator *Id-1* inhibits transcriptional activity of DNA constructs containing G4 (Cordier-Bussat et al., 1995). Islet-specific activation of the glucagon gene is also conferred by the intervening sequence of G4, and by the enhancer-like elements G2 and G3 which interact with yet unknown complexes present in different islet cell lines. G1 contains three A-boxes and an E-box motif, which does not possess a functional role (Cordier-Bussat et al., 1995; Morel et al., 1995). The most proximal A-box binds the homeodomain factor *isl-1* which was originally isolated by its capacity to bind the insulin minienhancer (Karlsson et al., 1990; Wang and Drucker, 1995). A second homeodomain protein, *cdx-2/3* binds to the distal A-boxes of G1 and transactivates the promoter (Laser et al., 1996). *Cdx-2/3* was previously shown to be implicated in insulin gene expresion as well as in the control of the intestinal sucrase-isomaltase gene (German et al., 1992; Suh et al., 1994). Thus, glucagon gene expression seems to be regulated at an endodermal and islet-specific level by common *cis*-acting elements and transcription factors; additional *trans*-acting proteins conferring α-cell specificity, however, remain to be isolated.

Regulation by Insulin, cAMP, and Calcium

Glucagon secretion has been studied extensively and shown to be regulated by a variety of physiological stimuli, hormones, and nutrients (for review see Lefèbvre, 1995). In contrast, much less is known on the factors that control glucagon biosynthesis and the relative importance of both control levels in the physiology and pathophysiology of glucagon. Among the stimuli that regulate glucagon secretion in normal α-cells, glucose, amino acids, insulin, and catecholamines are the most significant. Whether glucose and amino acids affect glucagon biosynthesis is unknown, whereas for insulin and cAMP analogues a transcriptional effect on glucagon gene expression has been demonstrated.

Insulin inhibits glucagon gene expression both *in vivo* and *in vitro* (Philippe, 1989, 1991a; Chen et al., 1989a,b; Philippe et al., 1995). In the islets of Langerhans, glucagon-producing α-cells are located at the periphery being exposed to insulin which is secreted by the centrally located β-cells and transported to the periphery by the blood (Bonner-Weir and Orci, 1982; Greenbaum et al., 1991). Insulin receptors are present on α-cells; binding of insulin stimulates phosphorylation of the receptor β-subunit as a first step in signal transduction (Kisanuki et al., 1995). Further indications for the inhibiting effects of insulin on glucagon secretion and biosynthesis were obtained by chronic hyperglycemic clamping in normal rats for five days which resulted in a 50% increase in insulin mRNA and an 81% decrease in glucagon mRNA levels. In insulin-dependent streptozotocin-treated diabetic

rats, glucagon mRNA levels increase despite hyperglycemia and are rapidly reduced by insulin treatment.

Regulation of glucagon gene expression by insulin is mediated by an insulin-response element (IRE) at the transcriptional level (Philippe, 1989, 1991a). The IRE (bp -249 to -261) is necessary and sufficient to confer insulin responsiveness to a heterologous promoter; it is contained within the enhancer-like box G3 and corresponds to the previously identified transcriptionally active domain A. (Philippe, 1991a; Philippe et al., 1995). Two islet-specific complexes interact with overlapping sequences of the A domain and integrity of their binding sites is critical for both the enhancer-activity of G3 and the insulin effects (Philippe et al., 1995). Domain B of G3 interacts with an ubiquitous CCAAT box binding protein; although this domain appears transcriptionally inactive by itself, protein binding to both domains A and B may be mutually exclusive. Thus, a potential mechanism for the modulation of glucagon gene expression by insulin may be explained by competition between different transcription factors for G3 binding. Interestingly, both proteins interacting with the IRE of the glucagon gene are islet-specific and have been proposed to bind also to upstream sequences of the insulin and somatostatin genes that are negatively regulated by insulin (Papachristou et al., 1989; Knepel et al., 1991; Koranyi et al., 1992). Characterization of the transcription factors which bind G3 will be necessary to elucidate the physiological interrelation between insulin and glucagon.

Analogues of the second messenger cAMP activate glucagon gene expression in primary pancreatic α cells, fetal intestinal L cells, and immortalized cell lines from pancreatic and intestinal origins (Drucker and Brubacker, 1989; Knepel et al., 1990b; Drucker et al., 1991, 1994; Gajic and Drucker, 1993). Cyclic AMP-dependent regulation is mediated at the transcriptional level through a CRE located upstream of G3 at position –291 to –298. The rat glucagon CRE consists of the palindromic octamer TGACGTCA flanked by additional sequence elements TCATT both 5′ and 3′ of the core element. Binding of the transcription factor CREB to the CRE octamer activates gene expression, whereas the interaction of to-date uncharacterized proteins with the flanking motifs result in a decrease of the CREB-mediated activation. The architecture of the rat glucagon CRE thus leads to an inferior response to cAMP analogues compared to other similar sites, but allows the modulation of CREB-mediated transcriptional activation by additional factors (Miller et al., 1993).

Recent studies indicate that the rat glucagon CRE integrates extracellular stimuli transduced by different second messengers (Figure 8). One mechanism is the generation of cAMP by β-adrenergic catecholamine-stimulation which activates protein kinase A to phosphorylate CREB at serine 119 (Pipeleers et al., 1985; Drucker et al., 1991). A second major effector of CREB in α-cells is membrane depolarization and the increase in intracellular calcium levels induced by, e.g., arginine or the neurotransmitter γ-aminobuyric acid (Rorsman et al., 1989; Wang and McDaniel, 1990; Schwaninger et al., 1993). Pharmacological experiments

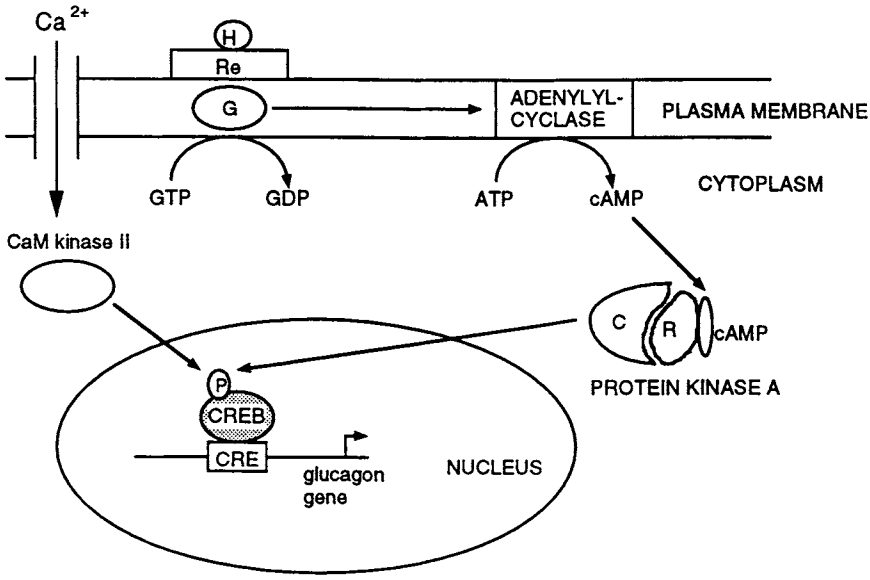


Figure 8. Model of the regulation of glucagon gene transcription by calcium and cAMP. Calcium influx induced by glucagon secretagogues via membrane depolarization leads to activation of the calcium/calmodulin-dependent protein kinase II (CaM kinase II). This enzyme phosphorylates the CRE-binding (CREB) protein at serine 119 increasing its transcriptional activity. Catecholamines and other hormones (H) may activate the cAMP second messenger pathway through G proteins (G). Binding of the ligand to its receptor (Re) induces dissociation of the α from the β and γ subunits which leads to the formation of cAMP from ATP after activation of the enzyme adenyl cyclase. cAMP binds to protein kinase A resulting in the dissociation of the catalytic from the regulatory subunit and the translocation of the catalytic subunit to the nucleus. The catalytic subunit phophorylates the CREB protein at the same amino acid as CaM kinase II which finally leads to transcriptional activation.

indicate that calcium influx activates the calcium/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylating CREB at serine 119 (Schwaninger et al., 1993). Increases in intracellular calcium levels and activation of the cAMP pathway result in synergistic effects on transcription. Both second messenger pathways lead to phosphorylation of the same amino acid in CREB; recent evidence obtained in pancreatic β-cells suggests, however, that full transcriptional activity of CREB depends on an additional dephosphorylation event at a different site (Schwaninger et al., 1993, 1995). This reaction is catalyzed by the phosphatase calcineurin which is stimulated by both second messengers.

Glucagon gene expression in pancreatic α-cells is negatively controlled by its own product glucagon as well as by various proglucagon-derived peptides (Kawai

and Unger, 1982). An excess of these peptides either infused or generated by proglucagon-producing tumors leads to a marked decrease in glucagon mRNA levels in the pancreas and intestine (Logothetopoulos et al., 1960; Bani et al., 1991; Brubacker et al., 1992,). The precise mechanisms of these changes are not completely understood; immunocytochemical and ultrastructural analyses indicate that one of the mechanisms involved may be a decrease in glucagon-producing cell number (Logothetopoulos et al., 1960; Bani et al., 1991). As exogenous glucagon is sufficient for the disappearance of α-cells in the rabbit endocrine pancreas *in vivo*, glucagon itself may be directly or indirectly responsible for this effect. Future studies will help to understand the mechamisms of this autoendocrine loop.

CONCLUSION

The application of molecular biology techniques on glucagon research has elucidated considerably its gene structure, expression, regulation, and physiological role. At the same time, this has led to a new interest in the structure and physiology of the glucagon-related peptides. A major line of interest in the future will be the identification of the factors implicated in the tissue and cell-specific expression of the glucagon and other islet hormone genes. Comparison of the control mechanisms involved in different endodermally-derived tissues will shed some light on the embryonic development of the endocrine pancreas and on islet cell differentiation. Secondly, as dysregulation of glucagon characteristic of diabetes is still incompletely understood, additional studies on the modulation of glucagon gene expression by insulin and/or glucose will be necessary to enlighten the physiology and pathophysiology of the islets of Langerhans.

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Chapter 8

Molecular Aspects of Familial Hypocalciuric Hypercalcemia

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FAMILIAL HYPOCALCIURIC HYPERCALCEMIA

Familial hypocalciuric hypercalcemia (FHH), also known as familial benign hypercalcemia, is, as its two names suggest, a benign disorder with autosomal dominant inheritance characterized by mild to moderate hypercalcemia. The hypercalcemia is present in childhood and does not respond to subtotal parathyroidectomy.

Advances in Molecular and Cellular Endocrinology
Volume 1, pages 229-239.

ISBN: 0-7623-0158-9

Serum calcium elevation is not severe, usually in the 10.5-12.5 mg/dl range. In contrast to hypercalcemia of other causes, hypercalcemia-induced morbidity is not typically seen (Law and Heath, 1985; Marx et. al., 1985). Before FHH was a well-recognized disorder, patients were often misdiagnosed with primary hyperparathyroidism.

Clinical studies of this disorder have demonstrated that both the kidneys and parathyroid glands respond abnormally to calcium in affected patients. In contrast to hypercalcemia of nonparathyroid origin, the parathyroid hormone level (PTH) is normal or minimally elevated, rather than depressed. Histologic examination of parathyroid glands from affected patients shows a spectrum from minimal pathologic change to mild chief cell hyperplasia (Thorgeirsson et al., 1981).

The kidneys of FHH patients exhibit an abnormal response to calcium. Patients with FHH who have undergone total parathyroidectomy are hypocalciuric at any given level of serum calcium compared with patients who are hypoparathyroid for other reasons, demonstrating that the relative hypocalciuria in FHH is not PTH dependent (Attie et al., 1983). In contrast to most other forms of hypercalcemia, renal complications are not seen in FHH. Specifically, the urine concentrating ability, the urinary excretion of cAMP, and the glomerular filtration rate are all preserved; moreover nephrocalcinosis and renal stones are generally absent (Marx et al., 1981).

Genetic linkage analysis of several large families with FHH demonstrated that the gene responsible for most cases of FHH was located on human chromosome 3q, flanked by genetic markers D3S1267 and D3S1303 (Chou et al., 1992; Pollak et al., 1994). Additional studies confirmed that chromosome 3q was the major, but not the only, genetic locus for FHH-causing genes, as discussed later.

NEONATAL SEVERE HYPERPARTHYROIDISM

In contrast to the mild hypercalcemia seen in FHH, neonatal severe hyperparathyroidism (NSHPT) is a disorder characterized by marked hypercalcemia, hyperparathyroidism, and skeletal undermineralization. This syndrome is generally lethal unless treated by total parathyroidectomy within the first few weeks of life (Marx et al., 1982). The parathyroid glands from affected neonates are grossly hyperplastic. A large proportion of the reported cases of NSHPT have occurred in families with other family members exhibiting the clinical features of FHH and many were the offspring of consanguineous FHH matings. This suggested the possibility that NSHPT could be the homozygous form of FHH (i.e., one copy of a mutant FHH allele causes FHH, and two copies cause NSHPT). The availability of genetic markers closely linked to the FHH locus on chromosome 3 allowed this hypothesis to be tested formally. Genotyping four NSHPT patients, their related FHH parents, and other family members demonstrated that two copies of a mutant FHH allele causes NSHPT (Pollak et al., 1994).

CALCIUM SENSING

Parathyroid cells respond to small changes in the extracellular ionized calcium concentration by altering the rate of PTH release in the opposite direction. This "calcium-sensing" property of the parathyroid gland serves to precisely regulate the extracellular ionized calcium concentration within a narrow range, normally 1.2-1.3 mM. In response to hypocalcemia, the secretion of PTH increases the serum Ca^{2+} level toward normal by effecting the release of Ca^{2+} from bone, increasing renal calcium reabsorption, and stimulating the production of 1,25-dihydroxyvitamin D [1,25(OH) $_2$-D]. The latter, in turn, increases intestinal Ca^{2+} absorption, reduces the growth of parathyroid cells, and inhibits PTH gene transcription (Nygren et al., 1988).

Parathyroid cells are not the only cell type which respond to alterations in extracellular calcium. The C-cells of the thyroid gland increase their release of calcitonin in response to an elevation in extracellular Ca^{2+}. Osteoclasts and certain cells of the renal tubule also exhibit calcium-sensing properties (Brown, 1991).

Studies carried out over the past 10 years suggested that the calcium-sensing ability of the parathyroid might be mediated by a cell surface receptor (Brown, 1991; Nemeth and Scarpa, 1986). An increase in extracellular Ca^{2+} activates phospholipase C, leading to the accumulation of inositol 1,4,5-triphosphate, and release of intracellular calcium from its stores. Thus Ca^{2+} itself appeared to be acting as a Ca^{2+} mobilizing hormone. Similar intracellular changes were seen in response to other cations, including magnesiun, gadolinium, and neomycin (Brown et al., 1990). These biochemical studies suggested that the calcium-sensing property of parathyroid cells was mediated by a G-protein coupled cell-surface receptor. Increasing levels of extracellular Ca^{2+} also produce a pertussis toxin-sensitive inhibition of cAMP accumulation in parathyroid cells. This suggested that a putative Ca^{2+} sensing receptor is also coupled to adenylate cyclase through an inhibitory G-protein.

An expression cloning strategy was used to clone a Ca^{2+} sensing receptor from bovine parathyroid. It had been previously demonstrated (Racke et al., 1991; Shoback and Chen, 1991) that injection of bovine parathyroid RNA into *Xenopus laevis* oocytes conferred upon the oocytes the ability to respond to extracellular calcium by causing the release of intracellular calcium from its stores, thereby opening cell-surface Ca^{2+}-activated chloride channels. Activation of these chloride channels leads to a current which provides an electrical readout allowing for screening successive RNA fractions for the ability to give oocytes calcium (or gadolinium) sensing activity. Using gadolinium as an agonist (to avoid a high extracellular Ca^{2+} induced increase in intracellular calcium that could result from using calcium itself), a single cDNA clone was isolated by this methodology (Brown et al., 1993).

Properties of this cDNA and its predicted protein product suggested it encoded the calcium-sensing receptor (CaSR). The pharmacological profile of the CaSR

protein as assessed by using CaSR RNA-injected oocytes was similar to that of dispersed parathyroid cells in culture, with an order of potency of agonists Gd^{3+}>Neomycin>Ca^{2+}>Mg^{2+}. The predicted protein product suggests three principal domains: a large (613 amino acid) extracellular amino terminus, seven membrane spanning segments, and a 222 amino acid intracellular carboxy-terminal tail. By Northern blot analysis, this gene is highly expressed in the parathyroid, thyroid, and kidney, and to a lesser extent in the brain and intestine (Ruat et al., 1995; Brown et al., 1993). The bovine sequence has nine potential N-linked glycosylation sites and a 21 amino acid N-terminal segment with characteristics of a signal sequence. Comparison of the bovine, rat, and human sequences shows greater than 90% homology at the amino acid level.

The extracellular tail of the receptor has no homology to consensus high-affinity calcium binding protein domains, consistent with the notion that the receptor senses extracellular calcium through low affinity binding sites. Among the superfamily of G-protein coupled receptors, the CaSR has significant homology only to the family of so-called metabotropic glutamate receptors (mGluRs) (Brown et al., 1993). Like the CaSR, the mGluRs have a large extracellular domain. It has been pointed out (O'Hara et al., 1993) that the extracellular domain of the CaSR and the mGluRs may be structurally similar to the bacterial periplasmic binding proteins involved in the sensing and transport of extracellular ligands into the cell.

DISORDERS OF CALCIUM SENSING

Several aspects of FHH and NSHPT suggested that they might be disorders of calcium sensing, perhaps caused by genetic defects in the CaSR. As noted above, both the kidney and the parathyroid gland do not respond normally to the hypercalcemia seen in FHH. If a person had one defective CaSR allele, parathyroid cells would perhaps require a higher extracellular calcium concentration for suppression of PTH secretion. Southern blot analysis demonstrated that the human homologue of CaSR was located on human chromosome 3, where most FHH and NSHPT genes map, consistent with this hypothesis.

The human CaSR was cloned by screening genomic and kidney cDNA libraries with a bovine CaSR probe. The genomic structure demonstrated that the transmembrane domains and the C-terminal tail were encoded by a single exon, while the extracellular N-terminal tail was encoded by five smaller exons. As a large number of G-protein coupled receptors (which generally lack a large N-terminal tail) are encoded for by a single exon, this may be of evolutionary significance. The CaSR gene structure may represent a fusion of a large low-affinity Ca^{2+} binding domain with another earlier receptor.

Pollak et al., (1993) used an RNase A protection assay to screen the coding sequence of the human CaSR for defects in affected members of FHH and NSHPT

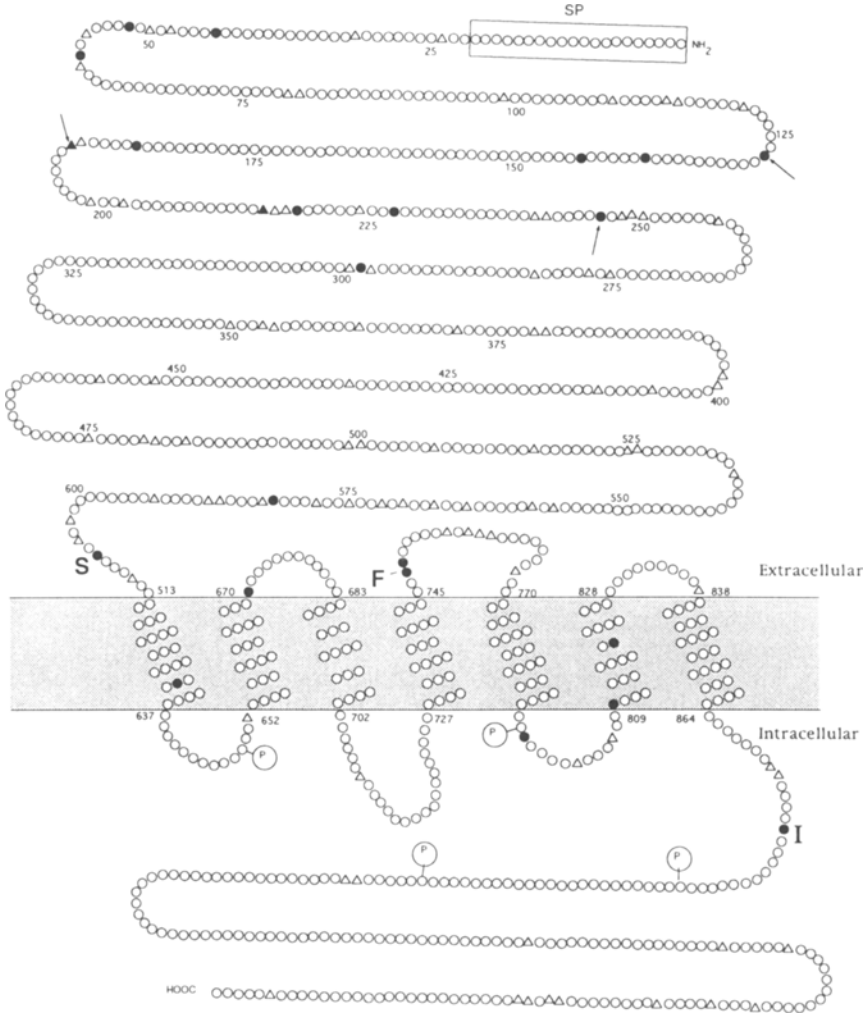

Figure 1. *Possible structure of the extracellular calcium sensing receptor is shown. Predicted PKC phosphorylation sites and signal peptide are labeled P and SP respectively. Reported mutations in the receptor are indicated by blackened amino arrows. S indicates a mutation creating a premature stop; F is a mutation leading to a frameshift; I is a mutation caused by an Alu sequence insertion.*

families. Initially they reported three missense mutations in three unrelated families which cosegregated with FHH. In one family, a child with NSHPT had two mutant alleles as predicted from the earlier genetic evidence. The expression of one of these mutant receptors in oocytes showed greatly diminished responsiveness to calcium.

This group and others have subsequently reported additional mutations in the CaSR which are presumed to cause the hypercalcemic phenotypes (Heath et al., 1994; Chou et al., 1995; Pearce, 1995a; Janicic, 1995). In most cases, the mutations are missense. The report of a stop codon in the extracellular domain in one family with FHH (Pearce et al., 1995c) suggests that a decreased number of receptors caused by one null allele is sufficient to cause a shift in the responsiveness of parathyroid cells to extracellular calcium, i.e., that the dominant phenotype of FHH is generally the result of "loss-of-function" rather than "gain-of-function" mutations. Mutations have now been reported at amino acids throughout the receptor (see Figure 1). The cause of the receptor malfunction in most of these mutant receptors has not been determined. It is reasonable to suspect that different receptor defects disrupt the receptor's function in different ways, including alteration of the affinity for calcium, abnormal protein expression, altered protein folding, and defective communication with G-proteins and, therefore, subsequent steps in signaling.

All of the mutations described in apparently unrelated families have been different. The relatively low incidence of FHH and the lack of any evidence of a so-called founder effect suggests that most FHH mutations are relatively recent events.

AUTOSOMAL DOMINANT HYPOCALCEMIA

Inherited hypocalcemia has been reported in kindreds with recessive, dominant, and X-linked patterns of inheritance (Ahn et al., 1986). Mutations which activate G-protein coupled receptors have been reported to cause human disease (Coughlin, 1994). This suggested the possibility that mutations which either activate the CaSR or increase its sensitivity to calcium could cause a hypocalcemic phenotype. This hypothesis proved to be correct. Three groups have now reported mutations in the extracellular domain of the CaSR which segregate with hypocalcemia in three families with autosomal dominant inheritance of this trait (see Figure 1; Pollak, 1994a; Pearce, 1995c; Perry, 1994). Specifically, hyocalcemia is associated with mutations Glu128Ala, Glu191Lys, and Gln246Arg. Pollak et al. (1994a) demonstrated that injection of RNA encoding mutant receptor Glu128Ala made oocytes hyperactive. The mutant receptor injected oocytes exhibited approximately fourfold and twofold higher levels of IP3 at 0.5 and 5.0 mmol/L calcium, respectively. These disorders can be thought of as a form of hypoparathyroidism. *In vivo*, the inappropriate stimulation of the receptor's signal pathway at low extracellular calcium levels inhibits secretion of PTH, resulting in frank hypocalcemia. Further reductions in serum calcium increase PTH, however, indicating that the abnormal receptor reduces the set-point of the parathyroid cell for extracellular calcium.

All three of these point mutations are in the extracellular domain of the receptor. In other G-protein coupled receptors, activating mutations have been described in

Table 1. Mutations in CaSR Causing Hypercalcemia or Hypocalcemia (in Bold)

Pro55Leu
Arg62Met
Arg66Cys
Glu127Ala
Thr138Met
Gly143Glu
Arg185Glu
Glu191Lys
Asp215Glu
Tyr218Ser
Arg227Gu,Leu
Gln246Arg
Cys582Tyr
Ser607 STOP
Ser657Tyr
Ala747 Frameshift
Pro748Arg
Arg795Trp
Phe806Ser
Val817Ile
877 Alu Insertion

the transmembrane domains. It will be of interest to see if any such naturally occurring transmembrane domain point mutations in the CaSR cause clinical disease, and if such a phenotype is different from the hypocalcemic phenotype described above. Location of mutations associated with FHN, NSHPT, and hypocalcemia are described in Table 1 and Figure 1.

PARATHYROID ADENOMAS

One of the most common causes of hypercalcemia is primary hyperparathyroidism. Most often, this is caused by parathyroid adenomas. Parathyroid hyperplasia and carcinoma are much rarer.

A sizable fraction of parathyroid adenomas appear to be monoclonal in origin (Arnold, 1988). Investigators have noted that these adenomas exhibit a shift in the calcium set-point for PTH suppression. This, together with the unregulated parathyroid growth in NSHPT, raised the possibility that somatic defects in CaSR could be an early event in the development of some parathyroid tumors. Search for such defects by RNase protection assays of DNA from more than 40 parathyroid tumors showed no evidence of mutations, however, suggesting that CaSR defects are not generally involved in the development of these tumors (Hosokawa et al., 1995).

OTHER FHH GENES

At least two other genetic loci for FHH exist. One group has demonstrated genetic linkage of a family with FHH to chromosome 19p (Heath et al., 1993), and another family has been described without linkage to either of these two loci (Trump et al., 1993). The responsible genes have not yet been identified. It will be of significant interest to see if these genes encode proteins located in the same signaling pathway initiated by CaSR activation.

PHYSIOLOGICAL IMPLICATIONS OF THREE INHERITED HUMAN DISORDERS

The human disease states which result from CaSR defects provide insight into its normal physiological role. The phenotype in the human CaSR "knockout," NSHPT, demonstrates that the CaSR plays a central and essentially nonredundant role in regulating calcium homeostasis. When both copies of the CaSR are defective, marked parathyroid hyperplasia is seen, suggesting that CaSR acts, directly or indirectly, as a growth suppresser in at least this one cell type.

The fact that one defective allele leads to the mild to moderate hypercalcemia of FHH is highly suggestive that a reduction in the number of normal receptors decreases the strength of the subsequent signal regulating PTH secretion. The lack of effects of hypercalcemia on renal function seen in FHH patients, together with its patterns of expression in this organ, yields information into CaSR's function in this organ. Patients with FHH exhibit a PTH-independent increase in renal calcium reabsorption. Thus CaSR functions in the kidney to directly regulate calcium excretion. *In situ* hybridization of the rat CaSR in the kidney demonstrates expression in the renal cortex and outer medulla (Riccardi et al., 1995). There is considerable overlap between regions of expression of the CaSR and PTH receptor in the cortical thick ascending limb of the kidney. Thus calcium appears to modify its own handling in the nephron in at least two ways: by altering the level of PTH, and by a direct interaction with the CaSR in the kidney. Similarly, patients with FHH, in contrast to other hypercalcemic states, exhibit no decrease in urine concentrating ability. Other forms of hypercalcemia produce a form of secondary nephrogenic diabetes insipidus. Sodium reabsorption in the thick ascending limb of the loop of Henle in the kidney is inhibited by Ca^{2+} (Hebert and Andreoli, 1984). Patients with defective Ca^{2+} sensing may be insensitive to this effect of Ca^{2+} CaSR is also expressed in the apical plasma membrane of tubular cells in the collecting duct, where CaSR-mediated signals may counter the vasopressin-mediated effect on water channel insertion.

As FHH, NSHPT, and hypocalcemia-associated mutations continue to be reported, structure-function information will continue to grow. The hypocalcemia-causing mutations in the extracellular domain may increase its affinity for calcium,

or cause the protein to adopt a configuration similar to that of an activated receptor. The mechanism by which these gain-of-function and loss-of-function mutations cause clinical phenotypes will be aided by *in vitro* studies of these mutant receptors.

CaSR AND THE BRAIN

Significant levels of CaSR expression are seen in bovine (Brown et al., 1993) and rat (Ruat et al., 1995) brain. The highest levels of gene expression are seen in the hypothalamus and corpus striatum. CaSR protein expression appears throughout the brain and to a significant extent is localized in nerve terminals. The function of CaSR in the brain is not yet understood. The fact that NSHPT patients who have undergone parathyroidectomy early in life lack obvious neurologic problems suggest that its function in the brain may not be of great importance. It is not known if the significant cerebral toxicity seen in both hypocalcemic and hypercalcemic states is mediated by the CaSR. Ruat et al. (1995) raise the possibility that CaSR may detect local changes in Ca^{2+} in synaptic clefts after neuronal depolarization and alter neuronal function accordingly.

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Chapter 9

Molecular Aspects of Steroid Receptor/DNA Binding

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Advances in Molecular and Cellular Endocrinology
Volume 1, pages 241-264.

ISBN: 0-7623-0158-9

INTRODUCTION

Regulation of gene transcription involves a large number of different DNA-binding transcription factors, which interact with specific DNA sequences in the promoters and enhancers of the regulated genes. Sequence specific DNA binding by transcription factors, and the subsequent regulation of gene transcription, forms the basis for interpretation of information stored in the DNA into developmental and physiological signals in the cell. The steroid hormones, glucocorticoids, progesterone, androgens, mineralocorticoids, and estrogens regulate different aspects of development, differentiation, growth, and metabolism by binding to the steroid receptors. Upon steroid hormone binding the receptors interact with DNA sequences, called response elements, near the regulated genes, and activate or repress gene transcription. Recognition of specific response elements, which is mediated by the DNA-binding domain, constitutes the basis for the unique effects of each hormone. Specific DNA binding by steroid receptors, as well as by other transcription factors, forms an important part of the specificity of gene regulation. In this review the general principles used by transcription factors, and specifically steroid receptors, to accomplish the needed specificity and affinity for DNA will be described.

STEROID RECEPTORS

A cell constantly receives signals from its environment that trigger the cell to perform different tasks, such as proliferation, differentiation, and metabolism. Long lasting changes in the cell require modification of the levels and pattern of cellular proteins, which is obtained by differential gene expression. The specific genes that are regulated by the received signals depend upon the set of transcription factors that have been activated by the stimuli. The external signals communicate with the transcription factors in several ways. Peptide hormones, neurotransmitters, growth factors, and cytokines activate receptor molecules in the plasma membrane, initiating a cascade of molecular events, often including protein phosphorylation and dephosphorylation, which eventually activate the transcription factor (Karin, 1994). Steroid receptors, glucocorticoid (GR), progesterone (PR), androgen (AR), mineralocorticoid (MR) and estrogen (ER) receptors form a distinct class of transcription factors that are directly activated by the binding of the signaling

molecule. Upon hormone binding the receptor interacts with specific DNA sequences near the regulated gene, called response elements, which results in activation or repression of gene transcription (Evans, 1988; Beato, 1989; Truss and Beato, 1993). Steroid receptors belong to the larger family of nuclear receptors that also includes receptors for thyroid hormone (TR), retinoids (RAR), and vitamin D3 (VDR). Many orphan nuclear receptors have been identified for which no ligands have been found as yet.

The steroid receptors are complex proteins, since determinants for all steps in the signaling pathway are included within one protein molecule; however the different functions are performed by distinct domains. The domain structure of the rat GR was initially studied using proteolytic fragments that retained the different functions, and immunodominant, DNA-binding, and ligand-binding domains were identified (Carlstedt-Duke et al., 1987). Cloning of the first receptors showed that the domain structure was conserved, and the functions of the three main domains were mapped in GR and ER (Figure 1). The C-terminal region of the receptor is responsible for ligand binding (Giguere et al., 1986; Kumar et al., 1986; Danielsen et al., 1987; Rusconi and Yamamoto, 1987) and contains regions that mediate nuclear localization (Picard and Yamamoto, 1987), binding of heat shock protein 90 (hsp90) (Denis et al., 1988), dimerization (Fawell et al., 1990), and transactivation (Hollenberg and Evans, 1988; Webster et al., 1988). The DNA-binding function is localized to a highly conserved central domain (Kumar et al., 1986; Danielsen et al., 1987; Hollenberg et al., 1987; Rusconi and Yamamoto, 1987), which also contains a nuclear localization signal (Picard and Yamamoto, 1987), and a dimerization region (Dahlman-Wright et al., 1991). Finally, the N-terminal region of the receptor contains a transactivation domain (Hollenberg and Evans, 1988; Tora et al., 1989).

The molecular events whereby the steroid receptors mediate their action are to a large extent similar for the different receptors (Figure 2). Unliganded steroid receptors are in an inactive form in a multiprotein complex with heat shock proteins, which dissociate from the receptor upon ligand binding. The hormone binds to the 250 residue long ligand-binding domain (LBD) and the ligand bound receptor binds

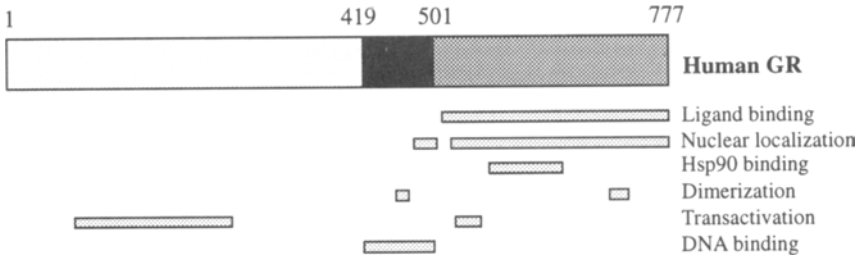

Figure 1. Domain structure of the human GR. Regions of the GR that mediate different functions are indicated. The location of the C-terminal dimerization domain is based on sequence homology with a dimerization domain identified in the ER.

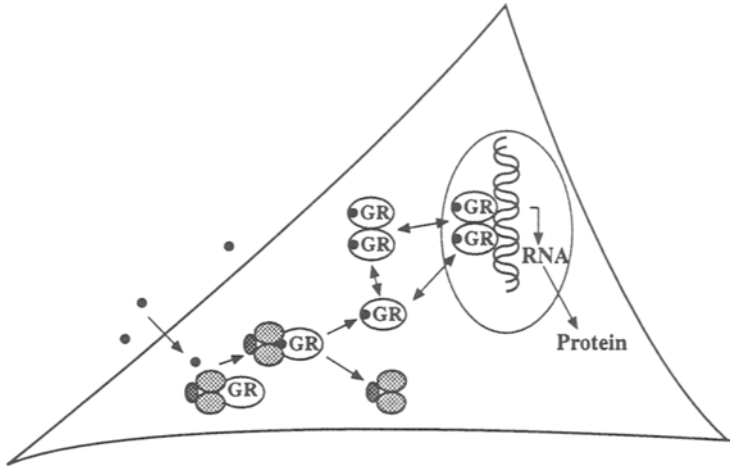

Figure 2. Model of the molecular mechanism of GR action. Glucocorticoids are indicated by filled circles, and heat shock proteins by shaded ovals.

as a dimer to specific DNA response elements close to regulated genes.The glucocorticoid (GRE) and estrogen response elements (ERE) are palindromic repeats of 6 base pair long half-site sequences, whereas many other nuclear receptors bind to direct repeats of the half-site sequence. The DNA bound receptor can, depending upon the gene, either activate or repress gene transcription.

PRINCIPLES OF DNA BINDING

DNA-Binding Motifs

DNA-binding proteins can be divided into different classes based on the structures of their DNA-binding domains (DBDs) (Wolberger, 1993; Burley, 1994). Three-dimensional structures are available for many DBDs, among them the helix-turn-helix (HTH), basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), and β-sheet motifs, as well as zinc fingers, and the high mobility group (HMG)-box (Table 1). Steroid receptors belong to the larger group of zinc finger proteins which contain zinc ions coordinated by cysteine or histidine residues (Berg, 1993).

Comparison of three-dimensional structures of protein-DNA complexes reveals some general principles of DNA binding (Wolberger 1993; Burley, 1994). Most DBDs use an α-helix, which is inserted into the major groove of the DNA, as a recognition element. However, the exact positioning of the α-helix in the major groove varies between the HTH, zinc finger, bZip, and bHLH classes of DNA-binding proteins. Arc and MetJ repressors belong to a distinct class that interacts with

Table 1. Classes of DNA-Binding Proteins

DNA-Binding Motif	*Subclass**	
Helix-turn-helix motif (HTH)	Prokaryotic	CAP
	Homeodomain	Engrailed
	Winged-helix	HNG-3
	Other eukaryotic	LFB1/HNF1
Zinc fingers	Cys_2His_2	Zif268
	Cys_6	GAL4
	Cys_4	GATA-1
	Nuclear receptor	GR
Basic leucine zipper (bZIP)		GCN4
Basic helix-loop-helix (bHLH)		Max
β-sheet motif	Prokaryotic	MetJ
	Eukaryotic	TBP
HMG-box		HMG-1

Note: * Classes of DNA-binding proteins are listed, and an example from each subclass is given.

the major groove using a β-sheet, and the TATA-binding protein (TBP) interacts with the minor groove of DNA by using β-sheets. Although the α-helix or β-sheet recognition elements from the main contacts with the DNA, other structural elements, such as additional α-helices and loops, also interact with the DNA.

Base Pair Interaction

Proteins interact with the DNA by forming extensive contacts both with bases and the DNA backbone. The specific recognition of a DNA sequence is due to interactions with the DNA bases. The four different bases have different sets of functional groups in the major groove, which allows discrimination between the bases (Figure 3) (Seeman et al., 1976). Hydrogen bonds are formed between the hydrogen bond acceptors or donors on the edge of the base, and the functional groups of the amino acid side chain. In some cases the carbonyl group of the protein backbone forms a hydrogen bond with a base. For example, GAL4 interacts with two of the three recognized bases by backbone carbonyl groups that form hydrogen bonds with cytosines (Marmorstein et al., 1992). The hydrogen bonds can be direct or mediated by bridging water molecules. The Trp repressor is an extreme example of the use of bridging water molecules for specificity, since no direct hydrogen bonds of importance for the sequence binding are formed (Otwinowski et al., 1988). A crystal structure of the Trp DNA binding site showed that the water molecules

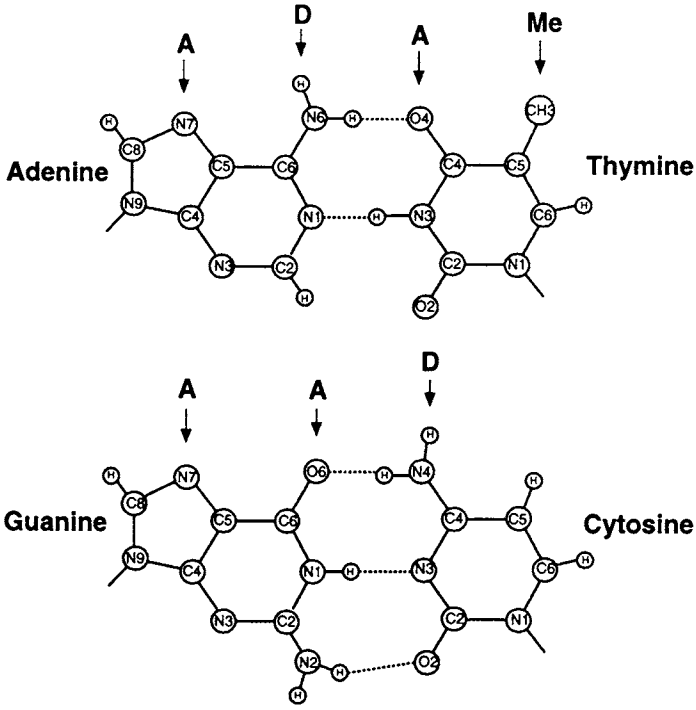

Figure 3. Schematic representation of base pairs. The atoms that can function as hydrogen bond acceptors (A) and donors (D) in the major groove are indicated. The methyl group of thymine is indicated by Me.

are already bound to the bases in the free DNA (Shakked et al., 1994). Water molecules can be regarded as noncovalent extensions of the DNA bases that together form the structure that the protein recognizes.

In addition to hydrogen bonds, hydrophobic van der Waals interactions contribute to specificity. Hydrophobic interactions are formed between the methyl group of thymine, and methyl groups of hydrophobic side chains, as well as the hydrophobic regions of the side chains of polar residues. Hydrophobic contacts contribute to base pair recognition in most DNA complexes. The interaction of TBP with the minor groove of the TATA-box is primarily due to hydrophobic base contacts (Kim et al., 1993a, b). The specific interaction between GATA-1 and the DNA in the major groove is also mainly hydrophobic (Omichinski et al., 1993), which is confirmed by the exclusion of water molecules from the protein-DNA interface (Clore et al., 1994).

DNA Backbone Interaction

The interactions with the functional groups of the bases are of primary importance for sequence specificity. In addition, the proteins contact the DNA backbone, mainly the phosphate molecules, but also the sugar groups. Interaction with the DNA backbone on both sides of the major groove fixes the position of the α-helix or β-sheet, and prevents rolling of the recognition element in the major groove. The backbone interactions increase the total contacts with the DNA, and thus the DNA affinity. Although the backbone interactions are of a nonspecific nature, they contribute to the specificity of binding by positioning the protein against the DNA, which allows the formation of the sequence specific contacts with the bases. Many different residues can interact with the DNA backbone. In addition to the basic residues arginine and lysine, which form electrostatic interactions with the phosphate groups, several polar residues, as well as the NH group of the protein backbone, form hydrogen bonds with the DNA backbone.

DNA Conformation

The local conformation of free DNA is base pair sequence dependent (Fedoroff et al., 1994) and most protein-DNA interactions result in some conformational change in the DNA (Travers, 1992). The affinity of a protein for a specific sequence can depend both on the intrinsic DNA conformation and whether the protein can change the DNA conformation to optimize the interaction. The prokaryotic 434 repressor binds with different affinity to the two 434 operators OR1 and OR2, which differ only in the base pair sequence at the center of the operator, which does not interact directly with the protein. Three-dimensional structures of both complexes show that the binding of the protein to the two sequences causes the DNA to adopt a specific conformation (Aggarwal et al., 1988; Shimon and Harrison, 1993). The energetic cost of achieving this conformation, which most probably is different for the two sequences, can contribute to the relative affinity of the repressor for the operators.

Dimers, Oligomers, and Extended Regions

To obtain high DNA affinity and specificity, a DNA-binding protein must interact with a long enough base pair sequence. In the *Escherichia coli* genome a binding site that reappears at random less than once must be at least 12 base pairs long (von Hippel and Berg, 1986). An α-helix or β-sheet that binds in the major groove cannot generally contact more than four bases. Different methods have been used by the DNA-binding proteins to increase the contacting surface (Burley, 1994; Wolberger, 1993). Many proteins bind to DNA as dimers which results in twice as many base contacts. The prokaryotic HTH proteins and the Cys_6 class of zinc finger proteins bind as homodimers, and the β-sheet proteins Arc and MetJ repressors

bind as tetramers or even higher oligomeric forms. The nuclear receptors, and the bZip and bHLH proteins bind either as homo- or heterodimers to the DNA. Cooperative interactions between the monomers can often further increase the affinity. Another solution to the problem has been developed by the Cys2.His_2 zinc finger proteins, which use coupled zinc fingers that each interact with three to four bases. The POU family of transcription factors use two covalently coupled DBDs, one HTH-like domain and one homeodomain, thus increasing the interaction surface. Some proteins that bind as monomers use an additional structural element to contact DNA. The homeodomains bind primarily as monomers, but increase the contact surface using an N-terminal arm, which interacts with bases in the minor groove. A similar mechanism is used by the zinc finger protein GATA-1, and the winged-helix protein HNF-3, which make additional contacts with the DNA using a C-terminal tail and two loops, respectively.

Thermodynamic Driving Forces

Protein-DNA interactions are ultimately determined by the free energy of binding and the thermodynamic driving forces. The free energy of binding is determined both by enthalpic and entropic contributions. The specific interactions between the protein and the DNA contribute to the enthalpy component, whereas entropy contributions can be regarded as being due to the release of water molecules or ions from the interacting surfaces of the protein and DNA. A protein-base hydrogen bond contributes about –1.5 kcal mol^{-1} to the binding energy (Lesser et al., 1993), which is due mainly to a favorable change in the enthalpy. Hydrophobic contacts with the thymine methyl group can account for between –0.6 and –1.6 kcal mol^{-1} of the binding energy (Takeda et al., 1989). It has been suggested that the major part of that energy may be due to the desolvation of the methyl group upon protein binding, which results in a favorable entropy change (Plaxco and Goddard, 1994). Enthalpy and entropy contributions to the free energy of binding can be estimated by determining the binding constant at different temperatures, that is by van't Hoff analysis, or by directly measuring the enthalpy change using microcalorimetry. The thermodynamics of some sequence specific DNA-binding proteins, including the GR DBD, have been studied using these methods (Record et al., 1991; Lundbäck et al., 1993, 1994; Ladbury et al., 1994). Most interactions appear to be entropy driven below 20 to 25 °C. Although the sequence specificity is probably mainly due to direct interactions and thus enthalpy changes, the actual driving force is related to desolvation of the interacting surfaces. Most specific protein-DNA interactions that have been studied are characterized by a large negative heat capacity change (ΔC_p). A similar situation is associated with protein folding (Dill, 1990). The large negative ΔC_p has been related to the hydrophobic effect: the removal of large amounts of nonpolar surface from water upon protein-DNA interaction (Ha et al., 1989). The amount of nonpolar surface that must be buried to account for the ΔC_p seems to be too large if the DNA and protein are considered

to be rigid bodies that interact. To account for the ΔC_p, it has been suggested that the binding is coupled to local folding, and conformational changes in the protein and/or DNA (Spolar and Record, 1994). This induced fit model for protein-DNA interaction could thus explain the large negative ΔC_p that characterizes specific protein-DNA interaction.

DNA Recognition Code

The amino acid-base contacts that contribute to the specificity of protein-DNA interactions cannot be resolved to a specific DNA recognition code, whereby certain DNA bases are always recognized by certain amino acids. However, comparison of different protein-DNA complexes shows that some amino acid-base interactions are repeatedly observed (Suzuki, 1994). For example, hydrogen bonds between arginine or lysine and guanine, aspartate or glutamate and cytosine, as well as asparagine or glutamine and adenine, are often found. Whether these interactions can be formed in a protein-DNA complex is influenced by other residues in the protein, and the position of the protein relative to DNA. The Cys_2His_2.zinc finger proteins have been used as models to investigate possible specificity rules (Nardelli et al., 1992; Desjarlais and Berg, 1993). Generally each of the residues in the zinc finger that contacts the DNA interacts with a specific base position, and mutation of the residue changes the specificity for that particular base position. However, the residues do not act completely independently, and some synergistic effects between residues are observed, which complicates the formulation of specificity rules. The phage display system has been used as an alternative strategy to study zinc finger specificity. Zinc finger peptides with some randomized residues are expressed on the phages, and peptides with desired specificities are selected. Using this method peptides interacting with five different DNA sequences were successfully selected, whereas no selection was obtained for six other sequences (Jamieson et al., 1994; Rebar and Pabo, 1994). Thus, the phage display system might be useful in generating proteins that are designed to bind specific DNA sequences, although it might not be possible to obtain sequence specific proteins for all DNA sequences.

DNA BINDING BY STEROID RECEPTORS

DNA-Binding Domain

The DNA binding of steroid receptors is mediated by the highly conserved DBD. The DBD was initially identified by proteolysis studies of the rat GR (Wrange et al., 1984). When the receptors were cloned, a 66 residue-long conserved region rich in cysteine, arginine, and lysine residues was found (Figure 4). A domain overlapping the conserved core which was needed for DNA binding was subsequently studied by deletion mutagenesis of GR and ER (Kumar et al., 1986;

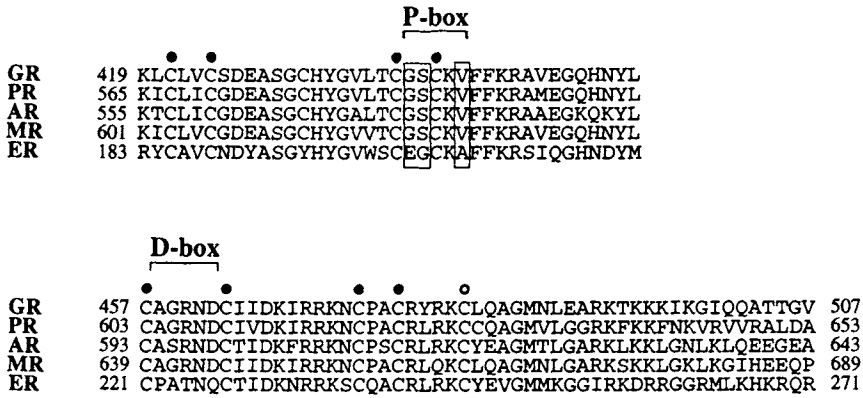

Figure 4. Amino acid sequences of steroid receptor DBDs. The nine conserved cysteine residues are indicated by circles; the eight cysteines that coordinate zinc ions by filled circles and the ninth cysteine by an open circle. The three P-box residues that determine half-site specificity, and the D-box that mediates dimerization are indicated. Residues that corresponds to 421-486 in GR form the highly conserved region. Sequences of human receptors are shown.

Danielsen et al., 1987; Hollenberg et al., 1987; Rusconi and Yamamoto, 1987). Experiments using chimeric GR and ER in which the conserved region was replaced by the corresponding segment in the other receptor confirmed that this region mediated specificity at the DNA binding level (Green and Chambon, 1987; Kumar et al., 1987). The three-dimensional structures of the GR and ER DBDs have been determined by nuclear magnetic resonance (NMR) and X-ray crystallography (Figure 5) (Härd et al., 1990; Schwabe et al., 1990, 1993a; Luisi et al., 1991; Baumann et al., 1993). The DBD is folded into a compact domain containing two subdomains each composed of the zinc binding site, an α-helix and an extended region. In each zinc binding site one zinc ion is coordinated by four cysteine residues. The α-helices extend from the zinc coordinating cysteines and end in the extended regions. The DBD binds to the palindromic response element as a dimer. The first α-helix in the DBD functions as a recognition helix and is inserted into the major groove of DNA as the receptor binds to the response element. The dimer interface is formed by residues in the C-terminal zinc binding site.

Zinc Coordination

Cloning of the nuclear receptors showed that the putative DBD contained conserved cysteine residues, which were suggested to be involved in zinc coordination by analogy with the cysteines and histidines in the *Xenopus* transcription

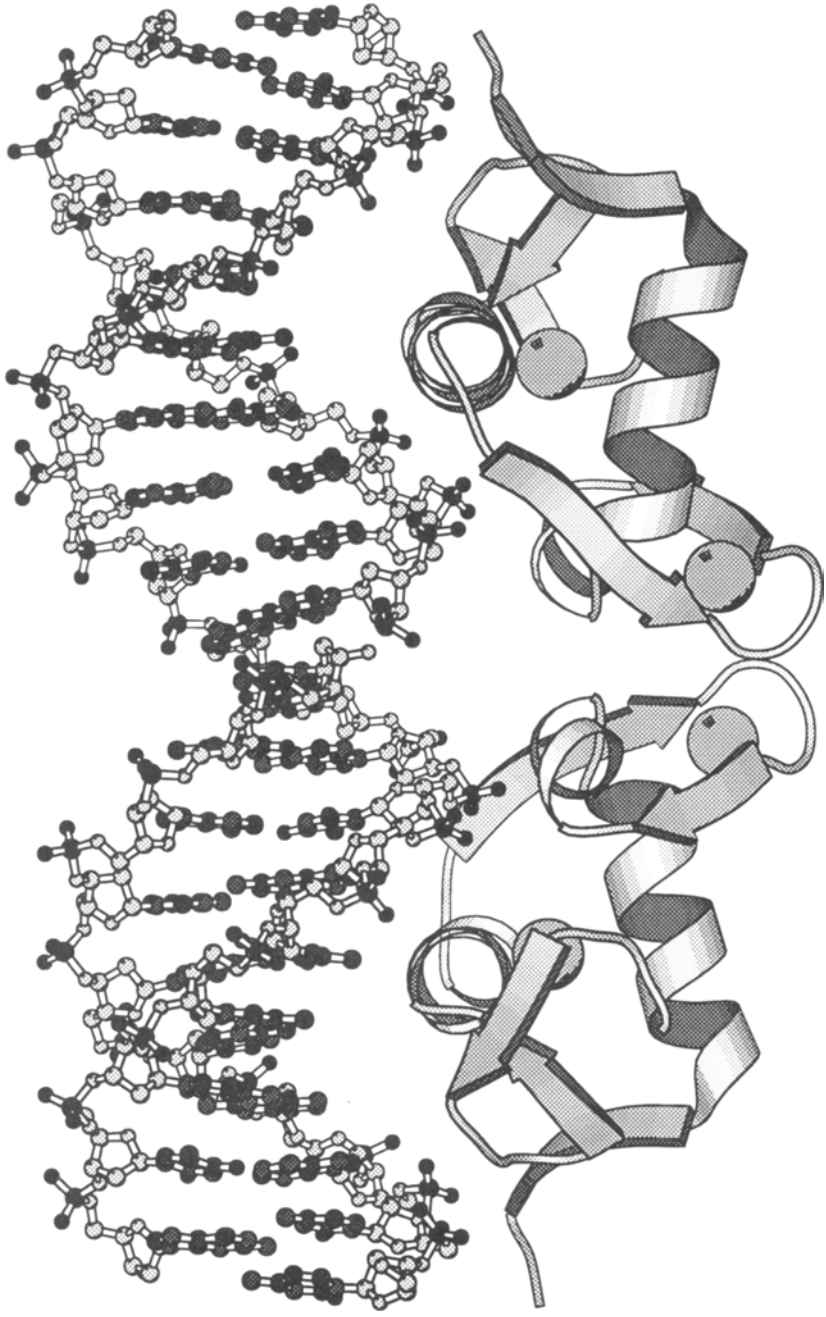

Figure 5. GR DBD bound to DNA. A schematic view of a GR DBD dimer bound to a GRE is shown. The recognition helices are positioned in the major grooves of the DNA, and the C-terminal zinc domains form a dimer interface. The zinc ions are indicated by spheres.

factor TFIIIA (Miller et al., 1985). A role for zinc in receptor function was suggested by the inhibition of DNA binding of GR and ER following the removal of metal by the metal chelator 1,10-phenanthroline (Schmidt et al., 1981; Sabbah et al., 1987), and by zinc ion potentiated binding of AR to nuclei (Colvard and Wilson, 1984). Subsequently, atomic absorption spectroscopy showed that the GR DBD contains two zinc ions (Freedman et al., 1988). The zinc ions in GR DBD can be removed by incubation at low pH in the presence of EDTA, which results in an apoprotein that is incorrectly folded, as shown by its susceptibility to proteolysis (Freedman et al., 1988; Zilliacus et al., 1992b) and by circular dichroism (Carlstedt-Duke et al., 1989; Pan et al., 1990). Furthermore, the apoprotein cannot bind DNA (Freedman et al., 1988; Carlstedt-Duke et al., 1989). DNA binding can be resorted by incubation with zinc ions at neutral pH (Freedman et al., 1988; Carlstedt-Duke et al., 1989).

EXAFS spectroscopy of the GR DBD, using Zn(II)tetrathiophenolate as a standard, established that the zinc ions are tetrahedrally coordinated (Freedman et al., 1988). Since the DBD contains nine conserved cysteine residues, and only eight are involved in zinc coordination, two different schemes for zinc coordination were suggested (Evans, 1988). The two schemes differed at the C-terminal zinc domain, which contains five conserved cysteines, and the most C-terminal cysteine (Cys-481 of human GR) was either included or excluded (Figure 4). The zinc coordination scheme, which excludes the most C-terminal cysteine, was subsequently supported by mutagenesis studies (Severne et al., 1988; Zilliacus et al., 1992b).

The zinc coordination scheme has further been studied by ^{113}Cd-^{1}H heteronuclear NMR spectroscopy using ^{113}Cd substituted GR and RAR DBDs. The ^{113}Cd-^{1}H heteronuclear coupling constant can be used to identify the metal binding ligands. The results showed that GR DBD contains two metal binding sites, and that Cys-476 is the fourth zinc coordinating cysteine in the C-terminal zinc domain (Kellenbach et al., 1991). However, due to resonance overlap, it was not possible to exclude unequivocally some role of Cys-481 in zinc coordination. In RAR it was possible to conclude that the most C-terminal cysteine residue is not involved in zinc coordination (Knegtel et al., 1993). The function of the conserved Cys-481 is not clear, except that it is part of the hydrophobic core of the DBD, and is included in an α-helix (Härd et al., 1990).

Hormone Response Elements

The DNA sequences that mediate the action of the steroid hormones and that are bound by the steroid receptors are called hormone response elements. The glucocorticoid response element was identified as a conserved sequence in glucocorticoid regulated genes (Scheidereit et al., 1986; Jantzen et al., 1987). Similarly, a conserved sequence was identified in estrogen regulated genes (Walker et al., 1984). Both sequences are partially palindromic and are composed of two 6 base pair long half-sites with a 3 base pair spacer (Figure 6). These sequences were

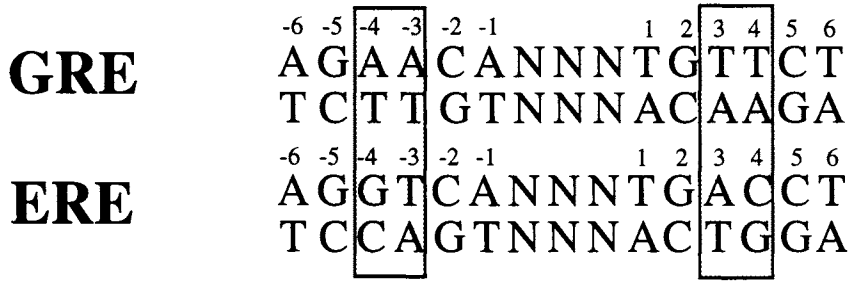

Figure 6. Hormone response elements. The GRE and ERE are palindromic repeats which differ in half-site sequence. The bases that are different in the two types of half-sites are highlighted.

sufficient to mediate gene induction by glucocorticoids and estrogens, respectively (Jantzen et al., 1987; Martinez et al., 1987; Strähle et al., 1987; Klein-Hitpass et al., 1988). In addition, the GRE can function as a recognition sequence for PR and AR (Strähle et al., 1987; Ham et al., 1988). Comparison of the optimal response elements for GR, PR, and AR shows no clear differences in the DNA-binding specificities of these receptors (Nordeen et al., 1990; Roche et al., 1992; Lieberman et al., 1993). However, the ER binds to a sequence that differs from the GRE at two positions in each half-site (Klock et al., 1987; Martinez et al., 1987).

Many other nuclear receptors, including TR, RAR, and VDR, bind to half-site sequences that are similar to the ERE half-site. However, the half-sites in the response elements for these receptors are arranged as direct repeats in contrast to the palindromic arrangement in the steroid hormone response elements (Näär et al., 1991; Umesono et al., 1991).

DNA Recognition

The crystal structures of GR and ER DBD bound to DNA allow analysis of the details of the receptor-DNA interaction (Luisi et al., 1991; Schwabe et al., 1993a, 1995). Comparison of the GR and ER DBD-DNA complexes shows similarities but also differences. Generally, conserved residues in the proteins interact with conserved bases in the response element. In the GR DBD-DNA complex Arg-447 donates two hydrogen bonds to G2 and Lys-442 donates one direct and one water mediated hydrogen bond to G(-5) (for amino acid and base pair numbering see Figures 4 and 6). In the ER DBD-DNA complex Arg-211 (which corresponds to 477 in GR) donates one direct and one water mediated hydrogen bond to G2 and Lys-206 (442 in GR) donates one hydrogen bond to G(-5). However, the conserved residue Lys-210 in ER (446 in GR) forms a hydrogen bond with T(-3) and G(-4) only in the ER DBD-DNA complex. In GR Lys-446 does not make base contacts,

but forms instead a salt bridge with Glu-450. The interactions that contribute to the specificity of the GR and ER are formed by Val-443 in GR and Glu-203 in ER. The GR specific Val-443 forms a hydrophobic contact with T3 and the ER specific Glu-203 accepts a hydrogen bond from C4. In addition to these base contacts the receptors form extensive phosphate contacts that further increase the binding affinity.

The receptor-DNA interactions seem to be partly flexible and show variation depending on the nature of both the protein and the DNA. Clearly, conserved residues in the GR and DBDs do not make exactly the same DNA contacts. In particular the conserved Lys-210 (446 in GR) interacts only with the bases in the ER DBD-DNA complex and although the conserved Lys-206 (442 in GR) and Arg-211 (447 in GR) contract mainly the same conserved bases in the two complexes the details of the interactions differ. Furthermore a crystal structure of ER DBD bound to a nonconsensus ERE in which (-5) in one of the half-sites is changed to an adenine shows that the receptor can adapt to this sequence by rearranging the side chain of Lys-206 (Schwabe et al., 1995).

The crystal structures of the GR and ER DBD-DNA complexes demonstrate the structural basis for the half-site specificity of the receptors, the discrimination between the GRE half-site TGTTCT, and the ERE half-site TGACCT. The specificity is mediated by the three P-box residues at the N-terminus of the recognition helix, Gly-439, Ser-440, and Val-443 in GR and Glu-203, Gly-204, and Ala-207 in ER (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evans, 1989; Zilliacus et al., 1991). Mutation of the P-box residues in one of the receptors to the corresponding residues in the other receptor changes the half-site specificity and allows recognition of the noncognate half-site. The P-box residues determine the half-site specificity partly by the base specific interactions formed by Val-443 in GR and Glu-203 in ER as shown in the crystal structures. In addition, mutational studies have suggested that negative steric interactions between noncognate residues and bases contribute to the specificity by decreasing the affinity of the receptor for the wrong response element (Zilliacus et al., 1992a, 1994). Introduction of all possible residues at GR position 439 in a chimeric GR DBD showed that all substitution mutants had a higher affinity than the ER specific Glu-439 for response elements containing the GRE specific T4 (Zilliacus et al., 1995). Molecular modeling suggested that the inhibitory property is related to a negative steric interaction between the ER specific glutamate carbonyl group and the GRE specific methyl group of thymine, which would contribute to the discrimination by ER DBD containing Glu at that position between GRE and ERE (Zilliacus et al., 1995). Unfavorable entropic contributions to the free energy of binding could also contribute to the specificity. This is suggested by the crystal structure of a mutant GR DBD containing the ER P-box residues bound to a GRE which showed that the noncognate interface between the protein and DNA was filled by water molecules (Gewirth and Sigler, 1995). The decreased affinity of the mutant protein for the noncognate response element could result from an unfavorable entropic contribu-

tion due to the immobilized water molecules. The DNA conformation could also contribute to the response element recognition as indicated by the fact that the GRE and ERE sequences in the resolved crystal structures show differences in the helical geometry (Gewirth and Sigler, 1995). For example the major groove of the GRE is wider than the major groove of the ERE. This results in a difference in the distance between the functional groups of the amino acids and the bases in the two complexes, which might determine whether interactions between conserved residues and bases in the two complexes can be formed or not.

Dimerization

The steroid receptors bind to DNA as dimers. Homodimeric DNA binding by GR, ER, and AR has been demonstrated by the formation of a complex of intermediary mobility in gel mobility shift assays, when two receptor fragments of different lengths were mixed (Kumar and Chambon, 1988; Tsai et al., 1988). Glycerol gradient centrifugation was also used to show that two GR molecules were bound to the response element (Wrange et al., 1989). The receptor dimers could be formed either by the ligand-bound receptors in solution, such that the preformed dimer binds to the response element, or the receptor could dimerize upon binding to the response element. The PR and ER have been demonstrated to dimerize in solution and bind to DNA as preformed dimers (Fawell et al., 1990; Rodriguez et al., 1990). Glycerol gradient centrigufation indicated that the GR also is a homodimer in solution, which can be further stabilized by crosslinking (Wrange et al., 1989). Using fractions from a sucrose gradient, it was shown that the dimeric form of GR bound with higher affinity to a GRE than a monomeric form (Cairns et al., 1991). Disruption of preformed GR dimers by dilution also resulted in a reduced affinity for GRE (Drouin et al., 1992). In conclusion, these results indicate that GR binds with high affinity to DNA as a preformed dimer.

The response elements for steroid receptors are palindromic sequences composed of two half-sites, which are always separated by a 3 base pair spacer. Changing the number of spacing base pairs in the response element abolishes the transactivation by the receptors (Umesono and Evans, 1989; Chalepakis et al., 1990; Nordeen et al., 1990; Dahlman-Wright et al., 1991). This is probably due to loss of binding to the response element since gel mobility shift assays showed that GR does not bind to a GRE with a 2 or 4 base pair spacer (Chalepakis et al., 1990), and ER does not bind to an ERE with a 0 or 6 base pair spacer (Mader et al., 1993b). The GR and ER DBDs bind to the response elements with correct spacing as cooperative dimers, indicating that the binding of the first monomer facilitates the binding of the second monomer (Tsai et al., 1988; Mader et al., 1993a). The cooperative binding is disrupted when the DBD binds to a response element with incorrect spacing, resulting in either noncooperative binding or monomer binding (Chalepakis et al., 1990; Dahlman-Wright et al., 1990, 1991; Schwabe et al.,

1993b). Thus, cooperative binding by the DBD is associated with recognition of correctly spaced half-sites.

Discrimination between half-site spacing by the GR is mediated by a five residue-long segment in the C-terminal zinc domain of the DBD, the D-box (Figure 4). Substitution of the D-box in a hybrid GR with the corresponding residues in TR relaxed the half-site specificity of the receptor, and allowed transactivation from response elements with either 3 or 0 base pair spaces (Umesono and Evans, 1989). The D-box determines the half-site specificity by mediating cooperative DNA binding of the DBD. GR DBD with the TR or VDR D-box has lost the ability to bind cooperatively to the GRE (Dahlman-Wright et al., 1991; Freedman and Towers, 1991). The GR D-box can also mediate cooperative binding to a response element with a 3 base pair spacer in the context of the TR DBD (Dahlman-Wright et al., 1993). The ER D-box also mediates cooperative binding to an ERE, since substitution of the D-box with the RAR D-box disrupts dimer formation (Mader et al., 1993a). The ER D-box can also confer cooperative DNA binding to the RAR DBD (Mader et al., 1993a). These results show that the GR and ER D-box sequences are important for dimerization on a GRE and ERE, and that the D-boxes also can mediate dimerization of chimeric receptors on these response elements.

The crystal structures of GR and ER DBD bound to DNA showed that the D-boxes mediate cooperative binding by forming protein-protein contact between the two DBD monomers (Figure 5) (Luisi et al., 1991; Schwabe et al., 1993a). Some of the dimer contacts are also formed by residues outside the D-box. Thus, the dimer interactions mediated by the D-box in the DBD position the receptor on the response element, to restrict binding to palindromic response elements with a 3 base pair spacer.

In addition to the dimerization interface in the DBD, additional regions of the receptors mediate dimerization. Deletions in the ER LBD identified a region that was needed for high affinity DNA binding and dimerization, and which was conserved in the nuclear receptors (Fawell et al., 1990). A 22 residue-long segment from the region could mediate high affinity DNA binding and dimerization of ER lacking most of the LBD (Lees et al., 1990). The GR LBD is also needed for high affinity DNA binding, suggesting that it also contains a dimerization region, which may be the same as the region in ER (Dahlman-Wright et al., 1992).

DNA Bending

Many transcription factors bend DNA upon binding to their response elements. DNA bending by transcription factors has been implicated in the activation of transcription by facilitating protein-protein interactions, enhancing DNA binding of other proteins, or by inducing conformational changes in the DNA needed for transcription (van der Vliet and Verrijzer, 1993). DNA bending has also been demonstrated for some nuclear receptors. Binding of ER DBD to an ERE bends the DNA, and partially purified full length ER induces an even larger bend (Nardulli

and Shapiro, 1992; Nardulli et al., 1993). However, no extensive DNA bend was observed in the ER DBD-DNA crystal structure (Schwabe et al., 1993a).

DNA Binding Enhancing Factors

Nuclear proteins have been reported to enhance DNA binding by steroid receptors (Edwards et al., 1989; Mukherjee and Chambon, 1990; Kupfer et al., 1993; De Vos et al., 1994; Onate et al., 1994). A protein that stimulates ER DNA binding has been purified from yeast and shown to be a 45 kDa single-stranded DNA-binding protein (Mukherjee and Chambon, 1990). DNA binding by purified PR is also enhanced by a single-stranded DNA-binding protein present in nuclear extracts (Onate et al., 1994). Interestingly, it was shown that the factor could be substituted by HMG-1, which could stimulate DNA binding by PR (Onate et al., 1994). The mobility of the PR-DNA complex was not changed in gel mobility shift assays although coimmunoprecipitation indicated that HMG-1 could be bound to the PR-DNA complex. HMG-1 binds nonspecifically to DNA and recognizes modified and bent DNA structures and can also further bend DNA. Thus, it is possible that HMG-1 enhances PR DNA binding by bending the DNA or stabilizing a specific DNA conformation that favors PR binding. It is not clear whether the factors that have been implicated in enhancing DNA binding by other steroid receptors are related to HMG-1. It is also possible that at least part of the DNA bending observed by nuclear receptors is due to HMG-1, since highly purified ER could not bend DNA (Nardulli and Shapiro, 1992).

Interaction With Chromosmal DNA

The DNA in the cell is incorporated into chromatin. Depending upon the translational and rotational positioning of the nucleosomes, binding sites for transcription factors can be in the linker sequence between the nucleosomes, or incorporated in the nucleosome either oriented towards the histones or facing outward. The exact positioning determines whether a transcription factor can bind to the sequence or not. The GR regulated mouse mammary tumor virus (MMTV) promoter is incorporated into six nucleosomes, and one of them is positioned at the GREs (Richard-Foy and Hager, 1987). GR can bind to the GREs although they are incorporated into the nucleosome (Perlmann and Wrange, 1988; Pina et al., 1990; Archer et al., 1991). The affinity of GR for one of the GREs was reduced only twofold compared with nucleosome free DNA (Perlmann, 1992). Since the affinity for random nucleosomes was drastically reduced compared with nucleosome free random DNA, the actual specificity for the GRE compared with nonspecific DNA was higher in the nucleosomal DNA (Perlmann, 1992). By determining the binding of GR to a GRE with different translational positioning in a nucleosome, it was shown that the affinity for the nucleosomal GRE was reduced 2.5-fold compared with free DNA, and that the affinity could vary 4.3-fold depending upon the exact

translational positioning in the nucleosome (Li and Wrange, 1993). Thus, positioned nucleosomes can modulate the binding of the receptor, allowing binding to specific response elements and excluding binding from others.

CONCLUSION

Specific DNA binding by transcription factors forms the basis for the specificity of gene regulation. The molecular determinants for sequence specific DNA binding by proteins has been studied extensively using both structural and functional methods. Several general mechanisms determine both specificity and affinity of DNA binding, including interactions with the bases and phosphate backbone of the DNA, the role of DNA conformation and the oligomerization of the protein, as well as thermodynamic principles. These determinants have been analyzed also for the members of the steroid receptor family. The problem of specificity and discrimination between closely related DNA sequences must be solved by the members of the steroid receptor family to accomplish the unique effects of the different steroids. The discrimination by GR and ER between response elements that differ in only a few base positions is an interesting example of this problem. Mutational and structural studies have shown that several mechanisms are involved in the discrimination process, including sequence specific base contacts, negative steric effects, and entropic driving forces. However only part of the specificity of different steroids is mediated by differential DNA binding specificity. For example no major differences in the DNA binding specificity has been noted for the GR, PR, AR, and MR, although the hormones mediate distinct effects. Thus, specificity within this group of receptors must be mediated at another level than DNA binding, possibly by differential transcription activity as well as modulation by chromatin and other transcription factors.

ACKNOWLEDGMENTS

This work was supported by grant 13X-2819 from the Swedish Medical Research Council and grant K-KV 9756-301 from the Swedish Natural Science Research Council.

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