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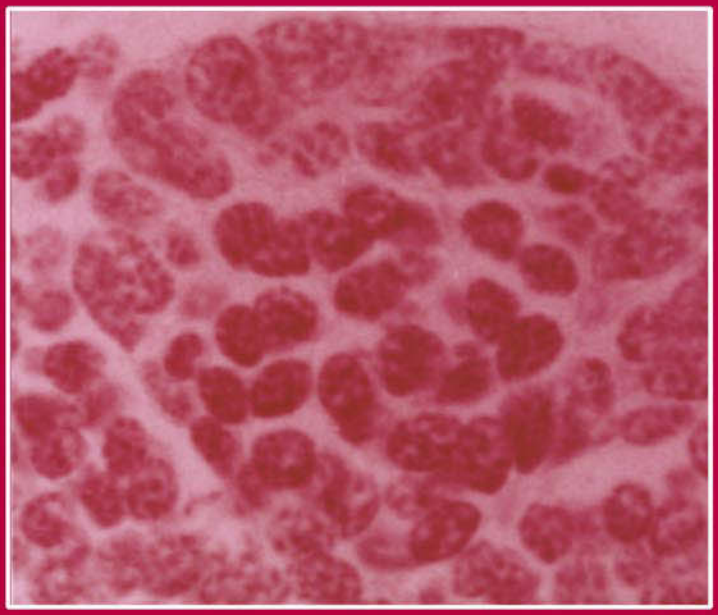
A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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A Survey of Cell Biology

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Edited by

Kwang W. Jeon
Department of Biochemistry
University of Tennessee
Knoxville, Tennessee

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Front cover photograph: Proliferation of new neurons and their migration in the cortex of mushroom bodies in adult house crickets. (For more details see Chapter 3, Figure 6.)

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CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Thomas Börner (1), *Institute of Biology, Humboldt University, D-10115 Berlin, Germany*

Myriam Cayre (137), *CNRS, LNB 5, Laboratoire de Neurobiologie,13402 Marseille Cedex 20, France*

Biao Ding (251), *Department of Botany, Oklahoma State University, Stillwater, Oklahoma 74078*

Gera D. Eytan (175), *Department of Biology, Technion—Israel Institute of Technology, Haifa 32000, Israel*

Wolfgang R. Hess (1), *Institute of Biology, Humboldt University, D-10115 Berlin, Germany*

Asuka Itaya (251), *Department of Botany, Oklahoma State University, Stillwater, Oklahoma 74078*

Philip W. Kuchel (175), *Department of Biochemistry, University of Sydney, Sydney, New South Wales, Australia*

Milton H. Saier, Jr. (61), *Department of Biology, University of California at San Diego, La Jolla, California 92093-0116*

Alain Strambi (137), *CNRS, LNB 5, Laboratoire de Neurobiologie,13402 Marseille Cedex 20, France*

Colette Strambi (137), *CNRS, LNB 5, Laboratoire de Neurobiologie,13402 Marseille Cedex 20, France*

Young-Min Woo (251), *Department of Botany, Oklahoma State University, Stillwater, Oklahoma 74078*

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Organellar RNA Polymerases of Higher Plants

Wolfgang R. Hess and Thomas Börner
Institute of Biology, Humboldt University,
D-10115 Berlin, Germany

The nuclear genome of the model plant *Arabidopsis thaliana* contains a small gene family consisting of three genes encoding RNA polymerases of the single-subunit bacteriophage type. There is evidence that similar gene families also exist in other plants. Two of these RNA polymerases are putative mitochondrial enzymes, whereas the third one may represent the nuclear-encoded RNA polymerase (NEP) active in plastids. In addition, plastid genes are transcribed from another, entirely different multisubunit eubacterial-type RNA polymerase, the core subunits of which are encoded by plastid genes [plastid-encoded RNA polymerase (PEP)]. This core enzyme is complemented by one of several nuclear-encoded sigma-like factors. The development of photosynthetically active chloroplasts requires both PEP and NEP. Most NEP promoters show certain similarities to mitochondrial promoters in that they include the sequence motif 5′-YRTA-3′ near the transcription initiation site. PEP promoters are similar to bacterial promoters of the $-10/-35$ σ^{70} type.

KEY WORDS: Plastids, Mitochondria, Transcription, Promoter, Gene regulation, Sigma factor, Linear plasmids.

I. Introduction

Chloroplasts and mitochondria are plant organelles that were acquired in two steps of endosymbiosis (Gray, 1992, 1993; Howe *et al.*, 1992; Whatley, 1993; Herrmann, 1997). The nature of the putative progenitors of chloroplasts (or, more generally, plastids) and mitochondria, free-living cyanobacteria-like organisms and bacteria from among the α-proteobacteria, is in harmony with many prokaryotic features of the organelles' gene expression systems. The process of eukaryotic cell evolution was accompanied by a

reduction of the plastid (plastome) and mitochondrial (chondrome) genomes to only 5–10% of their original size and by the transfer of a substantial number of genes to the nucleus, including genes encoding proteins operating in the transcriptional machineries of these organelles. However, there is also evidence that certain genes were recruited to fulfill new functions in the interplay of two or three genomes of the eukaryotic cell. A recently discovered example of the latter might be nuclear genes for organellar RNA polymerases (RNAPs) of the bacteriophage type. Such genes were first detected in yeast (Greenleaf *et al.,* 1986) and recently also found in higher plants (Cermakian *et al.,* 1996, 1997; Hedtke *et al.,* 1997; Weihe *et al.,* 1997). Whereas in bacteria one core RNAP consisting of several subunits transcribes all genes, the coevolution of nuclear, plastidic, and mitochondrial genomes has led to a strikingly different situation: Plastids need (at least) two different polymerases for the transcription of their genes—one of the eubacterial type [plastid-encoded RNAP (PEP)] and one nuclear-encoded polymerase (NEP) whose gene was most probably generated by duplication of the phage-type gene encoding the mitochondrial RNAP (Hedtke *et al.,* 1997; Gray and Lang, 1998; Maliga, 1998).

This review focuses on the RNAPs and the nucleotide sequences recognized by them, the promoters, in higher plant mitochondria and plastids. Aspects of regulation of transcription and of posttranscriptional steps of gene expression as well as data obtained from algae and lower plants are dealt with only if closely related to the topic of this article. Readers interested in more details on these aspects are referred to the excellent reviews of Herrmann *et al.,* (1992), Igloi and Kössel (1992), Gray *et al.,* (1992), Gruissem and Tonkyn (1993), Mullet (1993), Mayfield *et al.* (1995), Tracy and Stern (1995), Binder *et al.* (1996), Link (1996), Sugita and Sugiura (1996), and Stern *et al.* (1997).

II. The Mitochondrial RNA Polymerase

A. The Core Enzyme

Apart from the special case of plasmid-derived sequences (see Section IID), mitochondrial genomes of all "non-green" organisms lack genes for RNAPs, with the only known exception being the freshwater protozoon *Reclinomonas americana* (Lang *et al.,* 1997). In this primitive eukaryote subunits of a multisubunit RNAP are mitochondrially encoded. Photosynthetic eukaryotes from which the complete sequence of the mitochondrial genome has been determined do not possess RNAP genes within this organelle [*Chlamydomonas eugametos,* Denovan-Wright *et al.,* (1998);

Chlamydomonas reinhardtii, GeneBank accession No. U03843; *Chondrus crispus,* Leblanc *et al.,* (1995); *Prototheca wickerhamii,* Wolff *et al.* (1994); *Marchantia polymorpha,* Oda *et al.,* (1992); *Arabidopsis thaliana,* Unseld *et al.,* (1997)]. Thus, the mitochondrial RNAP of plants, including algae, is encoded by a nuclear gene or genes. Promoters of the eubacterial δ^{70} type are absent from plant chondromes suggesting that this enzyme is different from the multisubunit RNAP found in all eubacteria and in *Reclinomonas* mitochondria. An exception might be the mitochondrial genome of the brown alga *Pylaiella littoralis,* which harbors sequences similar to those of eubacterial δ^{70}-type promoters and, surprisingly, an open reading frame potentially encoding an RNAP of the single-subunit type (Delaroque *et al.,* 1996; Rousvoal *et al.,* 1998; see section IVA).

The mitochondrial RNAP of baker's yeast (*Saccharomyces cerevisiae*) and some animals has been well characterized (Clayton, 1992). The core enzyme consists of a single subunit most closely related to the single-subunit polymerases of the bacteriophages T3, T7, and SP6. The yeast protein is an ~145-kDa polypeptide encoded by the gene RPO41 (Greenleaf *et al.,* 1986; Kelly *et al.,* 1986; Masters *et al.,* 1987). Homologous genes were detected in *Neurospora crassa* (Chen *et al.,* 1996) and *Schizosaccharomyces pombe* (accession No. Z99126). More generally, similar genes seem to be widespread among eukaryotes, as was shown by an analysis of short amplified T7-like sequences from a variety of organisms, including several alga as well as wheat (*Triticum aestivum*) and rice (*Oryza sativa*) among higher plants (Cermakian *et al.,* 1996). Full-length cDNAs with significant similarity to the fungal and bacteriophage genes for RNAPs were recently described for humans (Tiranti *et al.,* 1997) and the dicotyledoneous plants *Chenopodium album* and *A. thaliana* (Hedtke *et al.,* 1997; Weihe *et al.,* 1997). The corresponding gene of *A. thaliana* has been cloned and characterized (Hedtke *et al.,* 1997). The putative transit peptides encoded by the *C. album* and the *A. thaliana* genes were attached to green fluorescence protein (GFP) and shown to target this protein *in vivo* (Fig. 1) and *in vitro* into mitochondria (Hedtke *et al.,* 1997, 1999), supporting the idea that these genes encode a mitochondrial protein. We refer here to these gene products as mitochondrial RNAPs, although no direct proof for this function has been provided.

In striking contrast to the situation in baker's yeast in which only one gene for an RNAP of the single-subunit type exists, the nuclear genome of Arabidopsis contains a small family of three such genes (Hedtke *et al.,* 1997; W. Schuster, personal communication; A. Weihe, personal communication). In accordance with the rules proposed by the Commission on Plant Gene Nomenclature for the designation of nuclear genes encoding plant RNAPs (*Rpa* for RNAP A or I, *Rpb* for RNAP B or II, and *Rpc* for RNAP C or III; Guilfoyle *et al.,* 1994) and in order to allow easy discrimination

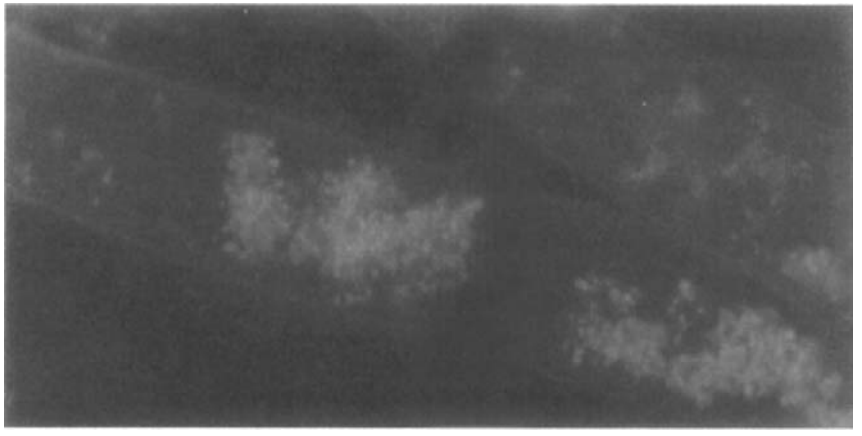

FIG. 1 Mitochondria in root hair cells from transgenic *A. thaliana* show fluorescence due to GFP targeted into the organelles by the transit peptide encoded by the putative gene for the mitochondrial RNAP of *C. album* (courtesy of A. Weihe and M. Meixner).

from chloroplast genes encoding RNAP subunits (*rpoA, B, C1, C2*), we suggest the designation "*RpoT*" genes (*R*NA *p*olymerase of the T3/T7 phage single-subunit type) for the plant genes encoding phage-type RNAPs including the core subunit of the mitochondrial RNAP. The "T" indicates the relationship to the T7- and T3-type RNAPs; the T7 polymerase forms a standard enzyme among the single-subunit RNAPs (Sousa, 1996). The different copies of genes belonging to this family in a plant species should be numbered accordingly *RpoT;1, RpoT;2* etc. In Arabidopsis, we refer to the previously mentioned gene encoding the putative mitochondrial RNAP as *RpoT;1* (named *RpoY* or *RpoMt* in Hedtke *et al.,* 1997). Another gene of this family in Arabidopsis, *RpoT;2* (GenBank accession No. AJ001037; W. Schuster, personal communication), has been shown to be expressed, and the encoded transit sequence targets GFP into mitochondria *in vivo* (A. Weihe and B. Hedtke, personal communication; W. Schuster, personal communication). Thus, Arabidopsis *RpoT;2* might represent a second gene for a mitochondrial RNAP. In addition, the nuclear genome of Arabidopsis contains another gene, *RpoT;3,* which belongs to the same family of genes coding for RNAPs of the single-subunit type, but its putative transit peptide targets translationally fused reporter proteins into plastids (see Section III). For a more general discussion of the origin and evolution of this family of RNAP genes in plants, see Section IVB, and Table II.

The predicted molecular masses of the plant mitochondrial RNAPs, including the transit peptides encoded by *RpoT;1,* are 111 and 112 kDa for the Arabidopsis and *Chenopodium* polymerase, respectively. The overall

degree of similarity between the Arabidopsis, the yeast, and the human enzymes increases from the NH_2 terminus to the COOH terminus (Fig. 2). This is consistent with a function of the carboxy terminus of phage-type RNAP in catalytic activity as shown for the T7 enzyme (Morris *et al.,* 1987; Patra *et al.,* 1992; Sousa *et al.,* 1993). The percentage of identical positions in the alignment shown in Fig. 2 is about 30% for all pairs. On the basis of the crystal structure of the T7 polymerase and mutational studies (Jeruzalmi and Steitz, 1998; Patra *et al.,* 1992; Sousa *et al.,* 1993), all amino acids reported to be essential for catalytic function are conserved in the three RNAPs. These include in particular the penultimate residue phenylalanine, shown to be essential for interaction with the incoming rNTP (Patra *et al.,* 1992), and the motifs A and C (Sousa, 1996), containing the structurally invariant Asp residues binding metal ions in the appropriate geometrical arrangement to catalyze a phosphoryl transfer reaction at the active center (Woody *et al.,* 1996). Additionally, the motif B and the T/DxxGR motif, the latter of which is thought to be involved in template-strand contacts (Joyce and Steitz, 1994), can be identified.

B. Mitochondrial Promoter Architecture

The transcription of mitochondrial genes has been most thoroughly investigated in man, mouse, *Xenopus laevis,* and yeast. The compact nature of the mitochondrial genomes in animals accords with the extremely small number of promoters and extensive cotranscription of physically tightly linked genes. In the mitochondrial genomes of mammals, transcript initiation occurs at only two sites, whereas about 20 promoters have been identified in the mitochondria of yeast (Edwards *et al.,* 1982; Shadel and Clayton, 1993; Tracy and Stern, 1995). Several *in vitro* studies pinpointed a conserved nonanucleotide sequence motif (5′-ATATAAGTA(+1)−3′; Osinga *et al.,* 1982) that is sufficient and essential for transcript initiation in yeast mitochondria. In contrast, the two promoters of animal mitochondrial genomes show only limited conservation, as do the promoters from other groups of organisms (Tracy and Stern, 1995).

In higher plants, mitochondrial promoters show only limited sequence homology. Frequently there is more than a single promoter for a single gene and often transcription initiation occurs at more than one nucleotide position, even when transcription is started from the same promoter. Extensive use of *in vitro* capping techniques, *in vitro* systems, and sequence analysis allowed the unequivocal identification of transcription initiation sites in *Zea mays* (Mulligan *et al.,* 1988a,b, 1991; Rapp and Stern, 1992; Rapp *et al.,* 1993; Caoile and Stern, 1997), *Glycine max* (Brown *et al.,* 1991), *Pisum sativum* (Binder *et al.,* 1995; Giese *et al.,* 1996), *T. aestivum* (Martin

et al., 1987; Covello and Gray, 1991), *Nicotiana sylvestris* (Lelandais *et al.,* 1996), *Solanum tuberosum* (Binder *et al.,* 1994; Lizama *et al.,* 1994; Giese *et al.,* 1996; Remacle and Marechal-Drouard, 1996), *Sorghum* (Yan and Pring, 1997), *Zea perennis* (Newton *et al.,* 1995), and *Oenothera berteriana* (Binder and Brennicke, 1993a). There is no evidence of a particular sequence motif that would be specific for tRNA-, rRNA-, or mRNA-encoding genes.

1. Dicots

With some analogy to the yeast mitochondrial nonanucleotide motif, the data revealed the existence of a conserved nonanucleotide motif, (−7)5′-CRTAaGaGA-3′(+2), in *Oenothera* (Binder and Brennicke, 1993a,b) encompassing the transcription initiation site that is represented by the penultimate nucleotide (G; Fig. 3). Thus, transcripts from these promoters always start with the dinucleotide GA (for convenience, we use the term "promoter" for consensus sequences near the transcription initiation site even in those cases in which their role in transcription and/or specific binding of RNAP has yet to be demonstrated). The same sequence motif can also be found upstream of the transcription start site of mitochondrial genes in *G. max* (Brown *et al.,* 1991), *N. sylvestris* (Lelandais *et al.,* 1996), and *P. sativum* (Binder *et al.,* 1995; Giese *et al.,* 1996) (Fig. 3). Frequently, a conserved five to seven-nucleotide AT-rich region is located a few bases upstream of the initiation site. No other conserved sequence elements in the vicinity of the transcription initiation site have been detected.

Although the identified motifs are likely to represent the basic structure of a mitochondrial promoter in a dicotyledonous plant, a few promoters do not follow this principle. The 26S rRNA of potato mitochondria is transcribed from a promoter that does not contain the complete nonanucleotide or the short, highly conserved CRTA motif (Fig. 3; Binder *et al.,* 1994). In potato the promoters for *atp9* and $tRNA^{Ser}$ and in *G. max,* a promoter for the small cappable RNA *scre*, do not correspond to the basic promoter structure of dicot mitochondria (Fig. 3).

In an *in vitro* system, transcription of the pea (*P. sativum*) *atp9* gene was correctly initiated at the conserved nonanucleotide motif (−7)5′-CRTAaGa*G*A-3′(+2), thus confirming the data inferred previously from the analy-

FIG. 2 Comparison of mitochondrial RNAPs from *A. thaliana* (EMBL accession No. Y08137), yeast (P13433), and humans (U75370). The alignment was constructed by the program ClustalW and refined manually. Amino acid residues that are positionally identical in all or two of three sequences or that are highly similar are labeled by an asterisk, colon, or dot, respectively. Functionally relevant positions mentioned in the text are shown in bold.

```
A. thaliana     MWR---NILGRASLRKVKFLS----------DSSS----------SGTHYPVNRVR--- 33
S. cerevisiae   MLRPAYKSLVKTSLLQRRLISSKGSKLFKPSPDSTSTILISEDPLVTGSSPTSSTTS--- 57
H. sapiens      MSALCWGRGAAGLKRALRPCGRPG----LPGKEGTAGGVCGPRRSSSASPQEQDQDRRKD 56
                *                 :  .           :.::          :.:    .

A. thaliana     GILSS-----VN------LSGV----RNG--LSINPVN--EMGGLSSFRHG-----QCY- 68
S. cerevisiae   GIISSNDFPLFNKNRKDAKSSISYQWKNPSELEFDPFNKSHASAVTSMTRTRD-VMQLWS 116
H. sapiens      WGHVELLEVLQARVRQLQAESVSEVVVNRVDVARLPECGSGDGSLQPPRKVQMGAKDATP 116
                    .               ..:     *   :   *      ..: .  :      :

A. thaliana     VFEG------YATAAQAIDSTD--PEDES------------SGSD------EVNELIT- 100
S. cerevisiae   LLEACLQSNLMKRAFSILESLYLVPEHKQRFIEDYNMYLNSFSKNDPNFPILKMNEKLTN 176
H. sapiens      VPCGRWAKILEKDKRTQQMRMQRLKAKLQMPFQSGEFKALTRRLQVEPRLLSKQMAGCLE 176
                :  .                        . .             .       :

A. thaliana     EMEKETERIRKKARLAAIPPKRVIAGMGAQKFYMLK---QRQVKMETEEWERAARECREI 157
S. cerevisiae   DLETSFKDVNYNDKTLAIMIHHALNFHSTTSSMLLKPIISAYLKMSVNG-IREIFSCLDI 235
H. sapiens      DCTRQAPESPWEEQLARLLQEAPGKLSLDVEQAPSGQHSQAQLSGQQQR-LLAFFKCCLL 235
                :   .      : :   :  .          .         . :. . :       .*  :

A. thaliana     L--AD----MCEQKLA-----PN--LPYMKSLFLGWFEPVR--------NAIQDDLDTFK 196
S. cerevisiae   LTISDLNILMNDLKVITPSQLPNSVRPILESLTLSP-TPVNNIENEEGLNKVEAENDS-K 293
H. sapiens      TDQLPLAHHLLVVHHGQRQKRKLLTLDMYNAVMLGWARQGAFKELVYVLFMVKDAGLTPD 295
                          :   :                 ::: *.               ::    : .

A. thaliana     IKKG--------KIP-YAP-----FM---EQLP--ADK-MAVITM------HKMMGLLMT 230
S. cerevisiae   LHKASNASSDSIKKPSLDPLREVSFHGSTEVLSKDAEKLIAVDTIGMRVIRHTLLGLSLT 353
H. sapiens      LLSYAAALQCMGRQDQDAG----TIERCLEQMSQEGLKLQALFTAVLLS-EEDRATVLKA 350
                : .          :           :    * :.  . *  *: *       .    :  :

A. thaliana     NAEGVGIVKL-VNAATQIG--EAVEQEVRIN--SFLQKKN------KKNATDKTINTEAE 279
S. cerevisiae   PEQKEQISKFKFDANDNVLKMKPTKNDDNNNSINFFEIYNSLPTLEEKKAFESALNIFNQ 413
H. sapiens      VHKVKPTFSLPPQLPPPVN-TSKLLRDVYAKDGRVSYPKLHLPLKTLQCFFEKQLHMELA 409
                  :     .:  :    :   .  .:   :   .           :   :. ::

A. thaliana     N---VSEE---IVAKE-----TEKARKQVTVLMEKN---KLR----QVKALVRK---H-- 316
S. cerevisiae   DRQKVLENRATEAARERWKHDFEEAKARGDISIEKNLNVKLWKWYNEMLPLVKEEINHCR 473
H. sapiens      SRVCVVSVEKPTLPSKEVKH-ARKTLKTLRDQWEKALCRALRETKNRLEREVYEGRFSLY 468
                .   * .      . :       .::         **     *     .:   * :

A. thaliana     ---------------DSFKP-WG-QEAQVKVG----ARLIQLLMEN------------ 341
S. cerevisiae   SLLSEKLSDKKSLNKVDTNRLGYGPYLTLIDPGKMCVITILELLKLNSTGGVIEGMRTAR 533
H. sapiens      PFLCLLD-------EREVVRMLLQVLQALPAQGESFTTLARELSARTFS----------R 511
                          :  :       :    *        :*   .

A. thaliana     AYIQP--------PAEQFDDGPP----DIR---PAFK---QN----FRTVTLENTK---- 375
S. cerevisiae   AVISVGKAIEMEFRSEQVLKSESQAFRDVNKKSPEFKKLVQNAKSVFRSSQIEQSKILWP 593
H. sapiens      HVVQRQRVSG-QVQALQNHYRKYLCLLASDAEVPEPCLPRQYWEELGAPEALREQP--WP 568
                  :.          : *                 *      *       .  :.:

A. thaliana     -TSR-RYG------CIECDPLVLKGLD---KSARH------------------------ 399
S. cerevisiae   QSIRARIGSVLISMLIQVAKVSVQGVDPVTKAKVHGEAPAFAHGYQYHNGSKLGVLKIHK 653
H. sapiens      LPVQMELGKLLAEMLVQATQMPCS-LDKPHRSSRLVP--VLYHVYSFRNVQQIGILKPHP 625
                 . : . *       ::   :  . :*   ::

A. thaliana     --------------MVIP-YLPMLIPPQNWTGYDQGAHFFLPSYVMRTHGAKQQRTVMKR 444
S. cerevisiae   TLIRQLNGERL-IASVQPQLLPMLVEPKPWVNWRSGGYHYTQSTLLRTKDSPEQVAYLKA 712
H. sapiens      AYVQLLEKAAEPTLTFEAVDVPMLCPPLPWTSPHSGAFLLSPTKLMRTVEGATQHQELLE 685
                          . .  :***  *  *..  .*..    : ::**  .  *   :
```

```
A. thaliana     TPKE-QLEPVYEALDTLGNTKWKINKKVLSLVDRIWAN-G-G--RIGGLVDREDVPIPVE 499
S. cerevisiae   ASDNGDIDRVYDGLNVLGRTPWTVNRKVFDVVSQVWNK-GEGFLDIPGAQDEMVLP-PAP 770
H. sapiens      TCPPTALHGALDALTQLGNCAWRVNGRVLDLVLQLFQAKGCPQLGVPAPPSEAPQPPEAH 745
                :     :. . :.*  **.  * :* :*:.:* :::   *     : .  ..   *  .

                                                                  T/DxxGR
A. thaliana     PEREDQEKFKNWRWESKK-AIKQNNERHSQRCDIELKLEVARKMKDEEGFYYPHNVDFRG 558
S. cerevisiae   PKNSDPSILRAWKLQVKTIANKFSSDR-SNRCDTNYKLEIARAFLGEK-LYFPHNLDFRG 828
H. sapiens      LPHSAAPARKAELRRELAHCQKVAREMHSLRAEALYRLSLAQHLRDRV-FWLPHNMDFRG 804
                  ..     :    .   . *   :  * *.:   :*.:*: : ..  :: ***:****

A. thaliana     RAYPIHPYLNHLGSDLCRGILEFCEGKPLGKSGLRWLKIHIANLYAGGVDKLAYEDRIAF 618
S. cerevisiae   RAYPLSPHFNHLGNDMSRGLLIFWHGKKLGPSGLKWLKIHLSNLF--GFDKLPLKDRVAF 886
H. sapiens      RTYPCPPHFNHLGSDVARALLEFAQGRPLGPHGLDWLKIHLVNLTG-LKKREPLRKRLAF 863
                *:**  *::****.*:.*.:* * .*: **  ** *****: **      .: . ..*:**

                                                                  A motif
A. thaliana     TESHLEDIFDSSDRPLEGKRWWLNAEDPFQCLAACINLSEALRSPFPEAAISHIPIHQDG 678
S. cerevisiae   TESHLQDIKDSAEKSLTGDRWWTTADKPWQALATCFELNEVMKMDNPEEFISHQPVHQDG 946
H. sapiens      AEEVMDDILDSADQPLTGRKWWMGAEEPWQTLACCMEVANAVRASDPAAYVSHLPVHQDG 923
                :*. ::** **:::.* * :**  *:.*:* ** *::: :.::   *   :** *:****

A. thaliana     SCNGLQHYAALGRDKLGADAVNLVTGEKPADVYTEIAARVLKIMQQDAEEDPETFPNATY 738
S. cerevisiae   TCNGLQHYAALGGDVEGATQVNLVPSDKPQDVYAHVARLVQKRLEIAAEKGDE---N--- 1000
H. sapiens      SCNGLQHYAALGRDSVGAASVNLEPSDVPQDVYSGVAAQVEVFRRQDAQRGMR------V 977
                :********** *  **  *** ..: * ***: :*  *    .  *:.. .

                motif B
A. thaliana     AKLMLDQVDRKLVKQTVMTSVYGVTYSSARDQIKKRLKERGTFEDDSLTFHASCYAAKIT 798
S. cerevisiae   AKILKDKITRKVVKQTVMTNVYGVTYVGATFQIAKQLSP--IFDDRKESLDFSKYLTKHV 1058
H. sapiens      AQVLESFITRKVVKQTVMTVVYGVTRYGGRLQIEKRLRELSDFP-QEFVWEASHYLVRQV 1036
                *::: . : **:******* *****  ..  ** *:*     *   .   . * * .: .

A. thaliana     LKALEEMFEAARAIKSWFGDCAKIIAS-------E----N-N------AVCWTTPLGLPV 840
S. cerevisiae   FSAIRELFHSAHLIQDWLGESAKRISKSIRLDVDEKSFKNGNKPDFMSSVIWTTPLGLPI 1118
H. sapiens      FKSLQEMFSGTRAIQHWLTESARLISHMG-----------------SVVEWVTPLGVPV 1078
                :.:::.*:* .:: *: *: :.*: *:                    * *.****:*:

A. thaliana     VQPYRKPGRH-LVKTTLQVLTLSR---ETDKVMARRQMTAFAPNFIHSLDGSHMMMTAVA 896
S. cerevisiae   VQPYREESKK-QVETNLQTVFISDP-FAVNPVNARRQKAGLPPNFIHSLDASHMLLSAAE 1176
H. sapiens      IQPYRLDSKVKQIGGGIQSITYTHNGDISRKPNTRKQKNGFPPNFIHSLDSSHMMLTALH 1138
                :****  .:   :   :* :  :          :*:*  .:.********.***:::*

                C motif
A. thaliana     CNRAGLSFAGVHDSFWTHACDVDIMNTVLREKFVELYEKPILENLLESF----------- 945
S. cerevisiae   CGKQGLDFASVHDSYWTHASDIDTMNVVLREQFIKLHEVDLVLRLKEEFDQRYKNYVKIG 1236
H. sapiens      CYRKGLTFVSVHDCYWTHAADVSVMNQVCREQFVRLHSEPILQDLSRFLVKRFCS----- 1193
                * : ** *..***.:****.*:. ** * **:*:.*:.  ::  * . :

A. thaliana     ---------QK--------S------------------------------------- 948
S. cerevisiae   KLKRSTDLAQKIIRIRKDLSRKLGRSTTLADEIYFEKKRQELLNSPLIEDRNVGEKMVTT 1296
H. sapiens      -------EPQK---------------------------------------------- 1197
                        **

A. thaliana     ---FPDIS----------------------FPPLPERGDFDLRKVLESTYFFN 976
S. cerevisiae   VSLFEDITDLDALELENGGDENSGMSVLLPLRLPEIPPKGDFDVTVLRNSQYFFS 1351
H. sapiens      --ILEASQLKET-------------------LQAVPKPGAFDLEQVKRSTYFFS 1230
                   :                          :  :*  * **:  : .* ***.
```

FIG. 2 (*continued*)

O. berteriana	*coxII*	TAACTAAAAATCT**CGTA**TGAGAATCAAAA	Binder and Brennicke, 1993a
	atpA	AAAGTTGATAAAT**CATA**AGAGAAGCAAAG	Binder and Brennicke, 1993a
	rrn18	AAGATTGAAATGT**CATA**AGTGATGTTCGA	Binder and Brennicke, 1993a
	$tRNA^{Phe}$	CACGACTTAATTT**CATA**AGAGAGAGAAGT	Binder and Brennicke, 1993b
P. sativum	*atp9*	CTCGACGAAATAATAG**CATA**AGAGAAGA	Binder *et al.*, 1995
	rrn18	CATCACAAATAT**CGTA**GGTGA	Giese *et al.*, 1996
N. sylvestris			
orf87-nad3....	*	GCAGCACTCGAAATAT**CATA**AGAGA	Lelandais *et al.*, 1996
G. maximum	*atp9*	CGAAATAG**CGTA**AGAGAAGAAAGT	Brown *et al.*, 1991
	scr b/c	TAAAATTT**CATA**AGAGAAGAAAGC	Brown *et al.*, 1991
	scr e	TTGTTTGGATTGATCGAGTGTAC	Brown *et al.*, 1991
S. tuberosum	*rrn26*	CAAGATCGAAAAGAATGCATTGGA	Binder *et al.*, 1994
	rrn18	CTACTTAAGCAAAAAT**CATA**AGAGAAGA	Giese *et al.*, 1996
	atp9	GTGTGAAGTCTACCGCCTGTCTAGCCTATG	Lizama *et al.*, 1994
dicot consensus promoter:		(5-7A/T)-**CRTA**aGaGA	

FIG. 3 Overview of known transcription initiation sites (underlined) and adjacent sequences from mitochondria of dicotyledons. The CRTA motif present in 10 of 13 compared promoters is shown in bold. The dicot consensus promoter deduced from this alignment is shown below. Lowercase letters indicate that a particular nucleotide occurs at that position in at least 75% of the compared sequences. *In contrast to other species, this promoter is present in two copies in the mitochondrial DNA of *N. sylvestris* and of some other *Solanaceae* together with the region containing *orf87-nad3-nad1* exonA (Gutierres *et al.*, 1997).

sis of *in vivo* initiated transcripts (Binder *et al.*, 1995). An additional sequence up to 18 nt upstream of this motif was found to be necessary for high efficiency *in vitro*. Promoters without the nonanucleotide motif did not function in the pea mitochondrial lysate (Binder *et al.*, 1995).

2. Monocots

In monocots the site of transcription initiation seems to be less conserved than that in dicots. Initiation may occur at A or G and sometimes even at T nucleotides (Fig. 4). The conservation of sequences at the transcription start site between dicots and monocots in many cases includes the sequence element 5′-CRTA-3′. However, determination of eight transcript 5′ termini in *Sorghum* revealed YRTA, a variant of the CRTA motif, in four of the analyzed sequences as a consensus sequence (majority consensus), whereas in the other four promoters this motif was further degenerated into AATA or CTTA (Fig. 4, secondary consensus; Yan and Pring, 1997).

The CRTA motif found in most promoters is the core sequence of a 12-nucleotide central domain that extends from −7 to +5. In addition, most promoter regions in mitochondria of monocots may have a small upstream

Z. mays	*rrn18*	TGAATTGA**CATA**GATAAATCTT	Mulligan *et al.*, 1988a
	rrn26	AAGAAAAT**CGTA**TAAAAATCAA	Mulligan *et al.*, 1988a
	atp1	AAAAGTAA**CGTA**TTAAAAAACA	Mulligan *et al.*, 1988a
	atp6	CCCATTTT**CATA**GAGAAAGATG	Mulligan *et al.*, 1988a
	atp1	AAAAGTAA**CGTA**TTAAAAAACA	Rapp and Stern, 1992; Rapp *et al.*, 1993
Z. perennis	*cox2upc*	CGAAAACT**CGTA**TAATAGTCTC	Newton *et al.*, 1995
	cox2cpc	AACGTGTG**CAT**GGAGAAAGCGA	Newton *et al.*, 1995
maize consensus		rADWNW**CRTA**KWDaAa	
Sorghum			
majority consensus		AATAAT**YRTA**NAAAA	Yan and Pring, 1997
secondary consensus		NTATCA**ATTA**NTTTT	Yan and Pring, 1997
T. aestivum			Covello and Gray, 1991
	atp1/atp9	CGAAATAG**CGTA**TAAAGTGATT	
	cox2	AGAAAACG**CGTA**TAGTAAGTAG	Covello and Gray, 1991
	cox3	AAATTTTG**CATA**GAATGATTGT	Covello and Gray, 1991
	orf25	CGAAAACG**CGTA**TAGTAAGTAG	Covello and Gray, 1991
wheat consensus		RAaaWHG**CRTA**tARtRagt	

FIG. 4 Transcription initiation sites in mitochondria of monocot plants. Consensus motifs are shown individually for *Sorghum, Triticum aestivum,* and the *Zea* sp. at the end of each block of sequences. Y, D, H, K, R, W are according to the accepted IUB abbreviations: Y [= T, C], R [= A, G], D = [G, A, T], H = [A, T, C], K = [G, T], and W = [A, T]. Other designations are as described in the legend to Fig. 3.

element in common, a purin-rich region located at position −12 to −9 (Rapp *et al.,* 1993; Tracy and Stern, 1995) resembling the structure of the *P. sativum atp9* promoter as deduced by Binder *et al.* (1995) from *in vitro* transcription studies.

The presence of more than one promoter and the use of multiple transcription initiation sites has been described more frequently for monocots than for dicots. Three inititation sites were reported for the *Z. mays cox2* and *cob* genes and six for the *atp9* gene (Mulligan *et al.,* 1988b). A similar observation was made for some plastid genes transcribed by the nuclear-encoded plastid RNAP, such as the *clpP* gene of *Nicotiana tabacum* (see Section III,E,4). The importance of the conserved sequence elements for effective transcription of mitochondrial genes in monocots has been confirmed by *in vitro* studies. *In vitro* transcription systems have been developed for *T. aestivum* (Hanic-Joyce and Gray, 1991) and *Z. mays* (Finnegan and Brown, 1987; Rapp and Stern, 1992). Hence, studies using site-directed point mutagenesis or linker-scanning and deletion mutagenesis of promoter sequences became possible. In *Z. mays,* the 19 nucleotides immediately

upstream of the transcription initiation site of the *atpI* gene, including a previously described motif of 11 nucleotides (Mulligan *et al.*, 1991), allowed efficient transcription to be initiated at exactly the same site as was deduced from the *in vitro* capping data (Rapp and Stern, 1992; Rapp *et al.*, 1993). Further functional investigation of the central and upstream domains in the maize mitochondrial *atpI* promoter showed that full activity required 26 bp and an unaltered spacing between the upstream and core elements (Caoile and Stern, 1997).

Promoters that deviate considerably from the majority of other promoters have also been described for monocot mitochondria. One such example is the *cox2* conditional (*cpc*) promoter (Fig. 4) of *Z. perennis* that cannot be transcribed by the *Z. mays in vitro* system and that requires the presence of a dominant nuclear allele (MCT) for activity *in vivo* (Newton *et al.*, 1995).

Although the data set on plant mitochondrial promoters is still far from complete, a few conclusions emerge. There are only short regions of sequence homology between the different promoter regions; of these, the most highly conserved is CRTA (or YRTA) immediately upstream of the initiation site. Transcription initiation occurs one to five nucleotides downstream of the CRTA motif. The YRTA element has not been described as a characteristic feature of plant nuclear promoters, but it has recently been reported for some promoters recognized by the nuclear-encoded RNAP in plastids (see Section III,E,2). A few promoters exhibit different sequence motifs, so it appears that plant mitochondria have more than one type of promoter. Certainly, forthcoming analyses will detect more "nonconsensus-type" promoters in plant mitochondria. There is no obvious relation between promoter structure and function of the respective gene (e.g., housekeeping vs respiration or rRNA genes vs genes encoding proteins). The variability in the sequence of promoters may indicate a high tolerance of the polymerase toward degenerated sequence motifs. This feature would discriminate the plant mitochondrial phage-type RNAP from the yeast mitochondrial and bacteriophage RNAPs that initiate highly sequence-specific transcription (Klement *et al.*, 1990; Raskin *et al.*, 1992; Rong *et al.*, 1998). Alternatively, undiscovered regulatory factors and/or the existence of more than one RNAP may compensate for differences in the respective promoter sequences.

C. Regulation of Transcription in Mitochondria and Regulatory Factors

Unlike the T3 and T7 RNAPs, mitochondrial RNAPs require transcriptional factors to recognize and bind to their promoters as well as to initiate

transcription efficiently. In various organisms, only one or two mitochondrial transcription factors (mtTF) have been described.

In *S. cerevisiae* two such proteins have been found. One of these factors, called sc-mtTFB (Schinkel *et al.*, 1987) or Mtf1p (Jang and Jaehning, 1991), has a molecular weight of 43 kDa and was shown to be essential for the initiation of transcription as a specificity factor (Schinkel *et al.*, 1987; Lisowsky and Michaelis, 1988; Jang and Jaehning, 1991). It does not bind to promoter elements on its own, forming instead a holoenzyme with the core polymerase capable of promoter recognition (Mangus *et al.*, 1994). Sequencing of the gene *MTF1*, encoding factor sc-mtTFB (Mtf1p), has revealed regions of sequence similarity to bacterial sigma factors (Jang and Jaehning, 1991; Shadel and Clayton, 1995). Two of the regions in Mtf1p essential for the interactions with the core subunit have been mapped to sequences in sigma-like factors known to be involved in binding to the core polymerases (Cliften *et al.*, 1997). Thus, the relatively weak sequence homologies to sigma-like factors seem to correlate with functional and structural similarities.

The second transcriptional factor from yeast mitochondria, sc-mtTFA (also designated ABF2, HM, p19/HM, and Mtf2), is a small protein of 19 kDa that binds DNA directly (Diffley and Stillman, 1991; Fisher *et al.*, 1991) via two HMG boxes (Fisher *et al.*, 1992). Properties of this protein are the unwinding of DNA and specific binding and bending of promoter-containing DNA (Diffley and Stillman, 1992; Fisher *et al.*, 1992). Thus, sc-mtTFA appears to provide a general stimulatory effect on transcription by enhancing accessibility of promoter regions for the core RNAP and perhaps other *trans*-acting factors by DNA binding. It is responsible for activating transcription rather than specificity, and it is not directly involved in promoter recognition in association with the core RNAP (Diffley and Stillman, 1991; Xu and Clayton, 1992).

Proteins closely related to sc-mtTFA have been described in several other organisms. Its homolog in *X. laevis*, xl-mtTFA, is similar to sc-mtTFA in sequence and function despite the fact that it is 30 kDa larger (Antoshechkin and Bogenhagen, 1995). It binds DNA directly and possesses the characteristic HMG boxes. Recently, it has been found to activate transcription from the *Xenopus* bidirectional promoter by binding as a homotetrameric nonspecific complex (Antoshechkin *et al.*, 1997). As in the case of yeast, *X. laevis* mitochondria contain a second, larger transcription factor (xl-mtTFB) with a molecular weight of 40 kDa. Despite showing no obvious sequence similarity to sc-mtTFB, it acts in a very similar way as a specificity factor (Antoshechkin and Bogenhagen, 1995). Other proteins functionally homologous to sc-mtTFB and xl-mtTFB have been isolated from *Kluyveromyces lactis* and *Saccharomyces kluyveri* (Carrodeguas *et al.*, 1997). These proteins share 40 and 56% identity with *S. cerevisiae* mtTFB, respectively,

and support specific transcription initiation *in vitro* with the *S. cerevisiae* mtRNAP. Moreover, their genes were able to substitute functionally the *MTF1* gene in *S. cerevisiae.* The available data suggest that proteins functionally homologous to sc-mtTFB and xl-mtTFB are widespread among eukaryots but belong to a highly divergent family and may therefore be difficult to identify from sequence data alone.

In contrast to yeast and *Xenopus,* there is only one mitochondrial transcription factor known from mammals (Clayton, 1991; Shadel and Clayton, 1993). This protein is similar to sc-mtTFA in abundance, size (25 kDa for the human factor mtTF1 or h-mtTFA and 23.5 kDa for the mouse protein m-mtTFA), and sequence because it contains HMG box DNA-binding elements (Fisher and Clayton, 1988; Parisi and Clayton, 1991; Montoya *et al.,* 1997). Indeed, it was shown that h-mtTFA can functionally replace sc-mtTFA both *in vitro* and *in vivo* (Parisi *et al.,* 1993). Interestingly, the mouse factor m-mtTFA is encoded by the same gene as a nuclear HMG box protein and is translated from an alternatively spliced mRNA (Larsson *et al.,* 1996). It remains to be investigated if there is indeed no human or mouse equivalent to sc-mtTFB and if h-mtTFA and m-mtTFA function both as specificity factors and as activating factors.

Considering the similarity of the core polymerases involved (see Section IIA), one might expect the existence of mtTFA- and mtTFB-like factors in plants. There might exist additional, tissue-specific proteins that regulate or modulate RNAP activity according to the needs of differently differentiated tissues and that are associated with the much higher number of mitochondrial promoters in plants than in animals or yeast. Evidence for transcriptional regulation in plant mitochondria was obtained from comparison of data obtained on the basis of a run-on system, with lysates from *Z. mays* mitochondria, and by the analysis of steady-state RNA levels (Finnegan and Brown, 1990; Mulligan *et al.,* 1991). Extensive cell type-dependent fluctuations in the accumulation of mtRNAs were observed in *Z. mays* (Li *et al.,* 1996). In young leaves of *T. aestivum,* the abundance of several mitochondrial gene transcripts was observed to be inversely regulated compared to those in the chloroplast, depending on the developmental stage of the particular cells (Topping and Leaver, 1990). Similarly, transcript levels of mitochondrial genes were higher in white, photosynthetically inactive leaves vs green leaves of the barley *albostrians* mutant (Börner and Hess, 1993). The higher level of transcripts was found to be correlated with an elevated level of mitochondrial relative to total leaf DNA (B. Hedtke *et al.,* unpublished results). In all these cases, the molecular mechanisms underlying the observed differences in transcription and transcript levels are not clear, nor is the contribution of posttranscriptional processing and mRNA stability understood. No plant factor with homology to animal and yeast mtTFA or mtTFB has been described. Circumstantial genetic

evidence was provided for a transcription factor encoded by the nuclear gene MCT that is essential for initiation at the nonconsensus *cox2* promoter of *Z. perennis* (Newton *et al.,* 1995). Direct biochemical evidence that mitochondrial transcription may require promoter-specific factors was reported by Young and Lonsdale (1997).

Using the well-characterized *atp9* promoter of *P. sativum* as a probe to search for DNA-binding proteins, two polypeptides of 32 and 44 kDa were purified by hydroxylapatite/phosphocellulose affinity chromatography and reversed-phase high-performance liquid chromatography from mitochondrial lysates of *P. sativum* (Hatzack *et al.,* 1998). These proteins bound specifically to conserved sequence elements of the pea promoter similar to the way they bound to the heterologous *atp1* promoter of *O. berteriana.* However, both pea proteins eluted from phosphocellulose columns at an ionic strength untypically low for DNA-binding proteins and binding of the 44-kDa protein to promoter sequences was independent of the presence of other proteins (Hatzack *et al.,* 1998). Sequence analysis of similarly sized proteins purified by a different approach, e.g., affinity chromatography, revealed no obvious similarity to any of the known mitochondrial transcriptional factors from animals or fungi (C. Thalheim, A. Brennicke, and S. Binder, personal communication).

D. Mitochondrial Plasmids: Plant Mitochondria May Contain Another Class of Single-Subunit-Type RNA Polymerases

Mitochondria of several fungal, as well as of some plant species, contain linear plasmids with long terminal inverted repeats and a variable number of open reading frames, including putative DNA polymerase and/or RNAP genes. The plasmids share these features with adenoviruses and certain linear bacteriophages (Palmer *et al.,* 1983; Kuzmin *et al.,* 1988; Meinhardt *et al.,* 1990; Chan *et al.,* 1991; Gessner-Ulrich and Tudzynski, 1992; Kempken *et al.,* 1992; Rohe *et al.,* 1992; Griffiths, 1995; Kempken, 1995). The RNAP genes of these linear plasmids belong to the gene family of single-subunit type RNAPs. Within this family, the genes of mitochondrial plasmids form their own distinct clade (see Section IV,A). Although evidence exists for the expression of such genes (Leon *et al.,* 1989; Vickery and Griffiths, 1993), polymerase activity has yet to be shown for a product of any of these genes.

In higher plants, open reading frames potentially coding for an RNAP have been identified on the S2 plasmid of *Z. mays* (Levings and Sederoff, 1983; Kuzmin *et al.,* 1988; Oeser, 1988) and on a linear plasmid occurring in the mitochondria of *Beta maritima* (Saumitou-Laprade *et al.,* 1989; G. Michaelis *et al.,* unpublished data; accession No. Y10854). According

to size, hybridization data, partial sequences, or restriction digest patterns, the linear plasmids in the mitochondria of certain sources from *Z. mays, Z. diploperennis, Z. luxurians,* and *S. bicolor* are highly similar to S2 (Weissinger *et al.,* 1982; Timothy *et al.,* 1983; Chase and Pring, 1985; Grace *et al.,* 1994; Sane *et al.,* 1994) and are thus also expected to bear RNAP genes.

Since these plasmids occur only in the mitochondria of a few higher plant species, their RNAP genes are not expected to play a general role in mitochondrial transcription. However, complete or partial integration of linear plasmids into the mitochondrial genome has been found both in fungi (Bertrand and Griffiths, 1989; Griffiths, 1995; Kempken, 1995) and in plants (Schardl *et al.,* 1984, 1985; Houchins *et al.,* 1986). In addition, polymerase genes without the sequence context of linear plasmids have been found inserted in the mitochondrial DNA of plants. They might originate from previous events of plasmid integration because their sequences resemble those of the linear plasmids genes. This concerns potential DNA polymerase-encoding reading frames in the genomes of rye (*Secale cereale;* Dohmen and Tudzyinski, 1994) and sugar beet (*Beta vulgaris*) mitochondria (Weber, 1996) as well as remnants of such a gene in the mitochondrial chromosome of *Marchantia polymorpha* (Weber *et al.,* 1995). An open reading frame closely related to putative RNAP genes of linear plasmids is inserted in the mitochondrial genome of sugar beet. Although transcribed, this open reading frame probably does not encode a functional RNAP because it is interrupted by a frameshift mutation (Weber, 1994). In contrast, a presumably plasmid-derived complete open reading frame for an RNAP of the single-subunit type was found in the mitochondrial genome of the brown alga *Pylaiella littoralis.* Expression of this gene was detected by reverse transcriptase-polymerase chain reaction (Rousvoal *et al.,* 1998). Further studies will be necessary to demonstrate if this gene indeed codes for an enzyme that transcribes mitochondrial genes. It remains questionable if this or other polymerase genes integrated into the chondrome play a role in the genetic apparatus of mitochondria or represent an intermediate step in the evolution of nuclear genes encoding organellar RNAPs of the single-subunit type (see Section IV).

III. The Chloroplast RNA Polymerases

A. The Plastome-Encoded Multisubunit Enzyme

Genes encoding proteins with significant sequence homology to the *Escherichia coli* RNAP subunits α, β, and β' were discovered in the chloroplast genomes of all nonparasitic plants and algae investigated, including

N. tabacum (Shinozaki *et al.,* 1986), *M. polymorpha* (Ohyama *et al.,* 1986, 1988), *Spinacia oleracea* (Sijben-Müller *et al.,* 1986; Hudson *et al.,* 1988), *Z. mays* (Ruf and Kössel, 1988; Hu and Bogorad, 1990; Igloi *et al.,* 1990; Maier *et al.,* 1995), *O. sativa* (Hiratsuka *et al.,* 1989; Shimada *et al.,* 1990), *P. sativum* (Cozens and Walker, 1986; Purton and Gray, 1989), *Euglena gracilis* (Little and Hallick, 1988; Yepiz-Plascencia *et al.,* 1990; Hallick *et al.,* 1993), *Hordeum vulgare* (Zeltz *et al.,* 1993), *Pinus thunbergii* (Wakasugi *et al.,* 1994), *Chlorella vulgaris* (Wakasugi *et al.,* 1997), *Odontella sinensis* (Kowallik *et al.,* 1995), *Cyanophora paradoxa* (Stirewalt *et al.,* 1995), and *Porphyra purpurea* (Reith and Munholland, 1993, 1995). *rpoA* encodes the 38-kDa α subunit, *rpoB* the 120-kDa β subunit, and *rpoC1* and *rpoC2* the 85-kDa β' and 185-kDa β'' subunits. In agreement with the endosymbiotic origin of plastids from cyanobacteria-like prokaryotes, this subunit structure of the core RNAP complex is homologous to the multisubunit RNAPs of cyanobacteria. The *rpoC* gene found in other eubacteria, including *E. coli,* is split into the *rpoC1* and *rpoC2* genes in plastids as in cyanobacteria (Bergsland and Haselkorn, 1991; Kaneko *et al.,* 1996). Therefore, the cyanobacterial and plastid β' and β'' subunits correspond to the amino- and carboxy-terminal domains, respectively, of the larger β' subunit encoded by *rpoC.* Not only the sequence but also the gene organization is conserved between bacteria and plastids: *rpoB, rpoC1,* and *rpoC2* (or *rpoC*) are colocalized in one operon, whereas *rpoA* forms part of another operon together with genes for ribosomal proteins (Sugiura, 1992). Part of the *rpoC2* gene was found to be deleted in several *cms* lines of *Sorghum,* providing a possible basis for cytoplasmic male sterility in this species (Chen *et al.,* 1993, 1995). *Plasmodium falciparum,* the flagellate parasite causing malaria in humans, also possesses the *rpoB–rpoC1–rpoC2* operon but not the *rpoA* gene in its vestigial 35-kb plastid genome (Gardner *et al.,* 1994; Wilson *et al.,* 1996; Wilson and Williamson, 1997). This genome is probably transcribed by an enzyme constituted from these and additional nuclear-encoded subunits, coinciding with previous reports on the high sensitivity of *P. falciparum* to rifampicin *in vitro* (Gardner *et al.,* 1991). All functional *rpo* genes are lacking in the plastid genome of the nonphotosynthetic parasitic plant *Epifagus virginiana* (Morden *et al.,* 1991).

Purified chloroplast RNAP preparations allowed the determination of enzymatic properties and subunit composition and elaboration of assays for factors and regulation (Kidd and Bogorad, 1980; Lerbs *et al.,* 1983a; Tewari and Goel, 1983; Hu and Bogorad, 1990; Hu *et al.,* 1991). The products of the *rpo* genes were shown to be present in enzymatically active preparations of RNAP from maize chloroplasts (Hu and Bogorad, 1990; Hu *et al.,* 1991). Recently, the plastid *rpo* genes have been overexpressed separately and the individual proteins shown to be accommodated in the *E. coli* enzyme instead of the bacterial RNAP subunits, hence demonstrating functional

equivalence (Severinov *et al.,* 1996). Termination of transcription by spinach chloroplast RNAP occurred at the *E. coli thra, rrnB, rrnC,* and *gene32* terminators at essentially the same sites as by the *E. coli* enzyme (Chen and Orozco, 1988). However, it is unclear how and when transcription in chloroplasts is terminated. The reader is referred to the reviews by Bogorad (1991), Igloi and Kössel (1992), and Link (1996) for additional data on termination and for more detailed information on the properties of the PEP. For a recent article on the evolution of the plastid-encoded RNAPs, see Howe (1996).

B. More Than One Type of RNA Polymerase Is Found in Higher Plant Plastids

There are several lines of evidence supporting the existence of more than one form of RNAP in plastids: a soluble and a DNA-bound polymerase activity, a rifampicin-sensitive and -insensitive form, and a PEP and a NEP polymerase. How the different forms relate to each other is not fully understood, but this has become more clear during the past few years. Two polymerases, a soluble and a more tightly DNA-bound enzyme [also named transcriptionally active chromosome (TAC)], were observed in chloroplasts of higher plants and *Euglena* (Hallick *et al.,* 1976; Briat *et al.,* 1979; Greenberg *et al.,* 1984; Reiss and Link, 1985). From these, the soluble form was suggested to transcribe predominantly tRNA genes, whereas the TAC would transcribe mainly the rRNA genes in *Euglena* chloroplasts (Narita *et al.,* 1985). Similar observations were made for the TAC and soluble polymerase activities of higher plant chloroplasts (Gruissem *et al.,* 1983a,b, 1986b). However, highly purified preparations of soluble chloroplast RNAP were able to transcribe all three classes of genes, i.e., rRNA, tRNA, and protein coding genes (Briat *et al.,* 1987; Rajasekhar *et al.,* 1991; Igloi and Kössel, 1992). The TAC from barley plastids contains the α subunit of the PEP (Suck *et al.,* 1996) and thus probably the complete PEP. Whether the nuclear-encoded activity is also a constituent of TAC remains to be studied. Reports describing chloroplast RNAP activities exhibiting different sensitivities to rifamycins such as rifampicin trace back to the early 1970s (Bogorad and Woodcock, 1970; Bottomley *et al.,* 1971; Smith and Bogorad, 1974). Two different soluble polymerase activities, called "A enzyme" and "B enzyme," were recently purified from mustard cotyledons (Pfannschmidt and Link, 1994). Whereas the A enzyme was abundant in chloroplasts from illuminated leaves, the B enzyme dominated in etioplasts. From these two activities, the B enzyme was found to be rifampicin sensitive, whereas the A enzyme was not. Based on these different sensitivities and on polypeptide compositions, the B enzyme was identified as PEP. In

contrast, the A enzyme, with at least 13 different polypeptides, resembled to some degree the larger protein complexes of nuclear RNAPs (Link, 1996). However, both the A and B forms of the chloroplast polymerase transcribe the same set of genes and use identical start sites, and neither was able to transcribe the 16S rRNA gene from the NEP promoter in tobacco (Kapoor *et al.,* 1997; Hajdukiewicz *et al.,* 1997; Pfannschmidt and Link, 1997). It was concluded that the A and B enzymes might consist of the same core complex of PEP-specific polypeptides but recruit different additional components in etioplasts and chloroplasts, respectively (Pfannschmidt and Link, 1997). It remains to be elucidated whether the A and B enzymes also occur in a DNA-bound form (TAC). The promoter usage of A and B suggests that they do not contain NEP activity, but direct evidence for this assumption is lacking.

C. The Nucleus-Encoded Plastid RNAP

Evidence for a fully nucleus-encoded transcriptional activity in plastids was essentially obtained using four approaches. Bünger and Feierabend (1980) and Siemenroth *et al.* (1981) reported the synthesis of RNA in ribosome-deficient plastids of heat-bleached rye leaves and in white tissue of the barley (*H. vulgare*) mutant *albostrians,* respectively. Plastome-encoded proteins are not synthesized in these leaves; therefore, these data were taken as evidence for the nuclear localization of the genes for chloroplast RNAP. Recently, the transcription of distinct sets of genes was demonstrated in ribosome-free plastids of *albostrians* barley (Hess *et al.,* 1993), *iojap* maize (Han *et al.,* 1993), and heat-bleached rye and barley seedlings (Falk *et al.,* 1993; Hess *et al.,* 1993). Using the ribosome-deficient plastids of the *albostrians* mutant, tentative evidence was obtained for the usefulness of tagetitoxin as a means of differentiating between PEP and NEP activity. Tagetitoxin is a potent inhibitor of chloroplast RNAP but not of the T7/T3 RNAPs (Mathews and Durbin, 1990). This drug did not inhibit run-on transcription in the ribosome-free plastids of *albostrians* barley; thus, it seems not to affect NEP activity and is specific for PEP (V. Rajasekhar and T. Börner, unpublished results).

Further strong evidence for the existence of a fully nuclear-encoded plastid RNAP derived from the genome analysis of the plastids of *E. virginiana.* This achlorophyllous parasitic plant was found to possess a reduced plastid genome of about 70 kb (Wolfe *et al.,* 1992b). In contrast to all other angiosperms investigated in this respect, it lacks the plastid genes *rpoB, rpoC1,* and *rpoC2,* whereas *rpoA* is present as a pseudogene (Morden *et al.,* 1991). Nevertheless, this reduced genome is transcribed (DePamphilis and Palmer, 1990; Ems *et al.,* 1995).

The third line of evidence stems from studies on chloroplast RNAP preparations. Using a sensitive antibody-linked polymerase assay, Lerbs *et al.* (1985) detected chloroplast RNAP activity among proteins that had been translated from purified polyA$^+$-mRNA. Consequently, a fully nuclear origin of the plastid RNAP was suggested. In a later study, separation of polymerase activity into three fundamentally different fractions was achieved. Whereas one fraction contained the bands characteristic of the multisubunit enzyme PEP, the other two contained only a single protein of about 110 kDa. Deduced from its molecular mass and from the fact that the RNAP fraction showed an enhanced activity with supercoiled rather than linear templates, both very characteristic for T7 RNAP, this protein was suggested to represent a T7/T3 phage-like chloroplast RNAP (Lerbs-Mache, 1993). This assumption was further supported by the finding that the enzyme recognized a T7 promoter (Lerbs-Mache, 1993).

Lastly, proof for the activity of a nuclear-encoded transcriptional activity in higher plant plastids was achieved by the sophisticated techniques of plastid transformation and homologous recombination, thereby deleting the *rpoB* gene (Allison *et al.,* 1996) and other *rpo* genes in tobacco plastids (Serino and Maliga, 1998; R. G. Herrmann, personal communication). The obtained transgenic plants transcribe plastid genes while lacking PEP activity.

All these findings indicated the existence of a plastid RNAP which is encoded by the nuclear genome (NEP) and might share certain properties with the RNAPs of bacteriophages. A good candidate for a gene encoding NEP was recently identified by Hedtke *et al.* (1997), who succeeded in cloning, together with the *RpoT;1* gene for the mitochondrial RNAP, a second similar gene, *RpoT;3,* from *A. thaliana.* The putative RNAPs encoded by *RpoT;1* and *RpoT;3* share 55% identical residues whereby similarity increases toward the COOH terminus. When the putative transit sequence encoded by *RpoT;3* was fused to GFP the reporter protein was imported into isolated chloroplasts but not mitochondria (Hedtke *et al.,* 1997). Transgenic Arabidopsis plants that harbor the respective construct in their nuclear genomes show an efficient import of GFP into plastids (Hedtke *et al.,* 1999). The protein has a predicted molecular mass of 113 kDa and a length of 993 amino acids (including the transit peptide), only slightly larger in size than the mitochondrial RNAP of Arabidopsis (see section II). Hence, size, intracellular localization, and sequence of this Arabidopsis enzyme are in good agreement with previous biochemical data on the spinach nuclear-encoded plastid RNAP (Lerbs-Mache, 1993). However, experimental data are missing that would link the biochemical activity of the RpoT;3 gene product to the existing data on NEP promoters and prove that it is indeed an RNAP. Nevertheless, it can be stated that, in striking contrast to their bacterial ancestors, the plastids (of higher plants)

not only need two RNAPs to transcribe their genes but also use entirely different and unrelated RNAPs, one of the common eubacterial multisubunit type and the other of the phage T3/T7 single-subunit type.

D. Organellar Primase

Plastids and probably plant mitochondria possess another RNAP, a primase (Nielsen *et al.,* 1991). Primases synthesize the (ribo)oligonucleotides necessary for initiation of DNA replication. The primers provide the free 3′-OH group, which serves as the starting point for the DNA polymerase. A putative chloroplast primase was successfully isolated from *P. sativum* by sequential column chromatography (Nielsen *et al.,* 1991). The oligonucleotides synthesized by this enzyme were short RNA molecules with a size range from 4 to about 60 nucleotides. This size is less than one would expect for an RNAP involved in transcription, but it is larger than the primers generated by primases. The primase activity was found to be rather weakly associated with DNA, a property that it shares with the primase of animal mitochondria but not with primases from other sources. The pea chloroplast enzyme showed a characteristic feature of primases in that it preferred single-stranded over double-stranded DNA as a template (Nielsen *et al.,* 1991; B. Nielsen, personal communication).

Interestingly, the enzymatic activity was shown to belong to a 115- to 120-kDa molecule and to be resistant to inhibition by tagetitoxin. Based on these facts, it was concluded that the chloroplast primase of pea is an enzyme distinct from the (chloroplast-encoded) RNAP (Nielsen *et al.,* 1991). However, in view of the similar molecular weight of the Arabidopsis NEP of 113 kDa (including the transit peptide; Hedtke *et al.,* 1997) and of the inability of tagetitoxin to hamper the activity of T3/T7-type RNAPs including NEP, further studies are required to discriminate the primase from NEP. Even in the very probable case that NEP is not identical with the analyzed primase activity, it might be worth searching for a possible role of NEP in replication. NEP activity could be involved in the initial priming of DNA synthesis at the origins, whereas a different primase activity might play a role in lagging-strand DNA synthesis. From the analysis of bacteriophage T7 DNA replication, it is known that the initial priming at the primary origin of replication is dependent on the T7 RNAP activity (Romano *et al.,* 1981). In yeast mitochondria, origins of replication and promoters share a conserved sequence motif of 11 bases that are accepted by purified RNAP preparations as a template for transcription initiation *in vitro* (Edwards *et al.,* 1982; Schinkel *et al.,* 1988). In view of these facts and the homology between the RNAPs of T3/T7 bacteriophages and yeast mitochondrial RNAP, a dual role for the yeast mitochondrial RNAP in

transcription and replication was suggested (Schinkel and Tabak, 1989) and might also occur in mitochondria and plastids of plants.

E. Chloroplast Promoters

1. PEP Promoters

In accordance with the existence of a bacterial-like RNAP in chloroplasts, $-10/-35$ σ^{70}-type promoters were observed to precede most of the transcription units in these organelles (Gruissem and Zurawski, 1985; Strittmatter *et al.,* 1985; Mullet, 1988; Gruissem and Tonkyn, 1993; Link, 1994). This type of promoter was found in plastids of all higher plants and algae and has been named Consensus Type (CT) promoter (Kapoor *et al.,* 1997). CT promoters not only have an architecture very similar to that of the eubacterial $-10/-35$ σ^{70}-type promoters (Hawley and McClure, 1983) but also function correctly in *E. coli* as was shown for the *Z. mays rbcL* (Gatenby *et al.,* 1981) and *atpB* (Bradley and Gatenby, 1985) as well as the *S. oleracea psbA* promoter (Boyer and Mullet, 1986). Variants of the $-10/-35$ σ^{70}-type promoters exist which are recognized by the multisubunit PEP, perhaps supplemented by different sigma factors. However, not all plastid genes are transcribed from $-10/-35$ σ^{70}-type promoters (Stern *et al.,* 1997). NEP needs completely different and differently located sequence motifs from those of $-10/-35$ σ^{70}-type promoters.

2. NEP Promoters

The $-10/-35$ σ^{70}-type promoters are highly active in photosynthetically competent chloroplasts. Consequently, they are relatively easy to investigate and have been the focus of research for many years. Investigation of plastid promoters used by NEP has, by contrast, only recently been initiated.

Direct evidence for the existence of specific sequences used as promoters by NEP was provided by experimental systems based on ribosome-deficient plastids (Hübschmann and Börner, 1998; Silhavy and Maliga, 1998a) or Δrpo plants lacking the chloroplast-encoded RNAP (Allison *et al.,* 1996; Hajdukiewicz *et al.,* 1997). A similar type of promoter is used in plastids of nongreen cells of a tobacco suspension culture, of an embryogenic culture of rice, and of cells treated with inhibitors of plastid translation (Kapoor *et al.,* 1997; Silhavy and Maliga, 1998b). NEP promoters do not belong to the eubacterial $-10/-35$ σ^{70} type and were therefore occasionally named NC (for nonconsensus) type II promoters (Kapoor *et al.,* 1997). Although some (or all?) NEP promoters are also active in differentiated green chloro-

plasts, they seem to play a dominant role during other developmental phases, for example, in plastids of nonphotosynthetic cells.

Figure 5 presents an overview of the identified NEP promoters. Several but not all of the NEP promoters that have been mapped in the plastome of monocot species have sequence homologies around the transcription initiation sites. Sequence comparison allows the conserved sequence element YATAGAATAA to be defined (Fig. 5, box I). Frequently, a shorter version of the same motif is found further upstream (ATAGAAT; Fig. 5, box II). NEP promoters in tobacco plastids have a similar box I motif near the transcription initiation site (ATAGAATRAA; the tentative tobacco consensus NEP promoter motif) as suggested first by Hajdukiewicz *et al.* (1997). A box II can be also identified in tobacco NEP promoters which seem to be less conserved than in monocots (Miyagi *et al.,* 1998; Fig. 5). Most recently, the importance of these boxes for promoter function has been verified experimentally (Liere and Maliga, 1999; S. Kapoor and M. Sugiura, personal communication). There seem to exist different types of NEP promoters in one and the same species. Box II is lacking from the *rpoB* promoter in maize (Silhavy and Maliga, 1998a) and from the *clp-511* promoter in tobacco (Hajdukiewicz *et al.,* 1997). A novel type II NEP promoter lacking the motifs of boxes I and II is represented by the tobacco *clpP-53* promoter. This promoter has been identified in tobacco and other species (Sriraman *et al.,* 1998b; see Section III,E,3).

Sequences that resemble enhancer- or silencer-like elements were detected in the 5′ region of *rpoB* genes from *Arabidopsis,* tobacco, and spinach (Inada *et al.,* 1997). Inada *et al.* determined that the 5′ region of the *Arabidopsis rpoB* gene, fused to a reporter gene, was delivered into the nongreen plastids of a suspension cultured tobacco cells. Under these conditions, transient transcription of the reporter gene was observed. Deletion of larger sequence stretches, including three motifs (I–III, respectively), did not prevent transcription but led to a decrease (motifs I and II) or increase (motif III) in activity. Thus, these motifes are not essential for transcription initiation but seem to modulate the strength of the promoter. Motif I (different in sequence from box I mentioned previously) was located near the transcription initiation site of spinach *rpoB* but more upstream in the case of the tobacco and *Arabidopsis* genes. Motifs II (shares the sequence, AGAA, with boxes I and II in Fig. 5) and III (a T-rich sequence) were identified 200 to several hundred nucleotides upstream of motif I.

The putative mitochondrial RNAP of plants and the putative NEP are closely related enzymes of the single-subunit type (Hedtke *et al.,* 1997). Consequently, some similarity between the promoters used by the two enzymes might be expected. Indeed, the YRTA motif—the part most conserved in dicot and monocot mitochondrial promoters—is also detectable in a similar position relative to the transcription initiation site in most

Z. mays	*atpB*	TATGCTTAAGTTA**AT-GAATA**TGTTTCATTCATATATAATGTGTACACCCTGT	Silhavy and Maliga, 1998a
	rpoB	GAAATCGTCTCTATTCATATGTATGAAATACATATA-TGAAATACGTATGTGG	Silhavy and Maliga, 1998a
	clpP	AATCTATGTATTA**ATAGAAT**CTATAGTATTCTTATAGAATAAGAAAAAAAAAAT	Silhavy and Maliga, 1998a
O. sativa	*clpP*	TTA**ATAGAAT**CTATAGTATTCTTATAGAATAAGAAAAAAACGT	Silhavy and Maliga, 1998b
H. vulgare	*clpP*	TTCCATCTATGTATTA**ATAGAAT**CTATAGTATTCATATAGAATAGAATAAGAATAAAA	Hübschmann and Börner, 1998
	rpl23	ATCATCCATACATAAC**GAAT**TGGTATGGTATATTCATACCATAACATAAGAACAATA	Hübschmann and Börner, 1998
consensus		YATA	
structure		----------**box II**--- 9-12 nt --**box I**-----AT-rich-----	
N. tabacum			
	clpP (-53)	AAAAAAAATTGTTACGTTTCCACCTCAAAGTGAAA	Sriraman *et al.*, 1998b
	clpP (-511)	CGAACCGCCCCAACCAGCAACCAGAGCTGTATGCATTATA**TGAA**CAGAAAGCAACCG	Hajdukiewicz *et al.*, 1997
	clpP (-173)	GCATAACC**AT**GAATA--GCTCCATACTTATTTATCAT**TA**GAAAG**A**CCTATTCGTAATA	Hajdukiewicz *et al.*, 1997
	rrn16 P2	CGGGCGA**ATAT**GAA-GCGCATGGATACAAGTTATGCCT**T**GGAATGAAAGACAATTCCGA	Allison *et al.*, 1996
	rrn16 P2	CGGGCGA**ATAT**GAA-GCGCATGGATACAAGTTATGCCT**T**GGAATGAAAGACAATTCCGA	Vera and Sugiura, 1995
	atpB	ATAGAA**ATA-GAA**AATAAAGTTCAGGTTCGAATTCCATAGAATAGATAATATGGATG	Kapoor *et al.*, 1997
	rpl32 P2	TGACATA**ATA-GAA**AT**AA**GAGTGCCGATCAAGTATCC---**GAA**GTCTAAAGAAAAATC	Vera *et al.*, 1996
	atpI (-208)	(-35) (-10)	Miyagi *et al.*, 1998
		*TAGCTAT***ATA**A**GAA**ATCCTTGATT*AATAATA*AA----CATA**ATA**AG**ATAAA**TAACTTA	
consensus		YaTa	
structure		--------**box II**------- ca.20 nt --------**box I**----------	

FIG. 5 Sequence comparison of selected NEP promoters. Transcription initiation sites that have been mapped unambiguously are underlined. Identity with a previously described conserved motif of NEP promoters in tobacco chloroplasts is indicated in bold (boxes I and II, respectively). This tentative tobacco NEP promoter consensus motif (ATAGAATRAA) accords to a comparison of 10 different transcription initiation sites (Hajdukiewicz *et al.*, 1997). Elements of a putative −10/−35 (σ^{70}-) CT promoter in the tobacco *atpI* (−208) promoter (Miyagi *et al.*, 1998) are indicated by italics. Partial similarity to the 5′-YRTA-3′ motif found in most mitochondrial promoters of monocots and dicots is double underlined (gaps have been inserted to enhance homology). Beneath the compared sequences of monocots and *Nicotiana,* respectively, the consensus structure of the respective group is shown.

plastid NEP promoters (Figs. 3–5). Initiation of transcription of one and the same gene may occur at different sites in plastids and mitochondria. However, there is only partial correspondence between the two organelles with respect to the first transcribed nucleotide. All known primary 5′ ends of transcripts are G or A in mitochondrial but T or A in NEP transcripts in plastids. It should be noted that, similar to the YRTA motif of plant organellar promoters, phage-type RNAPs of yeast and bacteriophage T3/T7 recognize sequences close to the transcription initiation site and transcripts start with G (Schinkel and Tabak, 1989).

3. Exceptional Promoters

A few plastid promoters from different plant species do not clearly belong to either of the types outlined previously. Internal promoters were suggested for the tRNA genes *trnR1* and *trnS1* of *S. oleracea* as well as *trnS* and *trnQ* (Neuhaus *et al.,* 1989, 1990) and *trnH* (Nickelsen and Link, 1990) from mustard. These genes do not seem to require 5′ upstream promoter elements for their expression; instead, they contain internal sequence elements that resemble blocks A and B of RNAP III transcribed nuclear tRNA genes (Galli *et al.,* 1981). Although bearing only a stretch of <10 bp upstream of the coding region, the spinach genes were transcribed *in vitro* by a soluble RNAP extracted from chloroplasts (Gruissem *et al.,* 1986a). An especially interesting case of an exceptional promoter is the tobacco *clpP* gene promoter, which leads to transcription initiation at position -53 relative to the start codon (Sriraman *et al.,* 1998b). This promoter is used by NEP but lacks any similarity to the other NEP promoters (Fig. 5). By studying mutated forms of this promoter *in vivo* fused to *uidA* as a reporter gene, it was found that *cis* elements downstream of the transcription initiation site were required for activity. Conservation of this unconventional NEP promoter throughout several land plants, including *Marchantia polymorpha,* provides evidence for the early evolutionary occurrence of NEP activity in plastids (Sriraman *et al.,* 1998b).

Another example of nonstandard sequence elements is the PC promoter, which is used to transcribe rRNA in spinach leaf chloroplasts. This promoter is different from both the $-10/-35$ σ^{70}-type and the NEP promoters that precede the *rrn16* gene in tobacco (Iratni *et al.,* 1997; see Section III,E,4).

4. Multiple Promoters: The Case of 16S rRNA

Some plastid transcripts are initiated at more than a single position; indeed, it seems rather common that plastid genes and operons are transcribed from two or even multiple promoters. Examples are the *psbK–psbI–psbD–psbC* operon of *H. vulgare* that is transcribed from at least three different promoters (Sexton *et al.,* 1990a,b; Christopher *et al.,* 1992), the *clpP* gene of tobacco

(four transcription initiation sites at −35, −95, −173, and -511 relative to the coding region; Hajdukiewicz *et al.,* 1997; Sriraman *et al.,* 1998b), the *atpI/H/F/A* operon (at −130/−131 and −208/−212; Hajdukiewicz *et al.,* 1997; Miyagi *et al.,* 1998), the *rpl32* gene (at −1101 and −1030; Vera *et al.,* 1992, 1996) in *N. tabacum,* and the *atpB* gene of *Z. mays* (at −298 and −601; Silhavy and Maliga, 1998a).

Promoter sequences and transcription initiation sites may differ greatly between plant species despite extensive sequence homologies. The DNA region preceding the *rrn16* gene is highly conserved between different plant species (Fig. 6). The promoter(s) located in this region has been subjected to detailed analyses in several species and interesting differences have been detected. Upstream of the chloroplast *rrn16* gene of *S. oleracea,* two different transcription initiation sites, P1 and P2, were mapped *in vitro* by using the *E. coli* RNAP (Lescure *et al.,* 1985). Surprisingly, investigation of *in vivo* primary transcripts revealed that no transcripts were initiated at P1 and P2. Instead, a single, third transcription initiation site, called PC, gave rise to *in vivo* transcripts in leaf chloroplasts (Baeza *et al.,* 1991; Iratni *et al.,* 1997). The PC promoter has no homology to the −10/−35 σ^{70}-type promoters nor to the tentatively identified promoters used by NEP in *N. tabacum* and in monocots (Fig. 6). Binding of proteins at or near PC may overlap with potential promoter sequences for P2. Indeed, evidence was presented for the involvement of a protein factor called CDF2 that is supposed to mediate the silencing of P1 and P2 and at the same time the activation of PC by sequence-specific DNA binding (Iratni *et al.,* 1994). In the nonphotosynthetic plastids of roots, however, PC is silent. Instead, transcription starts from a more upstream promoter preceding the *trnV* gene that in these circumstances is cotranscribed with the *rrn16* gene (Iratni *et al.,* 1997). Interestingly, this may resemble the situation found in the

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                                P1 (E.c.)                PC (NEP?; -141)                          P2 (E.c.)
S. o. CGTGATTGAATGAGAATGAATAAGAGGCTCGTGGGATTGACGTGAGGGGGTA-GGATGGCTATATTTCTGGGAGCGAACTCCAGGCGAATAT
      *** ** ********** ********************************* * ***************************** *********
N. t. CGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATAT
                                      (-35 box)                (-10 box)    P1 (PEP; -116)

S. o. GAAGCGCATGGATACAAGTTATGCCTTGGAATGAAAGACAAT-CCGA
      ****************************************** ****
N. t. GAAGCGCATGGATACAAGTTATGCCTTGGAATGAAAGACAATTCCGA
                                    P2 (NEP; -64)
```

FIG. 6 Comparison of regions upstream of the *rrn16* gene between *Spinacia oleracea* (*S.o.;* Briat *et al.,* 1982; Iratni *et al.,* 1994) and *Nicotiana tabacum* (*N.t.;* Vera and Sugiura, 1995; see Fig. 5). Consensus σ^{70}-type elements are underlined and italicized. Various mapped transcription initiation sites are indicated in bold and underlined text and have been labeled according to the RNAP using this site. *E.c., Escherichia coli* enzyme *in vitro;* NEP, nuclear-encoded plastid RNAP; PEP, plastid DNA-encoded RNAP. Positions that are conserved in both sequences are marked by an asterisk. The NEP and PEP sites are numbered with regard to the first nt of the mature 16S rRNA.

vestigial plastid genome of *E. virginiana.* In this genome, the region upstream of *rrn16* has been deleted during evolution, including the gene *trnV* and most of the *rrn16* promoter (Wolfe *et al.,* 1992a). Because the *rrn16* gene is actively transcribed (DePamphilis and Palmer, 1990), the initiation of transcription from the region corresponding to the *trnV* promoter in other species was suggested for *Epifagus* (Wolfe *et al.,* 1992a). However, sequence analysis reveals the existence of a possible NEP promoter in the 5′ region preceding the ribosomal RNA operon, which might also serve to initiate transcription of this gene in *Epifagus.*

Very different to the situation in *S. oleracea* is the promoter usage upstream of *rrn16* in *N. tabacum,* although there is an extreme nucleotide conservation in this region between both species (Fig. 6). Two promoters are active *in vivo* and the corresponding transcription initiation sites were mapped at −64 and −116 (Vera and Sugiura, 1995) or at −62 and −113/−114, respectively (Allison *et al.,* 1996). Although also called P1 and P2, they are not equivalent to P1 and P2 in *S. oleracea* (Fig. 6).

The tobacco promoter located upstream of P1 at −116 is of the −10/−35 σ^{70} type and corresponds in sequence to the *S. oleracea* P2 promoter, although the first transcribed nt is not identical. In contrast, tobacco P2 is at position −62/−64, considerably closer to the 5′ end of the mature 16S rRNA, belonging to the NEP promoter class (Allison *et al.,* 1996). Transcripts from both P1 and P2 promoters can be detected in RNA from green chloroplasts. Hence, both types of polymerases (NEP and PEP) contribute to the pool of rRNA that accumulates in leaf chloroplasts. However, the steady-state level of transcripts from the tobacco P2 NEP promoter is enhanced in proplastids of heterotrophic cells, and it is the sole *rrn16* promoter used in plants in which one of the plastid *rpo* genes has been knocked out (Δrpo plants; Allison *et al.,* 1996; Serino and Maliga, 1998). A *rrn16* promoter of the −10/−35 σ^{70} type was described previously for *P. sativum* (Sun *et al.,* 1989). This promoter's sequence is nearly identical to those of the promoters that precede P2 in *S. oleracea* or P1 in *N. tabacum,* respectively. It remains to be seen whether this eubacterial-type promoter is actually the sole *rrn16* promoter in *P. sativum* or whether there also exists a promoter with homology to either the tobacco NEP (P2) or the spinach PC promoter. Another situation exists in *Arabidopsis* plastids, in which active transcription was found from both the PC promoter (identical to spinach) and the P1 promoter (identical to *Nicotiana*). In experiments in which heterologous *rrn16* promoters were transferred into *Nicotiana* plastids, these were found to be used like the original tobacco promoters (Sriraman *et al.,* 1998a). These results, as well as data providing direct biochemical evidence for the presence of different promoter-binding proteins in spinach and tobacco (Iratni *et al.,* 1997), indicate the existence of

transcription factors that are specific for the respective promoter and species.

F. Division of Labor between Plastid RNA Polymerases

The intriguing observation that plastid genes are transcribed by two different types of RNAPs—the eubacterial-type PEP and the phage-type NEP—and that at least PEP appears to exist in different forms (soluble, TAC, and rifampicin sensitive and resistant) raises two central questions: What is the function of these polymerases? and Why do plastids need a seemingly more complex transcription machinery than their cyanobacterial ancestors?

There is good evidence for PEP using the promoters of the −10/−35 σ^{70} type which are not recognized by NEP (Hajdukiewicz *et al.,* 1997; Hübschmann and Börner, 1998). Conversely, NEP promoters seem not to be used by PEP (although this has not been proven rigorously). According to our current knowledge of the distribution of NEP and PEP promoters among the operons and genes in higher plant plastids and from the analysis of transcripts accumulation in plastids lacking PEP (Table I), one may tentatively answer the first question. NEP transcribes (mainly) housekeeping genes, whereas PEP transcribes mainly photosynthesis genes but also house-keeping genes (Fig. 7).

A number of photosynthesis genes seem to possess only PEP promoters (Hajdukiewicz *et al.,* 1997). Plastomes contain "mixed" operons with photosynthesis and housekeeping genes as well as genes coding for other functions (Tracy and Stern, 1995) which may be transcribed by both RNAP types. PEP promoters are also found upstream of housekeeping genes (e.g., *rrn16;* see Section III,E,4), again indicating transcription by PEP and NEP. A few genes (*rpoB* and *rpl23*) may be transcribed solely by NEP because their transcription started at the same nucleotide in PEP-free plastids and in normal green, PEP-containing chloroplasts (Hajdukiewicz *et al.,* 1997; Hübschmann and Börner, 1998). More genes that are fully dependent on NEP might be identified in future.

Thus, NEP activity should be essential for normal plastid function. On the other hand, it is evident from the albinotic phenotype of Δrpo plants lacking PEP that NEP alone is not sufficient to provide all the gene products needed for chloroplast development. Photosynthesis genes/operons have PEP promoters and usually no NEP promoters. However, transcripts from photosynthesis genes are also observed in PEP-free plastids (Table I; W. Hess and T. Börner, unpublished data) demonstrating that the activity of NEP is not exclusively restricted to the transcription of housekeeping genes. These transcripts seem to be initiated at NEP promoters far upstream of the photosynthesis genes, are of low abundance, and are not necessarily

TABLE I

Summary of Plastid Genes Transcribed by NEP[a]

Gene	Accumulation level	Promoter mapping	Reference
rrn16	Low	+	Siemenroth *et al.* (1981), Allison *et al.* (1996)
rrn23	Low	−	Siemenroth *et al.* (1981), Han *et al.* (1993)
atpI/H	Normal-low	−	Hess *et al.* (1993), Allison *et al.* (1996)
		+	Hajdukiewicz *et al.* (1997)
atpA	Low	−	Han *et al.* (1993)
psaB (precursors)	Low	−	Han *et al.* (1993)
trnK-matK (precursors)	Normal	−	Vogel *et al.* (1997)
trnfM-G (precursors)	Normal	−	W. Hess *et al.* (unpublished data)
rpoB, rpoC1	High	−	Hess *et al.* (1993), Zeltz *et al.* (1993),
		+	Silhavy and Maliga (1998a)
rps15	Normal	−	Hess *et al.* (1993)
rps2	Normal	−	W. Hess *et al.* (unpublished data)
rpl16–rps11-rpoA operon *rpoA operon*	High	−	W. Hess *et al.* (unpublished data), Allison *et al.* (1996)
rpl23–rpl2	High	+	Hess *et al.* (1994), Hübschmann and Börner, (1998)
clpP–rps12–rpl20	High	+	Hübschmann *et al.* (1996), Hübschmann and Börner (1998)
clpP	High	+	Han *et al.* (1993), Hajdukiewicz *et al.* (1997), Silhavy and Maliga, (1998a)
rps12–rps7	High	−	Hübschmann *et al.* (1996)
rps16	Normal	−	Hajdukiewicz *et al.* (1997), W. Hess *et al.* (unpublished data)
ycf3	Normal	−	Hess *et al.* (1994)
atpB	Normal-low	+	Hajdukiewicz *et al.* (1997), Silhavy and Maliga (1998a)
ndhB	Normal	−	Hajdukiewicz *et al.* (1997)
ndhF	Normal	−	Hajdukiewicz *et al.* (1997)
accD	High	+	Hajdukiewicz *et al.* (1997)
rpl33–rps18	High	−	Hajdukiewicz *et al.* (1997)
ycf2	High	−	Hajdukiewicz *et al.* (1997)
petB–petD	Normal-low	−	Han *et al.* (1993)

[a] The data are based on the accumulation of transcripts in ribosome-free plastids of the *Hordeum vulgare albostrians* mutant and the *Z. mays iojap* mutant as well as on mapping data obtained from the Δrpo plants of *Nicotiana tabacum*. The accumulation level has been classified relative to that in wild-type leaves as normal (approximately 50–150% of wild type), high (>150%), and low (<50%). Genes not listed may also be transcribed by NEP but have not been studied.

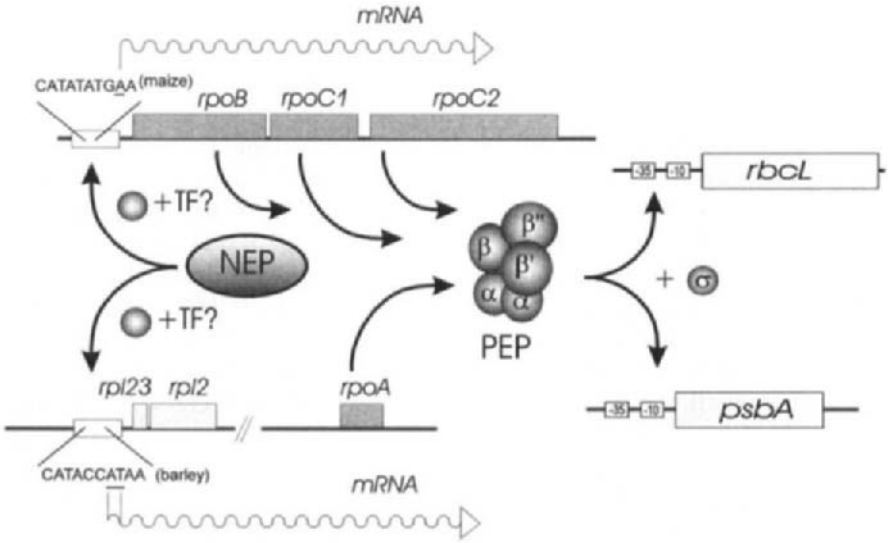


FIG. 7 Specificity and function of NEP and PEP. Different promoters each recognized by the respective enzyme are the basis for the division of labor between the two plastid RNAPs. NEP, a single-subunit-type RNAP, transcribes mainly genes of the genetic apparatus, including the PEP genes *rpoA, rpoB, rpoC1,* and *rpoC2* (dark gray). Hence, it is ensured that NEP maintains the basic functions of the plastid in all tissues and cell types. NEP may be complemented by regulatory factors (TF). The enhanced transcription of PEP genes by NEP in specific cells is a precondition for the differentiation of proplastids into photosynthetically functional chloroplasts because the major photosynthetic gene transcripts are generated by PEP subsequently. This enzyme recognizes eubacterial-like promoters (−10/−35 boxes) and is complemented by sigma factors. Two examples for NEP transcription initiation sites are included (see the legend to Fig. 5 for additional details). A large number of genes/operons contain PEP and NEP promoters and both RNAPs are active in parallel at least in green leaves.

translated (or not with high efficiency). Therefore, PEP-lacking plastids seem not to be able to construct their photosynthetic apparatus. NEP might be the major RNAP in plastids of nongreen tissue. White cells of suspension cultures transcribe their genes from NEP promoters but not from promoters of the −10/−35 σ^{70} type (Vera and Sugiura, 1995; Vera *et al.,* 1996; Kapoor *et al.,* 1997; Miyagi *et al.,* 1998; Silhavy and Maliga, 1998b). In root cells of spinach, *rrn16* is transcribed only from a NEP-type promoter (Iratni *et al.,* 1997). It remains to be investigated to what extent PEP is active in nongreen tissues.

Higher plants have numerous nongreen tissues in which different types of plastids have to fulfill different functions. These plastids (proplastids, leucoplasts, chromoplasts, amyloplasts, and elaioplasts) are photosynthetically inactive, i.e., they do not need to transcribe those genes that encode

products that are exclusively needed for photosynthesis. Thus, the primary function of NEP could be to transcribe (at a comparatively low rate) those genes that are necessary to maintain plastids as a compartment for diverse metabolic pathways, such as the biosynthesis of amino acids and fatty acids, certain phytohormones, etc. One should recall that the overwhelming majority of these pathways are exclusively driven by nuclear-encoded enzymes in higher plants: This coincides with a primary role for a fully nuclear-encoded RNAP in maintaining the metabolic activity of these nonphotosynthetic plastids and with a secondary role in keeping proplastids ready to develop into chloroplasts. Indeed, PEP-lacking plants have plastids and plastid DNA in (nearly) normal amounts (Hess *et al.,* 1993) and ribosomes (P. Maliga, personal communication; R. G. Herrmann, personal communication). Accordingly, NEP transcribes $tRNA^{Glu}$ (Walter *et al.,* 1995): This is essential because of the dual role that this tRNA plays in providing glutamic acid for the synthesis of aminolevulinic acid (for the formation of tetrapyrroles including heme) and in the translation of plastid mRNAs.

As soon as chloroplast or etioplast development starts, NEP activity seems to increase or certain NEP promoters are specifically activated in order to enhance transcription of the *rpo* genes and perhaps other housekeeping genes. In agreement with this scenario is the observation that the highest accumulation of the *rpoB* transcript (synthesized by NEP) is observed at the bases of cereal leaves (Baumgartner *et al.,* 1993; Inada *et al.,* 1996). The lowest part of the cereal leaves contains proplastids and plastids in a very early stage of development toward photosynthetically active chloroplasts. Later in chloroplast differentiation, PEP dominates transcription, providing the capacity necessary for the expression of genes involved in photosynthesis (Mullet, 1993). NEP will remain active in fully developed chloroplasts to transcribe genes which do not have PEP promoters and also genes which are at the same time transcribed by PEP (Allison *et al.,* 1996; Hajdukiewicz *et al.,* 1997; Hübschmann and Börner, 1998; Iratni *et al.,* 1997). The differential activation of NEP and PEP and their promoters, respectively, should be regulated. The spinach CDF2 and RPL4 proteins or the product of the virescent gene V1 of rice could be involved in this regulation (Iratni *et al.,* 1997; Trifa *et al.,* 1998).

G. Regulation of Plastid Gene Activity: Sigma Factors and Other Mechanisms

Transcription of plastid genes is affected by light quality and quantity (mainly in leaves) and cell type (mainly photosynthetically active vs inactive). The activity of plastid genes might be controlled by different means.

Effects of DNA supercoiling on transcription rates were described for *Z. mays* chloroplasts. Differences in the template-negative superhelical densities for *rbcL/atpB/E* promoters of *Z. mays* resulted in varied transcription rates (Stirdivant *et al.,* 1985). The transcription of relaxed DNA templates by partially purified RNAP was investigated by Zaitlin *et al.,* (1989).

There are a few indications for base-specific methylation of plastid DNA (Gauly and Kössel, 1989). Although certainly not one of the major regulatory mechanisms, DNA methylation may influence differential expression of plastid genes via interference with the transcriptional apparatus. Transcription in amyloplasts of sycamore culture cells was suppressed depending on the degree of methylation (Ngernprasirtsiri *et al.,* 1988). The selective methylation of chloroplast DNA was suggested to participate in the control of photosynthesis genes by cell-specific expression in mesophyll vs bundle sheath cells of greening *Z. mays* leaves (Ngernprasirtsiri *et al.,* 1989). The transition from chloroplasts to chromoplasts in ripening tomato fruits was also found to be correlated with an increase in 5-methylcytosine from undetectable levels to 4.6 mol% of the base composition (Kobayashi *et al.,* 1990). The transcriptional activity determined in run-on transcription assays decreased in parallel to only 8% in the chromoplasts of the ripened fruit compared to the activity of green leaf chloroplasts. Interestingly, DNA isolated from the chromoplasts had a low template activity when subjected to run-off *in vitro* transcription assays using soluble RNAP prepared from intact chloroplasts or *E. coli* RNAP. None of the enzymatic activities involved in the methylation of plastid DNA has been characterized. Thus, the role of DNA methylation during transcriptional regulation of plastid genes has not been elucidated in full. Whereas it seems to be of no importance in leaf plastids, it may play a role in those cases in which the transcription by PEP ceases during developmental differentiation of active green leaf chloroplasts into chromoplasts or amyloplasts. Another factor that seems to influence the transcription rate from plastid genes is the redox status of the plastid. Using different redox reagents and electron transport inhibitors, effects on plastid (and mitochondrial) gene expression were described that were suggested to result from their action on organelle transcription rather than on RNA processing or translation (Hughes and Link, 1988; Allen *et al.,* 1995). However, in contrast to processes related to efficiency of translation or RNA stability (Levings and Siedow, 1995; Liere and Link, 1997), experimental evidence for a specific effect of the redox status on transcription of chloroplast genes is lacking.

For a discussion of transcriptional and posttranscriptional processes in the regulation of plastid gene expression, the reader is referred to Allison *et al.* (1995), Gruissem and Tonkyn (1993), Link (1994, 1996), and Stern *et al.* (1997).

Clearly, the regulated transcription of plastid genes is accomplished by protein factors. A few polypeptides potentially involved in plastid gene regulation have been described. Transcription of *psbD-C* from one of several initiation sites seems to be regulated by a common blue light signaling pathway in land plant chloroplasts (Hoffer and Christopher, 1997). A sequence-specific binding factor, AGF, was found at the blue light-responsive *psbD-C* promoter in barley (Kim and Mullet, 1995). In rice, the affinity of a 36-kDa polypeptide to the same promoter was characterized biochemically (To *et al.,* 1996). The DET1 gene product of Arabidopsis was shown to repress the *psbD-C* blue light responsive promoter in a developmental and tissue-specific manner, suggesting the existence of a specific photosensory system that regulates these sequence-specific binding factors and is controlled by DET1 (Christopher and Hoffer, 1998). Chloroplast proteins were also described which bind specifically to the *rbcL/atpB* intergenic region in maize (Lam *et al.,* 1988).

In bacteria, activation of different sets of genes is achieved by the use of several sigma factors which complete the core RNAP to the holoenzyme and enable the enzyme complex to recognize the promoter, to interact with it, and to initiate transcription. As in bacteria, several sigma factors seem to be involved in the transcription of the plastid genome. In the gree alga *Chlamydomonas reinhardtii,* sigma-like activities were first described by Surzycki and Shellenbarger (1976). For higher plants, factors that provide transcription specificity and promoter-specific binding of the polymerase were analyzed first in spinach (Lerbs *et al.,* 1983b) and mustard (Bülow and Link, 1988; Tiller *et al.,* 1991). Due to their similarity in size to bacterial sigma factors and their ability to provide sequence specificity to a core polymerase extract, the term "sigma-like factors" (SLF) was introduced. Using antiserum to a cyanobacterial sigma factor, immunological evidence was obtained for SLFs in purified preparations of chloroplast RNAP from *Z. mays, O. sativa, C. reinhardtii,* and *Cyanidium caldarium* (Troxler *et al.,* 1994). Three different SLFs were isolated from mustard and termed, according to their size, SLF67, SLF52, and SLF29 (Tiller *et al.,* 1991; Tiller and Link, 1993a). During the past 2 years, genes or cDNAs have been cloned with homology to bacterial genes encoding sigma factors and therefore most likely encode plastid sigma factors. For the red alga *C. caldarium,* a single nuclear DNA sequence potentially encoding a plastid SLF was described (Liu and Troxler, 1996; Tanaka *et al.,* 1996). Similar cDNAs were isolated from *S. alba* (Kestermann *et al.,* 1998), rice (Tozawa *et al.,* 1998), and *A. thaliana* (Isono *et al.,* 1997; Tanaka *et al.,* 1997; Fig. 8). In Arabidopsis, three different cDNAs for putative plastid SLFs have been found in different laboratories. The molecular masses of the unprocessed polypeptides are 56 (SigA), 64 (SigB), and 65 kDa (SigC; Tanaka *et al.,* 1997). Unfortunately, apparently identical sigma factors were named differently: SigA (accession

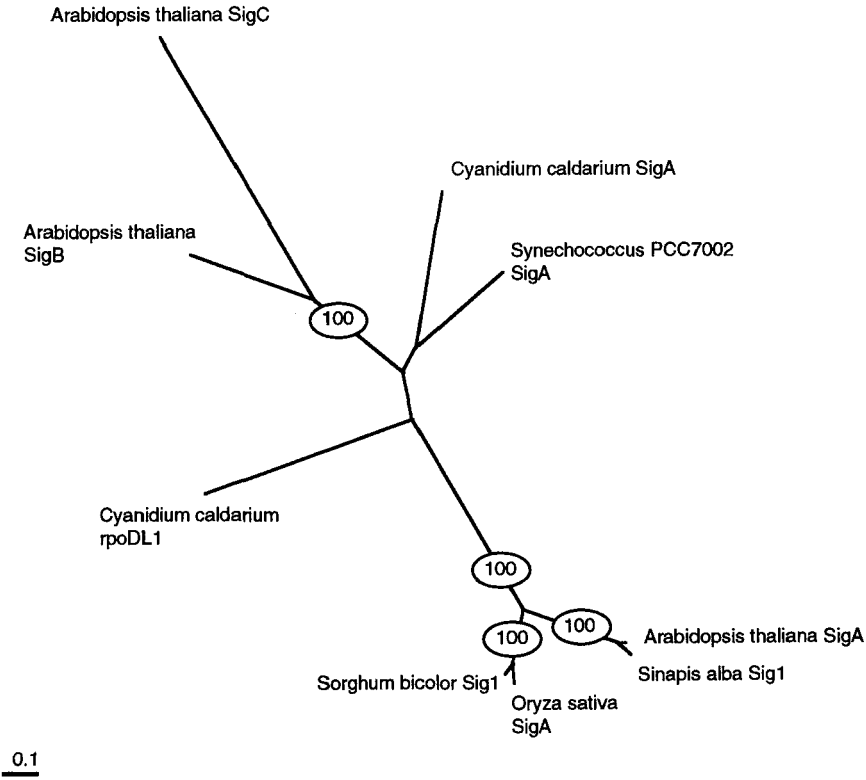


FIG. 8 Molecular phylogenetic tree of plant sigma factors. A total of 223 positions from the central part of a ClustalW alignment were considered. The tree has been calculated based on maximum likelihood distances using the program PUZZLE, Version 4.0 (Strimmer and von Haeseler, 1996, 1997), and the Jones model of sequence evolution. Numbers at the nodes indicate QP support values for the branch distal to it and are shown when they are 100%. The following sequences have been compared: *Arabidopsis thaliana* SigA (accession Nos. D89993, AF015542, AB004821, Y14252, AF024590, and Y14567), SigB (AB004293, AB004820, and Y15362), and SigC (D89994, AF015544, and AB004822); *Sinapis alba* Sig1 (Y15899); *Oryza sativa* SigA (AB005290); *Sorghum bicolor* Sig1 (Y14276); *Cyanidium caldarium* rpoDL1 gene product (L42639) and SigA (D83179). The sequence of the major sigma factor of *Synechococcus* PCC7002, SigA (U15574; Caslake and Bryant, 1996), was chosen as the outgroup. The tree topology did not change when SigA of *C. caldarium* was defined as the outgroup.

no. D89993), SigB (AB004293), and SigC (D89994; Tanaka *et al.*, 1997) correspond to Sig2 (AB004821), Sig1 (AB004820), and Sig3 (AB004822; Isono *et al.*, 1997). Small gene families encoding plastid sigma factors are probably not restricted to *A. thaliana* because in *O. sativa* multiple SLF genes seem to also exist (Y. Tozawa *et al.*, personal communication). A

few additional SLF sequences isolated in other laboratories or from more species have been submitted to GeneBank.

Phylogenetic trees based on sequence comparisons show that the three Arabidopsis factors may represent basic types of higher plant sigma factors (Fig. 8). All other plant sigma factor sequences published to date (from *S. bicolor, O. sativa,* and *S. alba*) are more similar to the SigA or Sig1 factor of Arabidopsis (Tanaka *et al.,* 1997; Isono *et al.,* 1997) than to SigB or SigC (Fig. 8). The available data suggest separate branches for the respective monocot and dicot SigA sequences, which belong to the same clade as *Arabidopsis* SigA. SigA may be considered the major plastid sigma factor because its mRNA seems to be the most abundant transcript for plastid sigma factors (Tanaka *et al.,* 1997). The SLFs of higher plant plastids highly resemble the sigma factors of cyanobacteria (Caslake *et al.,* 1997; Gruber and Bryant, 1997). Hence, it is likely that they cooperate exclusively with the PEP complex. This view is further supported by their expression mode that coincides with chloroplast biogenesis (Tanaka *et al.,* 1997; Kestermann *et al.,* 1998; Tozawa *et al.,* 1998) and increasing PEP activity (see Section IIIF). Why there is more than a single SLF is not clear. It might reflect the requirements of different PEP promoters; in addition, it is an interesting parallel to cyanobacteria, which possess up to six different sigma factors (Gruber and Bryant, 1997, 1998).

There is evidence for a control of chloroplast transcription by phosphorylation of putative sigma factors. Transcriptional activity of fractions purified from mustard etioplasts and chloroplasts was affected by phosphorylation and dephosphorylation (Tiller and Link, 1993b). Purified RNAP fractions from mustard chloroplasts contain a serin-specific protein kinase activity that phosphorylates SLFs *in vitro,* in particular the factors SLF29 and −52 (Baginsky *et al.,* 1997). This kinase, designated plastid transcription kinase (PTK), exists in a soluble form and in a form tightly linked to the core polymerase complex (PEP). The catalytic activity was assigned to a 54-kDa protein that appears as part of a heterotrimeric complex, from which the other two proteins were previously identified as putative polymerase (PEP) subunits (Pfannschmidt and Link, 1994). PTK was shown to affect promoter binding and the efficiency of transcription of the *psbA* gene *in vitro.* Because PTK was severely affected by treatment with phosphorylases and dephosphorylases, it might be regulated by phosphorylation (Baginsky *et al.,* 1997; Link *et al.,* 1997). The involvement of extraplastidic protein phosphatases in the regulation of the blue light-responsive *psbD-C* promoter of barley was suggested as a result of studies using inhibitors of eukaryotic protein phosphatases and kinases, okadaic acid, and K252A (Christopher *et al.,* 1997).

Virtually nothing is known about possible regulatory proteins of NEP. One may anticipate the existence of nuclear-encoded proteins with similari-

ties to mitochondrial transcription factors MTFA and MTFB. Surprisingly, the chloroplast ribosomal protein L4 appears to be a possible candidate as a protein regulating NEP in spinach. It copurifies in various protocols with the 110-kDa RNAP of spinach, which most likely represents NEP (Trifa *et al.,* 1998). Furthermore, the bacterial L4 protein has an RNA-binding domain (Zengel and Lindahl, 1993), and it regulates the transcription of its own operon as a repressor (Lindahl *et al.,* 1983; Zengel and Lindahl, 1990, 1992; Sha *et al.,* 1995). The nuclear-encoded plastid homolog possesses an elongated highly acidic domain as do many DNA-binding proteins, including transcription factors, and therefore might be even more likely to represent a regulatory factor for NEP (Trifa *et al.,* 1998).

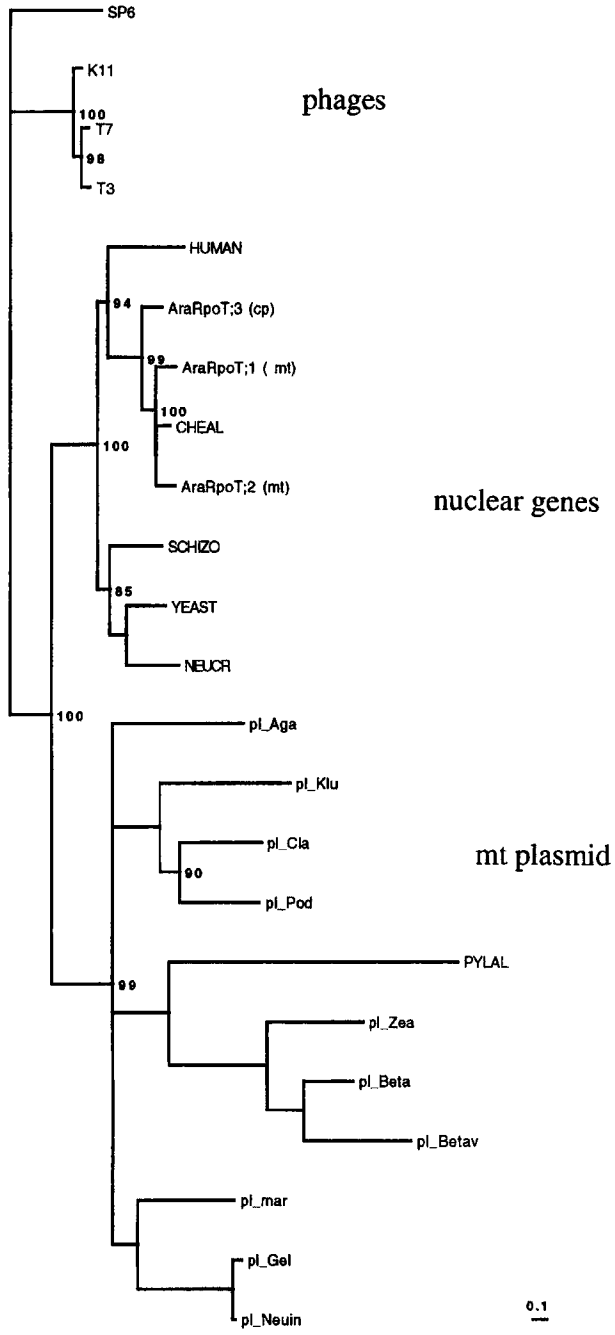
IV. Evolution of Plant Phage-Type RNAPs

A. Theories on the Origin of Organellar RNA Polymerase Genes

There are two main questions regarding the evolution of the higher plant phage-type RNAPs: When and from what kind of ancestral molecule did these enzymes develop? When did the development of at least two different nucleus-encoded enzymes that are differently targeted, one into mitochondria and the other into plastids, begin?

The RNAPs of plant mitochondria, the NEP of plastids, the mitochondrial RNAPs of humans and yeast, as well as the RNAPs encoded by linear mitochondrial plasmids and the bacteriophage polymerases all belong to the single-subunit type and are all phylogenetically related to each other. They differ considerably from the multisubunit RNAPs, including the archaebacterial, eubacterial, and eukaryotic, nuclear-localized enzymes (Cermakian *et al.,* 1997). This is reflected in phylogenetic trees as shown in Fig. 9. The unrooted phylogenetic tree has three main branches: one containing all genuine bacteriophage polymerases, one encompassing the plasmid-encoded polymerases and the *Pylaiella* sequence that is expressed from a gene inserted in the mtDNA, and one with all the nuclear genes including those of plants. A similar topology has been found when using maximum parsimony and maximum likelihood distances (Cermakian *et al.,* 1997; Hedtke *et al.,* 1997). There obviously exist three related clades of genes for single-subunit-type RNAPs. Each clade should be of monophyletic origin with a hypothetical sequence ancestral to all three clades.

The origin of all genes for single-subunit-type RNAPs was recently discussed in detail by Cermakian *et al.* (1997). These authors suggest an ancestral DNA polymerase or a reverse transcriptase gene as the progenitor of


SP6
K11
100
T7
98
T3
phages
HUMAN
94
AraRpoT;3 (cp)
99
AraRpoT;1 (mt)
100
CHEAL
100
AraRpoT;2 (mt)
nuclear genes
SCHIZO
85
YEAST
NEUCR
100
pl_Aga
pl_Klu
pl_Cla
90
pl_Pod
mt plasmid
PYLAL
99
pl_Zea
pl_Beta
pl_Betav
pl_mar
pl_Gel
pl_Neuin
0.1

this superfamily of RNAP genes. This suggestion is based mainly on the similarity of the T7 enzyme with DNA polymerase I (Klenow fragment) and reverse transcriptases with regard to short sequence motifs and three-dimensional structure.

It is obvious that the endosymbiontic ancestors of mitochondria and plastids should have contained the genes for their eubacterial multisubunit polymerase. In the chloroplast genomes of photosynthetically active plants, the respective *rpo* genes are still present. The recent finding of eubacterial-type *rpo* genes in the mitochondrial genome of the primitive flagellate *Reclinomonas americana* lends strong support to the idea that the mitochondria had these genes at the beginning of their evolution within the eukaryotic cell (Cermakian *et al.*, 1996). It seems plausible that in most but not all organisms the single-subunit type of RNAP took over the transcription of all mitochondrial genes at an early stage during the evolution of eukaryotes, with the *rpo* genes subsequently being lost.

It may be of importance in this context that the mitochondrial genome of the brown alga, *Pylaiella littoralis,* has the consensus sequences typical for eubacterial promoters upstream of some genes but lacks eubacterial *rpo* genes (Delaroque *et al.*, 1996). Instead, the chondrome of this alga harbors a gene for an RNAP of the type found on linear mitochondrial plasmids (Rousvoal *et al.*, 1998; see Section IID). It remains to be shown unambigously if $-35/-10$ σ^{70}-type promoters are indeed used and which type of RNAP operates in the mitochondria of this organism. If the plasmid-type RNAP were the principal transcriptase in the mitochondria of *P. littoralis,* then this would represent a special case of the evolution of the

FIG. 9 Molecular phylogenetic tree of bacteriophage, nuclear-encoded organellar, and plasmid-encoded mitochondrial RNAPs. The tree has been calculated based on maximum likelihood distances using the PUZZLE program (1000 puzzling steps; Strimmer and von Haeseler, 1996, 1997) and the Jones model of sequence evolution. Support values are shown at nodes when >80%. For the alignment (courtesy of A. Weihe), only the most conserved sequence motifs have been considered (total length: 470 positions). The following sequences have been compared: T7, bacteriophage T7 (P00573); T3, bacteriophage T3 (P07659); SP6, bacteriophage SP6 (P06221); K11, bacteriophage K11 (P18147); NEUCR, mtRNAP of *Neurospora crassa* (P38671); YEAST, mtRNAP of *Saccharomyces cerevisiae* (P13433); CHEAL, RpoT of *Chenopodium album* (Y08067); HUMAN, mtRNAP of *Homo sapiens* (U75370); SCHIZO, mtRNAP of *Schizosaccharomyces pombe* (Z99126); AraRpoT;1, AraRpoT;2, and AraRpoT;3, see Table II; PYLAL, *Pylaiella maritima* (AF034976); pl_Beta, plasmid *Beta maritima* (Y10854); pl_Betav, plasmid *Beta vulgaris* (Z34298); pl_Gel, *Gelasinospora* sp. (L40494); pl_Klu, *Pichia kluyveri* (Y11606); pl_mar, plasmid maranhar from *Neurospora crassa* (P33540); pl_Aga, plasmid from *Agaricus bitorquis* (P33539); pl_Pod, plasmid pAL2-1 from *Podospora anserina* (X60707); pl_Neuin, plasmid kalilo from *Neurospora intermedia* (P33541); pl_Cla, plasmid pC1K1 from *Claviceps purpurea* (P22372); pl_Zea, plasmid S-2 from *Zea mays* (P10581).

mitochondrial transcriptional apparatus rather than a step toward the use of T3/T7-like RNAPs as known from most eukaryotes. It is highly improbable that the nuclear genes for the mitochondrial single-subunit RNAP originated from one of the plasmids frequently found within the mitochondria of fungi and plants. These linear plasmids have their closest (known) relatives in bacteriophages (certain linear DNA phages), as was concluded from similarities in structure and mode of replication and from comparison of genes encoding DNA polymerases (Kempken *et al.,* 1992; Rohe *et al.,* 1992). Moreover, another *E. coli* phage (N4) was found to have an RNAP gene which is more closely related to the RNAP genes of linear mitochondrial plasmids than to those of phages T3/T7 and mitochondria (S. Willis, L. Rothman-Denes, and A. Weihe, personal communication). The considerably weaker phylogenetic relationship of plasmid-borne RNAPs to the nuclear-encoded RNAPs of mitochondria than to the RNAPs of T3/T7 bacteriophages argues against the evolution of nuclear genes encoding mitochondrial RNAPs from the plasmid genes. Hence, it is probable that the genes for organellar RNAPs and the RNAP genes on linear mitochondrial plasmids originated from two different sources, one similar to the T3/T7 group of phages and the other similar to linear phages such as N4.

There are several possible ways in which phage genes could have become mitochondrial RNAP genes during the evolution of the eukaryotes. For a thorough discussion of the origin of these genes, see Cermakian *et al.* (1997). One possibility is that a single-subunit-type RNAP gene or genes might have already been present in the genome of the eukaryotic progenitor cell prior to endosymbiosis. Alternatively, the endosymbiont that gave rise to mitochondria might have harbored a phage similar to T3/T7 bacteriophages either as a virus or as a provirus. However, there is no particular reason to assume that RNAP genes should have persisted in the nucleus or the endosymbiont for a prolonged period without having a necessary function. On the other hand, several phage genomes were found integrated in the genome of *Rhodobacter sphaeroides* (Vlcek *et al.,* 1997), i.e., phage-derived sequences may persist over considerable periods of time in bacterial genomes, perhaps long enough to take over by chance the transcription of an important cellular gene.

Shortly after, or even before, the endosymbiosis was established, the RNAP of such a temperate phage might have taken over an important, indispensible function in the endosymbiont. Such a function could be the transcription of one or a few genes or supporting replication by providing the primer. As a modification of these hypotheses, such a phage might have even been able to infect the early protoeukaryotic cell (still having many features of bacterial cells) around the time the α-proteobacterium that evolved into mitochondria was taken aboard.

Among various bacteriophages studied, only a few (T3, T7, SP6, and K11) possess an RNAP gene of this type. None of the bacterial hosts of these phages belongs to the supposed progenitor of mitochondria, the α-proteobacteria. Instead, the natural hosts of these phages all are enterobacteriaceae, and even under laboratory conditions only related members of the γ subdivision of proteobacteria can be infected. Moreover, these bacteriophages are virulent phages that destroy their host bacteria shortly after infection. Nevertheless, it may be worthwhile to screen α-proteobacteria for (temperent) phages having an RNAP gene of the T3/T7 type.

B. Evolution of Higher Plant Organellar RNA Polymerases

While it remains an enigma how and why a single-subunit-type RNAP has taken over the transcription of mitochondrial DNA, it seems more clear how the several *RpoT* genes present in Arabidopsis and some other higher plant species (Table II) are interrelated and especially how the NEP of

TABLE II

Sequences of Nuclear Genes Encoding Phage-Type RNAPs in Plants That Are Published or Submitted to GenBank

Species	GenBank accession Nos. and type of sequence	Gene	Subcellular localization	Reference
Arabidopsis thaliana	Y09006 (gene), Y08137 (cDNA), Y09432 (gene)	*RpoT;1*	Mitochondria	Hedtke *et al.* (1997)
Arabidopsis thaliana	AJ001037 (gene)	*RpoT;2*	Mitochondria	W. Schuster (personal communication), A. Weihe (personal communication)
Arabidopsis thaliana	Y08722 (gene), Y08463 (cDNA)	*RpoT;3*	Plastid	Hedtke *et al.* (1997)
Chenopodium album	Y08067 (cDNA)	*RpoT*	Mitochondria	Weihe *et al.* (1997)
Hordeum vulgare	Y11600 (partial cDNA)	*RpoT*	Unknown	Weihe *et al.* (1997)
Oryza sativa	U34283, Y11599 (partial cDNAs)	*RpoT*	Unknown	Cermakian *et al.* (1996), Weihe *et al.* (1997)
Zea mays	AJ005343 (gene)	*RpoT*	Unknown	Young *et al.* (1998)
Triticum aestivum	U34402 (partial cDNA)	*RpoT*	Unknown	Cermakian *et al.* (1996)

the plastids might have evolved. An overview on the phage-type RNAP sequences from higher plants that have been described and/or are accessible in data banks is displayed in Table II.

That the Arabidopsis *RpoT;1, RpoT;2,* and *RpoT;3* arose by gene duplication events from the original gene encoding the mitochondrial RNAP is evident (i) from the high sequence similarity of the coding regions, (ii) from the identical position of all 18 introns in the three genes (Fig. 10), and (iii) from their location in one clade together with the genes for mitochondrial RNAPs from organisms other than plants (Fig. 9; Cermakian *et al.,* 1997; Hedtke *et al.,* 1997).

Before or after the duplication, an RNAP originally imported exclusively into mitochondria might have acquired the ability to be cotargeted into plastids. Cotargeting of proteins into mitochondria and plastids occurs (Chow *et al.,* 1997) and further examples might still be found. Later, transport of each RNAP might have become more specific for one organelle only, resulting in a situation observed with the Arabidopsis RNAPs encoded by the *RpoT;1* and *RpoT;3* genes (Hedtke *et al.,* 1997). If this gene duplication occurred relatively late in the evolution of plants, cotargeting of an *RpoT*-encoded RNAP into both organelles might still be detectable in some cases. The transit peptide encoded by the Arabidopsis *RpoT;2* gene is able to target GFP into the mitochondria but a cotargeting into plastids cannot be ruled out completely (W. Schuster, personal communication). The methods used to investigate the localization of the Arabidopsis proteins encoded by *RpoT;1* and *RpoT;3* in the mitochondria and plastids, respectively, would not detect a low percentage of mistargeted protein. Thus, investigation of targeting of *RpoT* products by more sensitive methods is urgently needed, as is the analysis of these processes in other species.

There are not nearly enough sequence data from different plants, including algae, available to answer the question as to when this duplication (or series of duplications) actually happened—in the evolution of angiosperms, much earlier, during algal evolution, or, in the extreme, during evolution of Arabidopsis? Arabidopsis is not the only angiosperm to possess more than one *RpoT* gene. Southern hybridizations between *C. album* DNA and the *Chenopodium RpoT* cDNA sequence as a probe (Weihe *et al.,* 1997) revealed the presence of at least two *RpoT* genes in this species. Preliminary data show the existence of different *RpoT* genes in tobacco, two of them nearly identical to each other (Börner *et al.,* 1999). Tobacco is an allotetraploid species and, therefore, one may expect a higher number of members of this gene family to be present in its genome.

Monocots contain more than one *RpoT* gene. Two different *RpoT* genes were found in wheat (T. M. Ikeda and M. W. Gray, personal communication) and *Z. mays* (D. Lonsdale, personal communication; D. Stern, per-

A

Intron:	↓1	↓2	↓3	↓4	↓5	↓6	↓7	↓8	↓9
RpoT;1	VEQEVRI	QVKVGAR	TLENTKT	LDKSARH	WTGYDQG	YEALDTL	REDVPIP	KLEVARM	IHQDGSC
RpoT;2	IEQEIRI	RAKVGSR	AKGSMNS	LEKSGRV	WSGYDKG	FEALDTL	RSDVPLP	KLSVARM	IHQDGSC
RpoT;3	IEQEVRI	QAKLGSR	TYPGSKL	LDKSAKH	WKGYDKG	FEALDTL	REDVPIP	KLSVARM	IHQDGSC
cons.	**iEQEvRI**	**qaKvGsR**	**t....k.**	**LdKSarh**	**W.GYDkG**	**fEALDTL**	**ReDVPiP**	**KLsVARM**	**IHQDGSC**

Intron:	↓10	↓11	↓12	↓13	↓14	↓15	↓16	↓17	↓18
RpoT;1	GRDKLGA	IAARVLK	LDQVDRK	YAAKITL	CAKIIAS	HLVKTTL	TDKVMAR	FAGVHDS	LENLLES
RpoT;2	GRDTLGA	IATRVLD	LNQVDRK	YAAKVTL	CAKIIAS	KLVKTSL	TDQVIVR	FAGVHDS	LENLLES
RpoT;3	GRDSPEA	ISRRVHE	ITQVDRK	YSAKVTL	CAKIIAS	HLIRTSL	GNTVDVR	FAGVHDS	LEDLLQS
cons.	**GRD.lgA**	**Ia.RVl.**	**l.QVDRK**	**YaAKvTL**	**CAKIIAS**	**hLvkTsL**	**td.V.vR**	**FAGVHDS**	**LEnLLeS**

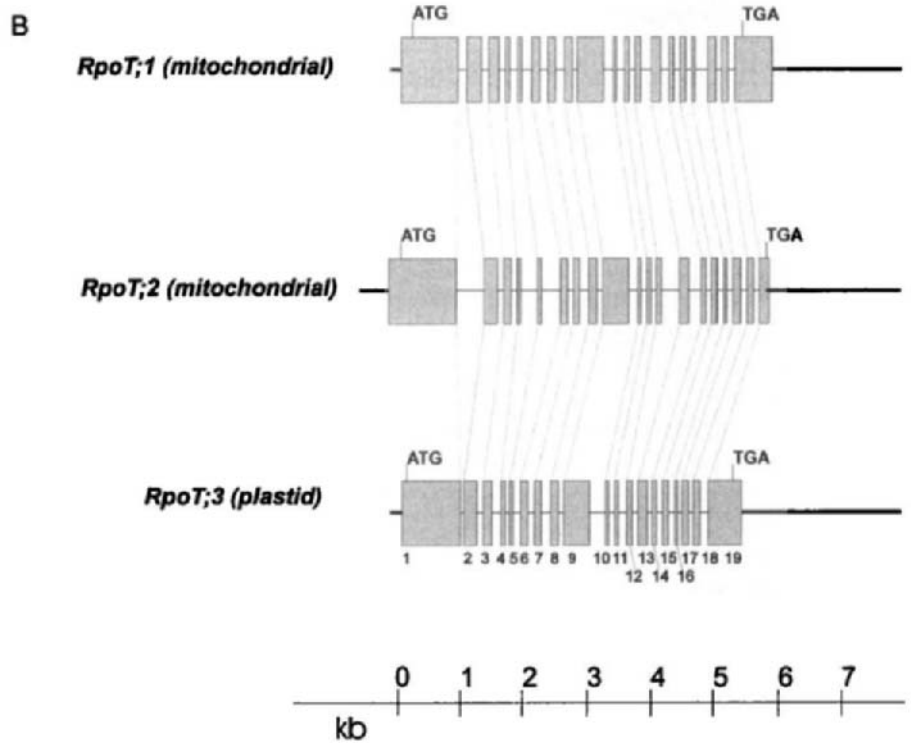


FIG. 10 (A) Intron localization along the aligned amino acid sequences of the three *Arabidopsis thaliana* single-subunit-type RNAPs. Sequence blocks of seven residues each surrounding the intron insertion sites are shown. The introns are numbered consecutively. The positions of I1–2 and I4–18 are highly conserved with respect to the protein sequences and represent identical insertion sites. The I3 site appears less conserved but from the total alignment it is likely that this intron resides at an identical site. For exact localization, compare to the RpoT;1 sequence in Fig. 2. (B) Genomic organization of the three RNAP genes of *A. thaliana*. Exon sequences are indicated by gray boxes and introns by a thin black line. The figure is drawn approximately to scale.

sonal communication). The sequence of one of the wheat *RpoT* genes is more close to the *RpoT;1* and *−2* genes than to the *RpoT;3* of Arabidopsis. The second wheat *RpoT,* however, seems not to be more closely related to any specific Arabidopsis homolog (T. M. Ikeda and M. W. Gray, personal communication). A detailed comparison of the sequences and intron–exon structure of *RpoT* genes from Arabidopsis and monocots provides at least an answer to the question of whether the present-day *RpoT* genes in dicots and monocots still have the structure of an ancestral gene that existed prior the divergence of monocots and dicots. Indeed, such a comparison shows that all 18 introns are located at exactly the same sites with regard to the encoded protein sequence in the three Arabidopsis genes, in a maize *RpoT* gene (Young *et al.,* 1998), and in a barley *RpoT* gene (W. Hess, unpublished observation). Consequently, it is clear that the currently known higher plant *RpoT* genes do represent the intron–exon structure of the ancestral *RpoT* gene and that this structure has been conserved for at least the past 200–270 million years, when the monocot–dicot divergence occurred (Wolfe *et al.,* 1989; Schneider-Poetsch *et al.,* 1998). However, concerning the time when the gene duplications took place, this information does not help much; it means only that it is equally possible that this had happened before the evolution of mono- and dicot plants started or thereafter. It is interesting to note that some of the introns found in the plant genes are also present at identical sites in the human gene for the mtRNAP (GeneBank accession No. AC004449).

In the evolutionary context of plants, it would be helpful to know when during evolution the NEP activity in plastids first occurred. No data are available regarding a potential NEP activity in plastids of mosses or ferns. A few data suggest the existence of NEP in algal chloroplasts. Previous reports on the detection of transcriptional activity in plastids of achlorophyllous mutants of *C. reinhardtii* lacking significant amounts of ribosomes in their plastids stressed the presence of a nuclear-encoded RNAP in *Chlamydomonas* plastids (Surzycki, 1969; Surzycki *et al.,* 1970). However, since the plastids in these mutants seemed to retain a few ribosomes, PEP synthesis might have occurred in sufficient amounts to explain the observed polymerase activity. Moreover, in contrast to similar experiments in tobacco (Allison *et al.,* 1996), in *C. reinhardtii* attempts to test the importance of plastid *rpo* genes by knock-out experiments failed to produce viable homoplasmic transformants (Rochaix, 1995; Fischer *et al.,* 1996). Thus, the *Chlamydomonas* PEP might transcribe genes of importance for the whole cell, whereas the tobacco PEP does not; alternatively, this result might be taken as indirect evidence for the lack of a NEP-like activity in *Chlamydomonas* plastids. Several lines of evidence suggested the existence of more than one RNAP activity in another alga, *Euglena gracilis* (Greenberg *et al.,* 1984; Narita *et al.,* 1985; see Section IIIB). It is currently not clear

whether these activities are due to PEP and NEP or to different forms of PEP–holoenzyme complexes generated, for example, by assembly with alternative sigma factors.

V. Concluding Remarks

The transcriptional machinery of mitochondria and plastids in higher plants is amazingly complex. In Arabidopsis, and presumably in other higher plants as well, there seem to be two closely related genes for mitochondrial RNAPs, both of the T3/T7 bacteriophage type. The mitochondrial genes and operons, respectively, are transcribed from promoters which resemble phage promoters in that their essential consensus sequences are very close to the transcription initiation site. Additional *cis* elements were identified further upstream which positively or negatively modulate the transcriptional activity of the respective gene. Moreover, mitochondrial genes exist which are transcribed from entirely different promoters. Virtually nothing is known about *trans*-acting factors involved in the regulation of mitochondrial gene activity and about the role the two polymerases might play. Urgently needed are studies of the function of the polymerases, the further dissection of promoter sequences, and the function of specific *cis* elements, of proteins facilitating promoter recognition, and of putative repressor and activator proteins. Though these types of investigation rely mostly on *in vitro* systems and are severely hampered by our inability to transform plant mitochondria, the recent discovery of genes for the mitochondrial RNAP(s) as well as the data obtained on promoter structure provide a solid basis for rapid development of this field of research.

Unfortunately, we have only a very limited knowledge about the regulation of the transcription of mitochondrial genes. In the case of plastids, construction of the photosynthetic apparatus during leaf development requires an activated transcription, together with an increase in the number of plastids per cell and chromosome copies per plastid, as well as a sometimes drastically changed transcript stability and translational efficiency. Mitochondria may react in a very similar way in tissues that need a particular high activity of respiration. Therefore, the study of components of the transcriptional apparatus in plant mitochondria has to be complemented by studies on the regulation of mitochondrial genes in different tissues of plants, in particular regarding the potential influence of the presence or lack of photosynthetic activity.

During the past few years, there has been a real breakthrough in our understanding of the sophisticated machinery operating in plastidic transcription. It is now evident that two different types of RNAP are needed

to transcribe the plastid genes. More than one sigma factor exists and these are encoded in the nucleus. Several types of plastid promoters have been identified. The $-10/-35$ σ^{70} type of promoters found upstream of most plastid genes/operons are used by PEP. Whether there are specific promoter subtypes interacting with certain sigma factors is one of many questions to be answered in the near future. The plastid-encoded polymerase is most active in green (but also etiolated leaf) tissue and transcribes genes encoding products necessary for photosynthesis and related processes and some, but not all, housekeeping genes. The second type of plastid RNAPs, the NEP, transcribes primarily housekeeping genes (but also some photosynthesis genes) and is active in both white and green tissues. We hypothesize that NEP activity provides a basic level of transcription of those plastid genes which are needed to keep the plastid compartment intact. This is necessary because (i) plastids house important metabolic pathways in addition to photosynthesis and (ii) proplastids have to be maintained in a status which permits their differentiation into specialized plastid types such as chloroplasts. NEP recognizes more than one type of promoter, including those which show a certain degree of similarity to the promoters in plant mitochondria. Several data strongly suggest that NEP is a single-subunit-type RNAP of the phage type. A respective gene has been identified in Arabidopsis which arose by duplication of the gene encoding the mitochondrial RNAP. There is a good chance that some of the most challenging questions concerning transcription in plastids will be answered in the near future. It has yet to be demonstrated whether NEP is indeed an RNAP of the T7/T3 type. Sequencing of nuclear *RpoT* genes in more species including algae will answer the questions of when and how often during the evolution of "green eukaryotes" was the gene for the mitochondrial RNAP duplicated, permitting evolution of a gene for a plastid enzyme. Systems for *in vitro* transcription have been established as have methods to study transcription in isolated plastids and to transform plastids. Hence, the research on *cis* and *trans* factors involved in the transcription of plastid genes should increase in the near future.

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Eukaryotic Transmembrane Solute Transport Systems

Milton H. Saier, Jr.[1]
Department of Biology, University of California at San Diego,
La Jolla, California 92093-0116

A comprehensive classification system for transmembrane molecular transporters has been proposed. This system is based on (i) mode of transport and energy-coupling mechanism, (ii) protein phylogenetic family, (iii) phylogenetic cluster, and (iv) substrate specificity. The proposed "Transport Commission" (TC) system is superficially similar to that implemented decades ago by the Enzyme Commission for enzymes, but it differs from the latter system in that it uses phylogenetic and functional data for classification purposes. Very few families of transporters include members that do not function exclusively in transport. Analyses reported reveal that channels, primary carriers, secondary carriers (uni-, sym-, and antiporters), and group translocators comprise distinct categories of transporters, and that transport mode and energy coupling are relatively immutable characteristics. By contrast, substrate specificity and polarity of transport are often readily mutable. Thus, with very few exceptions, a unified family of transporters includes members that function by a single transport mode and energy-coupling mechanism although a variety of substrates may be transported with either inwardly or outwardly directed polarity. The TC system allows cross-referencing according to substrates transported and protein sequence database accession numbers. Thus, familial assignments of newly sequenced transport proteins are facilitated. In this article I examine families of transporters that are eukaryotic specific. These families include (i) channel proteins, mostly from animals; (ii) facilitators and secondary active transport carriers; (iii) a few ATP-dependent primary active transporters; and (iv) transporters of unknown mode of action or energy-coupling mechanism. None of the several ATP-independent primary active transport energy-coupling mechanisms found in prokaryotes is represented within the eukaryotic-specific families. The analyses reported provide insight into transporter families that may have arisen in eukaryotes after the separation of eukaryotes from archaea and bacteria. On the basis of the reported analyses, it is suggested that the

[1] Phone: 619-534-4084; fax: 619-534-7108; e-mail: msaier@ucsd.edu.

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horizontal transfer of genes encoding transport proteins between eukaryotes and members of the other two domains of life occurred very infrequently during evolutionary history.

KEY WORDS: Transport systems, Protein families, Eukaryotes, Phylogeny, Energy coupling, Transporters.

I. Introduction

Transport systems serve the cell in numerous capacities (Paulsen *et al.*, 1998a,b; Saier, 1998, 1999). First, they allow entry of all essential nutrients into the cytoplasmic compartment and subsequently into organelles, allowing metabolism of exogenous sources of carbon, nitrogen, sulfur, and phosphorus. Second, they provide a means for the regulation of metabolite concentrations by catalyzing the excretion of end products of metabolic pathways from organelles and cells. Third, they mediate the active extrusion of drugs and other toxic substances from either the cytoplasm or the plasma membrane. Fourth, they mediate uptake and efflux of ionic species that must be maintained at concentrations that differ drastically from those in the external milieu. The maintenance of conditions conducive to life requires a membrane potential, requisite ion concentration gradients, and appropriate cytoplasmic concentrations of all essential trace minerals that participate as cofactors in metabolic processes. Such conditions are required for the generation of bioelectricity and for enzymatic activity. Fifth, transporters participate in the secretion of proteins, complex carbohydrates, and lipids into and beyond the cytoplasmic membrane, and these macromolecules serve a variety of biologically important roles in protection against environmental insult and predation, in communication with members of the same and different species, and in pathogenesis. Sixth, transport systems allow the transfer of nucleic acids across cell membranes, allowing for genetic exchange between organisms, thereby promoting species diversification. Seventh, transporters facilitate the uptake and release of pheromones, alarmones, hormones, neurotransmitters, and a variety of other signaling molecules that allow a cell to participate in the biological experience of multicellularity. Finally, transport proteins allow living organisms to conduct biological warfare, secreting, for example, antibiotics, antiviral agents, and antifungal agents that may confer upon the organism a selective advantage for survival purposes. Thus, from a functional standpoint, the importance of molecular transport to all facets of life cannot be overestimated.

The importance of transport processes to biological systems was recognized more than half a century ago (Gale and Taylor, 1947; Mitchell, 1949). Thanks largely to concerted efforts on the part of Jacques Monod and coworkers at the Pasteur Institute in Paris, who studied the mechanism of action of the *Escherichia coli* lactose permease, the involvement of specific carrier proteins in transport became established (Rickenberg *et al.,* 1956; Cohen and Monod, 1957). Since these early studies, tremendous progress has been made in understanding the molecular bases of transport phenomena, and the *E. coli* lactose permease has frequently been at the forefront. Initially, transport processes were characterized from physiological standpoints using intact cells. Cell "ghosts" in which the cytoplasmic contents had been released by osmotic shock proved useful, particularly as applied to human red blood cells and later to bacteria. Work with such systems provided detailed kinetic descriptions of transport processes, and by analogy with chemical reactions catalyzed by enzymes, the proteinaceous nature of all types of permeases became firmly established. Subsequent biochemical experimentation provided extensive documentation of this suggestion (Kaback, 1974).

With the advent of gene sequencing technologies, the primary structures of permeases first became available. Hydrophobicity analyses of these sequences revealed the strikingly hydrophobic natures of various types of integral membrane transporters (Kyte and Doolittle, 1982; Büchel *et al.,* 1980; Lee and Saier, 1983; Overath and Wright, 1983). Current multidisciplinary approaches are slowly yielding three-dimensional structural information about transport systems. However, since only a few such systems have yielded to X-ray crystallographic analyses (Deisenhofer and Michel, 1991; Unwin, 1995; Tsukihara *et al.,* 1996), we still base our views of solute transport on molecular models that provide reasonable pictures of transport systems and the processes they catalyze without providing absolute assurance of accuracy (Goswitz and Brooker, 1995; Varela and Wilson, 1996; Kaback *et al.,* 1997).

It is well recognized that any two proteins that can be shown to be homologous (i.e., that exhibit sufficient primary and/or secondary structural similarity to establish that they arose from a common evolutionary ancestor) will prove to exhibit strikingly similar three-dimensional structures (Doolittle, 1986). Furthermore, the degree of tertiary structural similarity correlates well with the degree of primary structural similarity. For this reason, phylogenetic analyses allow application of modeling techniques to a large number of related proteins and additionally allow reliable extrapolation from one protein member of a family of known structure to others of unknown structure. Thus, once three-dimensional structural data are available for any one family member, these data can be applied to all other members

within limits dictated by their degrees of sequence similarity. The same cannot be assumed for members of two independently evolving families.

Similar arguments apply to mechanistic considerations. Thus, the mechanism of solute transport is likely to be similar for all members of a permease family, and variations upon a specific mechanistic theme will be greatest when the sequence divergence is greatest. By contrast, for members of any two independently evolving permease families, the transport mechanisms may be strikingly different. Knowledge of these considerations allows unified mechanistic deductive approaches to be correctly applied to the largest numbers of transport systems, even when evidence is obtained piecemeal from the study of different systems.

The capacity to deduce and extrapolate structural and mechanistic information illustrates the value of phylogenetic data. However, another benefit that may result from the study of molecular phylogeny is to allow an understanding of the mechanistic restrictions that were imposed on an evolving family due to architectural constraints. Specific architectural features may allow one family to diversify in function with respect to substrate specificity, substrate affinity, velocity of transport, polarity of transport, and even mechanism of energy coupling. By contrast, architectural constraints imposed on a second family may not allow functional diversification. Knowledge of the architectural constraints imposed on a permease family provides a clear clue as to the reliability of functional predictions for uncharacterized but related gene products revealed, for example, by genome sequencing. Conversely, the functional diversity of the members of a permease family must be assumed to reflect architectural constraints, and thus phylogenetic/functional analyses lead to architectural predictions.

Finally, phylogenetic analyses provide valuable information about the evolutionary process itself. One can sometimes glean clues regarding the time of appearance of a family, the biological source in which the family arose, and the pathway taken for the emergence of the family during evolutionary history. One can also ascertain whether or not two distinct families arose independently of each other.

During the past decade, our laboratory has devoted considerable effort to the phylogenetic characterization of permease families (Saier, 1994, 1996). This work has led us to formulate a novel classification system, superficially similar to that implemented years ago for enzymes by the Enzyme Commission (EC). We call ourselves the Transport Commission (TC). In contrast to the EC, which based its classification system solely on function, we have chosen to classify permeases on the basis of both function and phylogeny. In this article, I describe our proposal, point out some of its strengths, and emphasize its flexibility for future assignments. I hope that the TC classification system will prove to be as useful as is the EC

system. Other treatises concerning the TC system have appeared (Saier, 1998, 1999).

A detailed description of the TC system can be found on our Web site (http://www-biology.ucsd.edu/~msaier/transport/titlepage.html). This site will be continuously updated as new relevant physiological, biochemical, genetic, biophysical, and sequence data become available. The scientific community is strongly encouraged to communicate novel findings and corrections to the author by e-mail, phone, fax, or mail.

II. Transport Nomenclature

Almost all transmembrane transport processes are mediated by integral membrane proteins, sometimes functioning in conjunction with extracytoplasmic receptors or receptor domains as well as with cytoplasmic energy-coupling and regulatory proteins or protein domains. Each such complex of these proteins and/or protein domains is referred to a transport system, transporter, porter, permease system, or permease. These are all equivalent terms that are in general use by members of the transport community. A permease (porter) is a protein or protein complex that catalyzes a vectorial reaction, irrespective of whether or not it also catalyzes a chemical or electron transfer reaction that drives the vectorial process. Thus, all transport systems are catalytic proteins or protein complexes analogous to enzymes or enzyme complexes. By definition, transporters facilitate vectorial rather than, or in addition to, chemical reactions.

Permease-mediated transport can occur by any one of three distinct but related processes. First and most simple is facilitated, equilibrative, or protein-mediated diffusion, a process that is not coupled to metabolic energy and therefore cannot give rise to concentration gradients of the transported substrate across the membrane. Two primary modes of facilitated transport have been recognized in biological systems: channel type and carrier type. In channel-type facilitated diffusion, the solute passes in a diffusion-limiting process from one side of the membrane to the other via a channel or pore which is lined by appropriately hydrophilic (for hydrophilic substrates), hydrophobic (for hydrophobic substrates) or amphipathic (for amphipathic substrates) amino acyl residue moieties of the constituent protein(s). The structures of several such channel proteins have been examined and elucidated using X-ray crystallographic techniques. In carrier-type facilitated diffusion, some part of the transporter is classically presumed to pass through the membrane together with the substrate. Whether or not this presumption is correct is not known because no classical carrier has

yielded to the analytical tools of the X-ray crystallographer. Carriers usually exhibit rates of transport that are several orders of magnitude lower than those of channels. Moreover, in contrast to most channels, they exhibit stereospecific substrate specificities. Although both channels and carriers may exhibit the phenomenon of saturation kinetics, this is a more common characteristic of carriers. Very few carriers have been shown to be capable of functioning by a channel-type mechanism, and the few that exhibit this capacity generally do so only after the protein has been modified, either by covalent or noncovalent ligand binding or by imposition of a large membrane potential. This observation leads to the suggestion that channels and carriers are fundamentally, not superficially, different.

If energy expenditure is coupled to transmembrane solute translocation, then a system catalyzing facilitated diffusion can become an active transporter. Such a system is considered to be a primary active transporter if a primary source of energy (i.e., a chemical reaction, light absorption, or electron flow) is coupled to the process. It is considered to be a secondary active transporter if a secondary source of energy [i.e., an ion electrochemical gradient, termed the proton motive force (pmf) in the case of protons or the sodium motive force (smf) in the case of sodium ions], generated at the expense of a primary energy source, is coupled to the process.

Active transporters can function by uniport, symport, or antiport. Uniporters, also called single species transporters or facilitated diffusion carriers, catalyze the transport of a single molecular species, and transport occurs independently of the movement of other molecular species. Symporters, also called cotransporters, catalyze the transport of two or more molecular species in the same direction. The fact that a single point mutation in a symporter can convert a carrier into a uniporter (Franco and Booker, 1994; King and Wilson, 1990; Krupka, 1994; Lolkema and Poolman, 1995; Varela and Wilson, 1996) emphasizes the superficial distinction between these two types of carriers. Antiporters, also called countertransporters, exchange transporters, or exchangers, catalyze the exchange of one (or more) molecular species for another. Antiport processes can be subdivided into two categories: antiport of like molecules (i.e., solute:solute antiport) and antiport of unlike molecules (i.e., solute:cation antiport). Many uniporters and symporters also catalyze solute:solute antiport, sometimes at rates that are substantially greater than those of uniport or symport. Some carriers catalyze solute:solute antiport at rates that exceed those of uniport or symport by three to five orders of magnitude, and uniport via these carriers is of little or no physiological consequence (Reithmeier, 1993). Such systems are said to be obligatory antiporters or exchangers.

Accelerative solute:solute countertransport has long been considered to be a diagnostic characteristic of carriers. Early transport kineticists concluded that its demonstration eliminated the possibility that a transporter functions by a channel-type mechanism and suggested that clear boundaries exist between carriers and channels (Mawe and Hempling, 1965; Stein, 1967). Subsequent observations that certain "carriers" could apparently be converted into "channels" by chemical treatment (Dierks *et al.,* 1990a,b; Brutovetsky and Klingenberg, 1994, 1996; Jezek *et al.,* 1994), by imposition of large membrane potentials (Schwarz *et al.,* 1994; Wallmeier *et al.,* 1992), or by ligand binding (Booth *et al.,* 1996) led many students of transport to consider these boundaries indistinct. Our phylogenetic analyses suggest that these examples are special cases and tend to reemphasize the importance of the channel versus carrier distinction.

A few carriers modify their substrates during transport. The best characterized such system is the bacterial phosphotransferase system (PTS) which phosphorylates its sugar substrates using phosphoenolpyruvate as the phosphoryl donor. Sugars taken up from the external milieu via the PTS are thus released into the cytoplasm as sugar phosphates. Any process in which the substrate is modified during transport is termed "group translocation." Although originally proposed in different form by Peter Mitchell as a general mechanism, its occurrence appears to be highly restricted in nature.

III. The Transport Commission System of Transporter Classification

A. Transporter Types: Primary Categorization

According to the proposed classification system, transporters are grouped on the basis of four criteria, and each of these criteria corresponds to one of the four numbers within the TC number for a particular permease. Thus, a permease-specific TC number has four components as follows: W.X.Y.Z., where W corresponds to the transporter type and energy source (if any) used to drive transport, X specifies the permease family (or superfamily), Y represents the phylogenetic cluster or subfamily in a family (or the family in a superfamily) in which a particular permease is found, and Z delineates the substrate(s) transported as well as the polarity of transport (in or out). Any two transport proteins in the same subfamily of a permease family that transport the same substrate(s) using the same mechanism are given the same TC number, regardless of whether they are orthologs (i.e., arose

in distinct organisms by speciation) or paralogs (i.e., arose within a single organism by gene duplication). Mode of regulation proves not to correlate with phylogeny and was probably superimposed on permeases late in the evolutionary process. Regulation is therefore not used as a basis for classification. Sequenced homologs of unknown function are not normally assigned a TC number, and functionally characterized permeases for which sequence data are not available are also not included. These deficiencies will be eliminated with time as more sequenced permeases become characterized biochemically and as sequences become available for the functionally but not molecularly characterized permeases. It should be noted that each primary category of porters has a one-digit TC number (W), each family (or superfamily) has a two-digit TC number (W.X), each phylogenetic cluster or subfamily has a three-digit TC number (W.X.Y), and each permease type has a four-digit TC number (W.X.Y.Z).

As noted previously, the primary level of classification in the TC system is based on mode of transport and energy-coupling source. Thirteen primary categories of permease proteins or types are currently recognized as follows:

1. Channel-type transporters: Proteins in this category have transmembrane channels which usually consist largely of α-helical spanners. Transport systems of this type catalyze facilitated diffusion (by an energy-independent process), allowing passage through a transmembrane aqueous pore or channel without evidence for a carrier-mediated mechanism. Outer membrane porin-type channel proteins are excluded from this category and have been placed in their own category (category 9). Because more than 12 distinct channel-forming peptides and proteins have been elucidated in three dimensions, the structural basis of channel formation is well understood.

2. Carrier-type transporters: Transport systems are included in this category if they utilize a carrier-mediated process to catalyze uniport (a single species is transported by facilitated diffusion in a process not coupled to the utilization of a primary source of energy), antiport (two or more species are transported in opposite directions in a tightly coupled process not directly linked to a form of energy other than chemiosmotic energy), and/or symport (two or more species are transported together in the same direction in a tightly coupled process not directly linked to a form of energy other than chemiosmotic energy). Three-dimensional structural data are not available for these porters or for the permeases included in categories 3–5, 8, 10, 98, or 99.

3. Pyrophosphate bond (usually in ATP) hydrolysis-driven active transporters: Transport systems are included in this category if they hydrolyze the terminal pyrophosphate bond in ATP, in another nucleoside triphos-

phate, or in pyrophosphate itself to drive the active uptake and/or extrusion of a solute or solutes. The transport protein may or may not be transiently phosphorylated, but the substrate is not phosphorylated.

4. PEP-dependent, phosphoryl transfer-driven group translocators: Transport systems of the bacterial phosphoenolpyruvate:sugar phosphotransferase system are included in this category. The product of the reaction, derived from extracellular sugar, is a cytoplasmic sugar phosphate.

5. Carboxylic acid-dependent, decarboxylation-driven active transporters: Bacterial transport systems that drive solute (e.g., sodium ion) uptake or extrusion by decarboxylation of a cytoplasmic substrate are included in this category.

6. Oxido-reduction-driven active transporters: Transport systems that drive transport of a solute (e.g., an ion) energized by the flow of electrons from a reduced substrate or protein to an oxidized substrate or protein are included in this category. Three-dimensional structural data, available for three of these enzyme complexes (see Section VI), resulted in an understanding of these proton and sodium ion pumps on a firm experimental basis. Hydride shift-driven proton pumps (e.g., H^+-translocating transhydrogenases) represent a unique example of an oxido-reduction-driven ion pump.

7. Light-driven active transporters: Transport systems that utilize light energy to drive transport of a solute (e.g., an ion) are included within this category. Two such systems (see Section VI) have been extensively studied from three-dimensional structural standpoints.

8. Mechanically driven active transporters: Transport systems are included within this category if they directly drive the movement of a cell, organelle, or other physical structure by allowing the flow of ions (or other solutes) through the membrane down their electrochemical gradients. The *E. coli* flagellar motor protein complex is considered an example of such a system, but F-type ATPases that couple ion movement to pyrophosphate bond formation or hydrolysis employing a mechanical device are not. These last systems utilize a mechanical process to couple transport to a chemical reaction (Junge *et al.*, 1997; Noji *et al.*, 1997). Although ion flux is used to drive flagellar rotation, rather than the other way around, it should be noted that the process can be considered to be reversible, and thus the distinction of which process drives which is semantic.

9. Outer membrane channel-type facilitators (porins): The proteins of this category exhibit transmembrane β strands that form β-barrels through which solutes pass. They are found in the outer membranes of gram-negative bacteria, mitochondria, and eukaryotic plastids. Porins represent a specific structural type of channel protein, and because of the large num-

bers of porin-type families, they are assigned a TC classification group distinct from other channel proteins.

10. Methyltransferase-driven active transporters. Transport systems are included in this category if they utilize methyl transfer to drive transport. A single well-characterized protein, the Na^+-transporting methyltetrahydromethanopterin:coenzyme M methyltransferase, is currently listed in this category.

11. Auxiliary transport proteins (98): Proteins that function with or are complexed to known transport proteins are included in this category. An example would be the membrane fusion proteins that facilitate transport across the two membranes of the gram-negative bacterial cell envelope in a single step driven by the energy source (ATP or the pmf) utilized by a cytoplasmic membrane transporter. Energy coupling and regulatory proteins that do not actually participate in transport represent other possible examples. In some cases auxiliary proteins are considered to be part of the transport system with which they function, and in such cases no distinct entry in category 98 is provided.

12. Transporters of unknown classification (99): Transport protein families of unknown classification are grouped under this number. Permeases within families maintained in the 99 class are of unknown mode of transport or energy-coupling mechanism, but at least one member of each of these families has clearly been shown to function as a transporter. These families will be classified elsewhere when the transport process and energy-coupling mechanism are characterized.

13. Putative transporters in which no family member is an established transporter (100): Putative transport protein families are grouped under this number and will either be classified elsewhere when the transport function of a member becomes established or will be eliminated from the TC classification system if the proposed transport function is disproven. These families include a member or members for which a transport function has been suggested, but evidence for such a function is not yet compelling.

The current index of transport system families is presented in Table I. There are more than 150 entries, each of which usually describes a single family or superfamily. Some of these families are large superfamilies with hundreds of currently sequenced members [e.g., the major facilitator superfamily (MFS); TC No. 2.1 (Pao *et al.*, 1998) and the ATP-binding cassette (ABC) superfamily; TC No. 3.1 (Saurin *et al.*, 1999)]. Others are small families with only one or two currently sequenced members. Most families, however, are of intermediate sizes, with between 5 and 100 sequenced members. In a few instances [e.g., the channel-forming amphipathic peptide (CAP) functional superfamily (TC No. 1.8) and the holin (Holin) functional superfamily (TC No. 1.11)], the entry includes

TABLE I

Families of Transport Proteins[†]

1. Channel-type transporters

1.1. The major intrinsic protein (MIP) family
1.2. The epithelial Na^+ channel (ENaC) family
1.3. The large conductance mechanosensitive ion channel (MscL) family
1.4. ATP-gated cation channel (ACC) family
1.5. The voltage-sensitive ion channel (VIC) family
1.6. The ligand-gated ion channel (LIC) family of neurotransmitter receptors
1.7. The glutamate-gated ion channel (GIC) family of neurotransmitter receptors
1.8. The channel-forming amphipathic peptide (CAP) functional superfamily
1.9. The ryanodine–inositol 1,4,5-triphosphate receptor Ca^{2+} channel (RIR-CaC) family
1.10. The chloride channel (ClC) family
1.11. The holin (holin) functional superfamily
1.12. The channel-forming colicin (colicin) family
1.13. The channel-forming δ-endotoxin insecticidal crystal protein (ICP) family
1.14. The α-hemolysin channel-forming toxin (αHL) family
1.15. The aerolysin channel-forming toxin (aerolysin) family
1.16. Animal inward rectifier K^+ channel (IRK-C) family
1.17. The organellar chloride channel (O-ClC) family
1.18. The channel-forming colicin V (colicin V) family
1.19. The channel-forming ε-toxin (ε-toxin) family
1.20. The transient receptor potential Ca^{2+} channel (TRP-CC) family
1.21. The yeast killer toxin K1 (YKT-K1) family

2. Carrier-type transporters (Uni-, Sym-, and Antiporters)

2.1. The major facilitator superfamily (MFS)
2.1.1. Sugar porter (SP) family
2.1.2. The drug:H^+ antiporter (14 spanner) (DHA 14) drug efflux family
2.1.3. The drug:H^+ antiporter (12 spanner) (DHA 12) drug efflux family
2.1.4. The organophosphate:P_i antiporter (OPA) family
2.1.5. The oligosaccharide:H^+ symporter (OHS) family
2.1.6. The metabolite:H^+ symporter (MHS) family
2.1.7. The fucose:H^+ symporter (FHS) family
2.1.8. The nitrate/nitrite porter (NNP) family
2.1.9. The phosphate:H^+ symporter (PHS) family
2.1.10. The nucleoside:H^+ symporter (NHS) family
2.1.11. The oxalate:formate antiporter (OFA) family
2.1.12. The sialate:H^+ symporter (SHS) family
2.1.13. The monocarboxylate porter (MCP) family
2.1.14. The anion:cation symporter (ACS) family
2.1.15. The unknown major facilitator (UMF) family
2.1.16. The aromatic acid:H^+ symporter (AAHS) family
2.1.17. The cyanate permease (CP) family
2.1.18. The polyol permease (PP) family
2.2. The glycoside–pentose–hexuronide (GPH):cation symporter family
2.3. The amino acid–polyamine–choline (APC) family

(*continued*)

TABLE I (*continued*)

2.4. The cation diffusion facilitator (CDF) family
2.5. The zinc (Zn^{2+})–iron (Fe^{2+}) permease (ZIP) family
2.6. The resistance–nodulation–cell division (RND) family
2.7. The small multidrug resistance (SMR) family
2.8. The gluconate:H^+ symporter (GntP) family
2.9. The L-rhamnose transporter (RhaT) family
2.10. The 2-keto-3-deoxygluconate transporter (KdgT) family
2.11. The citrate–Mg^{2+}:H^+ (CitM)–citrate:H^+ (CitH) symporter (CitMHS) family
2.12. The ATP:ADP antiporter (AAA) family
2.13. The C4-dicarboxylate uptake (Dcu) family
2.14. The lactate permease (LctP) family
2.15. The betaine/carnitine/choline transporter (BCCT) family
2.16. The telurite-resistance/dicarboxylate transporter (TDT) family
2.17. The proton-dependent oligopeptide transporter (POT) family
2.18. The amino acid/auxin permease (AAAP) family
2.19. The Ca^{2+}:cation antiporter (CaCA) family
2.20. The inorganic phosphate transporter (PiT) family
2.21. The solute:sodium symporter (SSS) family
2.22. The neurotransmitter:sodium symporter (NSS) family
2.23. The dicarboxylate:cation (Na^+ or H^+) symporter (DCS) family
2.24. The citrate:Na^+ symporter (CSS) family
2.25. The alanine:Na^+ symporter (ASS) family
2.26. The branched chain amino acid:Na^+ symporter (LIVSS) family
2.27. The glutamate:Na^+ symporter (ESS) family
2.28. The bile acid:Na^+ symporter (BASS) family
2.29. The mitochondrial carrier (MC) family
2.30. The cation–chloride cotransporter (CCC) family
2.31. The anion exchanger (AE) family
2.32. The silicon transporter (Sit) family
2.33. The NhaA Na:H^+ antiporter (NhaA) family
2.34. The NhaB Na^+:H^+ antiporter (NhaB) family
2.35. The NhaC Na^+:H^+ antiporter (NhaC) family
2.36. The monovalent cation:proton antiporter-1 (CPA1) family
2.37. The monovalent cation:proton antiporter-2 (CPA2) family
2.38. The K^+ transporter (Trk) family
2.39. The nucleobase:cation symporter-1 (NCS1) family
2.40. The nucleobase:cation symporter-2 (NCS2) family
2.41. The nucleoside uptake permease (NUP) family
2.42. The aromatic amino acid permease (ArAAP) family
2.43. The serine/threonine permease (STP) family
2.44. The formate–nitrate transporter (FNT) family
2.45. The metal ion transporter (MIT) family
2.46. The benzoate:H^+ symporter (BenE) family
2.47. The divalent anion:Na^+ symporter (DASS) family
2.48. The reduced folate carrier (RFC) family

TABLE I (*continued*)

2.49. The ammonium transporter (Amt) family
2.50. The triose phosphate translocator (TPT) family
2.51. The nucleotide–sugar transporter (NST) family
2.52. The Ni^{2+}–Co^{2+} transporter (NiCoT) family
2.53. The sulfate permease (SulP) family
2.54. The mitochondrial tricarboxylate carrier (MTC) family
2.55. The acetyl-coenzyme A transporter (AcCoAT) family
2.56. The tripartite ATP-independent periplasmic transporter (TRAP-T) family
2.57. The equilibrative nucleoside transporter (ENT) family
2.58. The phosphate:Na^+ symporter (PNaS) family
2.59. The arsenical resistance-3 (ACR3) family
2.60. The organo anion transporter (OAT) family
2.61. The C_4–dicarboxylate uptake C (DcuC) family

3. Pyrophosphate bond (ATP, GTP, and P_2) hydrolysis-driven active transporters
3.1. The ATP-binding cassette (ABC) superfamily
3.2. The H^+- or Na^+-translocating F-type, V-type, and A-type ATPase (F-ATPase) superfamily
3.3. The P-type ATPase (P-ATPase) superfamily
3.4. The arsenical (Ars) efflux family
3.5. The type II (general) secretory pathway (IISP) family
3.6. The type III (virulence-related) secretory pathway (IIISP) family
3.7. The type IV (conjugal DNA–protein transfer or VirB) secretory pathway (IVSP) family
3.8. The mitochondrial protein translocase (MPT) family
3.9. The chloroplast envelope protein translocase (CEPT) family
3.10. The H^+-translocating vacuolar pyrophosphatase (V-PPase) family
3.11. The bacterial competence-related DNA transformation transporter (DNA-T) family

4. Phosphotransferase systems
4.1. The PTS glucose–glucoside (Glc) family
4.2. The PTS fructose–mannitol (Fru) family
4.3. The PTS lactose–cellobiose (Lac) family
4.4. The PTS glucitol (Gut) family
4.5. The PTS galactitol (Gat) family
4.6. The PTS mannose–fructose–sorbose (Man) family

5. Decarboxylation-driven active transporters
5.1. The Na^+-transporting carboxylic acid decarboxylase (NaT-DC) family

6. Oxidoreduction-driven active transporters
6.1. The proton-translocating NADH dehydrogenase (NDH) family
6.2. The proton-translocating transhydrogenase (PTH) family
6.3. The proton-translocating quinol:cytochrome c reductase (QCR) superfamily
6.4. The proton-translocating cytochrome oxidase (COX) superfamily
6.5. The Na^+-translocating NADH:quinone dehydrogenase (Na-NDH) family

7. Light-driven active transporters
7.1. The ion-translocating bacteriorhodopsin (BR) family

(*continued*)

TABLE I (*continued*)

7.2. The proton-translocating reaction center (RC) family

8. Mechanically-driven active transporters

8.1. The H^+- or Na^+-translocating bacterial flagellar motor (Mot) family

9. Outer membrane porins (β structure)

9.1. The general bacterial porin (GBP) family
9.2. The chlamydial porin (CP) family
9.3. The sugar porin (SP) family
9.4. The *Brucella–Rhizobium* porin (BRP) family
9.5. The *Pseudomonas* OprP porin (POP) family
9.6. The OmpA–OprF porin (OOP) family
9.7. The *Rhodobacter* PorCa porin (RPP) family
9.8. The mitochondrial and plastid porin (MPP) family
9.9. The FadL outer membrane protein (FadL) family
9.10. The nucleoside-specific channel-forming outer membrane porin (Tsx) family
9.11. The outer membrane fimbrial usher porin (FUP) family
9.12. The autotransporter (AT) family
9.13. The alginate export porin (AEP) family
9.14. The outer membrane receptor (OMR) family
9.15. The raffinose porin (RafY) family
9.16. The short-chain amide and urea porin (SAP) family
9.17. The outer membrane factor (OMF) family
9.18. The outer membrane auxiliary (OMA) protein family
9.19. The glucose-selective OprB porin (OprB) family

10. Methyltransfer-driven active transporters

10.1. The Na^+-transporting methyltetrahydromethanopterin:coenzyme M methyltransferase (NaT-MMM) family

98. Auxiliary transport proteins

98.1. The membrane fusion protein (MFP) family
98.3. The cytoplasmic membrane–periplasmic auxiliary-1 (MPA1) protein with cytoplasmic (C) domain (MPA1-C or MPA1 + C) family
98.4. The cytoplasmic membrane–periplasmic auxiliary-2 (MPA2) family
98.6. The TonB-ExbB-ExbD/TolA-TolQ-TolR (TonB) family of auxiliary proteins for energization of outer membrane receptor (OMR)-mediated active transport
98.7. The phosphotransferase system enzyme I (EI) family
98.8. The phosphotransferase system HPr (HPr) family
98.9. The rBAT family of putative transport accessory proteins
98.10. The slow voltage-gated K^+ channel accessory protein (MinK) family

99. Transporters of unknown classification

99.1. The polysaccharide transporter (PST) family
99.2. The MerTP mercuric ion (Hg^{2+}) permease (MerTP) family
99.3. The MerC mercuric ion (Hg^{2+}) uptake (MerC) family
99.4. The nicotinamide mononucleotide (NMN) uptake permease (PnuC) family
99.5. The K^+ uptake permease (KUP) family
99.6. The L-lysine exporter (LysE) family
99.7. The chromate ion transporter (Chr) family
99.8. The ferrous iron uptake (FeoB) family

TABLE I (*continued*)

99.9. The low-affinity Fe^{2+} transporter (FeT) family
99.10. The oxidase-dependent Fe^{2+} transporter (OFeT) family
99.11. The copper transporter-1 (Ctr1) family
99.12. The copper transporter-2 (Ctr2) family
99.13. The metal ion (Mn^{2+}–iron) transporter (Nramp) family
99.14. The cadmium resistance (CadD) family
99.15. The putative amide transporter (Ami) family
99.18. The peptide uptake permease (PUP) family
99.19. The Mg^{2+} transporter (Mgt) family
99.20. The low-affinity cation transporter (LCT) family
99.21. The membrane targeting and translocation (Mtt) family

100. Putative transporters of unknown classification

100.1. The metal homeostasis protein (MHP) family
100.2. The Ca^{2+} homeostasis protein (CHP) family
100.3. The putative bacterial murein precursor exporter (MPE) family
100.4. The YahN family of putative transporters
100.5. The KX blood-group antigen (KXA) family
100.6. The toxic Hok/Gef protein (Hok/Gef) family
100.7. The putative bacteriochlorophyll delivery (BCD) family
100.8. The canalicular bile acid transporter (C-BAT) family
100.9. The urate transporter (UAT) family

[1] See our web site (address on page 65) for current families, their descriptions, and primary references describing them. This web site will be continuously updated in the future.

a number of functionally related families of peptides or proteins. In these cases, insufficient degrees of sequence similarity are observed between members of the different families included within the functional superfamily to establish homology. Table I therefore includes about 200 families of transport systems.

All of the families currently included in Table I will undoubtedly expand with time, and new families will be identified. The availability of new protein sequences will occasionally allow two or more currently recognized families to be placed together under a single TC number. In a few cases, two families are already known for which some evidence is available suggesting that these families are related, e.g., the monovatent cation:proton antiporter-1 (CPA1) and CPA2 families (TC Nos. 2.36 and 2.37) and the nucleobase:cation symporter-1 (NCS1) and NCS2 families (TC Nos. 2.39 and 2.40). This evidence is usually based on (i) limited sequence similarities; (ii) common function; and/or (iii) similar protein size, topology, and structure. When "missing link" sequences or three-dimensional structural data become available so that proteins of two families can be unequivocally grouped together within a single family, the lower TC number will be adopted for all of the family members, and the higher TC number will be abandoned.

The complete index (Table I) and representative tables describing some of the families to be discussed in this article will be presented later. The complete classification system is available on our Web site (http://www-biology.ucsd.edu/~msaier/transport/titlepage.html). It will be updated continuously as new information becomes available. Anyone noting errors or incomplete listings is encouraged to contact the author providing the missing information and references by e-mail, fax, phone, or mail.

In almost all cases, members of a transporter family utilize a single energy-coupling mechanism, thus justifying the use of transport mode and energy-coupling mechanisms as the primary basis for classification. However, a few exceptions have been noted. First, the arsenite (Ars; TC No. 3.4) efflux permease of *E. coli* consists of two proteins, ArsA and ArsB. ArsB is an integral membrane protein which presumably provides the transport pathway for the extrusion of arsenite and antimonite (Silver *et al.,* 1993). ArsA is an ATPase that energizes ArsB-mediated transport. However, when ArsB alone is present, as in the case of the arsenical resistance pump of *Staphylococcus aureus,* transport is driven by the pmf (Bröer *et al.,* 1993). The presence or absence of the ArsA protein thus determines the mode of energy coupling.

Such promiscuous use of energy is exceptionally rare and has been documented in only a very few instances. When such an effect is reported, we shall classify the permease in accordance with the more complicated energy-coupling mechanism [in this case, as an ATP-driven primary active transporter (Class 3) rather than as a secondary carrier (Class 2)]. The potentially different energy-coupling mechanisms will be described in the table characterizing that family (see our Web site).

Examples of secondary carrier families in which promiscuous transport modes have been reported include the mitochondrial carrier (MC) family (TC No. 2.29) and the triose phosphate translocator (TPT) family (TC No. 2.50). Proteins of both families are apparently restricted to eukaryotic organelles. Members of both families normally catalyze carrier-mediated substrate:substrate antiport and are therefore classified as secondary carriers. However, treatment of MC family members with chemical reagents, such as *N*-ethyl maleimide or Ca^{2+} (Dierks *et al.,* 1990a,b; Brutovetsky and Klingenberg, 1994, 1996; Jezek *et al.,* 1994), or imposition of a large membrane potential ($\Delta\Psi$) across a membrane into which a TPT member has been incorporated (Schulz *et al.,* 1993; Wallmeier *et al.,* 1992; Schwarz *et al.,* 1994) has been reported to convert these antiport-catalyzing carriers into anion-selective channels capable of functioning by uniport. Another secondary carrier that may be capable of exhibiting channel-like properties is the KefC protein of *E. coli* (Booth *et al.,* 1996), which is a member of the CPA2 family (TC No. 2.37). "Tunneling" of ions and other solutes through carriers with little or no conformational change has been discussed

(Frölich, 1988). Again, the more complicated carrier-type mechanism, which appears to be relevant under most physiological conditions, provides the basis for classifying these proteins (i.e., as Class 2 carriers rather than Class 1 channels).

B. Transport System Families: General Features

1. Characteristics of Transporter Families Found in Living Organisms

Table II summarizes some key characteristics of the transporter families we have identified. The table provides the family TC number, the abbreviations of the families, and the substrates of transporters included within each family. Substrates that are common to one transporter are separated by commas, whereas substrates of different transporters within the family are separated by semicolons. Thus, in the major intrinsic protein (MIP) family (TC No. 1.1), aquaporins generally transport water but not organic compounds, whereas glycerol facilitators generally transport short, straight chain polyols but not water. A few members of the family may transport both (Park and Saier, 1996).

Table II also includes the size ranges of the individual protein members of the families and the numbers of (putative) transmembrane α-helical segments (TMSs) included within the permease polypeptide chains. These characteristics are usually constant for a particular family except in the cases of the largest superfamilies such as the MFS (TC No. 2.1) and the ABC superfamily (TC No. 3.1). When a homo- or heterooligomeric structure has been established for an intact permease, this fact is also indicated. Finally, the organismal kingdoms (bacterial, archaeal, and/or eukaryotic) in which members of the family have been identified, the approximate number of members that have been identified in each family, and representative examples of well-characterized members are also provided. The table is largely self-explanatory.

2. Distribution of Transporter Families in the Three Domains of Life

It is estimated that the three major domains of life—bacteria, archaea, and eukarya—diverged from each other about 3.5 billion years ago (Doolittle, 1997; Feng *et al.*, 1997). Prior to that time, only organisms resembling bacteria probably existed on earth (Doolittle, 1997). Families of proteins that appeared before this domain divergence event could easily have been transmitted vertically to the members of all three domains, but protein families that appeared after divergence would, in the absence of horizontal

TABLE II

Characterization of Families of Transport Systems

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
Channel-forming proteins and peptides							
1.1	MIP	H_2O; glycerol, urea, polyols	220–310	$(6)_{2 \text{ or } 4}$	B, A, E[b]	>100	Aquaporins (Aqp1 Hsa); glycerol facilitators (GlpF Eco)
1.2	ENaC	Na^+; cations	640–700	$(2)_3$	E (animals)	>20	Epithelial Na^+ channels; degenerins; peptide-gated ionotropic receptors
1.3	MscL	Ions (slightly cation selective)	130–140	$(2)_6$	B	>10	Large mechanosensitive ion channels (MscL Eco)
1.4	ACC	Cations (monovalent cations; Ca^{2+}	380–600	$(2)_n$	E (animals)	>10	ATP-gated cation channels ($P2X_1$ Hsa)
1.5	VIC	Na^+; K^+; Ca^{2+}	Widely varied	$(6)_4$; $(24)_1$; $(2)_n$; often heterooligomeric	E, A, B	>200	Voltage-sensitive Na^+ channels; Voltage-sensitive Ca^{2+} channels; K^+ channels sensitive to voltage, Ca^{2+} or cyclic nucleotides
1.6	LIC	Cations or chloride	400–500	$(3–5)_5$; often heterooligomeric	E (animals)	>100	Acetyl choline or serotonin-activated cation channels; glycine, glutamate, or GABA-regulated Cl^- channels
1.7	GIC	Monovalent cations and Ca^{2+}	800–1000	$(3–5)_5$	E (animals)	>10	Glutamate-regulated ionotropic channels

1.8	CAP functional superfamily (>12 families)	Ions	25–75	$(1 \text{ or } 2)_n$	B, A, E	>100	Maganins; cecropins; melittin, defensins, bacteriocins
1.9	RIR-CaC	Ca^{2+}	5000 or 2500	$(6)_n$	E (animals)	>10	Ryanodine receptor Ca^{2+} channels; inositol 1,4,5-triphosphate receptor Ca^{2+} channels
1.10	ClC	Cl^-	400–1000	(10–12)	B, A, E	>30	Voltage-gated Cl^- channels (ClC1 Hsa)
1.11	Holin functional superfamily (>15 families)	Proteins; small molecules	70–150	$(2\text{–}4)_n$	B, phage, plasmids	>50	Lysis protein S
1.12	Colicin	Ions; small molecules	500–700 (150–180 for the channel domain)	$(4?)_n$	B, plasmids	>10	Colicin E1
1.13	IPC	Ions; small molecules	500–1300 (~220 for the channel domain)	$(6?)_n$	B	>50	Cry3A insecticidal δ-endotoxin (*Bacillus thuringiensis*)
1.14	αHL	Ions; small molecules	300–400	$(2\beta)_7$	B	>10	α-Hemolysin (*Staphylococcus aureus*)
1.15	Aerolysin	Ions; small molecules	440–490	$(2\beta)_7$	B, E (plants)	>7	Aerolysin (*Aeromonas hydrophila*)
1.16	IRK-C	K^+	390–430	$(2)_n$	E (animals)	>10	Inward rectifier K^+ channels (ATP-activated or G protein regulated)

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
1.17	O-ClC	Cl^-	240–440	$(2)_n$	E (animals)	>10	Organellar voltage-sensitive Cl^- channels
1.18	Colicin V	Ions; small molecules	100	$(1–2)_n$	B (plasmid)	>1	Colicin V of *E. coli*
1.19	ε-Toxin	Ions; small molecules	330	?	B	~10	ε-Toxin of *Clostridium perfringens*
1.20	TRP-CC	Ca^{2+}	800; 1300	$(6)_n$	E (animals)	>10	Transient receptor potential Ca^{2+} channel
1.21	YKT-K1	Cation selective	300	?	E (yeast)	>1	Yeast killer toxin of *Saccharomyces cerevisiae*
Carrier-type facilitators							
2.1	MFS	Various small molecules	400–600	12 or 14	B, A, E	>300	Lactose permease (LacY) of *E. coli;* drug efflux permease (EmrD) of *E. coli*
2.2	GPH	Sugars	~500	12	B, E (plants)	>20	Melibiose permease (MelB) of *E. coli*
2.3	APC	Amino acids, polyamines, choline	440–630	12	B, A, E	>100	Lysine permease (LysP) of *E. coli*
2.4	CDF	Cd^{2+}, Co^{2+}, Ni^{2+}	300–750	6	B, A, E	>10	Heavy metal uptake and efflux permeases of bacteria, eukaryotic plasma membranes, and mitochondria (CzcD of *Ralstonia eutropha*)
2.5	ZIP	Zn^{2+}, Fe^{2+}	220–430	8	E	>10	Zinc uptake transporter (Zrt1) of *S. cerevisiae*

2.6	RND	Heavy metal ions; multiple drugs; oligosaccharides	950–1100	12	B	>20	Drug efflux pump (AcrB) of *E. coli*
2.7	SMR	Multiple drugs	110	$(4)_3$	B	>10	Drug efflux pump (Smr) of *S. aureus*
2.8	GntP	Gluconate	450	12–14	B	>10	GntP of *Bacillus subtilis*
2.9	RhaT	Sugars	280–340	10	B	>5	RhaT of *E. coli*
2.10	KdgT	2-keto-3-deoxy gluconate	400	10–12	B	>5	KdgT of *Erwinia chrysanthemi*
2.11	CitMHS	Citrate	400	12	B	>5	CitM of *B. subtilis*
2.12	AAA	ATP, ADP	500	12	B, E (plants)	>10	ATP/ADP exchange translocase of *Rickettsia prowazekii*
2.13	Dcu	C_4-dicarboxylates	440	12	B (G−)	>5	DcuA of *E. coli*
2.14	LctP	Lactate	510–516	12	B, A	>10	LctP of *E. coli*
2.15	BCCT	Glycine betaine; carnitine; choline	480–680	12	B	>10	CaiT of *E. coli*
2.16	TDT	Tellurite; dicarboxylates	320–440	10	B, A, E	>10	Tellurite uptake permease (TehA) of *E. coli*
2.17	POT	Peptides; nitrates; amino acids	450–600	12	B, E	>30	DtpT of *Lactococcus lactis*
2.18	AAAP	Amino acids and their derivatives	400–710	11	E (animals, plants, yeast, fungi)	>30	Aux-1 of *Arabidopsis thaliana*
2.19	CaCA	Ca^{2+}	460–1200	10–12	B, A, E	>30	ChaA of *E. coli*
2.20	PiT	Inorganic phosphate	410–680	10–12	B, A, E	>20	PitA of *E. coli*
2.21	SSS	Sugars; amino acids; vitamins; nucleosides; inositols; iodide; urea	400–700	12–15	B, A, E	>30	PanF of *E. coli*

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
2.22	NSS	Neurotransmitters; amino acids; osmolytes	600–700	12	E (animals), B	>50	Serotonin:Na^+ symporter of *Homo sapiens*
2.23	DCS	C_4-dicarboxylates; acidic and neutral amino acids	420–580	10–12	B, E	>20	GltP of *E. coli*
2.24	CSS	Di- and tricarboxylates	450	12	B	>10	CitS of *Klebsiella pneumoniae*
2.25	ASS	Alanine, glycine	440–540	8–12	B	>10	DagA of *Alteromonas haloplanktis*
2.26	LIVSS	Branched chain amino acid	~440	12	B	>10	BraB of *Pseudomonas aeruginosa*
2.27	ESS	Glutamate	~400	12	B	>5	GltS of *E. coli*
2.28	BASS	Bile acids	360–480	12	B, E (animals)	>10	Bile acid uptake system of *Rattus norvegicus*
2.29	MC	ATP/ADP; P_i; organic anions; OH^-, carnitine/acyl carnitine; basic amino acids; FAD	300	6	E (mito)	>100	ATP/ADP exchanger of *H. sapiens*
2.30	CCC	K^+, Na^+, Cl^-	1000–1200	12	B, E (animals, plants, yeast)	>30	NaCl/KCl cotransporter of *R. norvegicus*
2.31	AE	Inorganic anions	900–1250	14	E (animals, yeast)	>20	Anion exchanger (AE1) of *H. sapiens*

2.32	Sit	Silicate	550	12	E (diatoms)	>6	Sit1 of *Cylindrotheca fusiformis*
2.33	NhaA	Na^+/H^+	~400	12	B	>10	NhaA of *E. coli*
2.34	NhaB	Na^+/H^+	~520	12	B	>5	NhaB of *E. coli*
2.35	NhaC	Na^+/H^+	~460	12	B	>5	NhaC of *Bacillus firmus*
2.36	CPA1	Na^+/H^+	500–900	10–12	B, A, E	>40	Nhe-1 of *R. norvegicus*
2.37	CPA2	Na^+/H^+ or K^+/H^+	330–630	10–12	B, A, E	>20	KefC of *E. coli*
2.38	Trk	K^+:H^+?	420–560	10–12	B, A, E	>10	K^+ uptake permease (TrkH) of *E. coli*
2.39	NCS1	Nucleobases	420–640	12	B, A, E	>10	CodB of *E. coli*
2.40	NCS2	Nucleobases	420–600	12	B, A, E	>10	UraA of *E. coli*
2.41	NUP	Nucleosides	390–660	12–14	B, E	>10	NupC of *E. coli*
2.42	ArAAP	Aromatic amino acids	~400	11	B		TyrP of *E. coli*
2.43	STP	Serine; threonine	~430	11	B		SdaC of *E. coli*
2.44	FNT	Formate; nitrite; acetate	250–630	6–8	B, A, E (yeast)	>10	Formate uptake and efflux permeases (FocA of *E. coli*)
2.45	MIT	Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}; Al^{3+}; Mn^{2+}	300–900	2 or 3	B, A, E	>10	Metal uptake and efflux permeases (CorA of *E. coli*)
2.46	BenE	Benzoate	400	12	B	>2	BenE of *Acinetobacter caleoaceticus*
2.47	DASS	Dicarboxylates; phosphate; sulfate	430–920	11–14	B, A, E	>20	SodiT1 of *Spinacia oleracea*
2.48	RFC	Reduced folate	500–600	12	E (animals)	>10	RFC of *Mus musculus*
2.49	Amt	Ammonium	390–620	12	B, A, E	>20	AmtB of *E. coli*
2.50	TPT	Triose-P; glucose-6-P, P_i	400–450	5–8	E (chloroplasts, plastids, yeasts)	>10	TPT of *Zea mays*
2.51	NST	Nucleotide sugars	320–340	8–12	E (organelles)	>10	UDP-galactose:UDP exchange transporter of *H. sapiens*

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
2.52	NiCoT	Ni^{2+}, Co^{2+}	300–400	8	B	>10	Ni^{2+} uptake permease (HoxN) of *Ralstonia eutropha*
2.53	SulP	Sulfate	430–900	10–13	B, E	>30	Sulfate permease of *H. sapiens*
2.54	MTC	Di- and tricarboxylates	~290	3–6	E (mito)	>10	Mitochondrial tricarboxylate carrier of *R. norvegicus*
2.55	AcCoAT	Acetyl coenzyme A, coenzyme A; peptides, antibiotics	420–650	12	B, E	>10	AcCoA:CoA antiporter; AmpG uptake permease of *E. coli*
2.56	TRAP-T	C_4-dicarboxylates; acidic amino acids	~1000 (three components)	12 + 4	B, A	>10	DctPQM of *Rhodobacter capsulatus*
2.57	ENT	Nucleosides	~450	11	E	>10	hENT1 of *H. sapiens*
2.58	PNaS	Inorganic phosphate	300–650	>10	B, E	>20	NPT2 of *R. norvegicus*
2.59	ACR3	Arsenite	400	10	B, A, E	>1	ACR3 of *S. cerevisiae*
2.60	OAT	Organic anions; prostaglandins; bile acids; bile conjugates	600–700	10–12	E (animals)	>10	Organic anion transporter of *R. norvegicus*
2.61	DcuC	Dicarboxylates	461	10–12	B	>10	DcuC of *E. coli*

Pyrophosphate bond hydrolysis-driven transporters							
3.1	ABC	All sorts of inorganic and organic molecules of small, intermediate, and large sizes, from simple ions to macromolecules	1000–2000 (multidomain; usually multisubunit)	10 or 12; variable	B, A, E	>300	MalEFGK of *E. coli;* MDR of *H. sapiens*
3.2	F-ATPase	H^+; Na^+	>4000 (multiple subunits)	$(2)_{12}$, $+(1)_2$, $+(5)_1$	B, A, E (chloroplasts; mito)	>100	F_0F_1-ATPase of *E. coli*
3.3	P-ATPase	Na^+; H^+; K^+; Ca^{2+}; Mg^{2+}; Cd^{2+}; Cu^{2+}; Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}; phospholipids (flipping)	600–1200 (sometimes multisubunit)	6–12	B, A, E	>100	KdpABC (K^+ uptake) of *E. coli*
3.4	Ars	Arsenite, antimonite, tellurite	~530 (multidomain, multisubunit)	12	B, A, E	>40	ArsAB (arsenite efflux) of *E. coli*
3.5	IISP	Proteins	>2000 (multiple subunits)	SecY (10)	B, A, E	>50	SecAYDEFG of *E. coli*
3.6	IIISP	Proteins	>2000 (multiple subunits)	6 integral membrane constituents	B	>10	YscNDRSTUC; LcrD of *Yersinia* species

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
3.7	IVSP	Proteins, protein–DNA complexes	>2000 (multiple subunits)	3 integral membrane constituents	B	>10	VirB4, B6, B7, B9, B10, B11 of *Agrobacterium tumefaciens*
3.8	MPT	Mitochondrial proteins	>2000 (multiple subunits)	9 integral membrane constituents	E (mito)	1 or 2	Tom and Tim proteins of *S. cerevisiae*
3.9	CEPT	Chloroplast proteins	>2000 (multiple subunits)	Several integral membrane constituents	E (chloro)	1–2	IAP proteins of *Pisum sativum* (pea)
3.10	V-PPase	H^+	~770	15	E (plant vacuoles), B	>5	V-PPase of *A. thaliana*
3.11	DNA-T	Single-stranded DNA	>1000 (multiple subunits)	3 subunits	B	>5	ComEA-EC-FA of *B. subtilis*
Phosphotransferase systems							
4.1	Glc	Glucose; *N*-acetyl glucosamine; α- and β-glucosides	~2000 (3 domains; dimeric)	$(8)_2$	B	~30	Glucose IICB-IIA of *E. coli*
4.2	Fru	Fructose; mannitol	~2000 (3 domains; dimeric)	$(6)_2$	B	~30	Fructose IIB'BC-IIAMH of *E. coli*
4.3	Lac	Lactose; cellobiose	~2000 (3 domains; dimeric)	$\sim(8)_2$	B	~20	Lactose IICB-IIA of *S. aureus*

4.4	Gut	Glucitol	~2000 (3 domains; dimeric)	$(8)_2$	B	~2	Glucitol IICBC'-IIA of *E. coli*
4.5	Gat	Galactitol	~2000 (3 domains; dimeric)	$\sim(8)_2$	B	~1	Galactitol IIC-IIB-IIA of *E. coli*
4.6	Man	Glucose, mannose, fructose, sorbose, etc.	~2000 (4 domains; probably dimeric)	$(6(IIC)+1(IID))_2$	B	~5	Mannose IIAB-IIC-IID of *E. coli*
Decarboxylation-driven active transporters							
5.1	NaT-DC	Na^+	~1000 (3 subunits)	11 (β)	B, A	~10	Oxaloacetate decarboxylase of *Salmonella typhimurium*
Oxidoreduction-driven active transporters							
6.1	NDH	H^+ (efflux)	14–40 subunits	Multiple integral membrane subunits	B, E (mito, chloro)	~10	NDH of *E. coli*
6.2	PTH	H^+ (efflux)	~2000 (1–3 proteins; 3 domains; dimeric)	$(12–14)_2$	B, E (mito)	~10	PTH of *E. coli*
6.3	QCR	H^+ (efflux)	2000–6000 multiple (3–11) subunits; dimeric	$(13)_2$	B, E (mito; chloro)	>20	Cytochrome bc_1 complex of *Paracoccus dinitrificans*
6.4	COX	H^+ (efflux)	2000–6000 multiple (3–13) subunits; dimeric	$(12–20)_2$	B, A, E, (mito)	>20	Quinol oxidase (Cyo) of *E. coli*

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
6.5	NaNDH	Na^+ (efflux)	Multiple subunits	?	B	>1	Na^+-translocating NADH-quinol reductase of *Vibrio alginolyticus*
Light-driven active transporters							
7.1	BR	Na^+ (efflux); Cl^- (uptake)	~250	7	A	~10	Bacteriorhodopsin of *Halobacterium salinarum*
7.2	RC	H^+ (efflux)	~850 (3 subunits)	5(L) +5(M) +1(H)	B, E (plant chloroplasts)	~20	Reaction center of *Rhodobacter sphaeroides*
Mechanically driven active transporters							
8.1	Mot	H^+; Na^+	500–1000 (2–4 subunits)	4(A) + 1(B)	B	~10	H^+ efflux-driven flagellar motor (MotAB) of *E. coli*
Outer membrane porins (β-structure)							
9.1–9.19	Various outer membrane porins		250–500	10–24 β strands	G- B; phage; E (mito, plastids)	>200	OmpF of *E. coli;* VDAC of *B. taurus;* AidA of *E. coli*
Methyl transfer-driven active transporters							
10.1	NaT-MMM	Na^+	~8 subunits; most integral membrane constituents	?	A	>2	Na^+-transporting methyltetrahydromethanopterin: Coenzyme M methyltransferase of *Methanobacterium thermoautotrophicum*
Transporters of unknown classification							
99.1	PST	Polysaccharides (export)	400–500	12	B	>10	RfbX1 of *E. coli*

99.2	MerTP	Hg^{2+} (uptake)	~200	2	B	~10	MerTP encoded on the IncJ plasmid pMERPH of *Shewanella putrefaciens*
99.3	MerC	Hg^{2+} (uptake)	~137	1	B	~10	MerC encoded on the IncJ plasmid pMERPH of *S. putrefaciens*
99.4	PnuC	Nicotinamide mononucleotide (uptake)	~320	7	B	~10	PnuC of *S. typhimurium*
99.5	Kup	K^+ (uptake)	600–800	12	B, E (yeast, plants)	~10	Kup of *E. coli*
99.6	LysE	Lysine (efflux)	190–240	6	B, A	~10	LysE of *Corynebacterium glutamicum*
99.7	Chr	Chromate; sulfate (uptake or efflux)	~400	10	B, A	~10	ChrA of *Alcaligenes eutrophus*
99.8	FeoB	Fe^{2+} (uptake)	~800	8–13	B, A	~10	FeoB of *E. coli*
99.9	FeT	Fe^{2+} (Co^{2+}, Cd^{2+}) (uptake)	~552	6	E (yeast)	~1	Fet4p of *S. cerevisiae*
99.10	OFet	Fe^{2+} (uptake)	~404	6	B, A, E	~10	Ftr1p of *S. cerevisiae*
99.11	Ctr1	Cu^{2+} (uptake)	~406	2–3	E (yeast)	~1	Ctr1p of *S. cerevisiae*
99.12	Ctr2	Cu^{2+} (uptake)	160–200	3	E	~10	Ctr2p of *S. cerevisiae*
99.13	Nramp	Mn^{2+} (uptake)	540–580	8–12	B, A, E	~2	Smf1p of *S. cerevisiae*
99.14	CadD	Cd^{2+} (efflux); quaternary amines (uptake)	190–210	5	B	~2	CadD of *S. aureus*
99.15	Ami	Short-chain aliphatic amides; urea (uptake)	170–210	6	B	~5	AmiS of *Pseudomonas aeruginosa*

(*continued*)

TABLE II (*continued*)

TC no.	Family	Substrates	Size range[a]	No. TMSs	Organisms	No. members	Example
99.18	PUP	Peptides, antibiotics (uptake)	~406	7	B	~2	SbmA of *E. coli*
99.19	Mgt	Mg^{2+}, Co^{2+} (uptake)	310–470	4–5	B, A	~10	MgtE of *Bacillus firmus*
99.20	LCT	Monovalent cations	~570	8–10	E (plants)	~1	LCT1 of *Triticum aestivum*
99.21	Mtt	Redox proteins	>800 (3 subunits)	9 or 10	B, A, E	10–20	MttABC of *E. coli*

[a] Size range (in number of amino acyl residues) when a single type of subunit is present or for the entire complex when several types of subunits are present.

[b] Abbreviations used: B, bacterial; A, archeal; E, eukaryotic.

gene transfer between domains, be restricted to just one domain. A family of proteins found only in one domain may have arisen (i) late in the evolutionary process, after divergence of the domains; (ii) early in the evolutionary process, before divergence, but the members of the family in the other two domains may have been lost; or (iii) early in the evolutionary process, before divergence, but the rate of sequence divergence of the members of the family may have been so great as to conceal the common ancestry of extant proteins within each domain. Additionally, a family which now appears to be restricted to one domain may eventually prove to be found in other domains when more sequence data become available.

In the remainder of this article, I examine families of transporters that are apparently restricted to eukaryotes. Twenty-eight families currently fall into this category (Table III), and of them about half are restricted to just one of the major eukaryotic kingdoms (animals, plants, and fungi). These families will be examined, and their properties and probable origins will be discussed. In a few cases, their routes of appearance can be surmised with a fair degree of reliability.

Table III summarizes the distribution of transporter families in the three domains of living organisms—bacteria, archaea, and eukaryotes. The families are grouped according to transporter type. Thus, among the 21 channel-type protein families analyzed, 7 are bacterial specific, 9 are eukaryotic specific, 1 is found in both the bacterial and eukaryotic kingdoms, and 4

TABLE III
Distribution of Transporter Types in the Three Domains of Life

	Domain						
Transporter type	B	A	E	BA	BE	BAE	Total
Channels	7	0	9	0	1	4	21
2° Carriers	20	0	11	2	11	17	61
1° Active transporters (total)	11	2	3	0	4	6	26
Energy source for 1° active transporters							
ATP hydrolysis	3		3			5	11
PEP phosphotransfer	6						6
Redox	1				3	1	5
Light		1			1		2
Methyl transfer		1					1
Mechanical	1						1
Porins	19		1				20
Unknown	7		4	4	1	3	19
Total	64	2	28	6	17	30	147

are found in all three kingdoms. Of the 9 eukaryotic-specific transporter families, 8 are found only in animals while the ninth is restricted to yeast.

Secondary active transporters (which do not utilize a primary source of energy) and primary active transporters (which do use a primary source of energy) exhibit a very different distribution. In both these categories, bacterial-specific families and ubiquitous families (found in all three domains of life or, in a few instances, in two of them) predominate, and eukaryotic-specific transporter families represent a minority of these families. Thus, while 9 of 21 channel families (43%) are eukaryotic specific, only 11 of 61 secondary carrier families (18%) and 3 of 26 primary carrier families (12%) are apparently restricted to eukaryotes. Transport via the three eukaryotic-specific primary active transporters is driven by pyrophosphate (diphosphate) bond hydrolysis. No other primary energy source is apparently used by the members of eukaryotic-specific transport families. Just 1 of 20 porin families (5%) and 4 of 19 transporter families of unknown mode of action (21%) are restricted to eukaryotes. Overall, 28 of the 147 recognized families (19%) are apparently restricted to eukaryotes (Table III).

Below, we shall examine each of these eukaryotic-specific transporter families. As noted previously, most of the eukaryotic-specific channel families are restricted to animals, and they therefore may have arisen after divergence of animals from plants and fungi (about 1 billion years ago). However, few of the other types of transporters are restricted to just one eukaryotic kingdom. Also of interest is the fact that a very substantial fraction of the total number of eukaryotic-specific transporter families include members that are found only in specific eukaryotic organelles. Thus, two major driving forces for the emergence of novel families of transporters in eukaryotes may have been (i) the evolution of an elaborate system of electrical communication dependent on various channel-type proteins in animals and (ii) the need for a novel intracellular, intercompartmental system of communication to allow coordination of the various activities in the compartmentalized eukaryotic cell. Evolution of the latter eukaryotic-specific intracellular communication system presumably preceded divergence of the three eukaryotic kingdoms in response to the needs of a highly compartmentalized cell, whereas the former animal-specific system of electrical communication arose late, after divergence of these kingdoms, to allow development of a highly complex form of multicellularity. Major evolutionary conclusions resulting from the analyses reported will be presented in Section VII.

3. Types of Transporters Found in Eukaryotes

As noted previously, transmembrane transporters fall into several major types depending on mode of transport and energy-coupling mechanism,

and these types provide the initial basis for classification of transporters according to the TC system. Channels and carriers are found universally throughout the living world, as are primary and secondary active transporters. However, the mode of energy coupling observed for primary active transporters may be kingdom specific.

Currently recognized transporters include simple proteins and large multisubunit complexes that either facilitate passive diffusion of molecules across membranes or use one or more types of energy to drive transport. Many potential energy-yielding reactions have already been shown to be coupled to transport. These include several distinct chemical reactions such as bond breakage reactions (e.g., decarboxylation and pyrophosphate bond hydrolysis), chemical group transfer reactions (e.g., hydride and methyl transfer), and electron flow. In addition, mechanical rotation, light absorption, and the flow of ions down electrochemical gradients can be used to drive transport. In the reverse direction, ion transport can function to drive flagellar rotation or ATP synthesis.

Surprisingly, only a restricted number of the recognized energy-coupling mechanisms that drive transport have been shown to exist in eukaryotes. Thus, the phosphotransferase-dependent group translocators are prevalent throughout the bacterial world, but not a single PTS-related gene has been sequenced from the archaeal or eukaryotic domain. Decarboxylation-driven transport and methyl transfer-driven transport also seem to be kingdom specific and apparently do not occur in eukaryotes. Bacteriorhodopsins that drive ion fluxes by absorbing light and bacterial flagellae that rotate in response to pmf-driven ion fluxes are also in eukaryotes. Moreover, hydride and electron transfer-driven transport processes are apparently restricted to mitochondria and chloroplasts which were clearly derived from endosymbiotic bacteria at a late stage in eukaryotic evolutionary history.

These facts led to the realization that eukaryotes have come to utilize only a restricted number of the available energy-coupling mechanisms to drive transport. Other mechanisms neither developed in the eukaryotic lineage nor were transferred to that lineage by horizontal gene transfer. The transporter families that apparently arose in eukaryotic organisms are related to those which existed before the development of the eukaryotic cell, namely, channel- and carrier-type transporters that catalyze facilitated diffusion as well as active transporters driven by either ion gradients or pyrophosphate bond hydrolysis. Photon absorption-, electron flow-, and hydride transfer-driven transport were undoubtedly brought into the eukaryotic kingdom when proteobacteria and cyanobacteria invaded the developing eukaryotic cell to give rise to mitochondria and chloroplasts, respectively.

Eukaryotic-specific transporter families thus fall into a limiting number of TC categories. Animals have clearly developed a wide spectrum of

channel protein families that are not found elsewhere, even among other eukaryotes. Secondary carrier, and a few ATP or pyrophosphate hydrolysis-driven transporters, may also prove to have arisen in eukaryotes. Finally, a few recognized transporter families of unknown transport mode or energy-coupling mechanism (category 99) are restricted to eukaryotes. Any one of these may prove to exhibit unique features. Later, I examine these eukaryotic-specific transporter families in detail.

4. Proof of Independent Origins of Selected Phylogenetic Families of Transporters

A lack of statistically significant sequence similarity is never proof of independent origin because sequence divergence can lead to proteins that are unrecognizably different. However, if two families of proteins can be shown to have arisen by intragenic duplication events because they exhibit recognizable internal repeat units, and these repeat units are different in size and topology, then one can clearly suggest independent evolutionary origins. These arguments have been applied to proteins of the MC family (TC No. 2.29) in which members bear an internally repeated 2-TMS unit, the MIP family (TC No. 1.1) in which members bear an internally repeated 3-TMS unit, the major facilitator (MF) superfamily (TC No. 2.1) in which members bear an internally repeated 6-TMS unit, and the resistance-nodulation division family (TC No. 2.6) in which the repeat units bear large hydrophilic domains as well as 6 TMSs in a 1 + 5 arrangement (Saier, 1994, 1996). On this basis, and also on the basis of consideration of the organismal distribution of mitochondrial carrier family members, we have suggested that this family arose in eukaryotes after the development of internal organelles. While the same cannot currently be established for most eukaryotic-specific families, examination of the latter families should provide insight into the nature of transporters that have either arisen in eukaryotes or most rapidly diverged from their origins since divergence of eukaryotes from prokaryotes. In either case, the properties of these families presumably reflect the unique needs of eukaryotic organisms.

IV. Eukaryotic-Specific Families of Transporters

A. Characteristics of Eukaryotic-Specific Channel Protein Families

Eukaryotes possess several channel-forming protein families that are found ubiquitously throughout the living world (Table I). These families include

the MIP family (TC No. 1.1; substrates: water and small neutral solutes), the voltage-sensitive ion channel (VIC) family (TC No. 1.5; substrates: K^+, Na^+, and Ca^{2+}), and the chloride channel (CIC) family (TC No. 1.10; substrate: Cl^-). Additionally, animals and bacteria both synthesize and process toxic channel-forming peptides that are used in biological warfare (proteins of the CAP functional superfamily; TC No. 1.8). Yeast and fungi similarly synthesize toxins and toxin peptides, one of which, the yeast killer toxin K1 (TC No. 1.21; Table II), has been well characterized.

Several families of channel proteins are found exclusively in eukaryotes, and excluding the yeast killer toxin K1 (TC No. 1.21) and the mitochondrial and plastid porins (TC No. 9.8), all of these families are found exclusively in animals (Tables IV and V).

1. The Epithelial Na^+ Channel Family (TC No. 1.2)

The first of the animal-specific channel families listed in Tables IV and V is the epithelial Na^+ channel (ENaC) family (TC No. 1.2). The ENaC family consists of more than 24 sequenced proteins (Le and Saier, 1996). All are from animals with no recognizable homologs in other eukaryotes or bacteria. The vertebrate proteins cluster tightly together on the phylogenetic tree, whereas the 11 sequenced *Caenorhabditis elegans* proteins, including the degenerins, are distantly related to the vertebrate proteins and to each other. The homologous *Helix aspersa* FMRF-amide-activated Na^+ channel is the first peptide neurotransmitter-gated ionotropic receptor to be sequenced. Protein members of this family all exhibit the same apparent topology, each with two amphipathic transmembrane-spanning segments separated by a large extracellular loop. The extracellular domains contain numerous highly conserved cysteine residues and are proposed to serve a receptor function. A structural model has been proposed but not established.

The generalized transport reaction for Na^+ channels is

$$Na^+ \text{ (out)} \rightleftharpoons Na^+ \text{ (in)},$$

and that for the degenerins is

$$\text{Cation (out)} \rightleftharpoons \text{cation (in)}.$$

2. ATP-Gated Cation Channel Family (TC No. 1.4)

Structurally and possibly evolutionarily related to the ENaC family is the ATP-gated channel (ACC) family. Members of the ACC family (also called P2X receptors) respond to ATP, a functional neurotransmitter released by exocytosis from many types of neurons (Alexander and Peters, 1997; North,

TABLE IV

Properties of Eukaryotic-Specific Channel Protein Families

TC no.	Family	Specificity	Kingdom(s)	Cell type	Subcellular localization	Receptor for	Comments
1.2	ENaC	Na^+	Animal	Epithelia	PM	Exogenous peptide neurotransmitters	May be related to the ACC family (TC No. 1.4)
1.4	ACC	Cations	Animal	Nerve cells	PM	Exogenous ATP, acting as a neurotransmitter	Structurally similar to ENaC family members
1.6	LIC	Cations or anions	Animal	Nerve and muscle cells	PM	A large number of small neurotransmitters	Pentameric channels
1.7	GIC	Monovalent cations; sometimes Ca^{2+}	Animal	Nerve cells	PM	Glutamate	Same structural features as for LIC family members
1.9	RIR-CaC	Ca^{2+}	Animal	Muscle cells; brain cells	Sarcoplasmic reticulum; endoplasmic reticulum	Ryanodine; inositol triphosphate	Channel domains may resemble those of the VIC family (TC No. 1.5)
1.16	IRK-C	K^+	Animal	Nerves	PM	K^+ (out), Mg^{2+} (in), ATP (in), G proteins	2 TMSs; may be related to the VIC family
1.17	O-ClC	Cl^-	Animal	Various tissues (e.g., brain, kidney)	Nuclear membranes; endoplasmic reticulum	Voltage-sensitive	2 TMSs
1.20	TRP-CC	Ca^{2+}	Animal	Nerves; various other cell types	PM	Heat; vanilloid receptor, intracellular Ca^{2+}	6 TMSs; structurally resemble the VIC family
1.21	YKT-K1	Cation selective	Yeast	—	PM	None	Yeast toxin; forms channels in the membranes of sensitive yeast

1996; Soto *et al.,* 1997). They have been placed into seven groups ($P2X_1$–$P2X_7$) based on their pharmacological properties. These channels, which function at neuron–neuron and neuron–smooth muscle junctions, may play roles in the control of blood pressure and pain sensation. They may also function in lymphocyte and platelet physiology. They are found only in animals.

The proteins of the ACC family are quite similar in sequence (>35% identity), but they possess 380–1000 amino acyl residues per subunit with variability in length localized primarily to the C-terminal domains. They possess two transmembrane spanners, one approximately 30–50 residues from their N termini and the other near residues 320–340. The extracellular receptor domains between these two spanners (of approximately 270 residues) are well conserved with numerous conserved glycyl and cysteyl residues. The hydrophilic C termini vary in length from 25 to 240 residues. They resemble the topologically similar ENaC proteins (TC No. 1.2) in possessing (i) N and C termini localized intracellularly, (ii) two putative transmembrane spanners, (iii) a large extracellular loop domain, and (iv) many conserved extracellular cysteyl residues. ACC family members, however, are not demonstrably homologous with them. ACC channels are probably hetero- or homomultimers and transport small monovalent cations (Me^+). Some also transport Ca^{2+}, and a few transport small metabolites.

The generalized transport reaction is

$$Me^+ \text{ (out)} \rightleftharpoons Me^+ \text{ (in)}.$$

3. Ligand-Gated Ion Channel Family of Neurotransmitter Receptors (TC No. 1.6)

The ligand-gated ion channel (LIC) family is a very large family of channel proteins. Members of the LIC family of ionotropic neurotransmitter receptors are found only in animals (Alexander and Peters, 1997; Hucho and Tsetlin, 1996; Hucho *et al.,* 1996; Unwin, 1993, 1995). They exhibit receptor specificity for acetylcholine, serotonin, glycine, glutamate, and γ-aminobutyric acid. The best characterized are the nicotinic acetylcholine receptors which are pentameric channels of $\alpha_2\beta\gamma\delta$ subunit composition. All subunits are homologous. The three-dimensional structures of the protein complex in both the open and closed configurations have been solved at 0.9-nm resolution (Unwin, 1995). The structures suggested include both β and α structures in the transmembrane region. The five subunits (each 400–500 amino acyl residues in length) are arranged in a ring with their "M2" transmembrane helical spanners lining the central channel and each M2 helix surrounded by a continuous rim of β-sheet. The five M2 segments come together in the middle of the membrane to form the channel gate,

TABLE V

Representative Well-Characterized Transporters of Differing Specifications or Ligand Sensitivities Found within the Eukaryotic-Specific Channel Protein Families

TC no.	Specificity	Kingdom(s)	Comment
	Epithelial Na^+ channel (ENaC) family (TC No. 1.2)		
1.2.1.1	Epithelial Na^+ channel	Animals	$\alpha\beta\gamma$ heterotrimeric epithelial Na^+ channel of *Homo sapiens* (α, spP37088; β, gbL36593; γ, gbL36592)
1.2.2.1	Degenerin	Worm	Degenerin of *Caenorhabditis elegans* (gbL34414)
1.2.3.1	Peptide neurotransmitter-gated ionotropic receptor	Snail	Phe-Met-Arg-Phe-NH_2-activated Na^+ channel of *Helix aspersa* (gbX92113)
	ATP-gated cation channel (ACC) family (TC No. 1.4)		
1.4.1.1	ATP-gated cation channel (purinoceptor or ATP-neuroreceptor)	Animals	$P2X_1$ of *H. sapiens* (gbX80477)
1.4.2.1	ATP-gated cation channel (purinoceptor or ATP-neuroreceptor)	Animals	$P2X_2$ of *Rattus norvegicus* (gbU14414)
	Ligand-gated ion channel (LIC) family of neurotransmitter receptors (TC No. 1.6)		
1.6.1.1	Nicotinic acetylcholine-activated cation-selective channel, α chain	Animals	Acetylcholine receptor, α_7 chain of *H. sapiens* (spP36544)
1.6.2.1	Serotonin (5-hydroxytryptamine-activated) cation-selective channel	Animals	Serotonin (5HT3) receptor of *H. sapiens* (spP46098)

1.6.3.1	Glycine-activated chloride channel, α chain	Animals	Glycine receptor α_1 chain of *H. sapiens* (spP23415)
1.6.4.1	Glutamate-inhibited chloride channel	Animals	Glutamate receptor of *Drosophila melanogaster* (gbU58776)
1.6.5.1	γ-Aminobutyric acid (GABA)-inhibited chloride channel, β_1 chain	Animals	GABA receptor β_1 chain of *R. norvegicus* (spP15431)
	Glutamate-gated ion channel (GIC) family of neurotransmitter receptors (TC No. 1.7)		
1.7.1.1	Glutamate ionotropic channel receptor (GIC), kainate-subtype	Animals	GluR-K1 of *R. norvegicus* (gbX17184)
1.7.1.2	GIC, AMPA subtype	Animals	GluR-D of *R. norvegicus* (gbM36421)
1.7.1.3	GIC, NMDA subtype	Animals	NMDA receptor of *H. sapiens* (gbL76224)
	Ryanodine-inositol 1,4,5-triphosphate receptor Ca^{2+} channel (RIR-CaC) family (TC No. 1.9)		
1.9.1.1	Ryanodine receptor Ca^{2+} release channel	Animals	Cardiac muscle RyR-CaC of *H. sapiens* (gbX98330)
1.9.2.1	Inositol 1,4,5-trisphosphate receptor Ca^{2+} release channel	Animals	Brain IP_3-CaC of *R. norvegicus* (spP29995)
	Animal inward rectifier K^+ channel (IRK-C) family (TC No. 1.16)		
1.16.1.1	ATP-activated inward rectifier K^+ channel, IRK1	Animals	IRK1 of *H. sapiens* (391 aa; spP48048)
1.16.1.2	G protein-enhanced inward rectifier K^+ channel 2, IRK2	Animals	IRK2 of *H. sapiens* (427 aa; spP48049)
1.16.1.3	G protein-activated IRK5 channel	Animals	IRK5 of *H. sapiens* (419 aa; spP48544)
	Organellar chloride channel (O-ClC) family (TC No. 1.17)		
1.17.1.1	Organellar chloride channel, p64	Animals	p64 of *Bos taurus* (spP35526)
1.17.1.2	Nuclear chloride channel-27	Animals	NCC27 of *H. sapiens* (gbU93205)
	Transient receptor potential Ca^{2+} channel (TRP-CC) family (TC No. 1.20)		
1.20.1.1	Transient receptor potential (TRP) protein	Animals	TRP protein of *Drosophila melanogaster* (1275 aa; spP19334)
1.20.2.1	Vanilloid receptor subtype 1 (VR1)	Animals	VR1 of *R. norvegicus* (838 aa; gbAF029310)

and the gate opens upon binding of acetylcholine to distant sites in the N-terminal domains of the two α subunits. These structural features are probably general for all members of the family. Ionotropic glutamate receptors exhibit limited sequence and topological similarity, but homology cannot be established on the basis of sequence similarity alone. They are included within the glutamate-gated ion channel (GIC) family (TC No. 1.7)

The channel protein complexes of the LIC family preferentially transport cations or anions depending on the channel (e.g., the acetylcholine receptors are cation selective, whereas glycine receptors are anion selective).

The generalized transport reaction is

$$\text{Ions (out)} \rightleftharpoons \text{ions (in)}.$$

4. Glutamate-Gated Ion Channel Family of Neurotransmitter Receptors (TC No. 1.7)

Members of the GIC family are heteropentameric complexes in which each of the five subunits is 800–1000 amino acyl residues in length (Alexander and Peters, 1997; Nakanishi *et al.*, 1990; Unwin, 1993). These subunits may span the membrane three or five times as putative α-helices with the N termini (the glutamate-binding domains) localized extracellularly and the C termini localized cytoplasmically. They may be distantly related to the LICs (TC No. 1.6), and if so, they may possess substantial β structure in their transmembrane regions. However, homology between these two families cannot be established on the basis of sequence comparisons alone. The subunits fall into six subfamilies: α, β, γ, δ, ε, and ζ.

The GIC channels are divided into three types: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-, kainate-, and *N*-methyl-D-aspartate (NMDA)-selective glutamate receptors. Subunits of the AMPA and kainate classes exhibit 35–40% identity with each other, whereas subunits of the NMDA receptors exhibit 22–24% identity with the former subunits. They possess large N-terminal, extracellular glutamate-binding domains that are homologous to the periplasmic glutamine and glutamate receptors (TC Nos. 3.1.3.2 and 3.1.3.4, respectively) of ABC-type uptake permeases (TC No. 3.1) of gram-negative bacteria.

All known members of the GIC family are from animals. The different channel (receptor) types exhibit distinct ion selectivities and conductance properties. The NMDA-selective large conductance channels are highly permeable to monovalent cations and Ca^{2+}. The AMPA- and kainate-selective ion channels are permeable primarily to monovalent cations with only low permeability to Ca^{2+}.

The generalized transport reaction catalyzed by GIC family channels is

$$Me^{+} \text{ (or } Me^{2+}\text{) (out)} \rightleftharpoons Me^{+} \text{ (or } Me^{2+}\text{) (in)}.$$

5. Ryanodine–Inositol 1,4,5-triphosphate Receptor Ca^{2+} Channel Family (TC No. 1.9)

Ryanodine (Ry)-sensitive and inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+}-release channels function in the release of Ca^{2+} from intracellular storage sites in animal cells and thereby regulate various Ca^{2+}-dependent physiological processes (Hasan and Rosbash, 1992; Lee, 1996; Michikawa *et al.*, 1994; Mikoshiba *et al.*, 1996; Tunwell *et al.*, 1996). Ry receptors occur primarily in muscle cell sarcoplasmic reticular (SR) membranes, and IP_3 receptors occur primarily in brain cell endoplasmic reticular (ER) membranes where they effect release of Ca^{2+} into the cytoplasm upon activation (opening) of the channel.

The Ry receptors are activated as a result of the activity of dihydropyridine-sensitive Ca^{2+} channels. The latter are members of the VIC family (TC No. 1.5). Dihydropyridine-sensitive channels are present in the T-tubular systems of muscle tissues.

Ry receptors are homotetrameric complexes with each subunit exhibiting a molecular size of more than 500 kDa (about 5000 amino acyl residues). They possess C-terminal domains with six putative TMSs. Putative pore-forming sequences occur between the fifth and sixth TMSs as suggested for members of the VIC family. The large N-terminal hydrophilic domains and the small C-terminal hydrophilic domains are localized to the cytoplasm. Low-resolution three-dimensional structural data are available. Mammals possess at least three isoforms which probably arose by gene duplication and divergence before divergence of the mammalian species. Homologs are present in *Drosophila melanogaster* and *C. elegans.*

IP_3 receptors resemble Ry receptors in many respects. First, they are homotetrameric complexes with each subunit exhibiting a molecular size of more than 300 kDa (about 2700 amino acyl residues). Second, they possess C-terminal channel domains that are homologous to those of the Ry receptors. Third, the channel domains possess six putative TMSs and a putative channel lining region between TMSs 5 and 6. Fourth, both the large N-terminal domains and the smaller C-terminal tails face the cytoplasm. Fifth, they possess covalently linked carbohydrate on extracytoplasmic loops of the channel domains. Finally, they have three currently recognized isoforms (types 1–3) in mammals which are subject to differential regulation and have different tissue distributions.

IP_3 receptors possess three domains: N-terminal IP_3-binding domains, central coupling or regulatory domains, and C-terminal channel domains. Channels are activated by IP_3 binding, and like the Ry receptors, the activities of the IP_3 receptor channels are regulated by phosphorylation of the regulatory domains, catalyzed by various protein kinases. They predominate in the ER membranes of various cell types in the brain but have also

been found in the plasma membranes of some nerve cells derived from a variety of tissues.

The channel domains of the Ry and IP_3 receptors comprise a coherent family that despite apparent structural similarities do not show appreciable sequence similarity to the proteins of the VIC family (TC No. 1.5). The Ry receptors and the IP_3 receptors cluster separately on the Ry–IP_3 Ca^{2+} channel (RIR-CaC) family tree. They both have homologs in *Drosophila.* Based on the phylogenetic tree for the family, the family probably evolved in the following sequence: (i) A gene duplication event occurred that gave rise to Ry and IP_3 receptors in invertebrates; (ii) vertebrates evolved from invertebrates, (iii) the three isoforms of each receptor arose as a result of two distinct gene duplication events, and (iv) these isoforms were transmitted to mammals before divergence of the mammalian species.

The generalized transport reaction catalyzed by members of the RIR-CaC family following channel activation is

$$Ca^{2+} \text{ (out or sequestered in the ER or SR)} \rightarrow Ca^{2+} \text{ (cell cytoplasm)}.$$

6. Animal Inward Rectifier K^+ Channel Family (TC No. 1.16)

Animal inward rectifier K^+ channels (IRK-C) possess the "minimal channel-forming structure" with only a P domain, characteristic of the channel proteins of the VIC family (TC No. 1.5), and two flanking transmembrane spanners (Ashen *et al.,* 1995; Hille, 1992; Salkoff and Jegla, 1995; Shuck *et al.,* 1994). They may exist in the membrane as homo- or heterooligomers. They have a greater tendency to let K^+ flow into the cell than out. Voltage dependence may be regulated by external K^+, by internal Mg^{2+}, by internal ATP, and/or by G proteins. The P domains of IRK channels exhibit limited sequence similarity to those of the VIC family, but this sequence similarity is insufficient to establish homology. Inward rectifiers play a role in setting cellular membrane potentials, and the closing of these channels upon depolarization permits the occurrence of long-duration action potentials with a plateau phase. Inward rectifiers lack the intrinsic voltage-sensing helices found in VIC family channels.

The generalized transport reaction catalyzed by IRK-C family proteins is

$$K^+ \text{ (out)} \rightleftharpoons K^+ \text{ (in)}.$$

7. Organellar Chloride Channel Family (TC No. 1.17)

Proteins of the organellar chloride channel (O-ClC) family are voltage-sensitive chloride channels found in intracellular membranes but not the plasma membranes of animal cells (Duncan *et al.,* 1997; Landry *et al.,* 1993;

Valenzuela *et al.*, 1997). They are found in human nuclear membranes, and the bovine protein targets to the microsomes, but not the plasma membrane, when expressed in *Xenopus laevis* oocytes. These proteins are thought to function in the regulation of the membrane potential and in transepithelial ion absorption and secretion in the kidney. They possess two putative TMSs with cytoplasmic N and C termini and a large luminal loop that may be glycosylated. The bovine protein is 437 amino acyl residues in length and has two putative TMSs at positions 223–239 and 367–385. The human nuclear protein is much smaller (241 residues). A *C. elegans* homolog is reported to be 260 residues long.

The generalized transport reaction believed to be catalyzed by proteins of the O-ClC family is

$$Cl^-\ (\text{cytoplasm}) \rightleftharpoons Cl^-\ (\text{intraorganellar space}).$$

8. Transient Receptor Potential Ca^{2+} Channel Family (TC No. 1.20)

The transient receptor potential Ca^{2+} channel (TRP-CC) family has also been called the store-operated calcium channel (SOC) family (Caterina *et al.*, 1997; Clapham, 1996; Hardie and Minke, 1993; Montell and Rubin, 1989; Wong *et al.*, 1989). The prototypical members include the *Drosophila* retinal proteins TRP and TRPL (Hardie and Minke, 1993; Montell and Rubin, 1989). SOC members of the family mediate the entry of extracellular Ca^{2+} into cells in response to depletion of intracellular Ca^{2+} stores (Clapham, 1996). The recently cloned vanilloid receptor subtype 1 (VR1), which is the receptor for capsaicin (the "hot" ingredient in chili peppers) and serves as a heat-activated ion channel in the pain pathway (Caterina *et al.*, 1997), is also a member of this family.

These proteins all exhibit the same topological organization. They consist of 800 (VR1) or 1300 (TRP proteins) amino acyl residues with six TMSs as well as a short hydrophobic "loop" region between TMSs 5 and 6. This loop region may dip into the membrane and contribute to the ion permeation pathway (Hardie and Minke, 1993). In these topological respects the members of the TRP-CC family resemble those of the VIC family although homology cannot be established on the basis of sequence similarity. The amino termini of TRP-CC proteins normally contain a proline-rich region and one or more ankyrin domains. VR1, for example, exhibits three such repeat domains in its amino-terminal hydrophilic segment (432 amino acids). It also has a hydrophilic C terminus that lacks recognizable motifs. The sequence similarity between VR1 and other TRP-CC family proteins is within and adjacent to the sixth TMS, including the

hydrophobic loop region. Unlike other TRP-CC family members, VR1 is not a SOC. Mammals appear to have multiple VR1 homologs.

The generalized transport reaction catalyzed by TRP-CC family members is

$$Ca^{2+}\ (out) \rightleftharpoons Ca^{2+}\ (in).$$

B. Characteristics of Eukaryotic-Specific Secondary Carrier Families

Of the 62 currently recognized families of secondary carriers, 11 are represented only in eukaryotes (Tables VI and VII). Of these, 4 are found only in animals but the remaining are represented in more than one eukaryotic kingdom. None is found only in plants, yeast, or protozoans. Several of these eukaryotic-specific families may be related to other families. For example, the amino acid/auxin permease (AAAP) family (TC No. 2.18) is probably distantly related to the APC family (TC No. 2.3), and the two eukaryotic-specific families, the triose-phosphate transporter (TPT) and nucleotide-sugar transporter (NST) families (TC Nos. 2.50 and 2.51, respectively) may be related to each other. In the cases of two families listed in Tables VI and VII [i.e., the neurotransmitter: sodium symporter (NSS) family (TC No. 2.22) and the acetyl-coenzyme A transporter (AcCoAT) family (TC No. 2.55)], uncharacterized bacterial homologs are known. Thus, the origins of a few of these proteins can be supposed.

1. Zinc–Iron Permease Family (TC No. 2.5)

Members of the zinc–iron permease (ZIP) family consist of 220–430 amino acyl residues with eight putative TMSs (Breitwieser *et al.,* 1993; Eide *et al.,* 1996; Eide and Guerinot, 1997; Eng *et al.,* 1998; Zhao and Eide, 1996). They are derived from animals, plants, and yeast. They comprise a diverse family, with several paralogs in any one organism (e.g., at least five in *C. elegans,* at least five in *Arabidopsis thaliana,* and two in *S. cerevisiae*). Several members have been functionally characterized. Several of these proteins transport Zn^{2+}, and two of them transport Fe^{2+}. One human protein member of the ZIP family is designated "growth arrest inducible gene product," but its presumed transport activity has not been identified. The energy source for transport has not been characterized, but these systems probably function as secondary carriers.

The generalized transport reaction for members of the ZIP family may be

$$Me^{2+}\ (out) + (pmf) \rightarrow Me^{2+}\ (in).$$

TABLE VI

Properties of Eukaryotic-Specific Secondary Carrier Families

TC no.	Family	Specificity	Kingdom(s)	Subcellular localization	Comment
2.5	ZIP	Heavy metals (Zn^{2+}; Fe^{2+})	Animals, plants, yeast	PM	8 putative TMSs
2.18	AAAP	Amino acids; auxin	Animals, plants, yeast, fungi	PM	12 TMSs; may be related to the APC family (TC No. 2.3)
2.22	NSS	Neurotransmitters, amino acids, osmolites	Animals (Bacteria)	PM	12 TMSs; uncharacterized bacterial homologs have been found
2.29	MC	ATP/ADP, P_i, organic anions, carnitine/acyl carnitine, basic amino acids, FAD, etc.	All eukaryotes	Mitochondria, peroxysomes	6 TMSs; arose by internal gene triplication
2.31	AE	Inorganic (and some organic) anions	Animals, yeast	PM	14 TMSs; serve as a cytoskeletal anchor protein
2.32	Sit	Silicate	Animals (diatoms)	PM	12 TMSs
2.48	RFC	Reduced folate	Animals	PM	12 TMSs
2.50	TPT	Triose phosphates, glucose 6-phosphate	Plants, yeast	Chloroplasts, plastids, yeast	5–8 TMSs; may be related to the NST family (TC No. 2.51)
2.51	NST	Nucleotide sugars (UDP-galactose, CMP-sialic acid, GDP-mannose)	Animal	Endoplasmic reticulum, Golgi	8–12 TMSs; may be related to the TPT family (TC No. 2.50)
2.54	MTC	Di- and tri-carboxylates	Animals, yeast, protozoa	Mitochondria	5–6 TMSs; not related to the MC family (TC No. 2.29)
2.55	AcCoAT	Acetyl CoA/CoA	Animals, yeast (bacteria)	Endoplasmic reticulum	12 TMSs
2.57	ENT	Nucleosides	Animals, yeast	PM	11 TMSs
2.60	OAT	Organic anions, prostaglandins, bile acids	Animals	PM	10–12 TMSs

TABLE VII

Representative Well-Characterized Transporters of Differing Specificities Found within Eukaryotic-Specific Secondary Carrier Families

TC No.	Specificity	Kingdom(s)	Comment
	Zinc (Zn^{2+})–iron (Fe^{2+}) permease (ZIP) family (TC No. 2.5)		
2.5.1.1	High-affinity zinc uptake transporter	Yeast, animals, plants	Zrt1 of *Saccharomyces cerevisiae* (spP32804)
2.5.1.2	Iron-regulated metal ion uptake transporter	Plants, animals, yeast	Irt1 of *Arabidopsis thaliana* (gbU27590)
	Amino acid/auxin permease (AAAP) family (TC No. 2.18)		
2.18.1.1	Auxin:H^+ symporter	Plants	Aux-1 of *A. thaliana* (gbX98772)
2.18.2.1	General amino acid permease 1	Plants	AAP1 of *A. thaliana* (pirA48187)
2.18.3.1	Proline permease	Plants	Prt1 of *A. thaliana* (gpX95737)
2.18.4.1	Neutral amino acid permease	Fungi	AAP1 of *Neurospora crassa* (pirS47892)
2.18.5.1	Vesicular GABA transporter	Animals	UNC-47 of *Caenorhabditis elegans* (spP34579)
	Neurotransmitter:sodium symporter (NSS) family (TC No. 2.22)		
2.22.1.1	Serotonin:Na^+ symporter	Animals	Serotonin transporter of *Homo sapiens* (gbX70697)
2.22.1.2	Noradrenaline:Na^+ symporter	Animals	Noradrenaline transporter of *H. sapiens* (spP23975)
2.22.1.3	Dopamine:Na^+ symporter	Animals	Dopamine transporter of *H. sapiens* (gbM95167)
2.22.2.1	Proline:Na^+ symporter	Animals	Proline transporter of *Rattus norvegicus* (spP28573)
2.22.2.2	Glycine:Na^+ symporter	Animals	Glycine transporter of *R. norvegicus* (pirA48716)
2.22.3.1	Betaine/GABA:Na^+ symporter	Animals	Betaine/GABA transporter of *H. sapiens* (spP48065)
2.22.3.2	GABA:Na^+ symporter	Animals	GABA transporter of *H. sapiens* (gbX54673)
2.22.3.3	Taurine:Na^+ symporter	Animals	Taurine transporter of *H. sapiens* (gbZ18956)
2.22.3.4	Creatine:Na^+ symporter	Animals	Creatine transporter of *Oryclologus cuniculus* (gbX67252)
2.22.3.5	Choline:Na^+ symporter	Animals	Choline transporter of *R. norvegicus* (spP28570)
	Mitochondrial carrier (MC) family (TC No. 2.29)		
2.29.1.1	ATP/ADP antiporter	Animals, plants, fungi	ATP/ADP carrier of *H. sapiens* (gbJ02683)
2.29.2.1	Oxoglutarate/malate antiporter	Animals	Oxoglutarate/malate carrier of *Bos taurus* (gbM60662)

2.29.3.1	Uncoupling protein	Animals	Uncoupling carrier of *B. taurus* (gbX14064)
2.29.4.1	Phosphate carrier	Animals, yeast	Phosphate carrier of *B. taurus* (gbX05340)
2.29.5.1	MRS protein	Yeast	MRS protein of *S. cerevisiae* (spP10566) (transport substrate unknown)
2.29.6.1	Peroxisomal carrier	Yeast	Peroxysomal carrier of *Candida boidnii* (pirS50283) (transport substrate unknown)
2.29.7.1	Tricarboxylate carrier	Animals	Citrate carrier of *R. norvegicus* (gbL12016)
2.29.8.1	Mitochondrial carnitine/acyl carnitine carrier (CAC)	Mammals	CAC of *R. norvegicus* (gbX97831)
2.29.8.2	Embryonic differentiation (DIF-1) protein	Animals	DIF-1 of *C. elegans* (pirS55056) (transport substrate unknown)
2.29.9.1	Mitochondrial basic amino acid carrier (BAAC)	Fungi	BAAC of *N. crassa* (gbL36378)
2.29.10.1	Flavin adenine dinucleotide (FAD) carrier (FADC; FLX1)	Yeast	FLX1 of *S. cerevisiae* (spP40464)
2.29.11.1	Amyloplast Brittle-1 (BT1) protein	Plants	BT1 of *Zea mays* (spP29518) (transport substrate unknown)
2.29.12.1	Grave's disease carrier (GDC) protein	Mammals	GDC of *B. taurus* (spQ01888) (transport substrate unknown)
2.29.13.1	Regulator of acetyl-CoA synthetase activity ACR1	Yeast	ACR1 of *S. cerevisiae* (spP33303) (transport substrate unknown)
	Anion exchanger (AE) family (TC No. 2.31)		
2.31.1.1	Anion exchanger	Animals	AE1 of *H. sapiens* (spP02730)
2.31.2.1	Na^+:HCO^-_3 cotransporter (NBC)	Animals	NBC of *H. sapiens* (gbAF007216)
	Silicon transporter (Sit) family (TC No. 2.32)		
2.32.1.1	Silicon transporter	Marine diatoms	Sit1 of *Cylindrotheca fusiformis* (gbU63417)
	Reduced folate carrier (RFC) family (TC No. 2.48)		
2.48.1.1	Reduced folate carrier (RFC)	Animals	RFC of *Mus musculus* (spP41438)
	Triose phosphate translocator (TPT) family (TC No. 2.50)		
2.50.1.1	Chloroplast TPT	Plants	TPT of *Z. mays* (spP49133)
2.50.2.1	Nongreen plastid TPT	Plants	TPT of *Brassica oleracea* (spP52178)
2.50.3.1	Sly41p (transport function unknown)	Yeast	Sly41p of *S. cerevisiae* (spP22215)
	Nucleotide–sugar transporter (NST) family (TC No. 2.51)		
2.51.1.1	UDP-*N*-acetyl glucosamine:UMP antiporter	Yeast, animals	Mnn2-2 of *Kluyveromyces lactis* (328 aa; gbU48413)

(*continued*)

TABLE VII (*continued*)

TC No.	Specificity	Kingdom(s)	Comment
2.51.2.1	UDP-galactose:UMP antiporter	Animals	UDP-galactose transporter isoform 1 of *H. sapiens* (322 aa; pirJC5024)
2.51.3.1	CMP-sialic acid:CMP antiporter	Animals	CMP-sialic acid transporter of *M. musculus* (336 aa; gbZ71268)
	Mitochondrial tricarboxylate carrier (MTC) family (TC No. 2.54)		
2.54.1.1	Mitochondrial tricarboxylate carrier (MTC)	Eukaryotes	MTC of *R. norvegicus* (pirI55210)
	Acetyl-coenzyme A transporter (AcCoAT) family (TC No. 2.55)		
2.55.1.1	Putative acetyl-CoA transporter	Animals	Acetyl CoA transporter of *H. sapiens* (gbD88152)
2.55.2.1	AmpG	Bacteria	AmpG of *E. coli* (spP36670)
	Equilibrative nucleoside transporter (ENT) family (TC No. 2.57)		
2.57.1.1	Equilibrative nucleoside transporter (ENT)	Animals, yeast	Placental hENT1 of *H. sapiens* (gbU81375)
2.57.1.2	Nucleolar protein, HNP36 (function unknown)	Mammals	HNP36 of *H. sapiens* (gbX86682)
	Organo anion transporter (OAT) family (TC No. 2.60)		
2.60.1.1	Organic anion (digoxin) transporter (OAT) (Na^+ independent)	Animals	OAT of *R. norvegicus* (spP46720)
2.60.1.2	Prostaglandin (and thromboxane) transporter (PGT)	Animals	PGT of *R. norvegicus* (spQ00910)

2. Amino Acid/Auxin Permease Family (TC No. 2.18)

The AAAP family includes more than two dozen proteins from plants, animals, yeast, and fungi. Individual permeases of the AAAP family transport auxin (indole-3-acetic acid), a single amino acid, or multiple amino acids (Bennett *et al.,* 1996; Fischer *et al.,* 1995; Rentsch *et al.,* 1996; McIntire *et al.,* 1997; Young *et al.,* 1998). Some of these permeases exhibit very broad specificities transporting all 20 amino acids naturally found in proteins. There are seven AAAP paralogs in *S. cerevisiae,* at least nine in *A. thaliana,* and at least five in *C. elegans.* These proteins, all from eukaryotes, vary from 376 to 713 amino acyl residues in length, but most are of 400–500 residues. Most of the size variation occurs as a result of the presence of long N-terminal hydrophilic extensions in some of the proteins. Some of the yeast proteins are particularly long. Variation in the loops and the C termini also occurs. These proteins exhibit 11 putative transmembrane α-helical spanners. They exhibit limited sequence similarity with an amino acid permease (RocE) of *Bacillus subtilis* (spP39137) which is a member of the large APC family (TC No. 2.3). Thus, the AAAP family may be distantly related to the APC family.

The generalized transport reaction catalyzed by the proteins of the AAAP family is

$$\text{Substrate (out)} + n\text{H}^+ \text{ (out)} \rightarrow \text{substrate (in)} + n\text{H}^+ \text{ (in)}.$$

3. Neurotransmitter:Sodium Symporter Family (TC No. 2.22)

Members of the NSS family catalyze uptake of a variety of neurotransmitters, amino acids, osmolytes, and related nitrogenous substances by a solute:Na^+ symport mechanism (Reizer *et al.,* 1994). Sometimes Cl^- is cotransported, and some exhibit a K^+ dependency. Most sequenced members are from animals, but bacterial homologs have recently been sequenced. These proteins are generally of 600–700 amino acyl residues in length and probably possess 12 transmembrane helical spanners.

The generalized transport reaction for the members of this family is

$$\text{Solute (out)} + \text{Na}^+ \text{ (out)} \rightarrow \text{solute (in)} + \text{Na}^+ \text{ (in)}.$$

4. Mitochondrial Carrier Family (TC No. 2.29)

Permease protein subunits of the MC family possess six TMSs (Ahringer, 1995; Fernández *et al.,* 1994; Fiermonte *et al.,* 1992; Indiveri *et al.,* 1997; Kuan and Saier, 1993; Liu and Dunlap, 1996; Sullivan *et al.,* 1991; Tzagoloff *et al.,* 1996). The proteins are of fairly uniform size of about 300 residues.

They arose by a tandem intragenic triplication event in which a genetic element encoding two spanners gave rise to one encoding six spanners. This event may have occurred about 2 billion years ago, after mitochondria became permanent specialized denizens within eukaryotic cells. Members of the family are found exclusively in eukaryotic organelles although they are nuclearly encoded. Most are found in mitochondria, but some are found in peroxisomes of animals and in amyloplasts of plants. Structurally characterized members of the MC family are dimers. Many of them preferentially catalyze the exchange of one solute for another (antiport). Fifteen paralogs of the MC family are encoded within the genome of *S. cerevisiae.*

The generalized transport reaction for carriers of the MC family is

$$S_1 \text{ (out)} + S_2 \text{ (in)} \rightleftharpoons S_1 \text{ (in)} + S_2 \text{ (out)}.$$

5. Anion Exchanger Family (TC No. 2.31)

Characterized protein members of the anion exchanger (AE) family are found only in animals (Burnham *et al.,* 1997; Espanol and Saier, 1995; Romero *et al.,* 1997). One AE homolog is found encoded in the *S. cerevisiae* genome, but it has not been characterized. The animal AE proteins consist of homodimeric complexes of integral membrane proteins that vary in size from about 900 amino acyl residues to about 1250 residues. Their N-terminal hydrophilic domains may interact with cytoskeletal proteins and therefore play a cell structural role. The membrane-embedded C-terminal domains may each span the membrane 14 times as α-helices. They preferentially catalyze anion exchange (antiport) reactions. There are three known paralogous isoforms (AE1–AE3) in animals such as mice and rats. The different isoforms have different tissue distributions. Renal Na^+:HCO_3^- cotransporters have been found to be members of the AE family.

The physiologically relevant transport reaction catalyzed by anion exchangers of the AE family is

$$Cl^- \text{ (in)} + HCO_3^- \text{ (out)} \rightleftharpoons Cl^- \text{ (out)} + HCO_3^- \text{ (in)},$$

and that for the Na^+:HCO_3^- cotransporters is

$$Na^+ \text{ (out)} + nHCO_3^- \text{ (out)} \rightarrow Na^+ \text{ (in)} + nHCO_{3-} \text{ (in)}.$$

6. Silicon Transporter Family (TC No. 2.32)

Marine diatoms such as *Cylindrotheca fusiformis* encode at least six silicon transport protein homologs which exhibit similar sizes (about 550 amino acyl residues) and topologies (12 putative TMSs) (Hildebrand *et al.,* 1997). The six homologs exhibit >80% sequence identity with each other and

therefore comprise a coherent family of closely related proteins. The six recognized silicon transporter (Sit) family members exhibit insufficient sequence similarity with other proteins in the databases to suggest homology. One characterized member of the family (Sit1) functions in the energy-dependent uptake of either silicic acid $[Si(OH)_4]$ or silicate $[Si(OH)_3O^-]$ by a Na^+ symport mechanism. The system is found in marine diatoms which make their "glass houses" out of silicon.

The generalized transport reaction is

$$\text{Silicate (out)} + n\text{Na}^+ \text{ (out)} \rightarrow \text{silicate (in)} + n\text{Na}^+ \text{ (in)}$$

7. Reduced Folate Carrier Family (TC No. 2.48)

Proteins of the reduced folate carrier (RFC) family are so far restricted to animals (Bowman *et al.*, 1989; Dixon *et al.*, 1994; Henderson, 1990; Williams *et al.*, 1994; Wong *et al.*, 1995). They have been sequenced from several mammals and from the worm, *C. elegans.* All exhibit a high degree of sequence similarity with each other. They are 500–600 amino acyl residues long and possess 12 putative TMSs. They take up reduced folate and its derivatives as well as the drug methotrexate. They lack statistically significant sequence similarity with carriers of the MFS (TC No. 2.1) or with other sequenced transporters.

Mammals possess at least three folate transporters: the RFC ($K_B \approx 100$ nM; $K_m \approx 1$ μM) described here as well as a lower affinity system and a higher affinity system. The RFC appears to transport reduced folate (but not folate) by an energy-dependent, pH-dependent, Na^+-independent mechanism. A folate:H^+ symport or folate:OH^- antiport mechanism has been proposed. Other anions may be able to substitute for OH^-.

The generalized transport reaction catalyzed by the proteins of the RFC family is probably

$$\text{Reduced folate (out)} + H^+ \text{ (out)} \rightarrow \text{reduced folate (in)} + H^+ \text{ (in)}.$$

8. Triose Phosphate Translocator Family (TC No. 2.50)

The functionally characterized members of the TPT family are derived from the inner envelope membranes of chloroplasts and nongreen plastids of plants (Fischer *et al.*, 1997; Flügge, 1992, 1995; Flügge and Heldt, 1991; Flügge *et al.*, 1992; Loddenkötter *et al.*, 1993, Schwarz *et al.*, 1994). However, homologs are also present in yeast. *Saccharomyces cerevisiae* has three functionally uncharacterized TPT paralogs encoded within its genome. Under normal physiological conditions, chloroplast TPTs mediate a strict antiport of substrates, frequently exchanging an organic three-carbon com-

pound phosphate ester for inorganic phosphate (P_i). Normally, a triose-phosphate, 3-phosphoglycerate, or another phosphorylated C_3 compound made in the chloroplast during photosynthesis exits the organelle into the cytoplasm of the plant cell in exchange for P_i. However, experiments with reconstituted translocator in artificial membranes indicate that transport can also occur by a channel-like uniport mechanism with approximately 10-fold higher transport rates. Channel openings may be induced by a membrane potential of large magnitude and/or by high substrate concentrations. Nongreen plastid TPT carriers, such as those from maize endosperm and root membranes, mediate transport of C_3 compounds phosphorylated at carbon atom 2, particularly phosphoenolpyruvate, in exchange for P_i. Glucose-6-P has also been shown to be a substrate of the plastid translocator. The chloroplast and nongreen plastid proteins are divergent in sequence and substrate specificity.

Each TPT family protein consists of about 400–450 amino acyl residues with five to eight putative TMSs. The actual number has been proposed to be six for the plant proteins as for mitochondrial carriers (TC No. 2.29) and members of several other transporter families. However, proteins of the TPT family do not exhibit significant sequence similarity with the latter proteins, and there is no evidence for an internal repeat sequence.

The generalized reaction catalyzed by the proteins of the TPT family is

$$\text{Organic phosphate ester (in)} + P_i \text{ (out)} \rightleftharpoons \text{organic phosphate ester (out)} + P_i \text{ (in)}.$$

9. Nucleotide–Sugar Transporter Family (TC No. 2.51)

Proteins of the NST family are found in the Golgi apparatus and the ER of eukaryotic cells (Abeijon *et al.,* 1996, 1997; Berninsone *et al.,* 1997; Descoteaux *et al.,* 1995; Eckhardt *et al.,* 1996; Ma *et al.,* 1997). Members of the family have been sequenced from yeast, protozoans, and animals. Animals such as *C. elegans* possess many paralogs of the NST family. Humans have at least two closely related isoforms of the UDP-galactose:UMP exchange transporter.

NST family proteins generally appear to function by antiport mechanisms, exchanging a nucleotide–sugar for a nucleotide. Thus, CMP-sialic acid is exchanged for CMP, GDP-mannose is preferentially exchanged for GMP, UDP-galactose and UDP-*N*-acetyl glucosamine are exchanged for UMP (and possibly UDP), and other nucleotide sugars (e.g., GDP-fucose, UDP-xylose, UDP-glucose, and UDP-N-acetyl galactosamine) are also transported in exchange for various nucleotides. Each compound appears to be translocated by its own transport protein. Transport allows the compound, synthesized in the cytoplasm, to be exported to the lumen of the Golgi

apparatus or the ER where it is used for the synthesis of glycoproteins and glycolipids. Comparable transport proteins exist for ATP which phosphorylates proteins and phosphoadenosinephosphosulfate, which is used as a precursor for protein sulfation. It is not known if these transport proteins are members of the NST family.

The sequenced proteins of the NST family are generally about 320–340 amino acyl residues in length and exhibit 8–12 putative TMSs.

The generalized transport reaction catalyzed by the proteins of the NST family is

Nucleotide–sugar (cytoplasm) + nucleotide (lumen)
$\rightleftharpoons$ nucleotide–sugar (lumen) + nucleotide (cytoplasm).

10. Mitochondrial Tricarboxylate Carrier Family (TC No. 2.54)

The mitochondrial tricarboxylate carrier family consists of a limited number of homologs, all from eukaryotes (Azzi *et al.*, 1993). A single member of the family has been functionally characterized and sequenced—the tricarboxylate carrier from rat liver mitochondria. It is 322 amino acyl residues in length with five or six putative TMSs. It does not exhibit internal repeats or show homology to proteins of the mitochondrial carrier family (TC No. 2.29). Homologs are found in *C. elegans, S. cerevisiae,* and *Leishmania major.* They are 285–293 amino acyl residues, with one exception, and are reported to possess three to six putative TMSs.

The rat liver mitochondrial tricarboxylate carrier has been reported to transport citrate, *cis*-aconitate, threo-D-isocitrate, D- and L-tartrate, malate, succinate, and phosphoenolypyruvate. *Trans*-aconitate, α-ketoglutarate, and malonate are not substrates. It presumably functions by a proton symport mechanism for the uptake of the variety of anionic substrates listed previously.

The generalized transport reaction catalyzed by the MTC protein of rat liver mitochondria is therefore probably

Anionic substrate (out) + nH^+ (out)
$\rightarrow$ anionic substrate (in) + nH^+ (in).

11. Acetyl-CoA Transporter Family (TC No. 2.55)

Two members of the AcCoAT family have been functionally characterized, but the precise biochemical functions of these proteins are not certain (Jacobs *et al.*, 1994; Kanamori *et al.*, 1997; Lindquist *et al.*, 1993; Park *et al.*, 1998; Saier *et al.*, 1998). One of these proteins is the putative acetylcoenzyme A transporter found in the ER and Golgi membranes of man

(Kanamori *et al.*, 1997). It is homologous to proteins in *C. elegans, S. cerevisiae,* and several gram-negative bacteria. The other protein, the homologous *E. coli* AmpG protein, probably brings peptides, including cell wall degradative peptides, and inducers of β-lactamase synthesis, into the cell. AmpG may be specific for cell wall degradation peptides but also transport penicillin and its derivatives. In *Haemophilus influenzae,* the gene encoding an AcCoAT homolog is found in a gene cluster concerned with lipopolysaccharide synthesis. A homolog from *Neisseria gonorrhoeae* has also been sequenced. These proteins are 425–632 amino acyl residues in length and exhibit 12 putative TMSs. The mechanism of energy coupling is not known, but the topology of these proteins suggests that they are secondary carriers. Furthermore, the acetyl-CoA transporter is expected to function by acetyl-CoA:CoA antiport, whereas the AmpG protein is most likely energized by substrate:H^+ symport. The AcCoAT family has therefore been placed within category 2. Structural similarities between peptides and coenzyme A are noteworthy.

Generalized transport reactions for the AcCoAT family may be

$$\text{Acetyl-CoA (cytoplasm)} + \text{CoA (lumen)} \rightleftharpoons \text{acetyl-CoA (lumen)} + \text{CoA (cytoplasm)}$$

$$\text{Peptide (out)} + n\text{H}^+ \text{ (out)} \rightarrow \text{peptide (in)} + n\text{H}^+ \text{ (in)}.$$

12. Equilibrative Nucleoside Transporter Family (TC No. 2.57)

A single member of the equilibrative nucleoside transporter (ENT) family has been functionally characterized (Crawford *et al.*, 1998; Griffiths *et al.*, 1997a,b; Yao *et al.*, 1997). This protein is designated hENT1 and is of human placental origin. It is 456 amino acyl residues long and possesses 11 putative TMSs. It is expressed in many human tissues. Homologs have been sequenced from yeast, nematodes, and mammals. *Caenorhabditis elegans* possesses at least five such homologs. Among these are the two smaller nucleolar "delayed early response" gene products, HNP36, sequenced from humans and mice (Williams and Lanahan, 1995). The hENT1 protein appears to exhibit broad specificity for nucleosides and cytotoxic nucleoside analogs used in cancer and viral chemotherapy.

The generalized transport reaction catalyzed by hENT1 is

$$\text{Nucleoside (out)} \rightleftharpoons \text{nucleoside (in)}.$$

13. Organo Anion Transporter Family (TC No. 2.60)

Proteins of the organo anion transporter (OAT) family catalyze the Na^+-independent facilitated transport of organic anions such as bromosulfobromophthalein and prostaglandins as well as conjugated and unconjugated bile

acids (taurocholate and cholate, respectively) (Chan *et al.*, 1998; Hagenbuch, 1997; Jacquemin *et al.*, 1994; Kanai *et al.*, 1995). These transporters are found exclusively in animals. Some exhibit a high degree of tissue specificity. For example, the rat OAT is found at high levels in liver and kidney and at lower levels in other tissues. These proteins consist of 600–700 amino acyl residues and possess 10–12 putative TMSs. They may catalyze electrogenic anion uniport or anion exchange.

The generalized transport reaction catalyzed by members of the OAT family is

$$\text{Anion (in)} \rightleftharpoons \text{anion (out)}$$

or

$$\text{Anion}_1\text{ (in)} + \text{anion}_2\text{ (out)} \rightleftharpoons \text{anion}_1\text{ (out)} + \text{anion}_2\text{ (in)}.$$

C. Characteristics of Eukaryotic-Specific Organellar Primary Active Transporter Families

Only three primary active transporter families are possible candidates for eukaryotic-specific families, and all three are driven by pyrophosphate bond hydrolysis. Two of these families include members that are multicomponent systems that transport proteins into mitochondria and chloroplasts, respectively (Tables VIII and IX). The system found in chloroplasts includes one protein that has bacterial homologs. At least some of the constituents of these transporters may therefore have bacterial origins. The third possible candidate for a eukaryotic-specific active transporter family is the H^+-translocating vacuolar pyrophosphatase (V-PPase) family (TC No. 3.19) (Tables VIII and IX). This last family has a partially sequenced bacterial homolog that is functionally uncharacterized. The V-PPase family may therefore have a bacterial origin. Its description is nevertheless included here.

1. Mitochondrial Protein Translocase Family (TC No. 3.8)

The mitochondrial protein translocase (MPT), which brings nuclearly encoded preproteins into mitochondria, is very complex with 19 currently identified protein constituents (Dekker *et al.*, 1997; Neupert, 1997; Schatz, 1996). These proteins include several chaperone proteins, 4 proteins of the outer membrane translocase (Tom) import receptor, 5 proteins of the Tom channel complex, 5 proteins of the inner membrane translocase (Tim), and 3 "motor" proteins. The 4 nonidentical integral membrane receptor proteins are called Tom70, Tom37, Tom22, and Tom20. Of these receptor

TABLE VIII

Properties of Eukaryotic-Specific Organellar Primary Active Transport Carrier Families

TC no.	Family	Specificity	Energy source	Kingdom(s)	Subcellular localization	Comment
3.8	MPT	Proteins	ATP	All eukaryotes	Mitochondria	Organellar protein import; 19 dissimilar subunits
3.9	CEPT	Proteins	ATP or GTP	Plants (animals; bacteria)	Chloroplasts	Organellar protein import; 5 dissimilar subunits identified; some constituents have animal and bacterial homologs
3.10	V-PPase	H^+	P_2	Plants (bacteria)	Vacuoles (tonoplasts)	15 putative TMSs; generates a pmf (positive inside) across the vacuolar membrane; exhibits some sequence similarity to c subunits of F-ATPases (TC No. 3.2)

TABLE IX

Representative Well-Characterized Members of the Three Eukaryotic-Specific Organellar Primary Active Transport Carrier Families

TC No.	Specificity	Kingdom(s)	Comment
	Mitochondrial protein translocase (MPT) family (TC No. 3.8)		
3.8.1.1	MPT	Yeast, fungi (animals)	MPT of *Saccharomyces cerevisiae* Tom70 (receptor; 617 aa; 1 TMS; spP07213) Tom37 (receptor; 327 aa; 2 TMSs; spP50110) Tom22 (receptor; 152 aa; 1 TMS; spP49334) Tom20 (receptor; 183 aa; 1 TMS; spP35180) Tom7 (channel regulator; 59 aa; spP53507) Tom6 (channel regulator; 61 aa; spP33448) Tom40 (channel core; 387 aa; 0 TMSs; spP23644) Tim23 (channel regulator; 222 aa; 4 TMSs; spP32897) Tim17 (channel regulator; 158 aa; 4 TMSs; spP39515) Tim44 (motor; 431 aa; 0 TMSs; spQ01852)
	Chloroplast envelope protein translocase (CEPT) family (TC No. 3.9)		
3.9.1.1	Chloroplast envelope protein translocase (CEPT); outer membrane complex	Plants, algae (bacteria?)	IAP75, 34, and 86 from *Pisum sativum* (pea) IAP75 (probable porin; 809 aa; pirS55344) IAP34 (GTP-binding protein; 310 aa; pirB55171) IAP86 (GTP-binding protein; 879 aa; pirS49910)
	H^+-translocating vacuolar pyrophosphate (V-PPase) family (TC No. 3.10)		
3.10.1.1	H^+-translocating vacuolar pyrophosphatase	Plant vacuoles	V-PPase of *Arabidopsis thaliana* (spP31414)

proteins, only Tom22 is essential for protein import. The receptor delivers the substrate protein to the outer membrane channel consisting of 5 hydrophobic proteins—Tom40, Tom38, Tom7, Tom6, and Tom5. Tom40 is the core subunit of this channel. The small Tom proteins may function in a regulatory capacity and are not essential.

The inner membrane channel-forming complex consists of at least five integral membrane Tim proteins—Tim33, Tim23, Tim17, Tim14, and Tim11—as well as a peripheral membrane protein, Tim44. Two chaperone proteins (mhsp70 and mGrpE) have been suggested to function together with Tim44. Tim44 (with or without the chaperone proteins) may function as an ATP-driven import motor that pulls the precursor polypeptide chain through the Tim channel into the matrix. The Tom and Tim proteins have homologs in yeast, fungi, and/or animals. Tim23, Tim17, and another *S. cerevisiae* protein, Tim22 (spQ12328), are homologous to each other.

The generalized transport reaction catalyzed by the MPT is

$$\text{Protein (cell cytoplasm)} \xrightarrow[\left(\begin{smallmatrix}\text{GTP}\\ \text{pmf}\end{smallmatrix}\right)?]{\text{ATP}} \text{protein (mitochondrial matrix).}$$

2. Chloroplast Envelope Protein Translocase Family (TC No. 3.9)

The vast majority of chloroplast proteins are encoded within the nucleus of the plant or algal cell (Cline and Henry, 1996; Kouranov and Schnell, 1996; Schnell *et al.*, 1994). These proteins are made as preproteins on cytoplasmic ribosomes and are then targeted to the appropriate organellar subcompartment. Some of the proteins that comprise the translocation apparatus in the envelope and thylakoid membranes of the chloroplast have been identified. A single assembly is thought to catalyze translocation across the two-membrane envelope, and the protein components of the envelope that have been identified and sequenced appear to be unique. By contrast, translocation across the thylakoid membrane may occur via three or four pathways. One of these pathways involves ATP hydrolysis and proteins of the type II general secretory pathway (TC No. 3.5). The SecY-dependent thylakoid membrane translocase is therefore not part of the translocase included under TC No. 3.9. A second thylakoid membrane translocase is GTP dependent and includes proteins homologous to eukaryotic and bacterial signal recognition particles. A third transthylakoid membrane translocation pathway appears to be pmf dependent but ATP and GTP independent.

Preproteins are normally recognized at the outer envelope and translocated in a single step across the outer and inner membranes through contact zones where the two membranes are maintained in close apposition. The targeting signal for envelope translocation is in the N-terminal "transit sequence." Translocation to the stroma occurs in at least two steps and requires the hydrolysis of both ATP and GTP. Subsequently, the transit sequence is removed in the stromal compartment.

Five proteins of the outer envelope import apparatus have been identified. Two of these are hsp70-type chaperones, and three of them (IAP34, IAP75, and IAP86) are believed to form a recognition and translocation complex in the outer membrane. IAP34 and IAP86 are related to each other in sequence and possess cytoplasmically exposed GTP-binding sites. IAP75 probably forms an outer membrane porin consisting largely of β structure that may serve as the outer membrane protein-conducting channel. IAP34 and IAP86 have sequenced homologs from several plants. They also have distant homologs in bacteria and slime molds. IAP75 has one sequenced homolog in the blue-green bacterium, *Synechocystis.*

The generalized transport reaction catalyzed by the chloroplast envelope protein import translocase is

$$\text{Protein (cell cytoplasm)} \xrightarrow[\left(\begin{smallmatrix}\text{GTP}\\ \text{pmf}\end{smallmatrix}\right)?]{\text{ATP}} \text{protein (chloroplast stroma).}$$

3. H^+-Translocating Vacuolar Pyrophosphatase Family (TC No. 3.10)

Proteins of the V-PPase family are found in the vacuolar (tonoplast) membrane of higher plants (Baltscheffsky and Baltscheffsky, 1993; Rea and Poole, 1993; Zhen *et al.,* 1997a,b). These enzymes pump H^+ upon hydrolysis of pyrophosphate, thereby generating a proton motive force, positive and acidic in the tonoplast lumen. These enzymes establish a pmf of similar magnitude to that generated by the H^+-translocating ATPases in the same vacuolar membrane.

The members of the V-PPase family are large proteins of about 770 amino acyl (aa) residues with 15 putative TMSs. The N termini are predicted to be in the vacuolar lumen, whereas the C termini are thought to be in the cytoplasm. These proteins exhibit a region that shows convincing sequence similarity to the regions surrounding the DCCD-sensitive glutamate in the C-terminal regions of the c subunits of F-type ATPases (TC No. 3.2). Several acidic residues in the *Arabidopsis* V-PPase have been shown to be important for function. Some plants possess closely related V-PPase isoforms. These enzymes have the enzyme commission number EC 3.6.1.1. A shorter homolog is found in the bacterium, *Thermatoga maritima* (272 aa; AF013268).

The generalized transport reaction catalyzed by V-PPases is

$$\text{Pyrophosphate } (P_2) + H_2O + nH^+ \text{ (cytoplasm)} \rightarrow \text{inorganic phosphate } (2P_i) + nH^+ \text{ (vacuolar lumen).}$$

D. Characteristics of a Eukaryotic Organellar Porin Family

Only a single family of recognized porin proteins is currently believed to be eukaryotic specific. This family is represented both in mitochondria and in plant plastids. It is therefore referred to as the mitochondrial and plastid porin (MPP) family.

Porins of the MPP family (TC No. 9.8) are found in eukaryotic organelles (Fischer *et al.*, 1995; Jeanteur *et al.*, 1991, 1994; Nikaido, 1992; Rauch and Moran, 1994; Schulz, 1996; Troll *et al.*, 1992). The organelles include mitochondria of many eukaryotes and chloroplasts and plastids of plants. The best characterized are the voltage-dependent anion-selective channel porins in the mitochondrial outer membrane. Three paralogs of the MPP family are encoded within the *S. cerevisiae* genome. Two of these, Por1 (the major porin) and Por2 (a minor porin), have been functionally characterized (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998; Mannella, 1997). Like many bacterial porins, the MPP family proteins probably consist of β-barrel structures. Although the bacterial and eukaryotic proteins do not exhibit significant sequence similarity with each other, porin proteins from bacteria appear to have undergone extensive sequence divergence. The bacterial and eukaryotic organellar proteins may therefore share a common origin.

E. Characteristics of Eukaryotic Transporter Families of Unknown Transport Mode or Energy-Coupling Mechanism

Four eukaryotic-specific families of transporters function in the uptake or efflux of inorganic cations (see Tables X and XI). These families generally consist of one or more proteins which is/are poorly defined from a functional standpoint. They are described below.

1. Low-Affinity Fe^{2+} Transporter Family (TC No. 99.9)

The low-affinity Fe^{2+} transporter (FeT) family consists of a single iron-deprivation inducible protein, Fet4p of *S. cerevisiae*, because no homologs are currently present in the databases (Dix *et al.*, 1994, 1997; Eide and Guerinot, 1997). *Saccharomyces cerevisiae* reduces Fe^{3+} to Fe^{2+} by an Fe^{3+} reductase localized to the external surface of the plasma membrane. The Fet4p permease then takes up Fe^{2+} into the cytoplasm with low affinity (K_m = 30 μM). It may also transport Co^{2+} and Cd^{2+}. The protein has 552 amino acyl residues and six putative TMSs. It presumably uses the pmf across the cytoplasmic membrane, but the energy-coupling mechanism is not established. A proton symport mechanism may or may not drive uptake.

TABLE X

Properties of Eukaryotic-Specific Transporter Families of Unknown Mode of Action or Energy-Coupling Mechanism

TC no.	Family	Specificity	Kingdom(s)	Polarity	Comment
99.9	FeT	Heavy metals (Fe^{2+}, Co^{2+}, Cd^{2+})	Yeast	Uptake	6 TMSs; just one member of the family has been sequenced
99.11	Ctr1	Cu^{2+}	Yeast	Uptake	2–3 TMSs; just one member of the family has been sequenced
99.12	Ctr2	Cu^{2+}	Animals, plants, yeast, protozoans	Uptake	3 putative TMSs; some may be in the PM; others in organelles
99.20	LCT	Monovalent cations	Plants	Uptake	8–10 TMSs; cation affinity, ~10 m*M*

TABLE XI

Representative Members of the Four Eukaryotic-Specific Transporter Families of Unknown Mode of Action or Energy-Coupling Mechanism

TC No.	Specificity	Kingdom(s)	Comment
	Low-affinity Fe^{2+} transporter (FeT) family (TC No. 99.9)		
99.9.1.1	Low-affinity Fe^{2+} transporter	Yeast	Fet4p of *Saccharomyces cerevisiae* (spP40988)
	Copper Transporter-1 (Ctr1) family (TC No. 99.11)		
99.11.1.1	High-affinity copper transporter	Yeast	Ctr1p of *S. cerevisiae* (spP49573)
	Copper transporter-2 (Ctr2) family (TC No. 99.12)		
99.12.1.1	Copper uptake transporter	Plants	CopT1 of *Arabidopsis thaliana* (gbZ49859)
99.12.1.2	Copper uptake transporter	Animals	hCTR1 of *Homo sapiens* (gbU83460)
99.12.1.3	Copper uptake transporter	Yeast	Ctr2p of *S. cerevisiae* (spP38865)
99.12.1.4	Copper uptake transporter	Yeast	Ctr3p of *S. cerevisiae* (spQ06686)
	Low-affinity cation transporter (LCT) family (TC No. 99.20)		
99.20.1.1	Low-affinity cation transporter	Plants	LCT1 of *Triticum aestivum* (gbAF015523)

The generalized transport reaction catalyzed by the Fet4p permease of *S. cerevisiae* is

$$Fe^{2+} \text{ (out)} \rightarrow Fe^{2+} \text{ (in)}.$$

2. Copper Transporter-1 Family (TC No. 99.11)

A single protein (Ctr1p) of *S. cerevisiae* has been characterized as a high-affinity Cu^{2+} uptake permease (Dancis *et al.*, 1994a,b; Eide and Guerinot, 1997). It has no homologs in the current databases. The protein (406 residues in length) possesses N-terminal repeats rich in serine and methionine that might be involved in copper binding, two or three putative TMSs (residues 153–173 and either 251–271 or 241–261 and 262–281), and a C-terminal region that is O-glycosylated. Ctr1p is probably present in the membrane as an oligomer. Its mechanism of energy coupling has not been investigated. The synthesis of Ctr1p is induced by copper deficiency and repressed by copper excess.

The generalized transport reaction catalyzed by Ctr1p is

$$Cu^{2+} \text{ (out)} \rightarrow Cu^{2+} \text{ (in)}.$$

3. Copper Transporter-2 Family (TC No. 99.12)

Four proteins, one from *A. thaliana* (CopT1; 169 aa residues), one from humans (hCTR1; 190 residues), and two from *S. cerevisiae* (Ctr2p, 189 residues; Ctr3p, 241 residues) have been cloned, sequenced, and expressed in mutant *S. cerevisiae* (Eide and Guerinot, 1997; Kampfenkel *et al.*, 1995; Zhou and Gitschier, 1997). They have at least three homologs in the worm, *C. elegans* (162, 252, and 253 residues), one in humans (143 residues), and one in the protozoan *Theileria parva* (accession No. L06323; 480 residues).

The *H. sapiens, A. thaliana, S. cerevisiae,* and *T. parva* proteins all have three putative TMSs and display N-terminal hydrophilic sequences homologous to the methionine and histidine-rich Cu^{2+}-binding domains of various copper-binding proteins, including the P-type copper transporting ATPases (TC No. 3.3). It is not clear that all these proteins are localized to the plasma membrane, but the majority of the evidence implicates them in Cu^{2+} uptake. The energy-coupling mechanism has not been investigated.

The generalized transport reaction presumably catalyzed by proteins of the Ctr2 family is

$$Cu^{2+} \text{ (out)} \rightarrow Cu^{2+} \text{ (in)}.$$

4. Low-Affinity Cation Transporter Family (TC No. 99.20)

A single member of the low-affinity cation transporter (LCT) family has been sequenced and functionally characterized (Schachtman *et al.,* 1997). This protein, LCT1 of *Triticum aestivum* (wheat), possesses about 570 aa residues, 8–10 putative TMSs, and a hydrophilic amino terminus containing sequences enriched in proline, glutamate, serine, and threonine. The transporter was shown to mediate low-affinity (~10 m*M*) uptake of Rb^+, Na^+, and Ca^{2+}, but not Zn^{2+}, in a yeast mutant strain deficient in K^+ uptake. *LCT1* is expressed in low abundance in wheat roots and leaves. The mode of transport and energy-coupling mechanism (if any) are not known.

The generalized transport reaction believed to be catalyzed by LCT1 is

$$\text{Cation (out)} \rightleftharpoons \text{cation (in)}.$$

V. Transporters Encoded within the Yeast Genome

The results summarized previously reveal that there are far more eukaryotic-specific families of secondary carriers, functioning by uniport, symport, or antiport, compared with primary carriers. Together with André Goffeau's group, our laboratory has recently analyzed the complete genome of *S. cerevisiae* for various types of transporters (Paulsen *et al.,* 1998b). Some of the results, summarized in Tables XII and XIII, reveal that the transporters encoded within this genome are predominantly secondary carriers with few channels and primary carriers.

Two hundred fifty-six transporters were identified as a result of the complete *S. cerevisiae* genome analyses. Of these, only 7 (3%) proved to be recognizable channel proteins, 42 (16%) proved to be primary carriers, and 196 (77%) proved to be secondary carriers. The remaining 11 (4%) of

TABLE XII
Types of Identified Yeast Transporters

Transporter type	Number	% of total
Channel	7	3
Secondary carriers	196	77
Primary carriers	42	16
Unknown	11	4
Total	256	100
2°/1° carriers	4.9	

TABLE XIII
Representation of Large Families of Transporters Encoded within the Yeast Genome

Family	Number	% of total
MF	78	31
MC	35	14
APC	24	9
ABC	22	9
P-ATPase	16	6
MF + ABC	100	40
Total	256	
MF/ABC	3.6	

the recognized transporters function by unknown transport modes and therefore cannot be assigned to one of the recognized transport modes (TC No. 1–10).

Yeast primary carriers are all ATP-hydrolysis or electron flow-driven transporters, and the electron transfer-driven H^+-translocating systems are found only in mitochondria. Other potential mechanisms of energy coupling (see Tables I and II) are apparently lacking. The statistical analyses reported in Table XII reveal that there are nearly five times as many secondary carriers as primary carriers in agreement with the observation that few eukaryotic-specific transporter families include members that function as primary carriers.

Only three families of channel proteins are recognized in yeast. These families are the cation-specific VIC family (TC No. 1.5), the CIC family (TC No. 1.10), and the MIP family (TC No. 1.1) of aquaporins and glycerol facilitators. All these families are ubiquitous, found in all the major domains of life. Except for the yeast killer toxin, which is not chromosomally encoded, all recognized eukaryotic-specific channel protein families are found exclusively in animals. This fact presumably reflects the need for channel protein-mediated forms of communication in multicellular metazoans.

Table XIII tabulates and provides statistics for the largest families of paralogs encoded within the *S. cerevisiae* genome. By far the largest family is the ubiquitous MF superfamily with 78 paralogs encoded within the yeast genome. This family alone accounts for 31% of all of the encoded transporters.

The second largest family is the MC family. The proteins of this family are all nuclearly encoded although the proteins are found exclusively in intracellular organelles. Fourteen percent of all recognized transporters encoded within the yeast genome (35 proteins) are MC paralogs. This family is believed to have arisen in eukaryotes as discussed previously.

The third largest family, with 24 *S. cerevisiae* paralogs, is the amino acid–polyamine–choline (APC) family. Most of the members of this family actively accumulate amino acids by either uniport or proton symport. It is important to note that the three largest families of transporters encoded on the yeast genome include only secondary carriers. Because the AAAP family with eight *S. cerevisiae* paralogs may be distantly related to the APC family, the combined number of APC–AAAP family paralogs may be 32, showing that the yeast representation of this superfamily may be almost as great as that for the MC family.

The fourth and fifth largest families include members that are ATP hydrolysis-driven transporters. These families are the ABC superfamily (TC No. 3.1) with 22 members and the P-type ATPase family (TC No. 3.2) with 16 members. All remaining families have 8 or fewer paralogs. Thus, although about 40 families of transporters are represented in yeast, the largest five families account for nearly 70% of all encoded transporters.

VI. Transport Proteins for Which High-Resolution Three-Dimensional Structures Are Available

Several transport proteins and transport protein complexes have been successfully studied by X-ray crystallography. Most of these proteins are bacterial proteins. They include many bacterial channel proteins, including colicins Ia and E1 of *E. coli,* the Cry 1Aa and Cry 3Aa crystal proteins of *Bacillus thuringiensis,* α-hemolysin of *Staphylococcus aureus,* and aerotoxin of *Aeromonas hydrophila* (see our web site for primary references). A number of outer membrane bacterial porins have also been solved (Saier, 1998b). High-resolution three-dimensional structural data are also available for bacteriorhodopsin of *Halobacterium salinarium* and photosynthetic reaction center complexes of photosynthetic bacteria. Cytochrome oxidase, a redox-driven proton pump of *Paracoccus dentrificans,* has also been solved (see Saier, 1998a,b and our web site for references). Only for a few eukaryotic transport proteins are high- or moderately high-resolution structures available, including the pentameric acetylcholine receptor which surprisingly, and in contrast to most bacterial transport proteins, appears to exhibit a mixture of α and β structure. The central channel is formed of one α-helix per subunit, and these five helices are probably surrounded by a shell of β structure (Unwin, 1995).

The large multicomponent mammalian e^- transfer complexes found in mitochondria have also been examined. Thus, complex III (quinol:cytochrome c reductase) and complex IV (cytochrome c oxidase) of *Bos taurus* have both been solved (Tsukihara *et al.,* 1995, 1996; Xia *et al.,* 1997). Notewor-

thy is the fact that no chemically driven primary carrier, no facilitator or secondary active transport carrier, and no group translocator has yet been solved in three-dimensions. It is therefore clear that much work will be required before an understanding of the most common and most important types of eukaryotic transport systems will be put on a firm structural basis.

VII. Concluding Remarks

The eukaryotic-specific transporter families identified to date can be classified into several categories. There are 10 eukaryotic-specific channel and porin protein families, 8 of which are found only in animals. These proteins are described in Tables IV and V. The nonmetazoan-specific channel is found within the yeast killer toxin K1 family (TC No. 1.21), represented by a single member. Its origin cannot be estimated. The other is the anion-selective mitochondrial and plastid porin family (TC No. 9.8). It probably arose from bacterial porins and diverged beyond recognition following association of the endosymbiotic bacteria that gave rise to mitochondria and plastids with the eukaryotic cell. Different bacterial porins have also diverged in primary structure beyond the point of recognition.

Of the eight animal-specific, channel-forming protein families described in Tables IV and V, all function in ion transport. Two pairs of these families are structurally similar and are probably related evolutionarily. These two pairs of families are the ENaC and ACC families (TC Nos. 1.2 and 1.4, respectively) and the LIC and GIC families (TC Nos. 1.6 and 1.7, respectively). Each of these pairs of transporters may have arisen in animals. Three of the other eight animal-specific families exhibit structural or marginal sequence similarity to the ancient VIC family (Tables I, II, and IV): the RIR-CaC, IRK-C, and TRP-CC families (TC Nos. 1.9, 1.16, and 1.20, respectively). They may have their origins in the VIC family; if so, they have diverged in sequence almost to a point that prevents recognition of sequence similarity. Finally, the organellar Cl^- channel family (TC No. 1.17) does not exhibit characteristics of any of the other families and therefore may have arisen independently.

There are 13 eukaryotic-specific secondary carrier families listed in Tables VI and VII. For two of these [NSS (TC No. 2.22) and AcCoAT (TC No. 2.55)], bacterial homologs have been identified, and consequently they may stem from ancient origins. Surprisingly, the NSS family has eukaryotic representation only in animals, although hundreds of sequenced family members are available in this kingdom. No recognizable homolog of this family is encoded within the complete genome of *S. cerevisiae* (Paulsen *et al.,* 1998b). A member of the AcCoAT family, however, is encoded within

the *S. cerevisiae* genome. The ER location of the animal acetyl coenzyme A/coenzyme A antiporter is worthy of note.

Another family listed in Tables VI and VII, the AAAP family (TC No. 2.18), is probably distantly related to the large and ubiquitous APC family (TC No. 2.3). What is surprising in this case is that the sequence-divergent AAAP family members are found only in eukaryotes, whereas the ubiquitous APC family members are found in every domain of life, and sequence similarities among the latter group of proteins are easily identifiable. If the AAAP and APC families arose from a common origin, one must propose that the eukaryotic branch of this superfamily that gave rise to the AAAP family must have diverged more rapidly from its ancestral system than did any of the APC members, many of which are found in eukaryotes.

For the remaining 10 eukaryotic-specific carrier families listed in Tables VI and VII, we can be reasonably sure that the MC family (TC No. 2.29) arose specifically in eukaryotes after the establishment of mitochondria as eukaryotic organelles as discussed previously. The arguments in favor of this assumption (the unusual internal gene triplication, the occurrence of these proteins exclusively in eukaryotes, and the high degree of similarity observed for the three repeat units in the carrier proteins) have been evaluated elsewhere (Kuan and Saier, 1993; Saier, 1994, 1996).

Of the remaining nine secondary carrier families, four are found exclusively in animals (Tables VI and VII). However, one of these four families, the NST family, may be distantly related to the TPT family, and the latter family is found in both yeast and animals. Thus, the Sit, RFC, and OAT families represent examples of possible recently arising (or extensively diverging) families found in a single eukaryotic kingdom. The fact that no plant-specific, fungal-specific, or protozoan-specific families have been identified may be due to either a deficiency of sequence data for these kingdoms relative to the animal kingdom or the interesting possibility that animals have evolved families of sequence-divergent permeases to a much greater extent than have the other eukaryotic homologs. Availability of more extensive genome sequence data should allow resolution of this interesting question.

The five remaining families in Tables VI and VII (ZIP, AE, MTC, ENT, and OAT) represent reasonable possibilities for families that may have arisen in eukaryotes. It is interesting to note that of the 13 secondary carrier families in Tables VI and VII, 5 are found exclusively in organelles, whereas 8 may be localized exclusively to the plasma membrane. Moreover, a unique, or nearly unique, organellar localization is observed for each of the five families of organellar transporters. These unexpected observations must have molecular explanations that are based in evolutionary theory. Mechanistically, the subcellular localizations observed for these proteins must reflect the presence of specific targeting sequences or structural features.

Only three primary carrier families, all pyrophosphate bond hydrolysis-driven systems, may be eukaryotic specific (Tables VIII and IX). While the MPT family (TC No. 3.8) may be eukaryotic specific, evidence has been presented that the other two families, the chloroplast envelope protein translocase family (TC No. 3.9) and the V-PPase family (TC No. 3.10), may have bacterial origins. Thus, eukaryotic-specific primary carriers are likely to be extremely rare and maybe nonexistent.

Finally, the four eukaryotic-specific transporter families of unknown mode of action include members that are all specific for inorganic cations (Tables X and XI). Very little can be said about the origins of these proteins. It will be of particular interest to determine if any of these families of proteins are characterized by unique modes of action or novel energy-coupling mechanisms.

In conclusion, it has been shown that eukaryotic-specific transporters (and indeed, eukaryotic transporters in general) are to a surprising degree mechanistically restricted. Thus, while animals have apparently proliferated channel protein families and secondary carriers, very few (if any) other types of families are restricted to eukaryotes. In addition to pyrophosphate bond hydrolysis and electron flow-driven proton pumps, there are no other types of transporters identified in eukaryotes. This fact is particularly surprising in view of the many distinct energy-coupling mechanisms that have evolved in prokaryotes (Tables I and II; Saier, 1998a,b; see our web site).

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Neural Plasticity in the Adult Insect Brain and Its Hormonal Control

Colette Strambi, Myriam Cayre, and Alain Strambi
CNRS, LNB 5, 13402 Marseille Cedex 20, France

Neural plasticity in the adult insect brain has not been widely explored and research on its control by insect morphogenetic hormones (ecdysteroids and juvenile hormone) is still in its infancy. Numerous examples of hormone-induced behavioral changes in adult insects will provide excellent models to explore the properties of underlying neurons and neural circuitry involved in the expression of adult behaviors. The recent discovery of a persistent neurogenesis modulated by hormones in the mushroom bodies of some adult insect species offers opportunities to develop research on the fate, functions, and possible involvement of new neurons in behavioral processes.

KEY WORDS: Neural plasticity, Hormones, Insects.

I. Introduction

Many examples of neural plasticity exist in both vertebrate and invertebrate nervous systems. In this review, neural plasticity refers to neurogenesis and to plasticity of neurites and synapses. All these phenomena naturally occur to modify the nervous system from an embryo to the adult.

In adult vertebrates, differentiation of neuronal networks is under hormone control. Numerous studies, recently reviewed (Kawata, 1995), have shown the roles of steroid hormones in these developmental processes and in neural plasticity. Thus, neurogenesis, neuronal migration, and cell death are regulated by steroid hormones. The occurrence of postnatal neurogenesis in the mammalian brain was first evidenced by Altmann (1963, 1967). Further work demonstrated that neurogenesis persists throughout adult life in the rat olfactory bulb and dentate gyrus (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). Moreover, recent data show neurogenesis occurring in the dentate gyrus of an adult primate, the marmoset monkey (Gould *et al.*, 1998). Similarly, neurogenesis persists in the telencephalon of song birds; birth and migration of new neurons still occur after sexual maturity and are observed in the adult brain. Newly generated neurons migrate to the high vocal center and are inserted into functional pathways (Bottjer *et*

al., 1986; Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Alvarez-Buylla *et al.,* 1992). Although gonadal steroids have been involved in the control of neurogenesis in the canary brain (Nordeen and Nordeen, 1989), further experiments suggested that in adult canary telencephalon, neurogenesis was independent of gonadal hormone levels (Brown *et al.,* 1993). Adrenal steroids influence the developmental processes of the rat dentate gyrus. They regulate neurogenesis (Cameron and Gould, 1994; Gould and Cameron, 1996) and are required for granule cell survival (Sloviter *et al.,* 1989; Gould, 1994). By contrast, adrenalectomy was seen to induce granule cell degeneration through programmed cell death (Sloviter *et al.,* 1989, 1993). However, removal of circulating adrenal steroids was also shown to increase the number of newly generated granule cells in the adult rat (Cameron and Gould, 1994). Thus, adrenal steroids have differential effects in the dentate gyrus where they are inducing or preventing both neuronal birth and death (Gould and McEwen, 1993). Recent experiments indicate that distinct populations of cells in the adult dentate gyrus undergo mitosis or apoptosis in response to adrenalectomy (Cameron and Gould, 1996). Although steroid hormones are clearly involved in the regulation of neurogenesis and apoptosis, their influence may not be direct. Recent findings indicate that other factors could be implied in the possible mechanisms underlying cell birth and death in the dentate gyrus (Gould and McEwen, 1993). Steroid hormones can also affect axonal and dendritic development. In the spinal nucleus of bulbocavernosus of adult rats, androgens induce growth of motoneurons somata and dendrites (Breedlove and Arnold, 1981; Kurz *et al.,* 1986); they also regulate the size and frequency of gap junctions between motoneurons (Matsumoto *et al.,* 1988). In the same way, gonadal hormones induce dendritic growth in the adult avian brain and formation of new synapses (de Voogd and Nottebohm, 1981; de Voogd *et al.,* 1985). This overview emphasizes the importance of hormones and especially steroid hormones in the development and plasticity of the adult vertebrate nervous system.

In insects, the patterns of neural reorganization during postembryonic development have been described in numerous species (Edwards, 1969; Pipa, 1978), and developmental changes in the anatomy of the nervous system as well as remodeling of neuronal networks were seen to be correlated with the molting cycle (Truman, 1988; Weeks and Levine, 1990). The morphogenetic hormones (juvenile hormones and ecdysteroids) control insect molts and the changes occurring in body form and behavior during the insect preimaginal life offer excellent opportunities to examine changes in neuronal structures. The isolation and synthesis of insect morphogenetic hormones, and the development of radioimmunoassays sensitive enough to permit individual determination of hemolymph hormone titers, allowed major advances in our knowledge of the hormonal control of nervous system development. Much attention has focused on the role of hormones during larval development and metamorphosis of the moth *Manduca sexta*

(a large insect), the endocrinology of which is well characterized (Fain and Riddiford, 1976; Bollenbacher *et al.,* 1981), and recently of *Drosophila melanogaster* (Truman, 1990). Neuroblast proliferation was studied during the postembryonic development of *Drosophila* (Truman and Bate, 1988) and was seen to be regulated by ecdysteroids in *Manduca* (Booker and Truman, 1987a,b). In *M. sexta,* morphological and functional changes that accompany metamorphosis in identified sensory neurons are induced by changes in hormone environment and both ecdysteroids and juvenile hormone act on the cell body to regulate growth of their central processes (Levine, 1989; Levine *et al.,* 1986). Similarly, the outgrowth of new dendritic arbors of larval motoneurons is dependent on ecdysteroids and can be prevented by juvenile hormone (Truman and Reiss, 1988). Dendritic regression of motoneurons was also seen to be triggered by ecdysteroids (Truman and Reiss, 1988; Weeks, 1987; Weeks and Truman, 1985, 1986). Modulation in neuropeptide content of identified motoneurons is hormonally regulated (Witten and Truman, 1996), and ecdysteroids were seen to switch the type of neuropeptide synthesized in some abdominal neurosecretory cells (Loi and Tublitz, 1993; Tublitz and Loi, 1993). Neuronal death plays an important role during embryonic and postembryonic development (Truman *et al.,* 1990, 1992). In *Manduca* (Truman and Schwartz, 1984) and *Drosophila* (Kimura and Truman, 1990), neuronal death is ecdysone regulated and coupled with a specific ecdysone receptor isoform (Robinow *et al.,* 1993; Truman *et al.,* 1994).

The occurrence of neural plasticity in adult insects was pointed out by some authors (Murphey, 1986a; Bulloch and Ridgway, 1989). Thus, in the cockroach *Periplaneta americana,* giant interneurons deprived of their ipsilateral cercal input recovered their ability to respond to wind (Vardi and Camhi, 1982). Similarly, plasticity was evidenced in the cercal sensory system of crickets (Murphey, 1986b). In the same way, it has been shown in the cricket auditory system that unilateral deafferentation, by preventing the development of the auditory nerve, resulted in dendritic sprouting and formation of new connections in the contralateral auditory neuropil (Hoy *et al.,* 1985; Schildberger *et al.,* 1986; Brodfuehrer and Hoy, 1988). Few studies have been devoted to the naturally occurring neural plasticity in adult insects and its hormonal control. Two reasons can explain why data are so scarse: (i) There was a strong belief in the static and hard-wired status of the adult nervous system and (ii) the nature and the physiological roles of insect morphogenetic hormones can be diverse among insects—thus, no single model can fit all insects.

II. Adult Insect Endocrinology

Two main morphogenetic hormones, ecdysteroids (ecdysone and 20-hydroxyecdysone) and juvenile hormones, control insect metamorphosis

(Sehnal, 1989; Gäde *et al.,* 1997; Riddiford, 1994). They are also produced in adults, in which they are involved in reproductive functions (Hagedorn, 1985, Wyatt and Davey, 1996).

A. Ecdysteroids

Ecdysone, extracted from silkworm pupae, was isolated in a crystalline pure form by Butenandt and Karlson (1954) and recognized as a steroid. Further works led to the identification of 20-hydroxyecdysone (Hoffmeister and Grützmacher, 1966; Horn *et al.,* 1966) and to numerous ecdysone-like substances called ecdysteroids (Rees, 1989). Among them, a homolog of ecdysone, makisterone A, was recognized as the molting hormone in Heteroptera and the major ecdysteroid in Hymenoptera and some Diptera (Feldlaufer *et al.,* 1986a,b). In larval insects, ecdysone biosynthesis takes place in ecdysial glands, and it occurs in ventral glands in apterygotes, the ring gland in Diptera, and the prothoracic gland in most insect groups (Herman, 1967). In adult insects, the presence of ecdysteroids in the ovaries was recognized more than 20 years ago (Onishi *et al.,* 1971; Hagedorn *et al.,* 1975; Lagueux *et al.,* 1977), and it is now well-known that ovarian follicle cells produce ecdysteroids *de novo* (Goltzené *et al.,* 1978). However, in some Lepidoptera, male gonads incubated *in vitro* release in the medium large amounts of ecdysteroids produced by the follicular sheath of the testes (Loeb *et al.,* 1984).

B. Juvenile Hormone

Secreted by two cephalic endocrine glands, the corpora allata, juvenile hormone (JH) was first discovered by Wigglesworth (1936). Its structure was elucidated by Röller *et al.* (1967) as a sesquiterpene having a 10,11-epoxide link. Several forms of JH have been recognized (Riddiford, 1994) that differ by the lengths of their side chain (C18 = JH I, C17 = JH II, C16 = JH III, and C19 = JH 0) or by having an additional 6,7-epoxide (JHB3; Richard *et al.,* 1989). Whereas in most insects JH III is the unique JH observed, JH 0, JH I, and JH II exist only in Lepidoptera (Schooley *et al.,* 1984), and JHB3 was found in higher Diptera (Richard *et al.,* 1989; Cusson *et al.,* 1991). Although mainly secreted by the corpora allata, other sources of JH have been found. In *Cecropia* silkmoth, JH is synthesized in the male accessory glands and then transfered to the female during mating (Shirk *et al.,* 1980, 1983). Similarly, the male accessory glands of *Aedes aegypti* produce JH I and JH III (Borovsky *et al.,* 1994).

C. Functions and Modes of Action of Insect Hormones

The functions of JH in the control of adult reproduction are fairly well-known (Wyatt and Davey, 1996). In adult female, the hormone stimulates

oocyte growth, vitellogenin synthesis in the fat body, and absorption of vitellogenin by the oocytes. By contrast, fewer reports concern the role of ecdysteroids in adult insects (Hagedorn, 1985, 1989). Moreover, although much evidence indicates that hormones affect adult behavior, only a few data focus on hormone actions on the nervous tissue.

Two kinds of action were recognized for JH: regulation and priming (Truman and Riddiford, 1974; Wyatt and Davey, 1996). The control of a function in a dose-dependent and immediate way is referred as to regulation (Truman and Riddiford, 1974) or releasing (Wyatt and Davey, 1996). The primer or modifier effect is slow to appear and prepares the tissue to change its responsiveness to a stimulus. Direct actions of JH at the cell membrane have been recognized in ovarian follicle cells and in male accessory glands; JH also modulates nuclear gene expression and recent reviews summarize the current knowledge of the mechanisms of action of JH (Riddiford, 1994; Jones, 1995; Wyatt and Davey, 1996). Despite numerous attempts, JH receptors have not been clearly identified; however, recent data suggest that USP can bind specifically to ligands of the JH family and could be a JH receptor (Jones and Sharp, 1997).

Although rapid actions of ecdysteroids at the cell membrane level have been observed (Robert *et al.*, 1986), most of the data concern ecdysteroid nuclear receptors. Recently isolated and cloned, the ecdysone receptor codes for three protein isoforms (Koelle *et al.*, 1991; Talbot *et al.*, 1993), two of which (EcR-A and EcR-B1) are mainly expressed in the central nervous tissue (Truman *et al.*, 1994). EcR proteins are activated to form heterodimers with another steroid receptor encoded by the *Drosophila* gene *ultraspiracle* (*usp*) (Yao *et al.*, 1992), and USP expression has also been observed in the nervous tissue (Oro *et al.*, 1992; Henrich and Brown, 1995).

D. Two Examples of Changes in Hormone Titers during Adult Life

Because hormone actions on the nervous system have been especially studied in the honeybee and in the cricket, we shall briefly review the life and changes in hormone titers occurring in these two models.

1. Hormone Titer Fluctuations in the Adult Honeybee

Among social insects comprising bees, ants, wasps, and termites, honeybees represent one of the best studied species. The colony usually consists of a reproductive queen which can live for several years, unreproductive worker bees having a 1- or 2-month life in summer, and drones having a short life and dying after mating. During their adult life, a clear division of labor exists among worker honeybees that was defined as age polyethism by

Winston (1987). In brief, young worker bees take care of the brood and are considered nurse bees during the first week of their life, then they perform different tasks in the hive, and finally, at the age of about 3 weeks, they start to forage outside the hive, becoming foragers.

Studies using bioassay indicated that JH titers were increasing during worker honeybee life (Fluri *et al.*, 1982). Further works using radioimmunoassays for direct measurement of blood JH levels, and *in vitro* studies of JH biosynthesis by corpora allata, demonstrated that JH III was the bee JH and that JH titers changed with aging. Whereas JH levels (Fig. 1a) were low in newborn bees, these titers were slightly increasing in 7-day-old nurses and became very high in 3-week-old foragers (Goodman *et al.*, 1993; Huang *et al.*, 1991, 1994; Robinson *et al.*, 1987, 1989).

By contrast to workers, queens present a peak of JH on their first day of adult life (Fig. 2a), and then JH titers drop and remain low throughout the queen life (Fahrbach *et al.*, 1995a). Circulating levels of JH in the drones (Fig. 3a), which were low at adult emergence peaked at the time of the first flight and then decreased (Fahrbach *et al.*, 1997).

Determination of ecdysteroid hemolymph titers demonstrated that in a queenright colony there were no measurable ecdysteroid titers in newborn workers, nurses, and foragers. Only in egg-laying workers from a queenless colony were ecdysteroids detectable. Queens had significantly higher levels of ecdysteroids than did laying workers, and among ecdysteroids makisterone A was the major compound (Feldlaufer *et al.*, 1986a,b; Robinson *et al.*, 1991).

2. Hormone Titer Fluctuations in the Adult Female Cricket

The adult crickets live for about 2 months. In females, the ovaries develop in about 5 days at 29°C in a 16-h light/8-h dark photoperiod. The panoistic ovaries contain about 150 ovarioles per ovary; the maturing terminal ovocytes are stored in the genital chamber before being laid in the soil in groups of 10–20. Because terminal ovocytes do not mature in a completely synchronous way, the waves of maturing ovocytes rapidly overlap. Thus, the first ovarian cycle is the most convenient period in which to analyze the titers of the two hormones involved in ovarian development.

As seen Figure 4b, after a weak elevation of JH titer, 1 day after emergence, two increases of JH levels occur, which are respectively related to previtellogenesis for the small increase and to vitellogenesis for the large increase (Renucci and Strambi, 1983; Renucci *et al.*, 1984).

During ovarian maturation, the follicular cells surrounding the follicles enlarge and secrete ecdysteroids (Fig. 4b), the titers of which increase in hemolymph, and a large peak of circulating ecdysteroids characterizes the end of egg maturation (Renucci and Strambi, 1981). In *Acheta domesticus,* analysis of JH production either *in vivo* or *in vitro* evidenced only JH III (Strambi *et al.*, 1984); among ecdysteroids, ecdysone appeared to be the

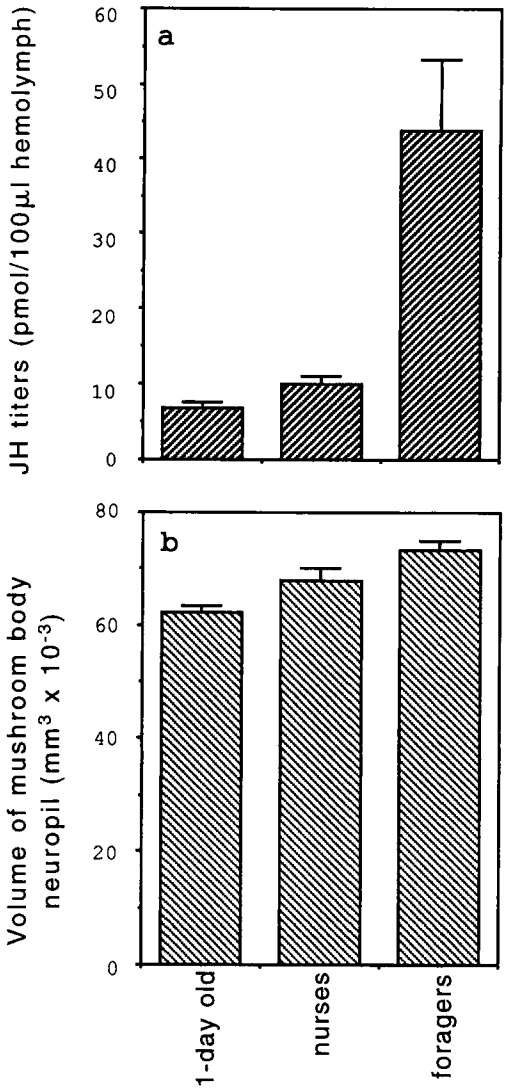

FIG. 1 (a) Blood JH titers in worker honeybees (mean value ± SE) (adapted from Huang *et al.*, 1991). (b) Median volumes of mushroom body neuropiles per hemisphere in worker honeybees (mean value ± SE) (adapted from Withers *et al.*, 1993).

major compound, although traces of 20-hydroxyecdysone were observed in hemolymph and ovarian extracts (Renucci *et al.*, 1987). In the house cricket, allatectomy performed during the last larval instar completely suppressed JH production and ovarian development; similarly, in females ovariectomized before emergence there was no trace of circulating ecdysteroids (Renucci and Strambi, 1981; Renucci *et al.*, 1990).

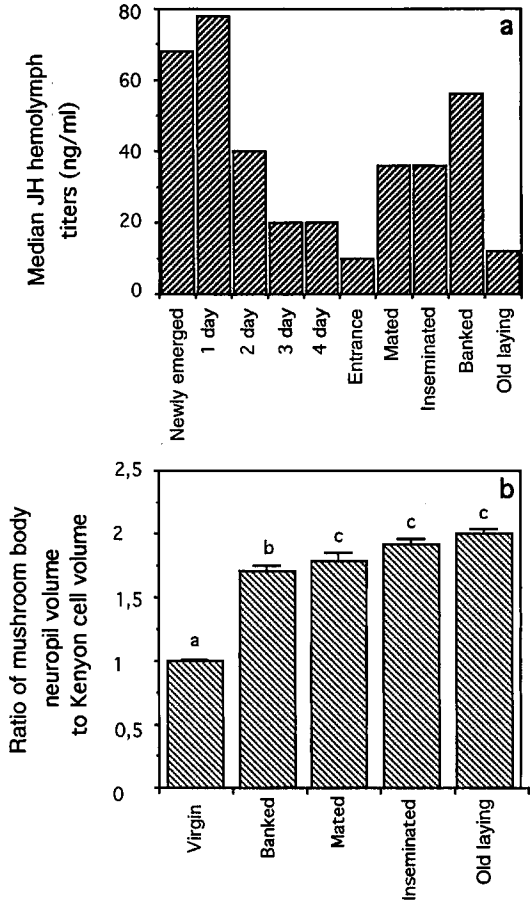

FIG. 2 (a) Median juvenile hormone titers for queen honeybees. (b) Mean ratio (± SE) of neuropil volume to Kenyon cell body region volume for queen honeybees. Only bars marked with different letters differed significantly ($p < 0.05$). "Banked" queens refer to newly emerged queens placed in a colony for the storage of queens (adapted from Fahrbach *et al.*, 1995a).

III. Behavioral Plasticity and Its Hormonal Control

A. Hormonal Modulation of Adult Behaviors

Hormone-induced behavioral changes are evidence of the actions of hormones on the nervous system. Numerous works have pointed out the role of hormones and especially JH on adult behaviors (Truman and Riddiford, 1974; Wyatt and Davey, 1996).

Sexual receptivity and its endocrine control has been reviewed by Ringo (1996). The data indicate a large variability in the JH control of receptivity

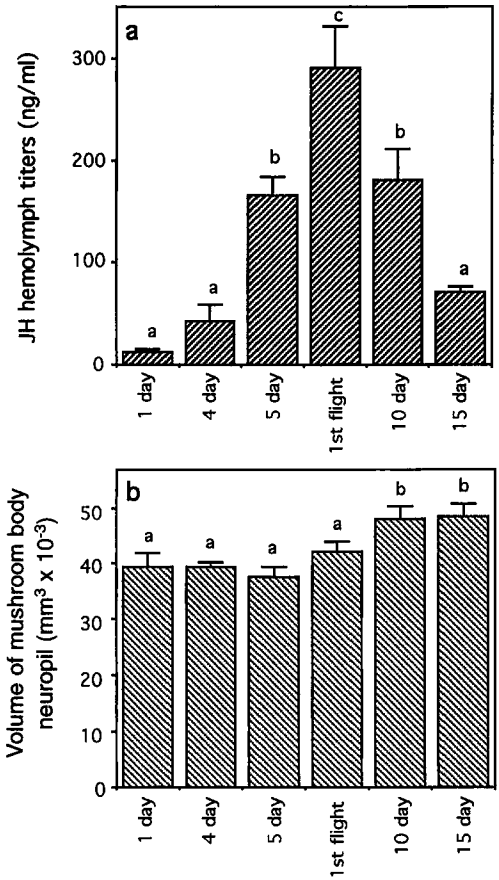

FIG. 3 (a) Blood JH titers for done honeybees at different stages of behavioral development. Bars indicate mean ± SE; those with different letters are significantly different from one another ($p < 0.05$). (b) Volume estimates for the neuropil of the mushroom bodies in drone bees. Bars with different letters are significantly different from one another ($p < 0.05$) (reprinted from *Neurosci. Lett.* **236,** Fahrbach, S. E., Giray, T., Farris, S. M., and Robinson, G. E., Expansion of the neuropil of the mushroom bodies in male honey bees is coincident with the initiation of flight, 135–138, copyright 1997, with permission from Elsevier Science).

between different species and even within the same family. In crickets, allatectomy increases response times and thresholds to the calling male but does not abolish receptivity (Renucci *et al.,* 1985; Loher *et al.,* 1992). In Acrididae receptivity is either controlled or not by JH depending on the family (Strong and Amerasinghe, 1977; Strambi *et al.,* 1997). Thus, in *Gomphocerus rufus,* allatectomized females become unreceptive to mating, this effect can be reverted by JH injection and feedbacks between corpora allata activity and behavior have been evidenced (Hartmann *et al.,* 1994).

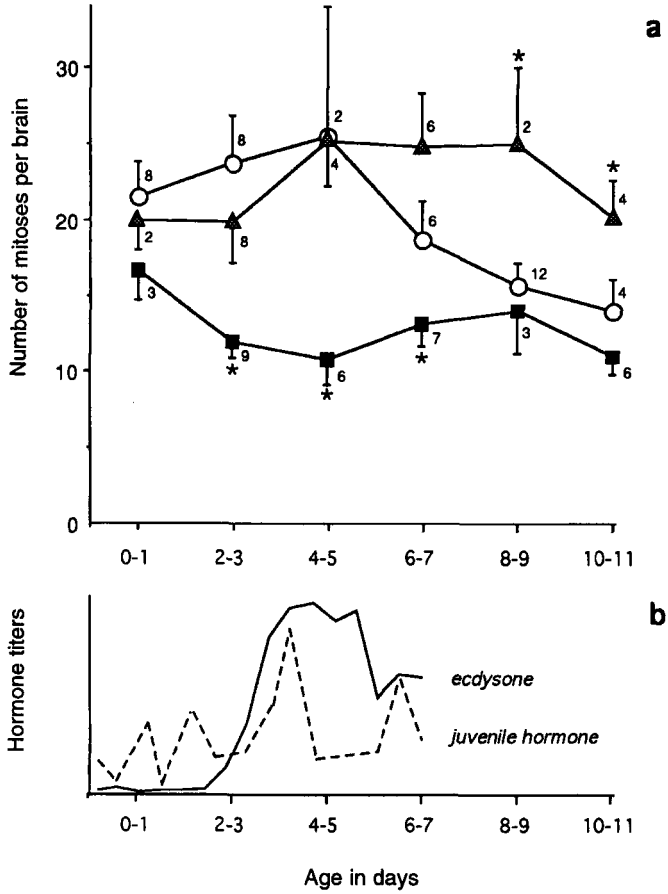

FIG. 4 (a) Mitotic index in control (○), ovariectomized (▲), and allatectomized (■) adult female house crickets, examined from emergence to Days 10 or 11 (numbers refer to sample size, asterisks indicate significant differences between operated and control females, $p < 0.05$). (b) Hemolymph JH and ecdysone titers in adult female house crickets during the beginning of imaginal life (reprinted from *J. Insect. Physiol.* **37,** Huang, Z. Y., Robinson, G. E., Tobe, S. S., Yagi, K. J., Strambi, C., Strambi, A., and Stay, B., Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis, 733–741, copyright 1991, with permission from Elsevier Science).

In *Drosophila,* the *apterous* mutation results in a reduced JH production and a lower receptivity which is partially restored by topical applications of a JH analog (Ringo *et al.,* 1991). Similarly in *Lucilia cuprina* and *Calliphora vomitoria,* female sexual receptivity increases after topical application of JH analogs (Barton-Browne *et al.,* 1976; Trabalon and Campan, 1985).

Pheromones are widely involved in the reproductive behavior of many insect species. The roles of JH in pheromone production have been clearly evidenced and the behavioral consequences of JH deprivation and/or supplementation can be found in the recent synthesis of Wyatt and Davey (1996). Similarly, in migratory species, JH has a striking influence on flight activity (Wyatt and Davey, 1996).

The behavioral program of *Culex pipiens* depends on JH. To become host responsive, newly emerged adults require a nectar meal which increases JH titer. After emergence, females prefer honey odors and the switch from honey to blood-host odor can be induced, without sugar feeding, by topical applications of JH analogs (Hancock and Foster, 1992). The influence of morphogenetic hormones on the establishment of dominance hierarchies has also been shown among foundresses of the paper was *Polistes gallicus* (Röseler *et al.,* 1984; Röseler, 1985).

In the female burying beetle *Nicrophorus orbicollis,* JH titers increase at the time when young larvae hatch; this peak in JH could possibly be associated with behavioral demands of parental care (Trumbo *et al.,* 1995; Trumbo, 1997).

Despite numerous models in which hormonally induced changes have been evidenced, only recent studies have examined the effects of hormones on the nervous system. Particular attention has been devoted to division of labor or age polyethism in the honeybee and to phonotactic and oviposition behavior in the house cricket. We shall briefly describe the behaviors modulated by hormones.

B. Age Polyethism in the Honeybee

Bees present identifiable behaviors that are regulated by hormones. As early as 1982, Fluri *et al.* had shown that old workers had higher JH titers than young workers, only recently has a clear correlation been demonstrated between JH titers and foraging. Experimental conditions can be developed to force young bees to become precocious foragers. In colonies consisting of a queen and 1-day-old worker bees, some individuals become foragers as soon as Day 4 after emergence. In these precocious foragers, JH titers are high and similar to those observed in control 3-week-old foragers (Robinson *et al.,* 1989; Huang *et al.,* 1991; Huang and Robinson, 1992). In a reverse way, depletion of nurse bees from a colony induces old workers to take care of the brood, thus becoming overaged nurses that present JH titers in the same low range as normally aged nurses (Robinson *et al.,* 1989). In response to experimentally induced or naturally occurring changes in colony age demography, behavioral reversion may also occur and forager bees can revert their behavior to nursing; in these reverted nurses, JH titers also drop to low values (Robinson *et al.,* 1992). Taken

together, these results suggested that JH was acting on the central nervous system to induce behavioral changes.

C. Phonotactic Behavior in the House Cricket

Female crickets recognize males and respond phonotactically to the calling song of a conspecific male. Their phonotactic response consists of orienting to and approaching the calling male (Stout *et al.,* 1976, 1983; Thorson *et al.,* 1982). The phonotactic response of *Acheta domesticus* begins 3 or 4 days after adult emergence. Using acoustic models of male calls, it was shown that the song intensity threshold of the female response decreased concomitantly with the increase in JH biosynthesis that peaks on Day 4 postemergence. Allatectomy of sexually responsive females resulted in a deterioration of their phonotactic orientation and sexual responsiveness to males; topical administration of JH III or JH analogs restored their responsiveness (Koudele *et al.,* 1987). Moreover, the attractive range of calling song syllable periods became broader with age, with the female becoming less selective; again, JH III treatment restored female selectivity (Stout *et al.,* 1989; Walikonis *et al.,* 1991).

D. Oviposition Behavior in the House Cricket

In *A. domesticus* (Destephano *et al.,* 1982), as in other crickets (Sugawara, 1986; Sugawara and Loher, 1986), egg-laying behavior consists of several behavioral sequences. The female first explores the egg-laying substrate, checking its moisture and granulometry through palpation movements with antennae and mouth parts; after a 180° turn, the female inserts its ovipositor into the soil, performing alternative movements which allow the eggs to slip between the four valves of the ovipositor and to be deposited in the soil. Up to 120 eggs per day can be deposited through successive egg-laying sequences. In contrast to the classical method, which consisted in collection of the eggs laid during given time periods, a special device was designed which continuously registered egg-laying movements during long periods of time. Briefly, it consisted of a radioactive ^{192}Iridium wire glued on the ovipositor; the γ-rays emitted were recorded using a scintillation probe connected to a computer (Renucci and Rage, 1985). Such a device demonstrated that ovipositor movements do not depend on the presence of mature eggs: Virgin females present ovipositor movements as soon as 2 or 3 days after emergence at the beginning of vitellogenesis; in the same way, females ovariectomized during the last larval instar lack ovaries but still perform ovipositor movements which appear in the same temporal delay as that in intact females (Sugawara, 1986; Renucci *et al.,* 1988). By contrast, allatec-

tomy performed during the last larval instar completely suppressed the egg-laying behavior, which could only be restored by corpora allata implantation or JH injections. However, when allatectomy was performed in adult females after the occurrence of the first rise of JH titer, egg-laying behavior was not affected and appeared at the same time as in controls (Renucci *et al.,* 1992). Because the reappearance of oviposition behavior in allatectomized females supplemented with JH took 1 to several days, this effect can be recognized as a primer effect of JH. The occurrence of a temporal delay between hormone supplementation and its behavioral effect necessarily evoked morphological and/or biochemical changes in the central nervous system under hormone influence.

IV. Neural Plasticity

A. Neuronal Plasticity

During insect metamorphosis, the hormonal regulation of neuronal reorganization has been described in sensory neurons (Levine *et al.,* 1986; Levine, 1989), motoneurons (Levine and Truman, 1985; Weeks and Ernst-Utzschneider, 1989), and interneurons (Waldrop and Levine, 1992). Despite more restricted data recorded during imaginal life, adult neuronal plasticity has been evidenced.

Hormones can affect sensory neurons. It has been shown that solitary adults of *Locusta migratoria,* usually characterized by high JH titers, had more olfactory sensilla on the antennae than gregarious phase insects (Greenwood and Chapman, 1984). In the cockroach *Blatella germanica,* JH treatment of last larval instar females led to retention of larval sensilla characteristics on the antennae and palps of the resulting adults that present a reduced electroantennogram response (Ramaswamy and Gupta, 1981).

Aging also alters sensory neurons. In *D. melanogaster,* the morphology of a mechanosensory neuron can change during aging and a decrease in the number of its side branches and varicosities occurs between emergence and 30 days of age (Corfas and Dudai, 1991).

Some neurons of adult crickets can sprout or retract processes from their somata, axons, and dendrites in response to lesions (Roederer and Cohen, 1983). Naturally occurring changes have also been reported. In *Teleogryllus oceanicus,* the omega neuron ON1, an intraganglionic interneuron that branches in the auditory neuropile of the prothoracic ganglion, presents an axon that ascends to the brain. An age-dependent loss of this ascending axon has been described: Present in 75% of young adults, it can be detected only in 30% of older animals (Atkins and Pollack, 1986).

The number of Kenyon cell fibers in mushroom body peduncles of adult *D. melanogaster* presents age-dependent changes. Thus, an increase in fiber number occurred during the first week of adulthood; after a plateau, a decline was observed during the third week of adult life. Sex-linked differences were also observed and significantly more fiber profiles were found in the peduncle in females than in males (Technau, 1984). Moreover, living conditions were seen to affect the number of Kenyon cell fibers. Flies reared in groups, in large cages, and submitted to various visual and olfactory stimulations presented 15% more Kenyon cell fibers than flies maintained in small plastic vials in the dark (Technau, 1984). Such changes may possibly be related to olfactory learning and, in fact, further works indicated that olfactory learning mutants did not present such an experience-dependent neural plasticity (Balling *et al.,* 1987). In the same species, the breeding of larvae also had profound effects on the number of Kenyon cell fibers in the peduncle. At hatching, in larvae reared in crowded conditions there was a larger number of fibers compared to isolated flies. Complementary experiments indicated that this effect could be due to a diffusible factor acting either to enhance or to suppress fiber number (Heisenberg *et al.,* 1995).

Changes in the axon volume have also been reported: The monopolar axons from the first optic neuropile of *Musca domestica* undergo daily fluctuations in diameter that have a circadian basis (Pyza and Meinertzhagen, 1995).

The development of cell culture studies allowed demonstration of a direct action of hormones on neuronal morphology. Mostly collected during insect metamorphosis, the data indicate that both ecdysteroids and JH can act on neurite outgrowth (Levine and Weeks, 1996). Thus, identified thoracic ganglia motoneurons of the moth *M. sexta,* maintained *in vitro,* increase their neurite outgrowth in the presence of 20-hydroxyecdysone, an effect that can be blocked by the addition of a JH analog (Prugh *et al.,* 1992). In contrast, in primary culture, the antennal lobe interneurons from the same insect are minimally responsive to ecdysteroids. Among the six identified neuronal types, only a local interneuron and a projection neuron responded to increasing concentrations of 20-hydroxyecdysone, and the response in the latter was a decrease in neurite outgrowth (Oland and Hayashi, 1993). The development of *in vitro* culture of adult Kenyon cells provided a useful tool to examine the possible hormone actions on dissociated mushroom body cells (Cayre *et al.,* 1998). From preliminary experiments it appears that addition of physiological concentrations of ecdysone to the tissue culture medium induces a significant increase in the percentage of neurons growing neurites and a significant lenghtening of their neurites (M. Cayre *et al.,* manuscript in preparation).

It must be remembered that neuromediators can also act as growth regulatory signals in the developing nervous system (Lauder, 1993), and

during the metamorphic development of *M. sexta,* serotonin has been seen to enhance the neurite growth of antennal lobe interneurons in primary cell culture (Mercer *et al.,* 1996). In adult insects, age-dependent changes in the concentration of neuroactive compounds have been identified. Glutamate and γ-aminobutyric acid concentrations, low at emergence, increase in 10-day-old honeybees (Fuchs *et al.,* 1989). In the same insect, an increase in dopamine levels was similarly observed during adult behavioral development, with forager bees having significantly higher dopamine levels than young bees (Taylor *et al.,* 1992). It remains to be determined whether dopamine can alter adult neuron morphology and if the increase in amine concentration is modulated by the changes in JH titers occurring during the worker bee life.

B. Synaptic Plasticity

Naturally occurring or experimentally induced synaptic plasticity has been evidenced in adults of different insect species. In the first optic neuropil of the fly, Fröhlich and Meinertzhagen (1982) reported a decrease in the number of photoreceptor synapses during the first 3 weeks of the adult life. In the honeybee, electroantennography recordings evidenced a maturation of the olfactory response in workers that takes place during the 4 days following emergence. Moreover, modifications of the environment induce a drastic modification of the olfactory response, which strongly decreases in bees that are socially and olfactory deprived (Masson and Arnold, 1984). Additional experiments demonstrated that olfactory deprivation reduced the volume of the antennal lobe and glomeruli as well as synape frequency (Gascuel and Masson, 1987). Illumination regime can also affect the size and frequency of synapses. Thus, selective wavelength deprivation has been shown to reduce the number of synapses of the honeybee photoreceptors with a sensitivity maximum in the deprived part of the spectrum (Hertel, 1983). In the housefly, short exposures to light after long periods in the dark induce an increase of small synapses in the lamina, and daily and circadian rhythms of synaptic frequency have been evidenced (Kral and Meinertzhagen, 1989; Rybak and Meinertzhagen, 1990; Pyza and Meinertzhagen, 1993).

Variations in the morphology of dendritic spines in mushroom bodies were also reported in distinct stages of behavioral development of honeybee workers: Foragers exhibit spines with markedly longer profiles areas and shorter stems than newly emerged and nurse bees (Coss *et al.,* 1980). A rapid dendritic stem shortening was also observed during the first orientation flight of the honeybee (Brandon and Coss, 1982). Taking into account our current knowledge of JH titer fluctuations in worker honeybees (see

Section II,C), one wonders if these changes in dendritic spine morphology could be related to JH.

Changes in the degree of excitatory and inhibitory inputs of an auditory neuron are modulated by JH. In the house cricket, JH has been seen to regulate the sensitivity of an auditory neuron's response to sound. Auditory neurons send their axons to relay interneurons located in the prothoracic ganglion. Among L-shaped prothoracic interneurons that receive inputs from auditory fibers and send their axons to the brain, the L1 auditory interneuron was shown to be necessary and sufficient for phonotactic response threshold (Atkins *et al.,* 1992; Stout *et al.,* 1985). In addition, the prothoracic neuron L3 was shown to respond selectively to the syllable period characteristic of the male calling song (Henley *et al.,* 1992). On the basis of these observations, the influence of JH on the activity of interneurons involved in conduction of the auditory message has been studied (Stout *et al.,* 1991, 1992; Stumpner *et al.,* 1995; Stout *et al.,* 1998). Applications of JH III to 1-day-old females caused both the threshold of phonotaxis and the threshold of the L1 auditory neuron to drop precociously to levels usually observed on Day 3. This effect of JH III on both phonotaxis and L1 electrical activity could be prevented by injection of transcription or translation blockers (Stout *et al.,* 1991). Similarly, JH III was seen to regulate L3 activity. A decrement in the selective response of L3 to a syllable period of 50–70 ms occurred in an age-dependent manner. Application of JH III to old females restored their L3 response to a level similar to that of young females (Henley *et al.,* 1992). Although the hormone response kinetics differed for the two interneurons (2 h for L1 and 2–4 days for L3), it was hypothesized that JH was inducing neurotransmitter receptor protein expression. Using *in situ* hybridization, expression of a nicotinic receptor mRNA was detected in L1 and L3 auditory interneurons. This expression was greater in old females whose phonotactic and L1 sound thresholds were at their minimal levels than in 1-day-old females with higher behavioral and auditory thresholds (Stout *et al.,* 1992, 1993; Atkins and Stout, 1994).

In the vertebrate nervous system, gonadal steroids modulate the levels of neuropeptides and amines (Kawata, 1995). Similarly, steroid-induced effects on transmitter steroid expression have been observed during avian embryogenesis (Anderson, 1989). During the postembryonic development of *M. sexta,* changes in neuropeptide content of identified motoneurons are hormonally regulated. The ecdysteroids induce changes in FMRFamide-like (Phe-Met-Arg-Phe-NH_2) immunoreactivity in neurons of the ventral chain (Witten and Truman, 1996). In the same species, some neurosecretory neurons alter their transmitter profiles during postembryonic development and this change is triggered by ecdysteroids (Loi and Tublitz, 1993; Tublitz and Loi, 1993). Recent data evidenced age-related changes in dopamine receptor density in the brain of the adult honeybee. Thus, D1-like dopamine receptor levels increase with age and are significantly higher in foragers than in newly

emerged bees (Kokai, and Mercer, 1997). The involvement of JH in the regulation of this change might be hypothesized but has not been explored.

C. Changes in Neuropil Volumes

Changes in brain volume were described in an adult Coleoptera in 1979 (Bieber and Fuldner, 1979). It is now recognized that, in adult insect brain, most neuropil regions can change in volume with aging or depending on living conditions (Heisenberg *et al.,* 1995). Most of the data concern the visual and olfactory neuropils and the mushroom bodies, known to be the main integrative area of the insect brain and involved in olfactory learning and memory.

1. Visual Neuropils

Localized in the optic lobes, the visual neuropils comprise three main neuropilar masses: an outer one called the lamina, a middle one called the medulla, and an inner neuropil called the lobula which, in Diptera, is subdivided into an anterior lobula and a posterior lobula plate (Strausfeld, 1976).

In *Drosophila* submitted to isolation and social deprivation, large differences with controls reared in flight cages were observed in the visual neuropils. Thus, there were larger volumes of lamina, medulla, and lobula neuropils in flies reared in rich environmental conditions. These changes were not restricted to the first week of adult life; they also occurred between Days 8 and 16 (Heisenberg *et al.,* 1995). Visual stimulation also modifies the volume of the lamina, medulla, and lobula plate. The lamina shows the clearest evidence of volumetric plasticity. Its volume increases during the first 24 h after emergence and it grows more in the light than in darkness, a change which seems to be mainly due to an increase in the terminals of the photoreceptor cell axons. Dark rearing of the flies for 1 day or more during the first 5 days after emergence results in an irreversible decrease in lamina volume (Barth *et al.,* 1997).

2. Olfactory Neuropils

The olfactory neuropils are part of the deutocerebrum. This paired structure receives the antennal nerve. Two distinct regions were recognized in deutocerebrum: the antennal lobe and the dorsal lobe (Kenyon, 1896). The antennal sensory fibers project into the antennal lobe. The olfactory receptor axons contact the highly branched dendrites of the output and local neurons of the antennal lobe to form a dense synaptic neuropil organized in units with spheroid or ovoid shape—the glomeruli (Masson and Mustaparta, 1990).

Changes in the volumes of the antennal lobe and glomeruli have been reported in the honeybee in which olfactory deprivation induces a decrease in volume of the antennal lobe and identified glomeruli (Masson and Arnold, 1984).

In the same species, the antennal lobe undergoes constant modifications throughout the lifetime of the adult worker bee. A significant increase in the total volume of the glomerular neuropils occurs during the first 4 days of adult life. Moreover, the growth of two particular glomeruli changes with the shift to foraging activity and their volumes enlarge. Interestingly, when nectar foragers were forced to revert to nursing behavior, the volume of another glomerulus [T4-2(1)], normally larger in nectar foragers, significantly decreased, evidencing an activity-dependent change in glomerular volume (Winnington *et al.,* 1996). In additional experiments, it was demonstrated that precocious foraging induced in young bees is accompanied by a premature enlargement of the T4-2(1) glomerulus. Because precocious foraging behavior induces a JH titer increase (Robinson *et al.,* 1989), JH could possibly be involved in this glomerular enlargement. However, bees treated with a JH analog, but prevented to forage, presented a weak but nonsignificant enlargement of the glomerulus, indicating that the premature increase in glomerular volume did not seem to be driven by JH titer alone but could depend on shifts in behavior (Sigg *et al.,* 1997). The role of behavioral changes on the honeybee brain plasticity has been recently emphasized since deprivation of the queen from a colony has a significant impact on the glomerular volume of the workers (Morgan *et al.,* 1998).

3. Mushroom Bodies

Since the early works of Dujardin (1850) and Kenyon (1896), mushroom bodies have been the subject of numerous anatomical investigations (Schürmann, 1987; Erber *et al.,* 1987; Heisenberg, 1994; Menzel *et al.,* 1994). Briefly, this paired structure, located in the protocerebrum, consists of densely packed intrinsic neurons: the Kenyon cells and differentiated neuropils. The neuropilar parts comprise a cup-like structure: the calyx (organized in a double calyx in several insect species), a peduncle, and two main arbors, the α and β lobes. In Hymenoptera (Mobbs, 1982), different parts have been recognized in the calyces, which are the input regions for visual and olfactory informations: the lips, which receive olfactory informations; the collar, receiving visual informations, and the basal ring neuropil, in which both visual and olfactory inputs converge (Fig. 5).

A correlation between the morphology of mushroom bodies and the complexity of behavior has been hypothesized for many years (Rensch, 1959; Neder, 1959; Howse, 1975). Sexual differences in the number of Kenyon cells, more numerous in females than in males of some Curculionidae, were supposed to be due to the rich behavioral repertory of the female

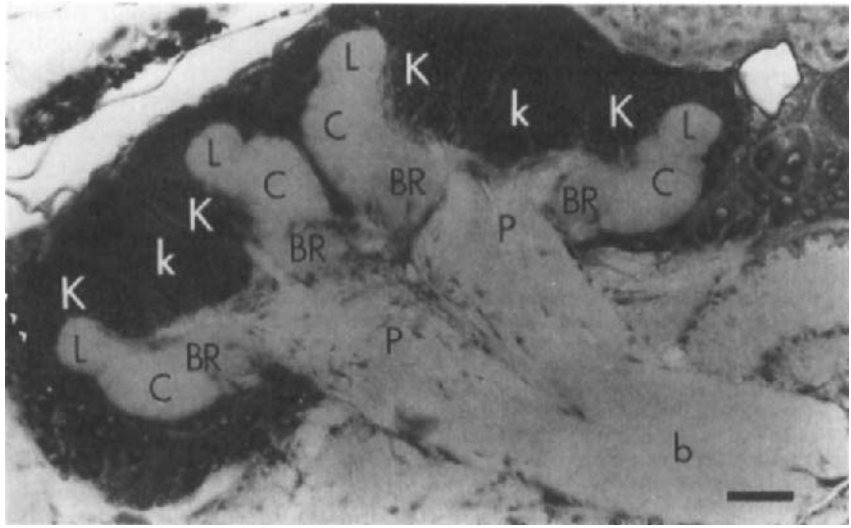

FIG. 5 Transverse section through the medial (right) and lateral (left) calyces of the mushroom bodies of a newly emerged honeybee queen showing the Kenyon cells (K) and the subregions of the neuropil. Arrowheads indicate the boundary of the Kenyon cell population of the lateral calyx. The calyces are formed from three concentric zones of neuropil: the lip (L), the collar (C), and the basal ring (BR). The peduncle (P) is formed of the axons of the Kenyon cells. These fibers bifurcate to form the α and β lobes (b). The α lobe (not seen in this section) would project perpendicularly out from the page. Scale bar = 50 μm (adapted from Fahrbach *et al.*, 1995a).

(Rossbach, 1962). Cast differences in the brain of social insects were also reported. In ants, mushroom bodies of workers are larger than those in other casts (Forel, 1874; Pandazis, 1930; Gronenberg, 1996). Similarly, in *Apis mellifera,* Jonescu (1909) indicated that mushroom bodies of drones were smaller than those of workers.

Analysis of mushroom body volumes recently evidenced striking differences between worker honeybees of different ages (Withers *et al.*, 1993; Durst *et al.*, 1994). Visual experience was suggested to play a role in this neural plasticity since among the different compartments of mushroom bodies, the collar, which receives visual inputs, was seen to increase at the time of the first orientation flight (Durst *et al.*, 1994). The putative role of JH in the observed changes was also explored. Compared to young nurse bees, foragers, which have high JH levels, present a larger volume of mushroom body neuropils (Fig. 1b) and a reduced volume of Kenyon cell somata. When induced to forage precociously, the precocious foragers showed an increase in JH levels and an enlargement of mushroom body neuropil volume, which was similar to those of normally aged foragers (Withers *et al.*, 1993). Moreover, when young bees were treated with methoprene (a JH

analog) and artificially forced to stay in the hive, mimicking the hormonal conditions of foragers without the flight experience, they also presented an enlargement of mushroom body neuropils (Withers *et al.,* 1995). These observations lead to the hypothesis that a relationship might exist between JH titer augmentation and increasing volumes of mushroom body neuropils (Fahrbach and Robinson, 1996). However, in queens (Fig. 2b), a similar enlargement of mushroom body neuropils was observed during the first week of adult life at a moment in which JH levels were low (Fahrbach *et al.,* 1995a). Recent comparisons between JH levels and mushroom body neuropil volumes in drones (Fig. 3b) also indicated that an increase in mushroom body neuropils occurred after the first flight, at a time when JH titers were decreasing (Fahrbach *et al.,* 1997). Taken together, these data induced the authors to conclude that, because expansion of the neuropils of mushroom bodies was maintained in the presence of low levels of JH, this expansion was probably associated with learning the location of the nest, a learning behavior which is common to workers, drones, and queens of the honeybee colony (Fahrbach *et al.,* 1997). Although the previous data do not support a direct relationship between JH level increases and increasing volumes of mushroom body neuropils, it must be noted that a rise in JH titer always preceded the development of mushroom body neuropils. Recently, allatectomy experiments appeared to deny any role to JH in the morphological changes observed in mushroom bodies, although the operation significantly delays the transition to forage (Sullivan *et al.,* 1996). However, surgical ablations of the corpora allata were not performed in pupae but in young adult bees, and the occurrence of a precocious JH biosynthesis cannot be completely ruled out.

In the ant *Camponotus floridanus,* it has been shown (Gronenberg *et al.,* 1996) that the overall brain volume of workers increased 15–20% between emergence and the age of 10 months. Moreover, volume changes differed among the different brain compartments and especially antennal lobe and mushroom bodies grew faster than the rest of the brain. In the course of this study, task-related changes were observed in the volumes of mushroom body neuropils and brood care because foraging activity seemed to significantly contribute to the development of mushroom bodies. Although no data exist regarding the possible changes in JH and ecdysteroid titers during adult life, the occurrence of a neuronal plasticity which evokes results obtained in the honeybee must be noted. Also, neither in honeybee (Fahrbach *et al.,* 1995b) nor in ants (Gronenberg *et al.,* 1996) is the increase in the size of mushroom body a consequence of neurogenesis; it must be a result of axonal and/or dendritic growth.

In *D. melanogaster,* experience-dependent structural plasticity not only induces changes in the number of Kenyon cell fibers (Technau, 1984; Balling *et al.,* 1987) but also affects the calyx volume. Flies reared in flight cages have significantly larger calyces than flies kept in isolation. Moreover, fe-

males have larger calyces if their partner is a female rather than a male (Heisenberg *et al.*, 1995). Recent experiments demonstrated that vision also affects mushroom bodies. Although there is a light-independent increase in the volume of calyces during the first 48 h of adult life, rearing flies in constant light increases calyx volumes compared to rearing animals in the dark. This light effect is not hormonally mediated since monocular-deprived flies develop a small ipsilateral calyx (Barth and Heisenberg, 1997). In the course of these experiments, the use of cAMP mutants led the authors to suggest that some of the structural changes observed could be linked to the cAMP signaling cascade, which is known to be involved in learning and memory (Davis, 1996).

D. Neurogenesis and Gliogenesis

Neuron and glial precursors, the proliferation of which has been extensively studied during embryogenesis and postembryonic development, can be present in some adult insects.

1. Neurogenesis

a. Neurogenesis in Adult Insects The occurrence of neurogenesis in the adult insect brain has not been widely studied. Ancient histological studies report the persistence of neuroblasts in the optic lobes of different adult species such as *Machilis polypoda* (Bauer, 1904), *Nepa cinerea* (Graichen, 1936), *Oncopeltus fasciatus* (Johansson, 1957), and *Tettigonia viridissima* (Panov, 1960). An age-related increase in Kenyon cell number was reported in a Coleoptera (Bieber and Fuldner, 1979). The persistence of neuroblasts in adult mushroom bodies was described in only a few species. In adults, as during preimaginal life, neuroblasts give birth by one asymetrical division to ganglion mother cells, which divide symetrically to produce neurons. Panov (1957, 1963) observed neuroblast divisions in *T. viridissima* and in three lepidopteran species. However, when examining the lepidopteran *Danaus plexippus,* Nordlander and Edwards (1970) failed to observe neurogenesis in adult mushroom bodies.

Recent data obtained from *Drosophila* indicated that the few [^{3}H]-thymidine-labeled cells described in mushroom body cortex of newly emerged flies (Technau, 1984) are probably late daughter cells of ganglion mother cells and do not correspond to adult neurogenesis because neuroblasts disappear during the pupal stage (Ito and Hotta, 1992).

In *Periplaneta americana,* Drescher (1960) observed one mitosis at the bottom of the calyx in a 2-month-old adult. Incorporation of tritiated thymidine in the same area was also described (Weiss and Edwards, 1974); however, this label can clearly be attributed to glial cells (Cayre *et al.*, 1996a).

The use of 5-bromo-deoxyuridine (BrdU) to monitor DNA replication allowed demonstration of a persistent neurogenesis in Gryllidae (Cayre *et al.*, 1994, 1996a). In *Acheta domesticus, Gryllus bimaculatus,* and *Gryllomorpha damaltina,* neuroblasts divide during adult life. By contrast, in another Orthopteran, *L. migratoria,* incorporation of BrdU occurred only in glial cells. Among other insect groups, adult neurogenesis in mushroom bodies was observed in Coleoptera, in which two neuroblasts persist in each Kenyon cell area, as described in Staphylinidae (Bieber and Fuldner, 1979). These neuroblasts give birth to new interneurons as revealed by BrdU labeling in *Tenebrio molitor* and *Zophobas* species as well as in the ladybird, *Harmonia axyridis* (Cayre *et al.*, 1996a). Similar studies performed in honeybees failed to show any neurogenesis in adult mushroom bodies (Fahrbach *et al.*, 1995b), a result which led Gronenberg *et al.* (1996) to conclude that in ant, another Hymenopteran, neurogenesis probably did not occur.

b. Hormonal Control of Neurogenesis In crickets, a group of neuroblasts persists at the apical part of mushroom body cortex (Figs. 6a and 6b; Cayre *et al.*, 1994, 1996a). As demonstrated by BrdU, the new neurons migrate into the mushroom body cortex, occurring between the preexisting interneurons (Fig. 6c). Observed throughout adult life, this neurogenesis is modulated by morphogenetic hormones. In control females, the mitotic index increases concomittantly to the large peak of JH related to vitellogenesis, and then decreases when maturing follicles produce large amounts of ecdysteroids. By contrast, allatectomy resulted in a low mitotic index and, in ovariectomized females, the mitotic rate fails to decrease and remains higher than that in control animals after Day 5 postemergence (Fig. 4a). JH injection into allatectomized females, as well as ecdysteroid injections into ovariectomized animals, demonstrated the antagonistic action of morphogenetic hormones on adult neurogenesis. Moreover, an effect of aging on this neural plasticity was evidenced since JH stimulatory effect was less efficient in 10-day-old than in 3-day-old females (Cayre *et al.*, 1994, 1997a). Taken together, these data suggest a constant remodeling of cricket mushroom bodies throughout its imaginal life.

The two morphogenetic hormones also affect brain metabolism and especially polyamine metabolism. Polyamines are ubiquitous polycations which, in vertebrates, are known to be involved in the control of nervous system development and the acquisition of behavioral competences (Slotkin and Bartolome, 1986). In *A. domesticus,* the brain polyamine levels are reduced after allatectomy and increased in ovariectomized females (Cayre *et al.*, 1993, 1995, 1997a). Injections of the lacking hormones restored the brain polyamine titers to control values, and studies on the key enzymes of polyamine biosynthesis have evidenced the role of hormones on polyamine metabolism (Cayre *et al.*, 1995, 1997a). The inhibition of polyamine biosynthesis induced by a specific and irreversible inhibitor of ornithine decarbox-

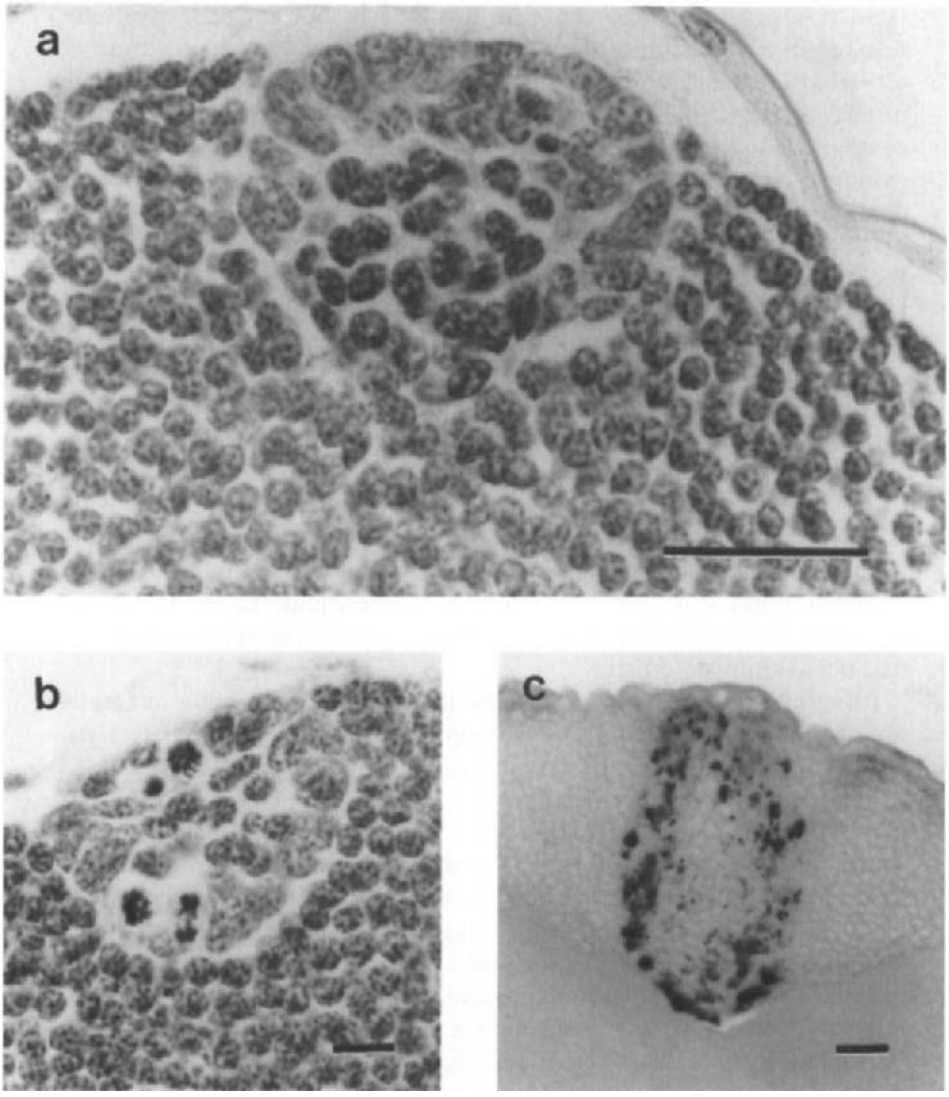

FIG. 6 Proliferation of new neurons and their migration in the cortex of mushroom bodies in adult house crickets. (a) Frontal section through the apex of mushroom body cortex. Above the densely packed Kenyon somata, a discrete cluster of distinct cells consists of large neuroblasts and ganglion mother cells. Feulgen–Rossenbeck nuclear staining. Scale bar = 25 μm. (b) Cell divisions within the proliferative area of mushroom bodies. Feulgen–Rossenbeck nuclear staining. Scale bar = 10 μm. (c) Mushroom body cortex of a 12-day-old female who was injected with BrdU at 1 and 6 days. Immunocytochemical detection of BrdU. The neuroblast progeny (labeled nuclei) has migrated into the cortex. Scale bar = 25 μm. (Adapted from Neurogenesis in adult insect mushroom bodies, Cayre, M., Strambi, C., Charpin, P., Augier, R., Renucci, M., and Strambi, A., *J. Comp. Neurol.*, Copyright © 1996 Wiley-Liss, Inc. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

ylase (ODC; the first key enzyme involved in polyamine biosynthesis) resulted in a depletion of brain polyamines to levels similar to those observed after allatectomy. During the same time, the mitotic rate in the proliferative area of mushroom bodies was strongly depressed to values comparable to those obtained after JH deprivation. Additional experiments demonstrated that, among the three main polyamines (putrescine, spermidine, and spermine), putrescine could mimick the effects of JH on neuroblast proliferation and the mitogenic action of JH necessarily required the presence of putrescine to occur (Cayre *et al.,* 1997b). Currently, the mechanism of action of the hormone can only be hypothesized according to data obtained in vertebrates. JH could act on the protooncogenes c-*myc* and *max* inducing activation of the ODC gene; the resulting putrescine production could regulate protooncogene expression and/or act on cyclins involved in cell cycle control (Cayre *et al.,* 1997b).

Together with the strong reduction of neuroblast proliferation, the inhibition of brain polyamine biosynthesis was shown to act on cricket oviposition behavior: The appearance of oviposition movement was delayed and its frequency significantly reduced in females fed a strong inhibitor of polyamine biosynthesis (Cayre *et al.,* 1996b). All these findings provide correlative data between hormonally induced changes in the central nervous system and behavioral consequences.

It is well-known that mushroom bodies are the brains main integrative area for messages arriving from the optic and antennal lobes. Clearly involved in the integration of external and internal informations, as well as in learning and memory (Heisenberg 1994), the mushroom bodies can be compared to the vertebrate hippocampus (Jarrard, 1995). Structural similarities (Mizunami *et al.,* 1993) and the demonstration of a form of long-term potentiation in the honeybee mushroom bodies (Oleskevich *et al.,* 1997) support this analogy. Moreover, the hormonally modulated neurogenesis observed in cricket mushroom bodies presents similarities with the cell proliferation and migration observed in a particular part of hippocampus—the dentate gyrus (Gould and Cameron, 1996). The fate of the newborn neurons of *Acheta* mushroom bodies remains unclear. They migrate into the mushroom body cortex and become undistinguishable from the classical Kenyon cells. Analysis of possible changes in mushroom body cortex volume failed to produce convincing results (M. Cayre, unpublished data); thus, the hypothesis of neuronal death was examined. To date, although pycnotic nuclei are present in mushroom body cortex, they cannot be considered to be due to apoptosis because the TUNEL method failed to evidence any apoptic cell (M. Cayre, unpublished data).

If the role of hormones on mushroom body neurogenesis is demonstrated in the adult cricket, the link between neurogenesis and the establishment of oviposition behavior is only correlative. Further work will be needed to

demonstrate the functional role of the hormonally controlled brain neurogenesis of *Acheta.*

2. Gliogenesis

Although neural plasticity and neurogenesis during embryogenesis and preimaginal life are fairly well-known, there are only a few data on gliogenesis. During the development of the *M. sexta* brain, glial cells were recognized as steroid targets (Bidmon *et al.,* 1991), and ecdysone receptor proteins were evidenced in glial cells of developing *Drosophila* brain (Truman *et al.,* 1994). Ecdysteroids stimulate proliferation of brain glial cells during the metamorphic development of *Manduca* (Kirschenbaum *et al.,* 1995). Moreover, glioblasts were described in the pars intercerebralis of adult *L. migratoria* (Vanhems and Girardie, 1983), and gliogenesis has been observed during adulthood in *L. migratoria* and *P. americana* (Cayre *et al.,* 1996a). However, the putative modulation of adult gliogenesis by morphogenetic hormones has not been examined.

V. Concluding Remarks

From the previous discussion, it appears that the same main processes are underlying preimaginal and adult neural plasticity: neurogenesis, as well as changes in neural arbors and synaptic connections, can be observed during the insect adult life. To date, a major difference between preimaginal and adult neural plasticity is the occurrence of programmed neuronal death. Patterns of neuronal death have been evidenced throughout embryogenesis, larval stages, and metamorphosis (Truman *et al.,* 1990, 1992). Even during the development of the pharate adult of *M. sexta* and *D. melanogaster,* programmed cell death takes place as the insect discards muscles and neurons that were specialized for eclosion behavior. The hormonal regulation of these phenomena has been clearly established (Schwartz and Truman, 1983; Truman, 1988; Kimura and Truman, 1990; Truman *et al.,* 1992). In contrast to preimaginal development, programmed neuronal death does not seem to occur during adulthood. Despite the decrease in mushroom body cortex volume observed in the worker honeybee (Withers *et al.,* 1993), no evidence for the possible occurrence of apoptosis could be obtained using the TUNEL method (Robinson *et al.,* 1997). Similarly, apoptic cells could not be recognized in the house cricket mushroom bodies (M. Cayre, unpublished data). However, it cannot be ruled out that the TUNEL method, considered to be well adapted to evidence neuronal cell death, is not the best suited method for adult insects.

Although extensive work has been devoted to the hormonal control of preimaginal neural plasticity, the roles of hormones in adult neural plasticity remain largely unexplored. Adult insects offer numerous opportunities to explore the molecular mechanisms underlying neural plasticity. The development of *in vitro* neuronal cultures will provide much information about mechanisms of hormone actions and will complete the important data collected during insect preimaginal development (Weeks and Levine, 1995; Levine and Weeks, 1996). The recent discovery of ecdysteroid receptors and the development of specific antibodies to their different isoforms are tools to study ecdysteroid receptor distribution and the hormonal regulation of receptor isoform expression. In vertebrates, sex steroid hormones are known to regulate genes encoding structural proteins involved in sprouting of neuronal processes and synaptic remodeling. Specific proteins are required for axonal growth and synaptogenesis, and among these, tubulin and actin are concerned (Kawata, 1995). In insects, ecdysteroids have been shown to stimulate tubulin and actin synthesis in a *Drosophila* cell line (Couderc and Dastugue, 1980; Sobrier *et al.,* 1986, 1989) and to increase the number of microtubules in a cell line from *Chironomus tentans* (Spindler-Barth *et al.,* 1992). In the same way, motoneurons of *M. sexta* cultured in the presence of 20-hydroxyecdysone have more microtubule-containing branches than motoneurons maintained in the absence of ecdysteroids (Levine and Weeks, 1996).

It must be remembered that, in contrast to vertebrates, the numerous molecules involved in neural plasticity are far from being identified in insects, and that molecular studies in species other than *Drosophila* are missing. However, the numerous examples of hormone-induced behavioral changes in adult insects provide excellent models to explore the properties of the underlying neurons and neural circuitry involved in the expression of behavior. The use of a simple behavior exhibited by *M. sexta,* the proleg withdrawal reflex, has been successfully studied, allowing exploration of its functional changes during metamorphosis and learning (Weeks *et al.,* 1997). Similar studies in adult insects will offer fruitful opportunities to investigate the mechanisms by which hormones can act on the nervous system to induce and modulate behaviors.

Note added in proof. Since the completion of the manuscript, several relevant data were published. The regulation by JH III of *A. domesticus* phonotactic response was recently reconsidered (Stout *et al.,* 1998a). The authors propose that phonotactic thresholds in female *A. domesticus* are regulated by JH III and other influences on both the threshold of the L1 auditory interneurons and on other, as yet unidentified, neurons. A correlation of nicotinic receptor-like mRNA expression with excitatory inputs into the L1 and L3 auditory neurons of *A. domesticus* was demonstrated. The influence of JH III might be expressed through control of the expression of excitatory neurotransmitter receptors in the L1 and L3 auditory interneurons (Stout *et al.,* 1998b).

In *D. melanogaster,* dopamine was seen to modulate female sexual receptivity, an effect which might be regulated via interactions with hormonal pathways since dopamine is required for normal ovarian maturation and fecundity (Neckameyer, 1998).

During optic lobe neurogenesis in the moth *M. sexta,* ecdysteroids control cell proliferation which, in cultured tissues, can be turned on or off simply by shifting ecdysteroid concentration (Champlin and Truman, 1998). In *D. melanogaster,* mushroom body neurons, isolated during metamorphosis and maintained in the presence of 20-hydroxyecdysone, had significantly greater total neurite length and branch number compared with that of mushroom body neurons grown without hormone (Kraft *et al.,* 1998). On the other hand, it has been demonstrated that ecdysteroids can reduce synaptic transmission at an intermolt stage of a crustacean tonic neuromuscular junction by acting at presynaptic site (Cooper and Ruffner, 1998).

Last, the recent discovery of neurogenesis in the adult human hippocampus (Eriksson *et al.,* 1998) will definitely rule out the conception of the immutability of adult brain structures. Moreover, this finding gives hope that structural brain repair through induced neurogenesis will possibly become of clinical use (Goldman, 1998).

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Mechanism of Action of P-Glycoprotein in Relation to Passive Membrane Permeation

Gera D. Eytan* and Philip W. Kuchel†
*Department of Biology, Technion—Israel Institute of Technology, Haifa 32000, Israel; and †Department of Biochemistry, University of Sydney, New South Wales, Australia

This review presents a survey of studies of the movement of chemotherapeutic drugs into cells, their extrusion from multidrug—resistant (MDR) cells overexpressing P-glycoprotein (Pgp), and the mode of sensitization of MDR cells to anticancer drugs by Pgp modulators. The consistent features of the kinetics from studies of the operation of Pgp in cells were combined in a computer model that enables the simulation of experimental scenarios. MDR-type drugs are hydrophobic and positively charged and as such bind readily to negatively charged phospholipid head groups of the membrane. Transmembrane movement of MDR-type drugs, such as doxorubicin, occurs by a flip-flop mechanism with a lifetime of about 1 min rather than by diffusion down a gradient present in the lipid core. A long residence time of a drug in the membrane leaflet increases the probability that P-glycoprotein will remove it from the cell. In a manner similar to ion-transporting ATPases, such as Na^+,K^+-ATPase, Pgp transports close to one drug molecule per ATP molecule hydrolyzed. Computer simulation of cellular pharmacokinetics, based on partial reactions measured *in vitro*, show that the efficiency of Pgp, in conferring MDR on cells, depends on the pumping capacity of Pgp and its affinity toward the specific drug, the transmembrane movement rate of the drug, the affinity of the drug toward its pharmacological cellular target, and the affinity of the drug toward intracellular trapping sites. Pgp activities present in MDR cells allow for the efficient removal of drugs, whether directly from the cytoplasm or from the inner leaflet of the plasma membrane. A prerequisite for a successful modulator, capable of overcoming cellular Pgp, is the rapid passive transbilayer movement, allowing it to reenter the cell immediately and thus successfully occupy the Pgp active site(s).

KEY WORDS: P-glycoprotein, Multidrug resistance, Flip-flop, Transmembrane movement, ABC transporter.

I. Introduction

The aim of this review is to present a comprehensive model describing the movement of anticancer drugs used in chemotherapy into cells, their extrusion from multidrug-resistant (MDR) cells overexpressing P-glycoprotein (Pgp), and the mode of sensitization of MDR cells to anticancer drugs by Pgp modulators. We do not attempt to cover the huge literature published on various aspects of Pgp role in MDR. The reader is referred to a few of the many reviews (Bradley *et al.,* 1988; Ling, 1992; Gottesman and Pastan, 1993; Ling, 1995; Higgins *et al.,* 1997; Sikic, 1997; Stein, 1997; Schinkel, 1998).

Resistance to multiple drugs, referred to as MDR, is a major impediment to the successful treatment of many human cancers. In some types of tumors, MDR is inherent whereas in others, such as small cell lung cancer, it is usually acquired. Experimental MDR, induced by selection for resistance to a single drug, involves the simultaneous development of cross-resistance to structurally diverse compounds with multiple targets within the cell. Drugs included in MDR include many therapeutically important natural products and their semisynthetic congeners. The two best described proteins mediating experimental MDR are the 170,000-kDa Pgp encoded by the *MDR1* gene (Roninson *et al.,* 1984; Gros *et al.,* 1986; Ueda *et al.,* 1986; Horio *et al.,* 1988; Chabner and Wilson, 1991; Gottesman and Pastan, 1993) and the 190,000-kDa MRP (Cole *et al.,* 1992; Cole and Deeley, 1993; Krishnamachary and Center, 1993). Although both Pgp and MRP are ATPase transporters located in the plasma membrane and belong to the ATP-binding cassette superfamily of transport proteins, they share less than 15% amino acid identity (Cole *et al.,* 1992). Pgp has been shown to mediate the ATP-dependent efflux of MDR drugs that can be inhibited *in vitro* by Pgp modulators, such as verapamil, reserpine and cyclosporin A and its analogue PSC-833. Interestingly, these Pgp modulators do not inhibit MRP-mediated drug efflux (Cole *et al.,* 1994). A 110-kDa protein, lung resistance protein (LRP), overexpressed in MDR cells was localized in the cytoplasm of Pgp-negative MDR cells of different histogenetic origins (Scheper *et al.,* 1993). Clinical investigations suggest that LRP overexpression in acute myelocytic leukemia (AML) and advanced carcinoma is associated with an unfavorable prognostic outcome (List *et al.,* 1996); however, whether LRP actually contributes to clinical resistance is unknown. Evidence suggests the existence of new unknown MDR mechanisms: depletion of energy in AML cells revealed MDR activity distinct from both Pgp and MRP (Hedley *et al.,* 1997) and studies in hematopoietic cells indicated a nonfunctional Pgp expression in some lines and the presence of an extended MDR mechanism not sensitive to Pgp in other cells (Marks *et al.,* 1996).

Pgp was the first identified protein capable of mediating MDR *in vitro* and is the best characterized protein mediating MDR. Pgp functions as an energy-dependent extrusion pump that expels hydrophobic cytotoxic agents from MDR cells. Pgp-mediated, drug-modulated ATPase activity was demonstrated in plasma membrane preparations (Sarkadi *et al.,* 1992; al-Shawi and Senior, 1993) and in reconstituted proteoliposomes (Ambudkar *et al.,* 1992; Sharom *et al.,* 1993; Shapiro and Ling, 1994; Urbatsch *et al.,* 1994; Sharom, 1995; Borgnia *et al.,* 1996). The amino acid sequence of Pgp reveals a tandemly duplicated molecule, each half containing six putative transmembrane domains and one cytoplasmically located nucleotide-binding site characterized by "homology A" and "homology B" consensus sequences (Walker *et al.,* 1982). Taking advantage of the noncompetitive inhibition of Pgp by vanadate, it has been shown that both Pgp nucleotide-binding sites are catalytically active (Urbatsch *et al.,* 1995); inactivation of either site abolishes Pgp activity (al-Shawi and Senior, 1993; al-Shawi *et al.,* 1994) and prevents activity of the other site (Senior and Bhagat, 1998).

Expression of mammalian Pgp is not limited to MDR tumor cells but is also found in a number of normal tissues (Thiebaut *et al.,* 1987; Arceci *et al.,* 1988; Sugawara *et al.,* 1988; Cordon-Cardo *et al.,* 1990). Little is known about the function of physiologically expressed Pgp and even less about its putative natural substrates. The localization of Pgp to the bile canaliculus, small intestine, colon, and proximal renal tubules led to the suggestion that Pgp is involved in the excretion of xenobiotics (Thiebaut *et al.,* 1987). A possible additional physiological role of Pgp is protection of the brain by the extrusion of neurotoxins across the blood–brain barrier. Pgp is highly expressed in brain capillary endothelial cells (Thiebaut *et al.,* 1987; Sugawara *et al.,* 1988) and prevents MDR-type drugs from accumulating in the brain (Bradbury, 1985). Schinkel *et al.,* (1994; Schinkel, 1998) have generated mice with a homozygous disruption of the *mdr*1a gene. The absence of Pgp from the blood–brain barrier in these mice resulted in elevated MDR-type drugs in their brain and a hypersensitivity to the centrally neurotoxic pesticide, ivermectin.

Pgp overexpressing cells display resistance to multiple cytotoxic hydrophobic agents, mostly of natural origin, including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, actinomycin D, taxoids, and hydrophobic peptides, such as gramicidin D, valinomycin, and dolastatin 10. The resistance conferred by Pgp can be reversed by a group of structurally and chemically unrelated compounds referred to as modulators or, alternatively, as modulators, chemosensitizers, Pgp inhibitors, resistance modifiers, and reversing agents (Skovsgaard, 1978; Tsuruo *et al.,* 1981).

MDR-type drugs and modulators compete on a common Pgp pharmacophore, indicating that the interaction of both MDR-type drugs and modula-

tors occurs at a common site on Pgp and ostensibly by a similar mechanism (Borgnia *et al.,* 1996). Upon reconstitution, Pgp molecules located inside out, with their ATPase site exposed to the medium, mediated an ATP-driven uptake of various substrates (Sharom *et al.,* 1993; Shapiro and Ling, 1995; Eytan *et al.,* 1996a). An ATP-driven, valinomycin-dependent K^+ uptake into proteoliposomes reconstituted with mammalian Pgp has been described (Eytan *et al.,* 1994). Pgp mediated the ATP-dependent uptake of the cation–ionophore complex into the proteoliposomes, where the radioactive cation was accumulated, thus circumventing the obstacle posed by the hydrophobicity of Pgp substrates in transport studies. Taking advantage of the high levels of Pgp expression in MDR Chinese hamster ovary cells and of this assay, both the ATPase and transport activities of Pgp were measured simultaneously under identical conditions and a stoichiometry of 0.5–0.8 ionophore molecules transported per ATP molecule hydrolyzed was observed (Eytan *et al.,* 1996a).

A similar but somewhat higher number of molecules of ATP hydrolyzed for every molecule of the MDR-type drug vinblastine was obtained by Ambudkar *et al.* (1997). They estimated carefully the number of human Pgp molecules expressed in mouse cells transfected with human MDR1 gene and exposed at the cell surface, the ATPase activity stimulated by vinblastine, and the rate of Pgp-mediated vinblastine extrusion from these cells. Thus, this stoichiometry and transport capacity of Pgp resemble various potent ion-translocating ATPases, such as Na^+,K^+-ATPase, which handle millimolar substrate concentrations.

The success of Pgp in lowering intracellular concentrations of a wide variety of hydrophobic drugs raises the following puzzling features:

1. Cellular Pgp successfully extrudes low drug concentrations (n*M* to μM concentration range) of a wide range of molecular structures. High-binding affinity such as exhibited by Pgp is usually assumed to indicate multiple binding interactions between the substrate and an enzyme, presumably leading to high recognition specificity. However, Pgp binds a wide range of structurally dissimilar drugs with high affinity.

2. MDR-type drugs are all at least partially hydrophobic and dissolve preferentially in organic solvents, such as octanol, rather than in aqueous media. Some of them are extremely hydrophobic, e.g., the substrate of the lactococcal multidrug transporter, TMA-DPH, exhibits a partition coefficient (octanol/water) of 2×10^5 (Bolhuis *et al.,* 1996). As such, MDR-type drugs are expected to diffuse freely across biological membranes (Stein, 1997). With its relatively slow transport activity, with an estimated turnover number of 15 sec^{-1} (Urbatsch *et al.,* 1994), PgP successfully removes these supposedly membrane-permeable drugs from MDR cells. This apparent

paradox led to suggestions of alternative mechanisms of Pgp-mediated drug extrusion from MDR cells, such as indirect drug pumping by alteration of the biophysical parameters of the cells (Simon and Schindler, 1994; Sikic *et al.,* 1997).

3. MDR modulators capable of reversing Pgp-mediated resistance in MDR cells are expected to behave similarly to MDR-type drugs, except that they are less cytotoxic. However, in contradistinction to MDR-type drugs, the modulators are not excluded from MDR cells by Pgp. Moreover, whereas modulators inhibit cellular Pgp and allow enhanced uptake of MDR-type drugs into MDR cells, most MDR-type drugs do not inhibit cellular Pgp and have no effect on drug uptake in MDR cells (Scala *et al.,* 1997). Structurally, MDR-type drugs and Pgp modulators are similar and no distinguishing molecular features of either group could be defined (Zamora *et al.,* 1988; Weaver *et al.,* 1993).

4. Although cellular Pgp reduces the apparent initial uptake rate of some MDR-type drugs (Sirotnak *et al.,* 1986; Cano-Gauci *et al.,* 1990; Shalinsky *et al.,* 1993), it has no effect on drug efflux from these cells (Sirotnak *et al.,* 1986; Bornmann and Roepe, 1994). The differential effect of Pgp activity on drug uptake as opposed to drug efflux led to the suggestion that Pgp prevents the cellular uptake of certain drugs by their extraction directly from the outer leaflet of the plasma membrane [see review by Stein *et al.* (1994)].

The goal of this review is to demonstrate that the basic assumption that the rate of the transmembrane movement of drugs is a sole function of their hydrophobicity does not hold for MDR-type drugs. Moreover, these drugs traverse membranes by a flip-flop mechanism rather than by diffusion down a concentration gradient located in the lipid core of the membrane. Incorporating experimental data measuring various parameters, such as the ATPase and transport activities of Pgp and the flip-flop rate of drugs, allows for an integrative model of drug kinetics in MDR- and drug-sensitive cells that, together with a computer simulation model, explains all the seemingly puzzling features of Pgp function detailed earlier. The essential features of the model are presented in Fig. 1. MDR-type drugs are hydrophobic and positively charged and as such bind readily to the negatively charged outer leaflet of the plasma membrane. Thus, in cells exposed to drugs, the low drug concentrations in the medium are in equilibrium with a pool of high drug concentrations present in the outer leaflet of the plasma membrane (denoted in the figure as K_1). The drug pool adsorbed to the cell surface is the direct source of drug influx into the cells. Drug transported from this pool into the cells is immediately replenished by drug from the medium. The transmembrane movement of MDR-type drugs, such as doxorubicin,

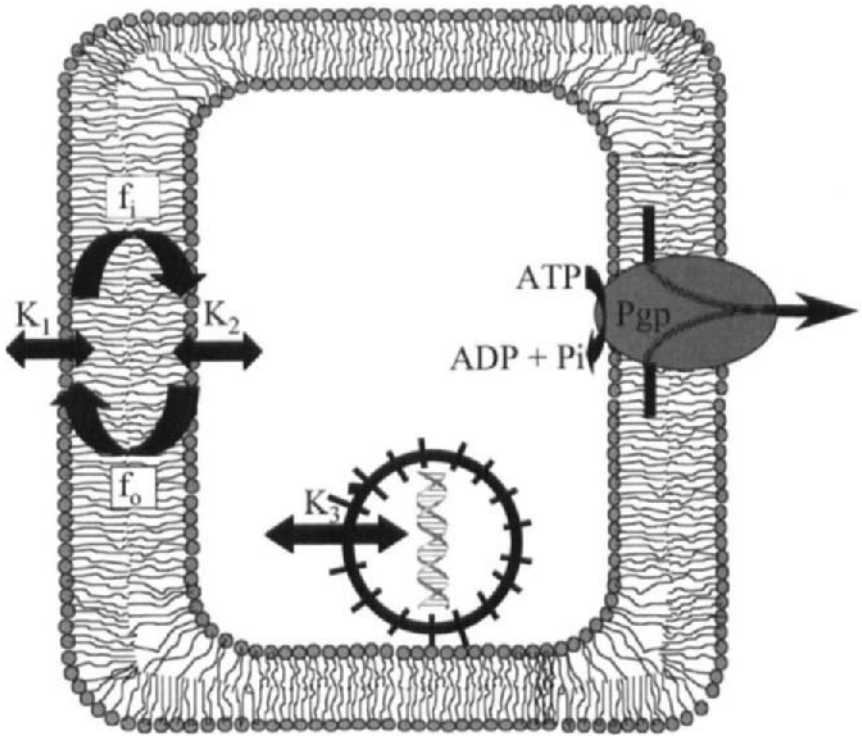

FIG. 1 Schematic diagram describing movement of MDR-type drugs in Pgp-overexpressing cells. MDR drugs added to the extracellular medium bind to and are in practical equilibration (K_1) with the drug pool in the outer leaflet of the plasma membrane. The drugs flip-flop across the plasma membrane (f_i and f_o). The drug pool in the inner leaflet of the membrane is in "effective" equilibrium with the drug pool in the cytoplasm (K_2). Drugs in the cytoplasm are largely bound and form an equilibrium (K_3) with intracellular molecular sinks represented here, for simplicity, as DNA. Pgp seems to extract its substrates from the inner leaflet of the plasma membrane and flip them outwards (see text).

across the plasma membrane, inward and outward, occurs with a lifetime of about a minute (denoted in the figure as f_i and f_o). The drug reaching the inner leaflet of the plasma membrane is equilibrated quickly with the drug pool in the cytoplasm. Because of the high affinity of MDR-type drugs for membranes, low concentrations of drugs in the cytoplasm are in equilibrium with relatively high drug concentrations in the inner leaflet of the plasma membrane (denoted in the figure as K_2). Drug reaching the cytoplasm is adsorbed by high-capacity molecular sinks, such as DNA capable of binding anthracyclines or tubulin capable of binding *vinca* alka-

loids. The drug binding, which can occur in multiple molecular sinks with a wide variety of characteristics, in terms of binding rate, affinity and capacity, is presented in Fig. 1, for simplicity's sake, as binding to a single molecular sink (denoted as K_3).

As detailed in Section VI, the authors adopt the suggestion made by Higgins and Gottesman (1992) that Pgp extracts its substrates directly from the inner leaflet of the plasma membrane, functioning as a "flippase." If such is the case, although Pgp seems to pump nanomolar and micromolar concentrations of drugs from the cytoplasm, it actually extracts its substrates from millimolar concentrations of drug present in the pool located in the inner leaflet of the plasma membrane. The computer simulation model presented in Section V demonstrates that, assuming a flip-flop rate of drugs observed in *in vitro* experiments, the Pgp-mediated ATPase and transport activities observed in MDR cells are sufficient to lower the cytoplasmic drug concentration to levels low enough to account for the resistance levels observed experimentally in these MDR cells. Moreover, the computer model helps clarify the important role played by the intracellular drug-binding sinks in determining cellular pharmacokinetics. Thus, the amount and affinity of drug-binding intracellular molecules largely determine the total amount of drug bound to cells and the time lag required to reach equilibrium of a drug with cells. Moreover, in case of intracellular drug sinks capable of slow binding of drugs, Pgp pumping activity results in the apparent slow uptake observed with certain drugs. No exceptional mechanisms such as Pgp-mediated drug pumping from the outer leaflet of the plasma membrane are required for the reduction in the apparent initial drug uptake.

The difference in cellular functions between MDR-type drugs and MDR modulators can be due to different rates of transmembrane movement. MDR-type drugs flip-flop slowly across membranes and, as a result, are removed efficiently from Pgp-overexpressing cells and cannot compete on the intracellular binding sites of Pgp. However, the modulators diffuse rapidly across membranes. Upon removal from MDR cells, they immediately reenter the cells, rendering their extrusion by Pgp futile. Their high intracellular concentrations allow efficient competition on the intracellular-binding sites. Indeed, assays of the rate of transmembrane movement of numerous MDR-type drugs and modulators have resulted in fast transmembrane movement of the modulators vs slow transmembrane movement of MDR-type drugs (Eytan *et al.,* 1996b).

Thus, as detailed next, MDR in Pgp overexpressing cells and its modulation can be attributed to Pgp functioning similarly to other well-characterized transporters, such as Na^+,K^+-ATPase.

II. Mechanism of Transbilayer Movement of Drugs

A. Solubility/Diffusion Model

The substrates of Pgp are large complex hydrophobic molecules that have been assumed to cross the membrane by the classical model of diffusion down a gradient formed in the lipid core of the membrane (Gottesman and Pastan, 1993; Simon and Schindler, 1994; Stein, 1997). This model is based on a correlation between the permeability of a variety of small molecules across the alga plasma membrane and their partition coefficient in an olive oil/water system. Based on the close correlation between hydrophobicity and membrane permeability, Collander, Stein, and others (Collander, 1954; Lieb and Stein, 1986; Walter and Gutknecht, 1986; Todd *et al.*, 1989; Xiang and Anderson, 1994) suggested that small nonelectrolytes cross membranes by partitioning into the exterior of the lipid core of the membrane, diffusing down a gradient formed in the hydrophobic core of the membrane, and equilibrating the drug into the water at the interior face of the membrane according to the partition coefficient (see Fig. 2). According to this proposal, the rate of transbilayer movement is characterized by a permeability coefficient that is a function to the partition coefficient (lipid/water) of the solute, the diffusion coefficient of the solute across the lipid core of the membrane, and a factor that incorporates the surface area of the cell and the thickness of the cell membrane. This approach has

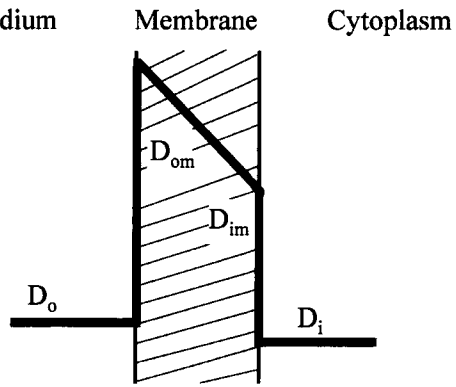

FIG. 2 The solubility/diffusion model describing the transmembrane movement of solutes. Drug present in the extracellular medium (D_o) dissolves into the outer surface of the lipid core of the plasma membrane to a concentration (D_{om}) determined by its hydrophobicity. The drug diffuses down a gradient formed within the membrane to the inner surface of the plasma membrane. The drug pool at the inner surface of the membrane (D_{im}) is in "effective" equilibrium with the drug pool in the cytoplasm (D_c).

proven accurate in describing the transbilayer movement of small nonelectrolytes (Stein, 1986, 1990). Stein (1997) has demonstrated that the same experimental approach can also be applied successfully to Pgp-type substrates, such as doxorubicin and daunorubicin. The good correlation between the experimentally determined permeability coefficient and the coefficient extrapolated from data obtained with small nonelectrolytes is surprising, as both these anthracyclines are complex molecules. The transport of these drugs is mediated by the small fraction of drug molecules that, under physiological conditions, are uncharged and not by most of the molecule population, which remains charged (Dalmark, 1981; Speelmans *et al.,* 1994). The solubility/diffusion model can be misleading, as it assumes solubility of the drugs in the lipid core of the membranes, whereas these amphipathic drugs are located close to the surface of the membrane leaflets in close association with the phospholipid head groups (see Section III).

B. Drug Transmembrane Movement by a Flip-Flop Mechanism

MDR-type drugs, such as doxorubicin, partition readily into membranes and are immobilized by their amphipathic nature at the interface(s) of the hydrophobic core of the membrane and the polar head groups of the phospholipids. Consequently, the transmembrane movement rate of MDR-type drugs is limited by transport of the drug molecules from one interface of the membrane to the other. Because these transport events are rather rare (see later), a proper name for them is flip-flop, in analogy to the flip-flop of phospholipids across membranes (McNamee and McConnell, 1973; Rothman and Dawidowicz, 1975; Wimley and Thompson, 1990, 1991; Haest *et al.,* 1997). Thus, the transmembrane movement of MDR-type drugs can be described as fast and massive incorporation of the drug into one membrane leaflet, flip-flop across the lipid core, and release from the opposing leaflet (see Fig. 3).

C. Flip-Flop of Doxorubicin across Liposome Membranes

As a first step toward characterization of the transbilayer movement of MDR-type drugs across biological membranes, doxorubicin movement within the membrane was studied in liposomes (Regev and Eytan, 1997). DNA served as an agent capable of rapidly and efficiently quenching the fluorescence of doxorubicin. As previously shown (Tarasiuk *et al.,* 1989; Frezard and Garnier-Suillerot, 1991; Speelmans *et al.,* 1994; Regev and Eytan, 1997) and presented in Fig. 4, doxorubicin fluorescence was

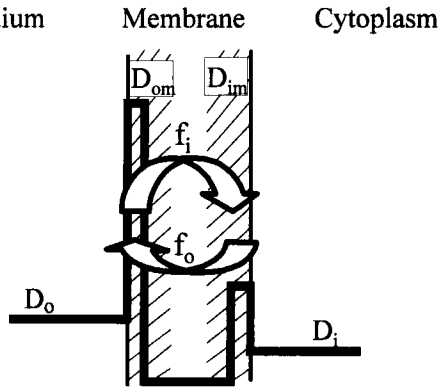

FIG. 3 The flip-flop model describing the transmembrane movement of solutes. Drug present in the extracellular medium (D_o) dissolves into the outer leaflet of the plasma membrane to a concentration (D_{om}) determined by multiple factors, including its hydrophobicity and amphipathic nature. The drug crosses the plasma membrane by rare flip-flop events (f_i and f_o). The drug pool in the inner leaflet of the plasma membrane (D_i) is in quasi-equilibrium with the drug pool in the cytoplasm (D_i).

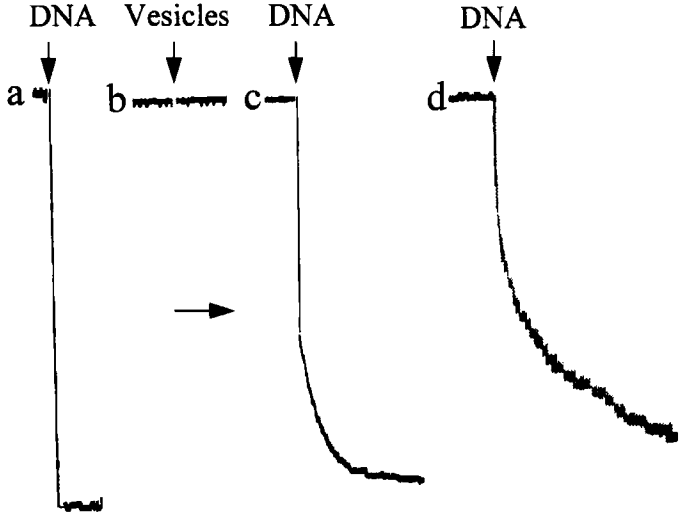

FIG. 4 Outward transbilayer movement of doxorubicin in vesicles. Doxorubicin (10 μM) was incubated at 23°C and its fluorescence was monitored continuously. After a brief incubation, either DNA (0.5 mg/ml) or unilamellar vesicles prepared from phosphatidylserine (trace b, 0.5 mg/ml) were added. Similar concentrations of doxorubicin and either unilamellar (trace c) or multilamellar vesicles (trace d) were equilibrated by overnight incubation at 23°C prior to the addition of DNA (0.5 mg/mgl). The horizontal arrow corresponds to either 5 (traces a–c) or 30 (trace d) min (adapted with permission from Regev and Eytan, 1997).

quenched upon mixing with DNA (trace a), but it was not changed upon mixing with liposomes (trace b), even after complete equilibration. Binding studies using [^{14}C]doxorubicin showed that after equilibration, 90% of the drug was bound to PS vesicles. The bound drug was distributed equally between the two phospholipid leaflets in association with the acidic head groups of the phospholipid (Nicolay *et al.,* 1984; Speelmans *et al.,* 1994). The volume encapsulated in the vesicles comprised less than 0.2% of the total volume, rendering negligible the contribution of the doxorubicin free within the lumen of the vesicles compared to the drug amount bound to the membranes. This was confirmed by repeating the transport studies described later in the presence of 2 *M* sucrose with no effect on the experimental results (Regev and Eytan, 1997).

Because of the high affinity of doxorubicin for DNA, the addition of DNA to doxorubicin, preequilibrated with vesicles, induced desorption of the drug from the vesicles and binding to the DNA, leading to fluorescence quenching. The fluorescence quenching occurred in two steps (Fig. 4, trace c). About 50–60% of the total fluorescence decrease was too fast to be recorded under our experimental conditions. The second phase of fluorescence decrease occurred slowly with a time course that could be fitted to an exponential decay with a lifetime of 1.1–1.3 min. The initial rapid fluorescence decrease observed in the first phase represents the quenching of doxorubicin, both free in the medium and bound to the outer leaflet of the vesicles. The slow phase of the fluorescence decrease is the outcome of the outward transbilayer movement of drug originally bound to the inner leaflet. When multilamellar vesicles were used (trace d) instead of unilamellar vesicles, the fast phase of fluorescence quenching was equivalent to 20% of the total drug fluorescence, corresponding to the drug, both free in the medium and bound to the outer leaflet of the outer membrane. Compared to unilamellar vesicles, the second phase of fluorescence decrease in multilamellar vesicles was much slower, could not be curve fitted to a single first order reaction, and presumably is the result of the outward movement of the drug across multiple membranes.

The flip-flop rate is an intrinsic characteristic of the membrane and the drug and should not be affected by the concentration of the various components, such as the membranes, the DNA, and the drug. Indeed, repeating the experiment with various concentrations of DNA and vesicles resulted in varying extents of fluorescence quenching, but the lifetime of the slow phase of fluorescence quenching remained 1.2 min (Fig. 5). Varying the concentrations of both the unilamellar vesicles and the drug also had no effect on the rate of the slow phase of fluorescence quenching (Fig. 6). The amount of drug participating in the transbilayer movement, measured as the extent of the slow phase of drug quenching, remained equivalent to half the total amount of drug bound to the unilamellar vesicles.

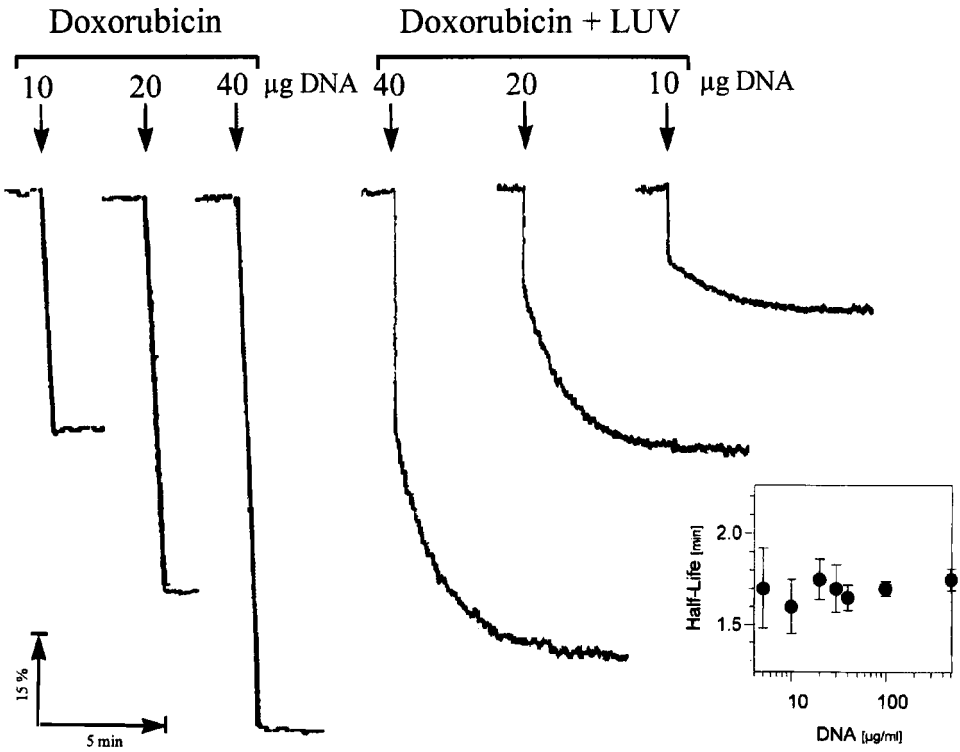

FIG. 5 Effect of DNA concentration on the transbilayer movement of doxorubicin. At the points marked by the arrows, DNA (concentrations as noted, μg/ml) was added to either free doxorubicin (10 μM) or doxorubicin (10 μM) preequilibrated overnight with unilamellar vesicles (LUV, 1 mg/ml). Doxorubicin fluorescence was monitored continuously. The slow phases of fluorescence quenching were fitted to first-order reaction curves, and the resulting half-lives were plotted in the insert as a function of DNA concentration (adapted with permission from Regev and Eytan, 1997).

Thus, factors expected to modulate the interaction rate of the drug with vesicles such as the concentrations of the DNA, the drug, and the vesicles had no effect on the slow phase of fluorescence quenching. In contradistinction, parameters affecting the fluidity of the membranes and expected to affect the movement of drugs within the membrane modulated the rate of the slow phase of fluorescence quenching. As is shown in Fig. 7, cholesterol, known to reduce membrane fluidity (Gasset *et al.*, 1988), inhibited the transbilayer movement rate of doxorubicin. Inclusion of 30% cholesterol in the vesicles increased the doxorubicin rate of flip-flop sevenfold. However, the membrane fluidizer, benzyl alcohol (Sinicrope *et al.*, 1992), accelerated the transbilayer movement. Furthermore, fluidizing the membrane by

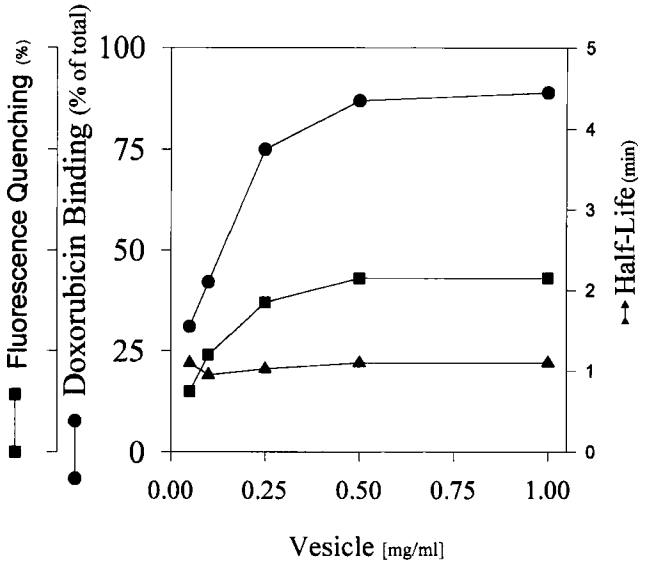

FIG. 6 Effect of vesicle concentrations on the transbilayer movement of doxorubicin. Various concentrations of unilamellar vesicles were equilibrated overnight with doxorubicin (phospholipid/drug molar ratio of 25). DNA (0.5 mg/ml) was then added, and the rates and extents of the slow phases of fluorescence quenching were determined by curve fitting. The extents of binding are expressed as the percentage of initial doxorubicin fluorescence and are compared to doxorubicin binding determined with [^{14}C]doxorubicin (adapted with permission from Regev and Eytan, 1997).

raising the temperature led to shorter doxorubicin transbilayer movement lifetimes in both the presence and the absence of cholesterol. The half-lives could be plotted in an Arrhenius-type plot yielding straight lines corresponding to an increase by a factor of 4–5 for each 10°C rise in temperature (Regev and Eytan, 1997). Presumably, this temperature dependence reflects mainly fluidization of the membranes and direct acceleration of the transbilayer movement rate.

Variation of the strategy employed to measure drug transport from the inner to the outer leaflet was used to measure the drug transport rate from the outer to the inner leaflet. In the latter case, drug transfer from the DNA to the membranes was monitored. For this purpose, doxorubicin was equilibrated with small amounts of DNA, resulting in intercalation of the drug into the DNA and a corresponding fluorescence quenching (Fig. 8). Upon subsequent addition of an excess of unilamellar vesicles, the lipid vesicles competed for the drug and partially relieved the fluorescence quenching. The fluorescence relief due to the transfer of the drug from the DNA to the membranes occurred in two phases. The first phase was fast

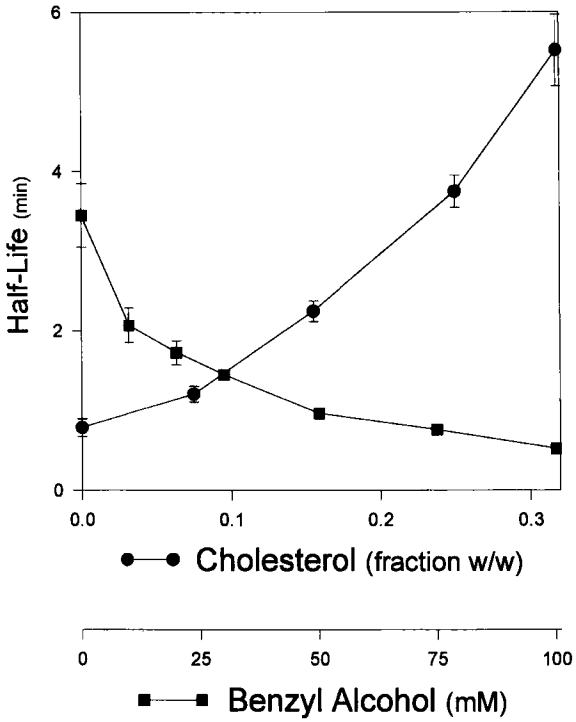

FIG. 7 Effect of cholesterol and benzyl alcohol on the rate of doxorubicin transbilayer movement. The half-life of doxorubicin transbilayer movement was determined as described in Fig. 4 (trace c) except that the vesicles were of various compositions of lipids. For the demonstration of the effect of cholesterol on doxorubicin transbilayer movement, the vesicles contained soybean phospholipids and various concentrations of cholesterol. Vesicles containing soybean phospholipids and cholesterol [0.7:0.3 (w/w), respectively] were used to assess the fluidizing effect of benzyl alcohol on the doxorubicin transbilayer movement rate (adapted with permission from Regev and Eytan, 1997).

and represents the transfer of doxorubicin from DNA to the outer leaflet of the unilamellar vesicles. Because of the inward movement of drug from the outer to the inner leaflet, the second phase could be fitted to an exponential function with a half-life of 1.1 min similar to the outward movement. As for the outward transbilayer movement, the relative concentrations of DNA and vesicles determined the extent of quenching relief, but had no effect on the half-life of the slow phase of fluorescence increase.

Slow flip-flop rates have been demonstrated by similar experimental approaches for other Pgp substrates, such as rhodamine 123 (Eytan *et al.*, 1996b; Shao *et al.*, 1997), (see Section VII), Hoechst 33342 (R. Regev and G. D. Eytan, unpublished results), or the substrate of the lactococcal

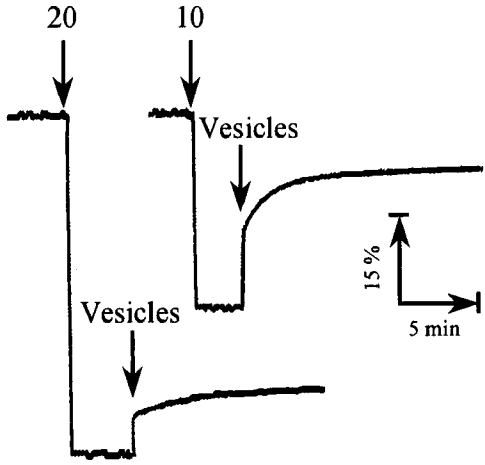

FIG. 8 Inward transbilayer movement of doxorubicin. The fluorescence of doxorubicin (10 μg/ml) was monitored continuously. The fluorescence was partially quenched by the addition of either 10 or 20 μg/ml DNA (marked by arrows). LUV (1 mg/ml) was then added. The slow phase of the relief of fluorescence quenching was curve fitted to a first-order reaction. The half-life of this relief was equivalent to the half-life of the outward movement of doxorubicin measured with vesicles of similar lipid composition (adapted with permission from Regev and Eytan, 1997).

multidrug transporter, TMA-DPH (Bolhuis *et al.*, 1996). Interestingly, the channel-forming ionophore, gramicidin A, which has been demonstrated to flip-flop across membranes with a lifetime of minutes (O'Connell *et al.*, 1990), is an MDR-type drug. Mammalian cells exhibiting the P-170-dependent Mdr phenotype displayed a collateral resistance to gramicidin D (Slovak *et al.*, 1988; Lincke *et al.*, 1990; Assaraf and Borgnia, 1994; Loe and Sharom, 1994). It is excluded from Pgp-overexpressing cells and exposure of drug-sensitive cells to it turns the cells into MDR cells (Assaraf and Borgnia, 1994; Loe and Sharom, 1994).

D. Flip-Flop of Doxorubicin across Erythrocyte Membranes

Upon equilibration of [^{14}C]doxorubicin under the experimental conditions described in Fig. 9 with either IOV or sealed ghosts, about 90% of the drug became membrane bound. Upon addition of DNA to IOV preequilibrated with doxorubicin, the pattern of fluorescence decrease was similar to that observed with liposomes containing 30% (w/w) cholesterol. The extent of the slow phase of the fluorescence decrease was equivalent to nearly 50% of the drug bound to IOV. However, the corresponding extent in sealed

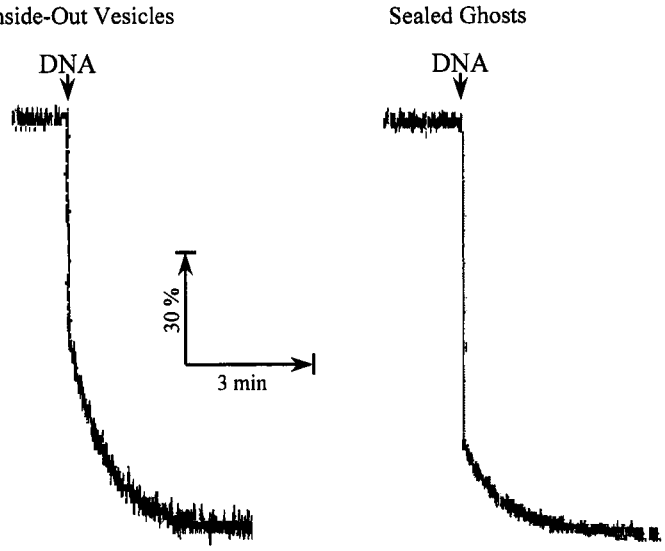

FIG. 9 Transbilayer movement of doxorubicin in erythrocyte membranes. Doxorubicin (10 μM) was incubated at 37°C with either inside-out vesicles prepared from erythrocyte ghosts (0.5 mg/ml) or sealed ghosts (1 mg/ml) prepared from fresh blood. Doxorubicin fluorescence was monitored continuously and DNA (1 mg/ml) was added. The vertical arrow represents 30% fluorescence of the total doxorubicin fluorescence (adapted with permission from Regev and Eytan, 1997).

ghosts was lower, presumably due to an unsealed fraction of the ghosts. The results described in Fig. 9 were reproduced after osmotic collapse of the vesicles in the presence of 2 *M* sucrose (Regev and Eytan, 1997). As with lipid vesicles, the half-life of the transbilayer movement in erythrocyte membranes was not affected by various concentrations of DNA, drug, and vesicles.

The similar flip-flop rate of doxorubicin across cholesterol-containing liposomes and erythrocyte membranes, which are considered to be relatively rigid plasma membranes, indicates that the flip-flop rate is determined by the lipid core of the membranes and is rather insensitive to the protein content of the membranes.

E. Effect of Membrane and Drug Charge on Drug Transmembrane Movement

The nature of both acidic and neutral phospholipids present in the vesicles had little effect on the transbilayer movement rate of doxorubicin. Thus,

substitution of the PS with either phosphatidylglycerol or cardiolipin did not alter the transbilayer movement rate (Regev and Eytan, 1997). Partial substitution of PC with phosphatidylethanolamine also had little effect on the drug transbilayer movement rate. As expected, total elimination of the acidic phospholipid resulted in the reduced binding of doxorubicin (Nicolay *et al.,* 1984; Frezard and Garnier-Suillerot, 1998). However, the transbilayer movement rate observed in neutral liposomes was twice as fast as that observed in acidic liposomes (Fig. 10). A similar effect could be observed at pH 9.7, which is above the p*K* of doxorubicin (pH 8.6). At this pH most of the drug is uncharged, and the transbilayer movement rate of doxorubicin is twice as fast. Thus, even in the absence of charge interaction between the polar head groups of the phospholipids and the drug, doxorubicin crosses membranes by a flip-flop mechanism.

Similar results were obtained when Speelmans *et al.* (1994) measured the permeability coefficient of doxorubicin in lipid vesicles. The transport of doxorubicin across pure PC membranes was twice as fast as across PC:PS (1:1) membranes. Similarly, Frezard and Garnier-Suillerot (1998) measured

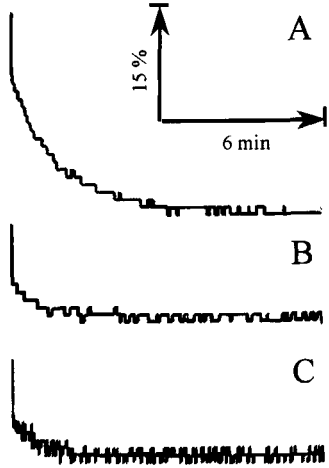

FIG. 10 Effect of pH and phospholipid charge on transbilayer movement of doxorubicin. The outward transbilayer movement rate of doxorubicin (trace A) was determined as described in Fig. 4 (trace c) with vesicles containing phosphatidylcholine:phosphatidylserine and cholesterol (0.35:0.35:0.3 weight ratio). The half-life of the first-order process describing doxorubicin influx was determined, by curve fitting, to be 1.25 min. The experiment was repeated (trace B) except that the pH of the medium was 9.7. Under these conditions, the half-life of doxorubicin transbilayer movement was 0.6 min. The experiment described in trace A was repeated (trace C) except that the vesicles contained only phosphatidylcholine and cholesterol [0.7:0.3 (w/w), respectively]. The half-life determined was 0.7 min (adapted with permission from Regev and Eytan, 1997).

the permeability of various anthracyclines across membranes as a function of bilayer charge and observed that the permeability coefficient of the membranes decreases as the amount of negatively charged phospholipids increases. They explained this observation as the result of a decrease in the quantity of neutral drug form at the head group region. Presumably, the increase in charged phospholipids induces a decrease in the amount of neutral drug form available for flip-flop (Frezard and Garnier-Suillerot, 1991).

The mechanism by which doxorubicin traverses membranes may be better understood by analogy to the well-studied slow transbilayer movement of gramicidin A (O'Connell *et al.*, 1990). This channel-forming ionophore is an amphipatic peptide anchored by a hydroxyl group at the membrane surface. It transverses the membrane slowly by rare flip-flop events. Doxorubicin is also an amphipatic molecule with a hydrophilic amine group present in the daunosamine moiety and hydroxyl groups limited to the same moiety of the drug (Fig. 11). Thus, for the following reasons the transbilayer movement of doxorubicin is best described in terms of distinct events of flip-flop across the membrane and not as diffusion down a continuous gradient located in the membrane core: (1) Transbilayer movement occurs with a half-life close to a minute, which is hard to reconcile with free diffusion across the short width of membranes. (2) The measurements of both the inward and the outward transbilayer movements of doxorubicin indicate that the amount of doxorubicin located in the membrane is divided into two similar-sized drug pools. Evidence of two similar-sized drug pools in the membrane is consistent with two similar compartments, presumably identical with the two membrane leaflets. (3) Doxorubicin is soluble in polar solvents, such as chloroform and octanol, but not in apolar solvents, such as benzene and carbon tetrachloride (Table I). Thus, doxorubicin is not soluble in the hydrophobic core of the membrane, but is rather located mainly at the membrane surfaces. (4) Doxorubicin bound to lipid mem-

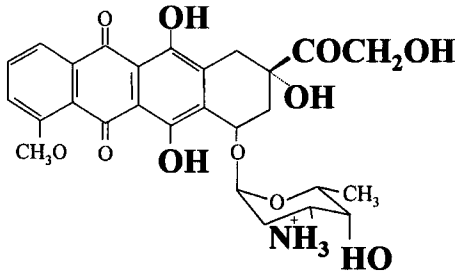

FIG. 11 Amphiphatic nature of doxorubicin. Hydrophilic groups are represented in bold letters.

TABLE I

Partition Coefficients of Doxorubicin in Various Organic Solvents

	Octanol	Chloroform	Benzene	Carbon tetrachloride
Dielectric constants	10.3	4.8	2.28	2.23
Partition coefficient				
pH 7.5	1	9	<0.05	<0.05
pH 8.6	8	17	0.2	<0.05
pH 9.7	>20	>20	0.4	<0.05

branes is primarily associated with the acidic head groups of the phospholipids, presumably due to its amphipathic nature and the electrostatic attraction of the basic amine group of doxorubicin to the acidic head groups of the phospholipids both in liposomes and in erythrocyte membranes (Nicolay *et al.*, 1984; de Wolf *et al.*, 1993). (5) The transbilayer movement of doxorubicin is not modulated by varying the concentrations of drug, vesicles, or DNA, but is correlated with membrane fluidity, indicating that the step limiting the movement is located in the membrane matrix. (6) Osmotic collapse of both vesicles and erythrocyte vesicles did not alter the time course of fluorescence decrease, indicating that drug efflux from the medium encapsulated in the vesicles did not contribute to the transport measurements presented here. (7) The decrease in doxorubicin fluorescence observed upon addition of DNA to multilamellar vesicles, preequilibrated with drug, exhibits prolonged and complex kinetics that are consistent with the drug crossing multiple membrane barriers. This prolonged and complex kinetics exclude the possibility that the two phases of fluorescence reduction, observed with unilamellar vesicles, are due to two modes of drug interaction with the vesicles or to phase separation in the liposomes.

The determination of solute transbilayer movement by the fluorescence quenching technique is limited to drugs whose fluorescence is altered upon transfer from the aqueous phase into the membrane bilayer. However, data obtained with multilamellar vesicles suggest an alternative approach to determining the transbilayer movement of solutes. Upon incubation with multilamellar vesicles, drugs initially bind very fast to the outermost leaflet and subsequently equilibrate more slowly throughout the vesicles by transbilayer movement. An estimation of the transbilayer movement rate of drugs can be obtained by determination of the binding rates of the drugs to the vesicles. The binding kinetics of two MDR-type drugs, doxorubicin and mitoxantrone, are presented in Fig. 12. The drug amount determined to be associated with the vesicles after prolonged incubation was similar to the amount predicted from equilibrium dialysis studies (Table II). After the initial fast binding of MDR-type drugs, further equilibration throughout

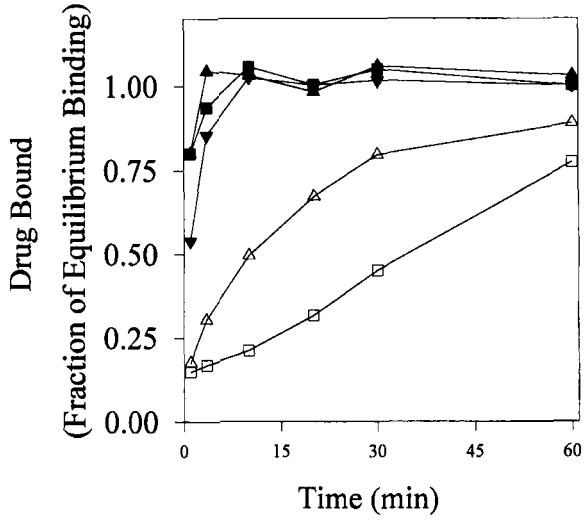

FIG. 12 Binding kinetics of MDR-type drugs and modulators to multilamellar vesicles. Multilamellar vesicles were incubated at room temperature with the MDR-type drugs, doxorubicin (△) or mitoxantrone (□) or with the MDR modulators, verapamil (▼), quinine (▲), or quinidine (■). After various further incubation periods, samples were withdrawn and centrifuged, and the amount of MDR-type drugs or modulators remaining in the medium was determined. The drug amount removed from the medium, together with the vesicles, represented the amount bound to the vesicles. The amount of drug bound at equilibrium was determined after a 3-h incubation and was similar to the values obtained by equilibrium dialysis (Table I). The first time point was after 1 min incubation (adapted with permission from Eytan *et al.*, 1996b).

the multilamellar vesicles proceeded slowly, with the equilibrium of doxorubicin and mitoxantrone reached after more than 0.5 and 2 hr, respectively.

The estimation of binding kinetics just described was achieved without washing the vesicles and therefore it could be applied only to drugs exhibiting high affinity toward the vesicles. An alternative, more sensitive, approach is to determine the drug amount associated with the vesicles after a wash by centrifugation. The latter procedure results in a loss of drug bound to the outer leaflet during the wash step, which lasts for about 1 min. The drug amount lost includes also the drug fraction capable of diffusing outward from the inner leaflets during the wash. Thus, the size of the fraction lost during the wash step constitutes an additional indirect measure of the transbilayer movement rate.

Binding rates of six classical MDR-type drugs, doxorubicin, daunomycin, rhodamine 123, taxol, vinblastine, and mitoxantrone, were analyzed (Fig. 13). Of the MDR-type drugs tested, mitoxantrone proved the slowest and taxol the fastest. The equilibration period was relatively long and varied

TABLE II
Solubility and Binding Properties of Drugs and Modulators[a]

	μM	Partition coefficient (octanol/aqueous phase)	Binding coefficient (lipid/aqueous phase)
Daunomycin	200	7.4	260
Doxorubicin	200	1.1	155
Mitoxantrone	200	7.2	450
Vinblastine	0.05	45.0	240
Taxol	0.02	12.0	480
Rhodamine 123	5	6.9	112
Quinidine	200	9.9	270
Quinine	200	9.1	250
Progesterone	0.02	360	925
Trifluoperazine	200	27	245
Verapamil	0.02	8.3	996
Verapamil	200	8.3	550

[a] Partition coefficients of drugs and modulators were determined after vigorous shaking in the two-phase system. Binding coefficients and the concentrations ratio of the drug in the lipid phase relative to the aqueous phase were determined by equilibrium dialysis of the drug, at the concentrations mentioned, with multilamellar vesicles. The binding coefficient is the calculated concentration ratio in the lipid bilayer and the aqueous medium.

between 1 and 3 hr; as expected from slow-moving drugs, the amount of drug lost during the wash step from vesicles at equilibrium was ~10%.

No clear correlation was observed among the following three characteristics of the various drugs: (1) partition coefficient into octanol (Table II), (2) binding to vesicles (Table II), and (3) the transbilayer movement rate in multilamellar vesicles. Thus, the slowest-moving MDR-type drug, mitoxantrone, exhibited a moderate partition coefficient, but was tightly bound to vesicles. The well-established MDR modulator, verapamil, exhibited a similar partition coefficient, but moved much faster across membranes and displayed the highest affinity toward multilamellar vesicles. Further research is required into the molecular characteristics determining the rate of transmembrane movement; presumably the rate is a function of the total hydrophobicity of the drug and its degree of amphipathy.

The barrier to MDR-type drug transport posed by the hydrophobic core of the plasma membrane divides the cellular environment into two topological domains: (1) the intracellular domain, which is close to equilibrium with the inner plasma membrane leaflet, and (2) the extracellular domain, which is close to equilibrium with the outer plasma membrane domain. It has been suggested that the function of Pgp may involve substrate transport from the lipid bilayer itself to the external medium ("vacuum cleaner" hypothesis) (Higgins and Gottesman, 1992). Within this hypothesis, Pgp

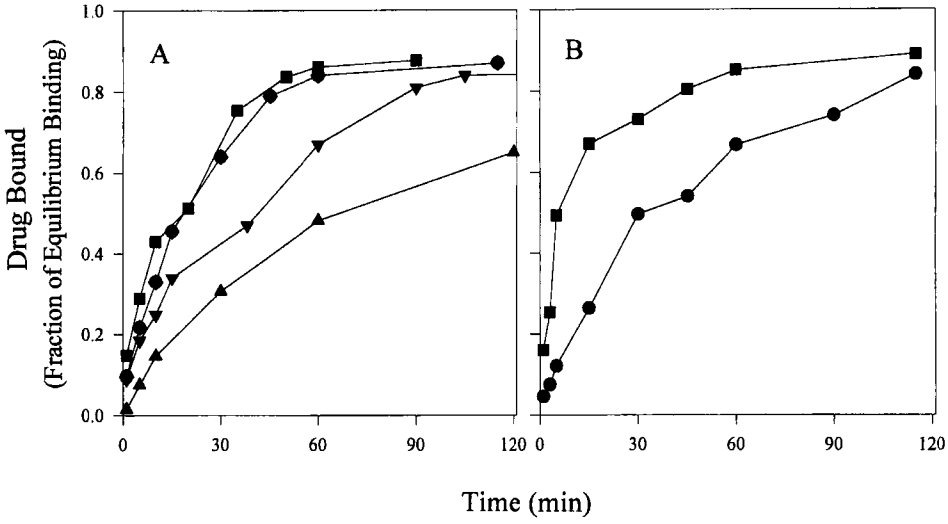

FIG. 13 Equilibration kinetics of MDR-type drugs with multilamellar vesicles. Multilamellar vesicles were incubated with a medium containing 60% (w/v) sucrose at room temperature. (A) The MDR-type drugs doxorubicin (▲), daunomycin (■), rhodamine 123 (●), or mitoxantrone (▼) were added to a 0.2 m*M* final concentration. (B) The MDR-type drugs taxol (■) and vinblastine (●) were added to a concentration of 20 n*M*. After various further incubation periods, samples were withdrawn and centrifuged through a 40% (w/v) sucrose cushion into an ether fraction. The amount of MDR-type drugs or modulators in the ether fraction was determined. The amount of drug bound at equilibrium was determined by equilibrium dialysis (adapted with permission from Eytan *et al.*, 1996b).

could function as a flippase, i.e., catalyzing the translocation of hydrophobic substrates present in the cell membrane from the inner to the outer leaflet (Higgins and Gottesman, 1992).

III. Drug Adsorption to the Plasma Membrane

Pgp substrates are large complex amphipathic molecules possessing hydrophobic moieties and basic charged groups. As such, the drugs partition readily into biological membranes. Although they are at least partially soluble in organic solvents, their octanol/water partition coefficient (i.e., the ratio of their solubility in octanol to their solubility in water) varies over a wide range, from about equal solubility of doxorubicin in octanol and water (Table II) to TMA-DPH, which exhibits a partition coefficient (octanol/water) of 2×10^5 (Bolhuis *et al.*, 1996). Upon incubation with

cells, the latter, highly hydrophobic, dye equilibrates rapidly with the outer leaflet of the membrane and within a second its concentration in the outer leaflet reaches 5 m*M*, whereas the extracellular free concentration is only 30 n*M*. This yields a lipid over medium partition coefficient of 10^5. The intercalation level into membranes of the more hydrophobic Pgp substrates, such as TMA-DPH (Bolhuis *et al.,* 1996) and hydrophobic analogues of doxorubicin (Israel *et al.,* 1987; Constantinides *et al.,* 1989; Burke *et al.,* 1990), is determined by their hydrophobicity. However, the intercalation into membranes of less hydrophobic Pgp substrates is most probably determined mainly by their capacity to interact with the polar head groups of the membrane phospholipids. Thus, doxorubicin with an octanol/water partition coefficient of about 1 exhibits a 1200-fold preference for artificial acidic membranes compared to aqueous medium (DOPC/DOPS (1/1) (Speelmans *et al.,* 1994). The high solubility of doxorubicin in the membranes is presumed to be due to electrostatic interaction with the acidic head groups of the phospholipids, but also to a combination of hydrophobic affinity of the hydrophobic moieties of the drug with the membrane core and attraction of the polar groups of the drug to the polar head groups of the phospholipids (Saeki *et al.,* 1992). Thus, doxorubicin exhibits a preferential partitioning into neutral membranes composed of pure DOPC of 300 compared to aqueous media (Speelmans *et al.,* 1994). As shown in Fig. 11, doxorubicin is an amphipathic molecule with a planar hydrophobic moiety and hydrophobic amine and hydroxyl groups limited to one molecular pole. Presumably, the hydrophilic groups bind and are immobilized close to the phospholipid head groups, while the hydrophobic moiety of the drug is inserted in the membrane core. Indeed, it has been shown to perturb the order of the phospholipid acyl chains (Burke and Tritton, 1985; de Wolf *et al.,* 1991, 1992). A comparative study of the interaction of various doxorubicin analogues with artificial lipid vesicles (Gallois *et al.,* 1998) revealed that while electrostatic interaction played a role in adsorption of all analogues, hydrophobic interactions played a major role only in the membrane intercalation of the more hydrophobic analogues.

The binding of Pgp substrates to membranes is rapid and reversible. Binding of even relatively hydrophilic drugs, such as doxorubicin, is too fast to be measured by conventional methods (Speelmans *et al.,* 1994; R. Regev and G. D. Eytan, unpublished results). However, the dilution of membranes preequilibrated with the highly hydrophobic Pgp substrate TMA-DPH results in the immediate desorption of excess dye (Bolhuis *et al.,* 1996). Thus, under all conditions, membrane concentrations of Pgp substrates in the membrane leaflets are close to those at equilibrium with their adjacent medium, the cytoplasm, or the extracellular medium.

Extrapolating the high affinity exhibited by Pgp substrates toward artificial membranes to physiological conditions leads to the realization that the

substrate fraction, i.e., free either in the cell cytoplasm or in the extracellular medium, is very low compared to the concentration in the plasma membrane. De Wolf *et al.,* (1993) have calculated, and shown experimentally, that in a hypothetical spherical cell with a diameter of 10 μm, surrounded by a plasma membrane, but containing no membranes at all in the cytoplasm, more than 70% of total internal doxorubicin would be bound to the cytoplasmic side of the membrane. In the cytoplasm of a real eukaryotic cell, the total membrane surface available for drug binding, relative to the volume of the cytoplasm, is even higher due to the presence of many organelles and vesicles. Thus, in most cell types, almost all Pgp substrates, especially the more hydrophobic ones, are bound to membranes or to other molecular sinks. The high affinity of various drugs to cellular targets, such as the binding of anthracyclines to DNA or *vinca* alkaloids to tubulin, further reduces the drug fraction free in the cytoplasm.

The direct source of drug taken up into cells is the drug pool bound to the cells and not drug freely that is soluble in the medium. Upon uptake of drug into the cells, the bound drug pool is replenished by the further binding of drug from the medium. Thus, *in vivo,* for example, in the blood where cell concentrations are high, the drug free in the plasma is negligibly low compared to drug bound to cells and plasma proteins.

Despite the crucial role played by drug bound to cells in cellular drug uptake, *in vitro* kinetics experiments are intentionally designed to ignore the drug pool bound to the outer leaflet of the cell plasma membrane. Drug bound to cells is removed by extensive washing of them. In some experiments, to further artificially reduce the amount of drug bound to cells, excess unlabeled drug is included in the wash medium. Finally, the residual amount of drug bound to the outer leaflet of the cells is usually subtracted as a background that is considered to be insignificant to the uptake process. Thus, by artificial means, the drug uptake into cells is converted into an apparent drug uptake from the medium directly into the cells, whereas drug uptake occurs in two steps, namely massive drug binding to the outer leaflet of the membrane and uptake from the bound drug pool into the cells.

IV. Stoichiometry of Pgp-Mediated ATP Hydrolysis to Drug Transport

A. Technical Problems in Determination of Stoichiometry

An important mechanistic question concerns the stoichiometry of ATP hydrolyzed to substrate transported by Pgp. A ratio close to 1:1, i.e., between

2:1 and 1:3, would be consistent with Pgp functioning similarly to the well-characterized, ion-transporting ATPases, such as Na^+, K^+-ATPase. Because Pgp has two ATP-binding domains (Urbatsch *et al.*, 1995), as many as two ATP molecules hydrolyzed per drug molecule transported might be justified by established transport mechanisms. Higher ATP to substrate ratios are not realistic at a mechanistic level, as it would require multiple cycles of ATP hydrolysis per substrate transported and a novel energy storage mechanism within Pgp would need to be postulated. Although both ATP-binding domains are catalytically active (Higgins, 1992; Urbatsch *et al.*, 1995) and are required for catalysis (Urbatsch *et al.*, 1998), kinetic evidence suggests that the two ATP-binding sites alternate in catalysis (Garrigos *et al.*, 1993; Urbatsch *et al.*, 1995) and that the upper limit of ATP molecules hydrolyzed per substrate molecule transported is one.

An ideal experimental system for the determination of ATP hydrolyzed to substrate transported would involve the purification of Pgp, reconstitution, and simultaneous assay of ATP hydrolysis and substrate transport. However, the following theoretical considerations and the amphipathic nature of Pgp substrates render such an experiment almost impossible. Under optimal conditions, a reconstituted vesicle contains one molecule of Pgp ATPase located with its ATPase site exposed to the medium. Reconstituted Pgp vesicles contain about 1 μl of encapsulated medium/mg lipids (Eytan *et al.*, 1994; Shapiro and Ling, 1994), which corresponds to vesicles with an average diameter of 100 nm (Chen and Wilson, 1984) and a calculated inner volume of 5×10^{-19} liters per single vesicle. The best estimate of the ATPase activity of a single Pgp molecule was obtained with plasma membranes containing 32% Pgp that were purified from CHO MDR cells (al-Shawi and Senior, 1993). The specific ATPase activity of Pgp was estimated to be 9 μmol/min/mg ($= 21\ sec^{-1}$) corresponding to 21 molecules of ATP hydrolyzed per Pgp molecule per second. Assuming a 1:1 ratio of substrate molecules transported per ATP molecules hydrolyzed, this turnover number corresponds to 21 molecules of substrate pumped per second. This amount of substrate in the tiny volume encapsulated within a single vesicle will yield an increase in substrate concentration of 65 μM per second or about 4 mM per minute. Pgp substrates are amphipathic and such high concentrations of most Pgp substrates would compromise membrane integrity, disrupting vesicles. Concentrations of less than 100 μM Pgp substrates, including cytotoxic drugs and modulators, induced the leakage of vesicle contents (Drori *et al.*, 1995). A similar problem is encountered in plasma membrane vesicles. The encapsulated volume of these vesicles is larger, but they contain many copies of Pgp capable of accumulating potentially high substrate concentrations that could lead to disruption of the vesicles from within. Thus, it is not surprising that the reported apparent efficiency of drug uptake into either reconstituted Pgp

or plasma membrane vesicles was extremely low, with the number of ATP molecules hydrolyzed required for transport of a single substrate molecule exceeding 50 (Frezard and Garnier-Suillerot, 1991; Doige and Sharom, 1992; Shapiro and Ling, 1995).

The apparent low efficiency of Pgp-mediated drug transport observed *in vitro* led several researchers to doubt whether Pgp functions as a drug pump and to suggest alternative roles for Pgp, such as cytoplasm alkalization leading to electrochemical exclusion of drugs from the cells (Roepe *et al.,* 1993; Simon and Schindler, 1994). However, an ever-growing number of reports show that alterations in the Pgp sequence not only affect its capacity to pump drugs, but also the spectrum and specificity of drugs pumped (Devault and Gros, 1990; Yang *et al.,* 1990; Gros *et al.,* 1991; Currier *et al.,* 1992; Dhir *et al.,* 1993; Kajiji *et al.,* 1994; Beaudet and Gros, 1995; Chen *et al.,* 1997; Ueda *et al.,* 1997). Thus, the correlation between Pgp structure and substrate specificity indicates direct pumping of drugs by Pgp.

As described earlier, the apparent low efficiency of Pgp-mediated transport in reconstituted vesicles is the result of technical problems. Alternative experimental approaches, either estimating drug transport in whole cells (Ambudkar *et al.,* 1997) or measuring the uptake of MDR-type drugs indirectly (Eytan *et al.,* 1996a), yielded a higher efficiency of transport with a stoichiometry of close to one ATP molecule hydrolyzed per drug molecule transported. A similar stoichiometry was observed for bacterial-binding, protein-dependent transport systems (also members of the superfamily of ABC transporters) (Bishop *et al.,* 1989; Mimmack *et al.,* 1989).

B. Determination of Stoichiometry in Whole Cells

Whole cells as objects of transport studies present the advantage of a large cytoplasmic volume compared with volume encapsulated within vesicles. The disadvantages of cells compared with reconstituted vesicles are the inability to measure drug-dependent ATPase activity in viable cells and the complexity of transport kinetics due to multiple compartments and drug-binding locations. Several laboratories assayed cellular Pgp-mediated transport (Spoelstra *et al.,* 1992; Nielsen *et al.,* 1994; Ghauharali *et al.,* 1996), but only Ambudkar *et al.* (1997) attempted to assay transport stoichiometry by estimating cellular drug transport and drug-dependent ATPase activity. A major obstacle to accurate estimation of stoichiometry in whole cells is that ATPase activity cannot be estimated readily in viable cells and the activity has to be deduced from *in vitro* essays. Ambudkar *et al.,* (1997) have carefully estimated the number of Pgp molecules actually present at the cell surface. For this purpose, they determined the maximal number of anti-Pgp monoclonal antibody molecules specifically bound to murine

cells expressing human Pgp. Flow cytometry of cells treated with varying amounts of antibody at a fixed cell number and with a fixed amount of antibody at various numbers of cells yielded an estimate of $\sim 2 \times 10^6$ Pgp molecules per cell. The drug-stimulated activity of Pgp ATPase was measured *in vitro* using crude preparations of plasma membranes. The component of drug efflux from cells mediated by Pgp was estimated as either (1) the drug uptake component due to inhibition of Pgp efflux by a Pgp modulator, verapamil, or (2) the energy-dependent component of drug efflux determined as the difference between efflux in the absence and the presence of glucose from energy-depleted cells. Combining all these parameters and extrapolation to conditions prevalent in the cells yielded a stoichiometry of 2.8 ATP molecules hydrolyzed per one vinblastine molecule transported by Pgp. Ambudkar *et al.,* (1997) concluded that due to the uncertainty introduced by their assumptions, this number should be treated as a rough estimate; however, it demonstrates clearly that ATP hydrolysis is linked directly to drug transport by Pgp.

C. Indirect Estimation of Stoichiometry in Reconstituted Pgp Vesicles

Estimation of the number of ATP molecules hydrolyzed per drug molecules transported requires determination of the rates of both Pgp-dependent drug transport and the ATP hydrolysis driving drug transport under identical experimental conditions. Pgp-mediated drug uptake was estimated as ATP-driven, valinomycin-dependent cation uptake into proteoliposomes reconstituted with Pgp (Eytan *et al.,* 1994, 1996a) using the strategy presented in Fig. 14. Valinomycin, a cation ionophore with a high affinity toward K^+ ions, is a substrate of Pgp. Presumably, Pgp molecules reconstituted with their ATP-hydrolyzing sites exposed to the medium catalyze the ATP-driven uptake of valinomycin. Because the concentration of K^+ ions in the medium is high, the ionophore is saturated with cations and the molecular species transported by Pgp is actually an ionophore–cation complex. Within the vesicles, the cations are released and the excess ionophore leaks out of the vesicles. Presumably, the passive transmembrane movement of uncharged ionophore is faster compared to the positively charged cation–ionophore complex in contradistinction to Pgp-mediated active transport, where positively charged molecules are preferable substrates.

Valinomycin allows passive uptake of K^+ ions into proteoliposomes, which can be measured using $^{86}Rb^+$ ions as a radioactive probe. ATP hydrolysis accelerates both the rate and the maximal level of valinomycin-dependent K^+ uptake into proteoliposomes reconstituted with Pgp (Eytan *et al.,* 1996a). The ATP-dependent transport, calculated as the difference

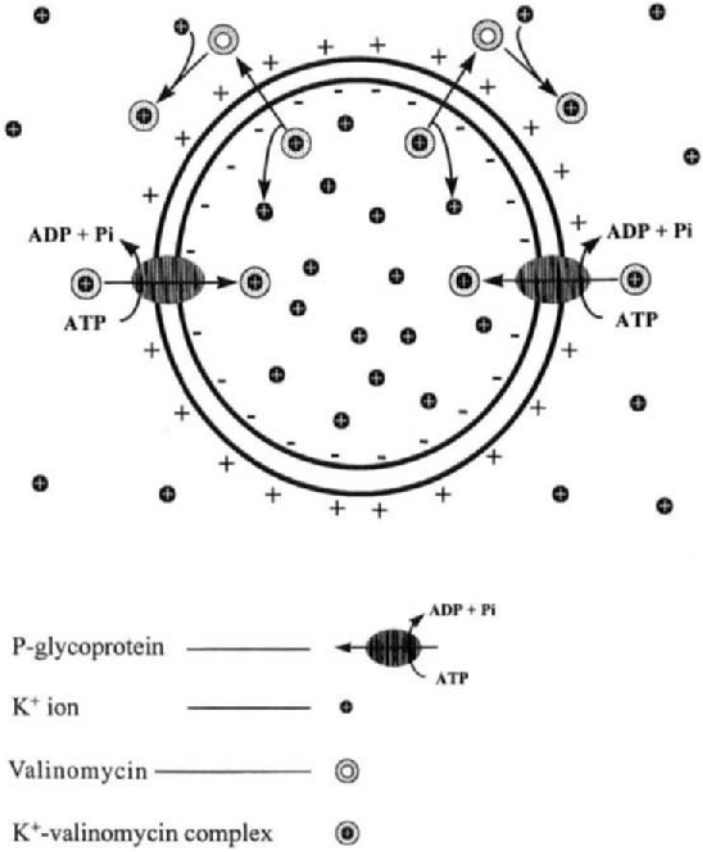

FIG. 14 The mechanism of ATP-driven and valinomycin-dependent Pgp-mediated cation uptake. Pgp actively transports cations into proteoliposomes by the following mechanism: Pgp catalyzes the ATP-dependent uptake of the K^+–ionophore complex into the vesicles. The actively transported K^+ ions remain within the vesicles and are monitored using the radioactive probe, $^{86}Rb^+$, while the hydrophobic ionophore leaks out of the vesicle. Thus, Pgp-mediated transport is measured indirectly as cation uptake (adapted with permission from Eytan *et al.*, 1996a).

between the uptake values obtained in the presence of ATP and those obtained in the presence of its nonhydrolyzable analogue AMPPCP, reached a maximum after 2 min (Fig. 15A) and was dissipated after a 30-min incubation. As expected for Pgp-mediated activity, the ATP-dependent uptake component was restricted to proteoliposomes reconstituted with a Pgp-rich fraction from Pgp-overexpressing cells and was absent from proteoliposomes reconstituted with a parallel fraction from the parental drug-sensitive subline (Fig. 15B).

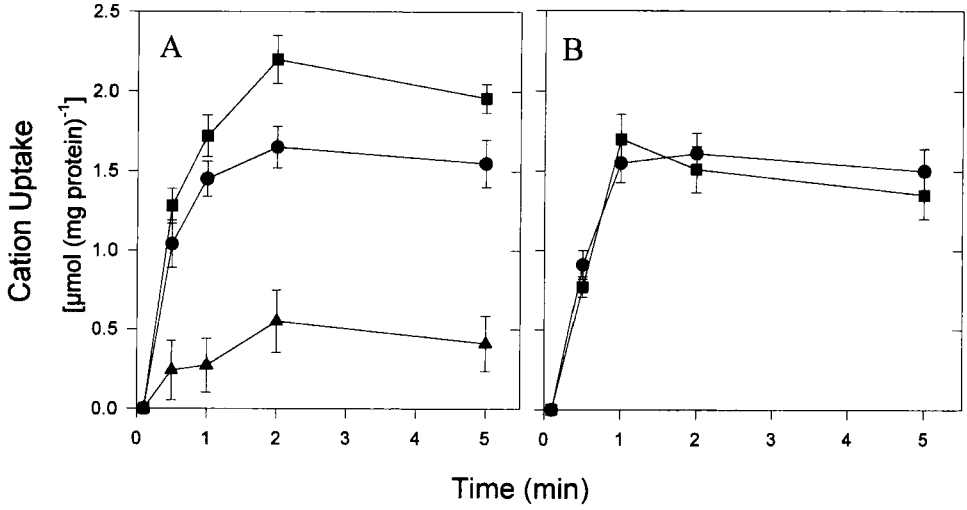

FIG. 15 Time course of cation uptake into reconstituted proteoliposomes. A plasma membrane fraction from Pgp-overexpressing cells (A) and a corresponding protein fraction from Pgp-poor cells (B) were extracted and reconstituted for cation uptake as described in the legend of Fig. 14. Cation uptake was measured in an assay medium containing valinomycin, $^{86}Rb^+$ as a radioactive probe for K^+, and 3 m*M* of either ATP (■) or AMPPCP (●). The buffer was preincubated for 2 min at 37°C, and the reaction was initiated by the addition of Pgp-reconstituted proteoliposomes. The ATP-dependent cation uptake (▲) was calculated by subtracting the values obtained in the presence of AMPPCP (adapted with permission from Eytan *et al.*, 1996a).

In order to discern between the ATP-dependent uptake and the ATP-independent ionophore-mediated passive equilibration of K^+ ions, reconstituted proteoliposomes were incubated for 3 min in a transport buffer containing valinomycin but lacking ATP. Under these conditions, K^+ ions were allowed to reach apparent equilibrium with the K^+ trapped in the proteoliposomes (see Fig. 15). At this point, ATP or AMPPCP was added and K^+ uptake was determined (Fig. 16). The ATP-dependent component of K^+ uptake was not affected by preincubation. Thus, the ATP-dependent uptake results from an authentic active K^+ ion uptake and not from effects on the ionophore-mediated equilibration of K^+ ions across the proteoliposome membrane. The ATP- and valinomycin-dependent K^+ ion uptake mediated by CHO Pgp was specific to ATP and did not occur with UTP, CTP, ADP, and nonhydrolyzable trinucleotides. The rate of drug transport determined by this procedure can only serve as a rough estimate of actual drug transport, as it is measured under conditions allowing massive passive transport of K^+ ions across the vesicle membranes, resulting in a large background of passive uptake and a leak of cations that are actively taken up.

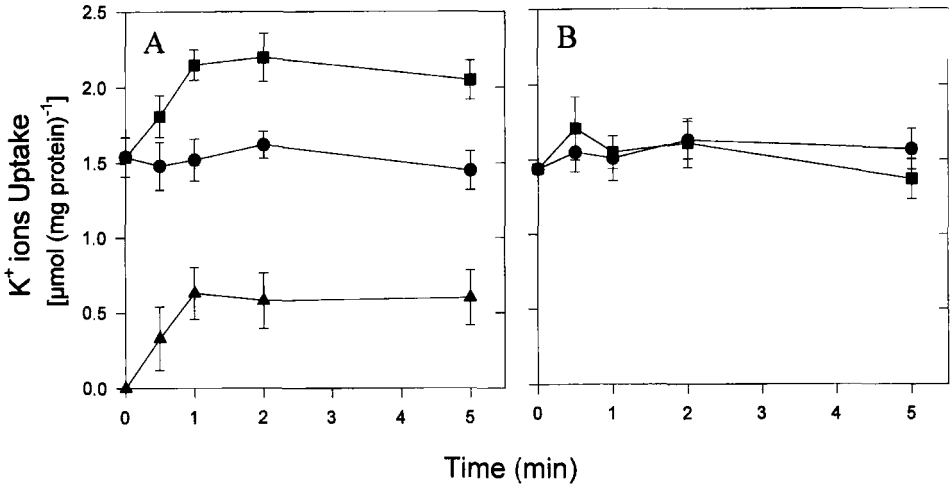

FIG. 16 The effect of preincubation of reconstituted proteoliposomes in the absence of ATP on the subsequent ATP-dependent uptake of cations. After preincubation for 3 min at 37°C, 5-µl proteoliposomes were reconstituted with either a Pgp-enriched membrane fraction (A) or a corresponding protein fraction from Pgp-poor cells (B) and further incubated for 3 min. At this time point, ATP (■) or AMPPCP (●) was added. The ATP-dependent cation uptake (▲) was calculated by subtracting the values obtained in the presence of AMPPCP (adapted with permission from Eytan *et al.*, 1996a).

The rates of drug transport measured must be compared with the ATPase activity of Pgp measured under identical experimental conditions. However, as shown in Fig. 17, the situation is not as straightforward as might be expected. Pgp exhibits a basal (i.e., with no substrate added) ATPase activity (Ambudkar *et al.*, 1992; al-Shawi and Senior, 1993; Sharom *et al.*, 1993; Shapiro and Ling, 1994; Urbatsch *et al.*, 1994; Borgnia *et al.*, 1996). This basal activity is stimulated by valinomycin and emetine, the selecting drug used to establish the specific Pgp-overexpressing cell line (Fig. 17A). However, a major problem is that the minimal valinomycin concentrations required for demonstrating stimulation of ATPase activity are higher than 0.1 μM, whereas appreciable ATP- and valinomycin-dependent cation uptake is already evident at a concentration of 0.1 μM valinomycin. Thus, the high basal activity demonstrated by Pgp presumably masks the ATPase activity required for valinomycin-dependent transport. Because basal ATPase activity has been shown to be inhibited competitively by gramicidin D (Borgnia *et al.*, 1996), it is due to a putative endogenous substrate present in all Pgp preparations. To overcome this obstacle, we looked for a Pgp inhibitor capable of reversibly repressing the basal ATPase activity without exerting a deleterious effect on the proteoliposomes. Poly-L-tryptophan

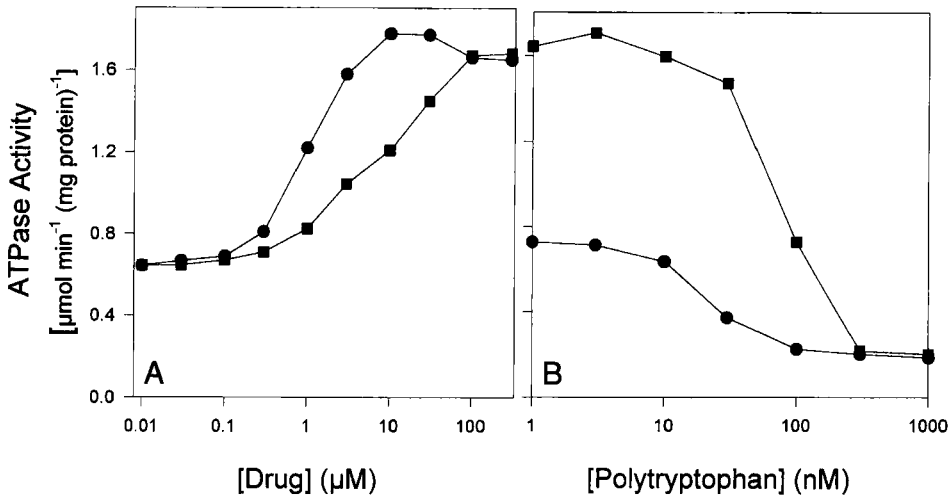

FIG. 17 Modulation of Pgp ATPase activity by emetine, valinomycin, and poly-L-tryptophan. Pgp was extracted from Pgp-overexpressing cells and was reconstituted into proteoliposomes. (A) The ATPase activity of these proteoliposomes was determined in the presence of various concentrations of either emetine (■) or valinomycin (●) for 1 h. (B) The ATPase activity of Pgp proteoliposomes determined in the presence of 100 μM (■) or absence (●) of valinomycin and various concentrations of poly-L-tryptophan (5.4 kDa) (adapted with permission from Eytan *et al.*, 1996a).

met these expectations; at concentrations lower than 100 nM it inhibited both the basal ATPase and the substrate-stimulatable activities of Pgp (Fig. 17B). These concentrations of poly-L-tryptophan had no harmful effects on the integrity of the proteoliposomes as revealed by the retention of encapsulated $^{86}Rb^+$ or calcein. Most importantly for this study, low concentrations of poly-L-tryptophan repressed the basal ATPase activity and, at concentrations required for mediation of ATP-dependent cation-uptake (see later), valinomycin was reactivated (Fig. 18). The Michaelis–Menten-type competitive inhibition exerted by poly-L-tryptophan on the stimulatory effect of valinomycin is presented in Fig. 18B as a Lineweaver–Burk plot.

In the presence of poly-L-tryptophan, low valinomycin concentrations mediated an increase in both ATPase activity and cation uptake (Fig. 6B), allowing determination that 0.5–0.8 cations were transported per ATP molecule hydrolyzed. A similar ratio was obtained by an alternative approach where inhibitors were used to inhibit both ATPase and cation uptake (Fig. 19); thus it was possible to estimate the apparent stoichiometry as the ratio of parallel reductions in cation uptake and ATP hydrolysis. Pgp ATPase as well as valinomycin- and ATP-dependent uptake were both eliminated by the established inhibitors of Pgp, such as vanadate and oligo-

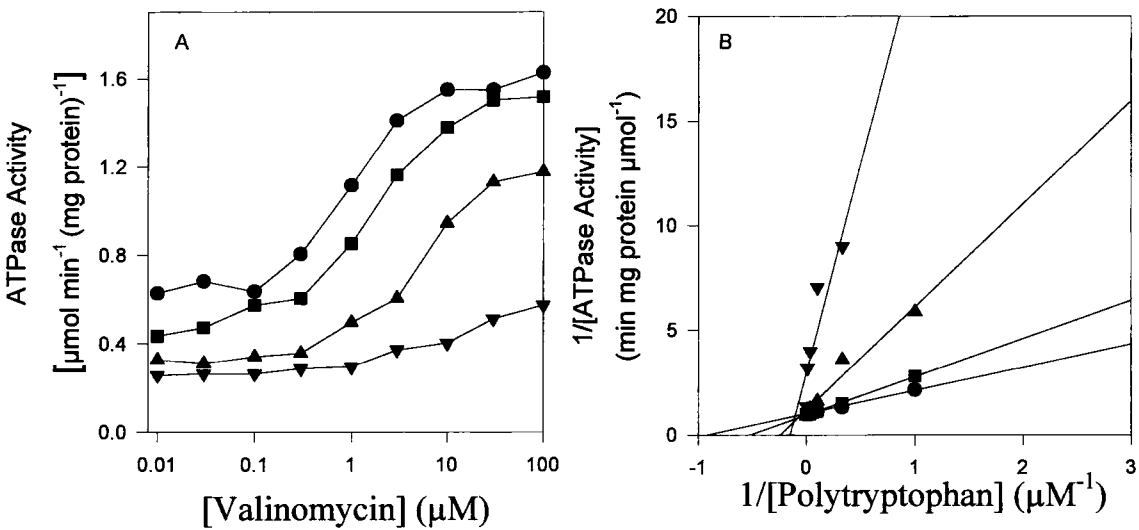

FIG. 18 Activation of poly-L-tryptophan inhibition of Pgp ATPase by valinomycin. (A) The ATPase activity of Pgp proteoliposomes was determined in the presence of various concentrations of valinomycin or emetine and with the following concentrations (μM) of poly-L-tryptophan (5.4 kDa): 0 (●), 1 (■), 10 (▲), and 100 (▼). (B) The same experimental data are presented as a Lineweaver–Burk plot (adapted with permission from Eytan *et al.*, 1996a).

mycin, and by high concentrations of poly-L-tryptophan (Eytan *et al.*, 1996a).

V. An Integrative Model of Drug Transport in MDR Cells

Despite extensive experimental data describing various aspects of drug transport in MDR cells, a comprehensive model integrating passive drug transport and the active Pgp-mediated efflux is badly needed for deeper insight into the MDR phenomenon. This section presents a model based on MDR-type drugs traversing membranes by a flip-flop mechanism (Figs. 1 and 20). Previous models (Demant *et al.*, 1990; Higgins and Gottesman, 1992; Simon and Schindler, 1994; Stein *et al.*, 1994; Stein, 1997) have attempted to explain the apparent paradoxes detailed in the introduction by attributing unique features to Pgp such as pumping from the outer leaflet of the plasma membrane. In our model, Pgp functions similarly to well-established ion-transporting ATPases, such as Na^+, K^+-ATPase and Ca^{2+}-ATPase(s), and it proves well capable of efficiently excluding MDR-type

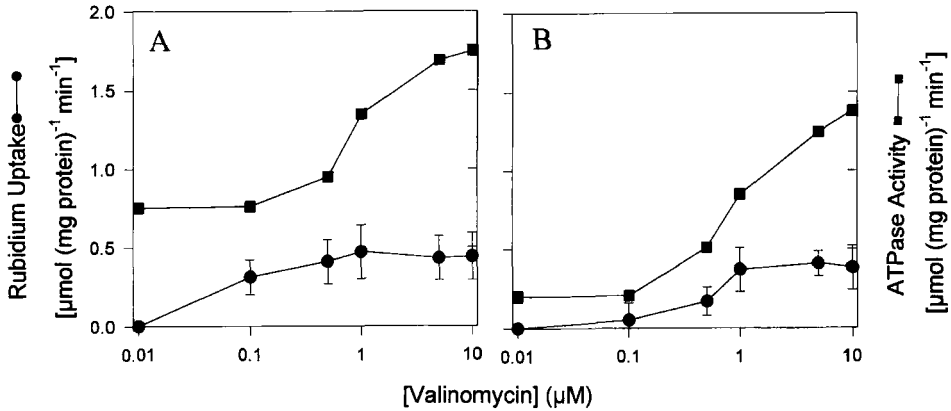

FIG. 19 Determination of stoichiometry of molecules of drug transported to ATP hydrolyzed by estimation of Pgp ATPase and cation uptake activities as a function of valinomycin concentration. Pgp was extracted and reconstituted, and both ATPase and cation uptake were measured simultaneously in uptake medium containing various concentrations of valinomycin, poly-L-tryptophan (5.4 kDa), and 3 m*M* of either ATP or AMPPCP. The cation uptake (●) and ATPase activity (■) were assayed for 0.5 and 60 min, respectively. The ATP-dependent cation uptake was calculated by subtracting the values obtained in the presence of AMPPCP. The ATPase rates presented were calculated by subtracting the corresponding values obtained in the absence of valinomycin (adapted with permission from Eytan *et al.*, 1996a).

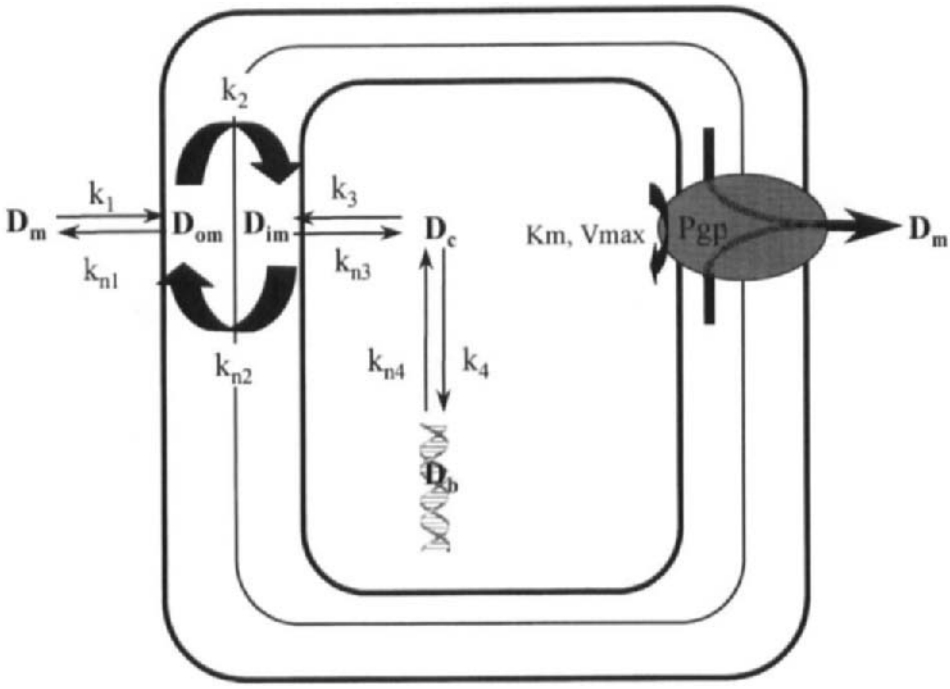

FIG. 20 Theoretical model used to describe the kinetics of drug uptake by, and extrusion from, MDR cells.

drugs from MDR cells, except that Pgp extracts its substrates directly from the inner leaflet of the plasma membrane and not from the cytoplasm. The model is based mainly on experimental data obtained *in vitro* on partial reactions, such as flip-flop of drugs across membranes, Pgp ATPase activity, stoichiometry of drug pumped by Pgp relative to ATP molecules hydrolyzed, and the pumping mechanism of Pgp. The purpose of the model is to allow better critical evaluation of the implications of experimental data on the pumping mechanism of Pgp. Thus, the model shows that in kinetic terms, Pgp activities present in MDR cells can confer resistance levels observed in the laboratory whether the drugs are extracted from the cytoplasm or from the inner leaflet of the plasma membrane. Moreover, the model indicates that kinetic studies measuring drug or dye concentration in the cytoplasm will not contribute to our determination whether Pgp pumps its substrates from the cytoplasm or directly from the plasma membrane. The model clearly indicates that Pgp can effectively extract drugs from the inner leaflet of the plasma membrane, but not from the outer leaflet. However, as detailed in Section VI, experimental data favor the

possibility that Pgp extracts substrates directly from the inner leaflet of the membrane and, thus, the version of the model preferred here is based on Pgp-mediated drug extraction directly from the membrane.

In case experimental data are not available, e.g., for the binding rate and affinity of drugs to cellular components, a range of possible constants was applied, the implications for Pgp-mediated cellular MDR were studied, and the results are discussed.

The model is based on a four-compartment system (see Fig. 20) where the compartments represent the extracellular medium (o), the outer leaflet of the plasma membrane (om), the inner leaflet of the plasma membrane (im), and the cytoplasm. The drug in the cytoplasm is either free (c) or bound to cellular components, e.g., DNA (D_b).

The simplification has been made that the effects of pH on drug uptake are ignored. The pH in the cytoplasm was assumed to be similar to that of the outer medium and no distinction has been made between charged and neutral drug molecules (although this could be incorporated easily into the model). The contribution of pH-dependent drug trapping in a pH 5.0 endosomal compartment has been shown to be inadequate to account for the drug efflux in MDR cells (Demant *et al.*, 1990) and was ignored in the present model. The model describes a standard drug uptake experiment where 10^6 cells/ml are exposed to 1 μM drug, such as doxorubicin. The parameters used for the model are as follows.

As described in Section III, most MDR-type drugs are hydrophobic and positively charged and therefore become incorporated readily into membranes. Binding of drugs to the plasma membrane and their release into the cytoplasm are fast, as compared to the rate of transbilayer movement (Speelmans *et al.*, 1994; Bolhuis *et al.*, 1996; Regev and Eytan, 1997). Drug absorption into the outer leaflet of the plasma membrane is described in terms of a first-order binding constant with a lifetime of 0.1 sec ($k_1 = 10$ sec^{-1}). The rate of drug release from the membrane into the outer medium is $k_{n1} = k_1 \times P$, where P is the partition coefficient of the drug, e.g., doxorubicin, into membranes compared with aqueous medium. Daunorubicin association with the outer leaflet of the plasma membrane of cancer cells has been determined by extrapolation to zero time to be equal to the drug amount present in two cell volumes. (Spoelstra *et al.*, 1992). Thus, a similar value has been used for our model. The high affinity of drugs, such as daunorubicin, toward membranes results in high drug concentrations in the plasma membrane. Thus, when cells are suspended in 1 μM doxorubicin, the drug concentration in the outer leaflet of the membrane is 40 mM (assuming $P = 40{,}000$ based on the amount of drug bound to the cell surface). Rate constants similar to those observed at the cell surface are assumed for the absorption and release of drugs from the cytoplasm to the plasma membrane and release ($k_3 = 0.25$ sec^{-1}; $k_{n3} = k_3/P$).

The transport across the plasma membrane occurs by a flip-flop mechanism with a lifetime of 1 min (see Section II).

The rate of Pgp-mediated drug efflux was assumed to equal Pgp ATPase activity. The apparent K_m of the cellular Pgp pump was estimated experimentally, assuming Pgp extracts its substrates from the cytoplasm, to be equivalent to 1.5 μM for daunorubicin (Spoelstra *et al.*, 1992) and 1.1 μM for vinblastine (Horio *et al.*, 1990). Therefore, the version of the model that is based on drug extraction from the cytoplasm is based on a Pgp K_m of 1 μM. However, the version based on Pgp-mediated direct extraction from the membrane must assume a Pgp K_m equivalent to the much higher drug concentrations encountered within the plasma membrane. The K_m used in our model was the drug concentration in the plasma membrane attained under equilibrium conditions when the drug concentration in the cytoplasm was 1 μM. Thus, the Pgp K_m is assumed to be either 1 μM or $P \times 1\ \mu M = 40$ mM when the drugs are extracted from the cytoplasm or directly from the membrane, respectively.

Because cellular ATP concentrations exceed the reported K_m concentrations of Pgp ATPase activity, of the Pgp is assumed to be saturated with ATP.

The V_{max} of Pgp ATPase activity in Pgp-overexpressing rodent and human cells generated in the laboratory ranges between 5 and 50 nmol $(10^9$ cells$)^{-1}$ sec^{-1}. Pgp ATPase activity observed in cells obtained from cancer patients is variable and lower compared to MDR cells generated in the laboratory. In the model, similar V_{max} values were assumed for drug extraction from the cytoplasm and directly from the membrane.

The number of cellular drug-binding sites varies with the drug type. The model described here, for doxorubicin uptake, was based on *in vitro* experiments of drug binding to chromatin so we assume an amount of DNA capable of binding 2.5 μmol of drug per 10^9 cells with half-saturation occurring at 5 μM drug (Dano, 1971; Sabeur *et al.*, 1979; Demant *et al.*, 1990). The large number of cellular-binding sites leads to extensive drug trapping within the cells. Association of doxorubicin with nuclear DNA entails either transport through the two membranes of the nuclear envelope and/or diffusion through the nuclear pores. Thus, doxorubicin trapping was assumed to be relatively slow and to occur with a lifetime of 15 min.

Other parameters used in the computer simulation are detailed in Fig. 21. The curves presented were generated by the simulation of cellular pharmacokinetics of a drug, similar to doxorubicin, using the computer program given in the Appendix.

The computer simulation of doxorubicin uptake into drug-sensitive and MDR cells is presented in Fig. 21. The curves were based on "best" experimental *in vitro* data and compare favorably with experimental data obtained from various cell types and drugs (Skovsgaard, 1978; Inaba *et al.*, 1979;

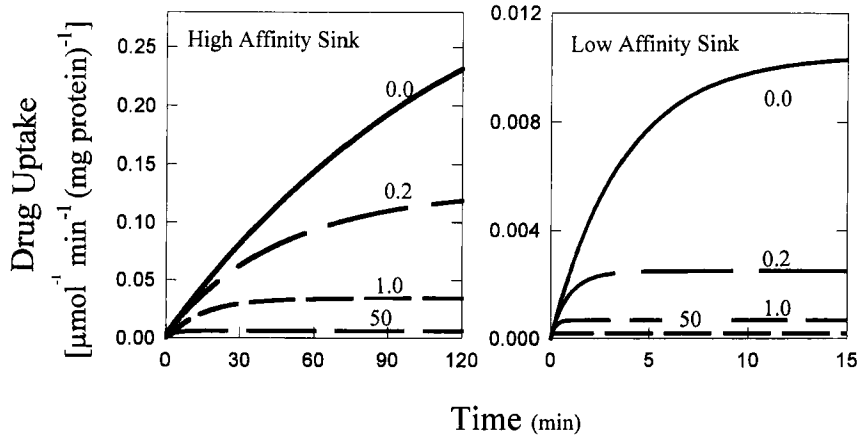

FIG. 21 Simulated time curves of doxorubicin uptake into cells containing either DNA (high-affinity sink) or a putative low-affinity, drug-binding sink (low-affinity sink). Solid lines are predictions for drug-sensitive cell system with no pump activity. Broken lines are predictions of an MDR cell system with various Pgp pump activity levels. In typical uptake experiments, drug bound to the surface of the cells is washed away and is not considered a cell-associated drug. Thus, the drug uptake presented includes the drug transported into the cells, but excludes the drug associated with the outer leaflet of the plasma membrane. The amount of DNA available for drug trapping in the cells is 2.5 μmol $(10^6 \text{ cells})^{-1}$, and the drug concentration required for half-saturation was 5 μM (high-affinity sink) an affinity similar to that of doxorubicin toward DNA (Dano, 1971; Sabeur *et al.*, 1979; Demant *et al.*, 1990) and 500 μM (low-affinity sink), respectively. Numbers denote the V_{max} of the Pgp pump activity in nmoles sec^{-1} $(10^9 \text{ cells})^{-1}$. Typical Pgp-overexpressing cells selected in the laboratory for drug resistance exhibit a pump activity of 5–50 nmol sec^{-1} $(10^9 \text{ cells})^{-1}$ (Ambudkar *et al.*, 1992; Spoelstra *et al.*, 1992; al-Shawi and Senior, 1993). The amount of Pgp observed in cells obtained from cancer patients is lower and more variable compared to Pgp-overexpressing cells produced in the laboratory (Beck *et al.*, 1996). The lifetimes corresponding to the various rate constants used for computer simulations were: k_1, 0.01 sec; k_{n1}, 250 sec; k_2, 60 sec; k_{n2}, 60 sec; k_3, 2.5 k_{n3}, 0.0001 sec; k_4, 900 sec; k_{n4}, and 225 sec. Cell volume (CV) equals 1500 μm^3 (Sehested *et al.*, 1987; Ramu *et al.*, 1989) introduced into the model as Fcv (volume of the cells as the fraction of total volume). The available cell fraction (Acf) equals 0.7, which is defined as the cell volume fraction capable of osmotic activity and presumably available to soluble drugs (DuPre and Hempling, 1978). The fraction of total cell volume occupied by the plasma membrane leaflets (Bov = Biv) equals 0.00005, as the volume of the plasma membrane was assumed to constitute 0.0001 of cellular volume (Sehested *et al.*, 1987) and to be distributed equally between the two leaflets.

Dalmark and Hoffmann, 1983; Cano-Gauci *et al.*, 1990; Frezard and Garnier-Suillerot, 1991; Dordal *et al.*, 1992; Stein *et al.*, 1994).

Association and dissociation of anthracyclines to membranes are fast, and the drug concentration in the cytoplasm, and the extracellular medium, is practically in equilibrium with drug concentrations in the adjacent leaflets of the plasma membrane. The kinetics of drug uptake into drug-sensitive

cells, in the absence of active efflux, are determined by the flip-flop rate across the plasma membrane and the amount of drug trapped within the cells. At equilibrium, the concentration of the drug free in the cytoplasm is equal to the concentration of drug in the extracellular medium. However, due to the massive drug trapping by DNA, the total intracellular concentration appears close to 400-fold higher than that in the medium. Thus, drug uptake into cells can be envisaged as a continuous process of passive influx of drug into the cytoplasm coupled to trapping, as drug binds to macromolecules that act as a sink.

Better insight into the mechanism of passive drug uptake is gained by the analysis of initial drug entry into the various cell compartments as presented in Fig. 22. Initially, the outer membrane leaflet is saturated with drug. Drug transported into the cell from the outer leaflet is replaced immediately by extracellular drug, and the amount of drug bound in the outer leaflet of the cell membrane remains constant, as long as the drug concentration in the medium is constant. Drug from the outer leaflet flips over to the inner leaflet of the plasma membrane where it is in "effective" equilibrium with the drug present in the cytoplasm. Thus, in drug-sensitive cells, drug concentrations in the inner leaflet, and in the cytoplasm, exhibit parallel kinetics. Interestingly, despite the flip-flop lifetime of 1 min, the initial rapid uptake into the inner leaflet is complete within 15 sec. Drug trapping within the cell keeps the drug concentration in the cytoplasm relatively low and consequently also in the inner leaflet of the membrane. These low drug concentrations are reached very rapidly, and drug concentrations in these compartments are elevated slowly as the drug-binding sites within the cell become saturated. Surprisingly, despite the slow trapping rate (the lifetime of the process was set in the model to 15 min) after only a few seconds, most of the drug associated with the cells is bound to DNA. The role of a high-affinity molecular sink, such as DNA for anthracyclines or tubulin for *vinca* alkaloids, in cellular drug kinetics can be appreciated when it is replaced by a putative low-affinity sink, such as intracellular membranes (Fig. 21). The latter situation simulates drug kinetics in cells exposed to drugs that exhibit high affinity toward their cellular target, but the cells do not contain massive amounts of high-affinity drug-binding sinks. Examples of such situations are cells exposed to antifolates that bind with high affinity to their target enzymes that are present in tiny amounts, but exhibit low affinity toward cellular components such as membranes. Comparison of the two panels of Fig. 21 reveals that the initial rate of the drug uptake rate into drug-sensitive cells is determined by the drug flip-flop rate across the plasma membrane and is not affected by the amount or affinity of cellular drug-trapping sites. The cellular drug traps determine the amount of drug associated at equilibrium with the cells and the time span required to reach equilibrium. In the absence of intracellular drug-

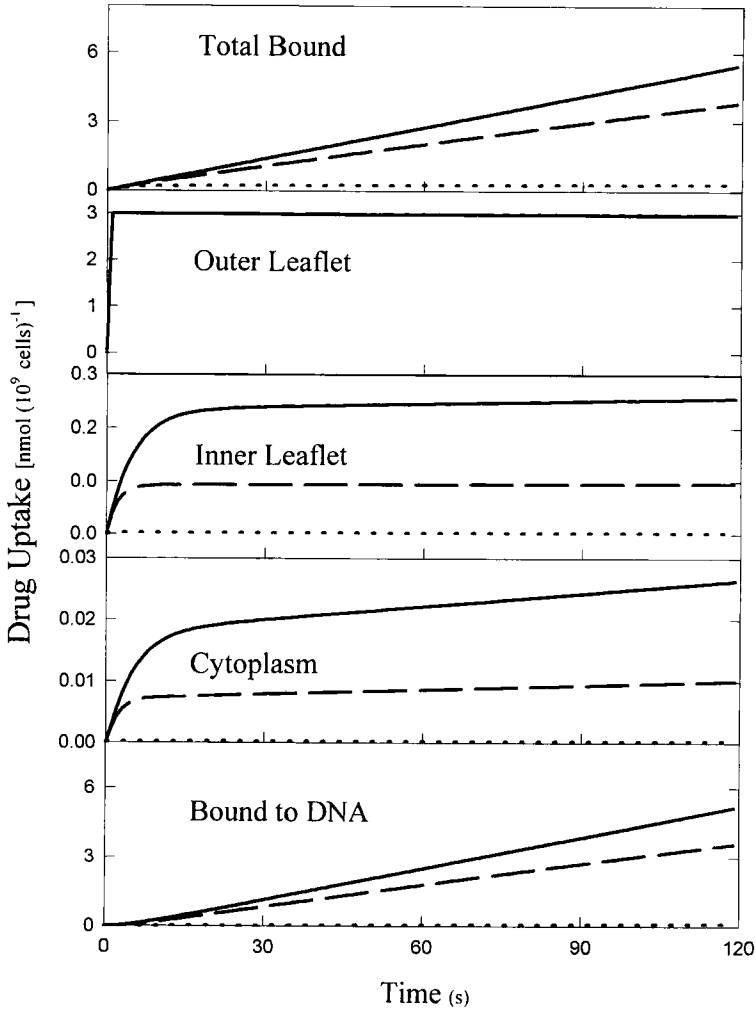

FIG. 22 Simulation of the time course of drug uptake into the various compartments of drug-sensitive and MDR cells. Computer simulation of drug uptake into drug-sensitive cells (solid lines) and MDR cells exhibiting either moderate [1 nmol $(10^9$ cells$)^{-1}$ sec^{-1}], broken lines) or high [50 nmol $(10^9$ cells$)^{-1}$ sec^{-1}, dotted lines] Pgp ATPase activity was performed using the parameters presented in Fig. 21.

binding sites, the equilibration rate of the drug that is free in the cytoplasm, and in the extracellular medium, is determined solely by its flip-flop rate across the membranes.

Incorporation of the phenomenon of Pgp-mediated active drug efflux into the model reveals that the Pgp activity levels measured in membranes

prepared from MDR cells are adequate to substantially reduce drug uptake into cells (Figs. 21 and 22). ATPase activities of 1 and 50 nmol $(10^9 \text{ cells})^{-1}$ sec^{-1}, observed in moderately and highly resistant cells, reduce cellular drug uptake 12- and 450-fold, respectively. As detailed in Section V, the source of the Pgp substrates, whether from the cytoplasm or the plasma membrane, has no effect on overall drug pharmacokinetics. The resistance level of cells is determined not by the total drug associated with the cells, but by the drug concentration free in the cytoplasm or the nucleus. Simulation of drug concentration free in the cytoplasm reveals a 13- and 650-fold reduction in moderately and highly resistant cells, respectively (Figs. 22 and 23). Analysis of the effect of Pgp on drug concentrations in the various cell compartments shows that Pgp extracting drugs directly from the plasma membrane reduces the drug concentration in the cytoplasm, as the amount of drug bound to DNA, and the drug concentration in the inner leaflet of

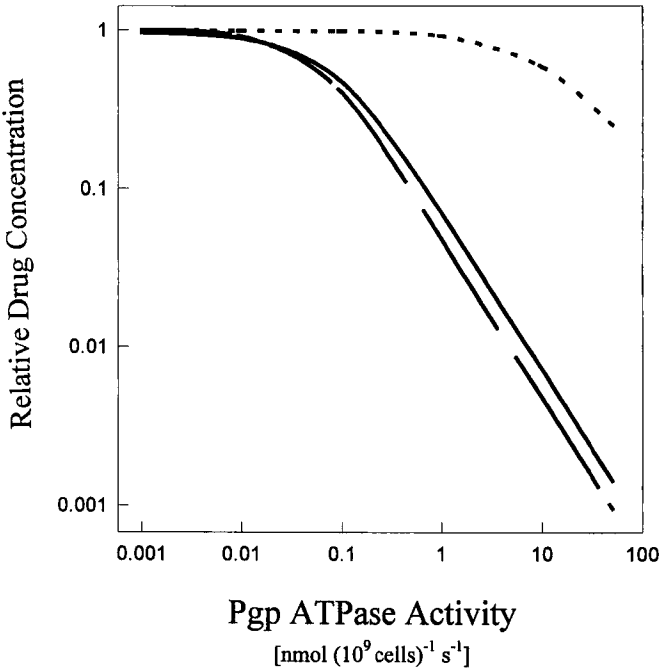

FIG. 23 Effect of cellular drug-binding capacity on predicted MDR. Drug concentrations free in the cytoplasm were predicted using the parameters described in Fig. 21 except that the affinity of the drug toward cellular drug-binding sites, e.g., DNA, was varied as follows: half-saturation similar to doxorubicin binding to DNA at 5 μM (solid lines), 1000-fold lower affinity (dashed lines), and 1000-fold higher affinity (dotted lines). The curves presented depict cellular drug uptake after quasi-equilibrium conditions had been attained.

the plasma membrane are reduced, but it has no effect on the drug associated with the outer leaflet of the plasma membrane.

Binding sites with high affinity toward a drug interfere with the Pgp-mediated efflux of that drug from MDR cells. As shown in Figs. 22 and 23, Pgp competes with intracellular drug-binding sites and, as a result, will afford less resistance toward drugs that bind with high affinity to intracellular sites, including sites distinct from their pharmacological targets. Thus, the level of cellular resistance toward a drug is a function of multiple factors, including the "trapping affinity" of the drug by cellular components.

A possible additional variable that determines cellular pharmacokinetics is the rate at which a drug reaches its intracellular binding sites. The situation described in Figures 21–23 is based on very slow binding of the drug to its main cellular trapping sites. We have examined the effect of the rate at which drug present in the cytoplasm is trapped by binding to intracellular sites on its long-term pharmacokinetics. For this purpose, the cellular drug uptake into cells with a very slow trapping rate (lifetime of 15 min) was compared with the uptake into cells with a fast tapping rate (lifetime of 0.1 sec). Surprisingly, despite the huge difference in the binding rate, no effect on long-term cellular pharmacokinetics was observed, neither on the passive drug uptake into sensitive cells nor on the efficacy of Pgp in reducing drug uptake by the cells (Fig. 24).

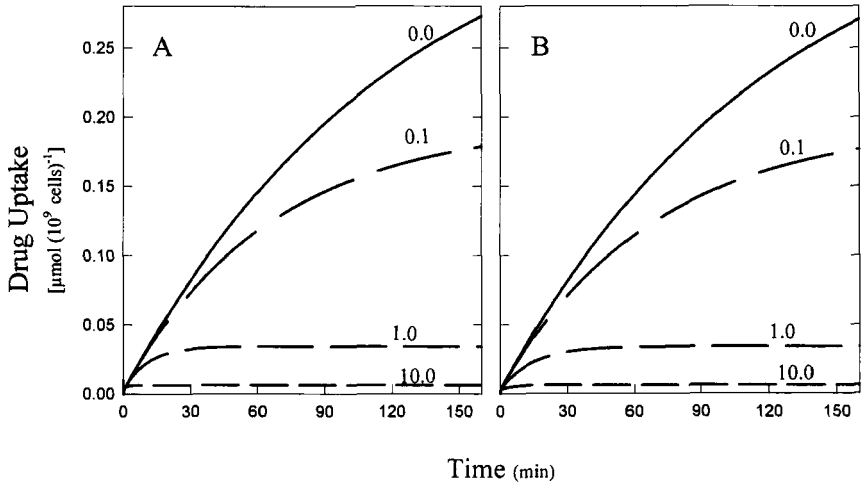

FIG. 24 Effect of drug intracellular binding rate on long-term cellular drug uptake. Drug uptake kinetics were predicted using the parameters described in the legend of Figure 21 except that the binding rate of the drug to the cellular molecular trap, k_4, occurred with a lifetime of 0.1 sec or 15 min for A and B, respectively. The numbers represent Pgp activity in nmoles $(10^9 \text{ cells})^{-1}$ sec^{-1}.

A major feature of the cellular pharmacokinetics model is the slow flip-flop of the drug across the plasma membrane. Figure 25 compares cellular pharmacokinetics of drugs with a flip-flop rate constant of either 60 or 1 sec^{-1}. Acceleration of the rate of transmembrane movement of the drug results in faster drug uptake into drug-sensitive cells and interferes with Pgp-mediated drug expulsion from MDR cells. The efficacy of Pgp in reducing drug concentrations in the cytoplasm is compromised by a high rate of transmembrane pumping of the drug (Fig. 26). Acceleration of both the drug transmembrane movement rate (k_2, k_{n2}) and the release from the inner membrane (k_3) can completely abolish the capacity of Pgp to reduce drug concentration in the cytoplasm (Fig. 26).

The efficacy of Pgp in conferring MDR on cells is dependent on (1) Pgp pumping capacity and affinity toward the specific drug, (2) the transmembrane movement rate of the drug, (3) affinity of the drug toward its pharmacological cellular target, and (4) affinity of the drug toward intracellular trapping sites, such as DNA for anthracyclines.

VI. Is the Multidrug Transporter a Flippase?

A. Theoretical Considerations

The hydrophobic nature of Pgp substrates and their range of chemically unrelated structures have led Higgins and Gottesman (1992) to suggest

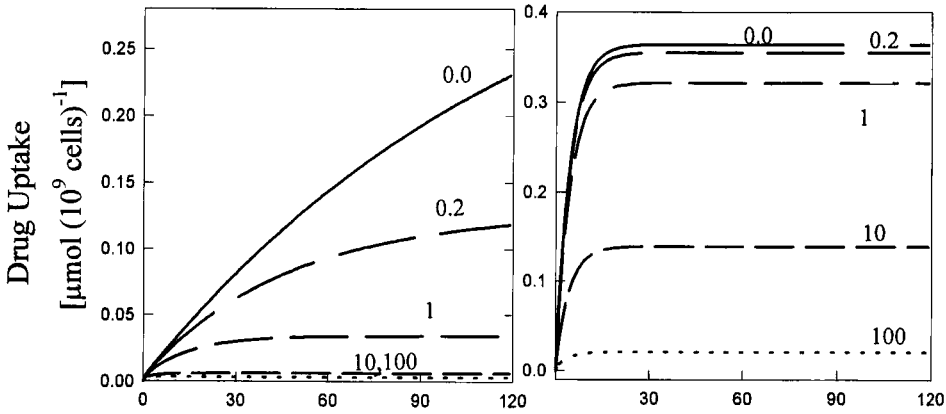

FIG. 25 Effect of rate of transmembrane movement of the drug on its rate of uptake into cells. Drug uptake into cells containing various levels of Pgp ATPase activities [numbers represent ATPase activity in nmol (10^9 cells)$^{-1}$ sec^{-1}, A] were predicted using the parameters of Figure 21 except that the transmembrane drug movements (k_2 and k_{n2}) occurred with a lifetime of 1 sec (B).

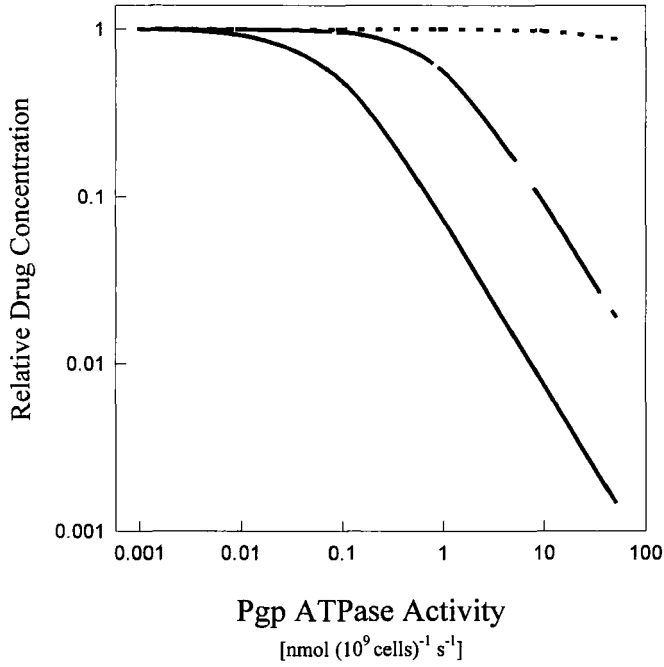

FIG. 26 Effect of transmembrane movement rate of the drug on its concentration in the cytoplasm. The time course was simulated using the parameters detailed in the legend of Figure 21 (solid lines) except that the flip-flop rate constant for the plasma membrane (k_1 and k_{n1}) had a lifetime of 1 (broken lines) and 0.01 (dotted lines) sec, and the drug release from the inner surface of the plasma membrane also occurred with a lifetime of 0.01 sec.

that Pgp functions as a flippase, extracting its substrates directly from the inner leaflet of the plasma membrane. Based on kinetic evidence, Stein (1997) suggested that Pgp extracts its substrates from both leaflets of the plasma membrane. Thus, three possibilities for the mechanism of Pgp pumping have to be considered: (1) Pgp functions similarly to ion transporting ATPases and extracts it substrates directly from the cytoplasm; (2) Pgp functions as flippase, extracting its substrates from the inner leaflet of the plasma membrane; and (3) Pgp extracts its substrates from the outer leaflet of the plasma membrane, thus largely preventing the drug from reaching the cytoplasm. Before considering experimental data supporting the various possibilities, let us evaluate, using the computer model, the implications of the various possibilities on cellular pharmacokinetics. We compared the effect of Pgp pumping from the various cell compartments on drug uptake into cells. We assumed a similar V_{max} for Pgp and a high-affinity K_m (1 μM) for the case in which Pgp extracts its substrates from the cytoplasm

or a low affinity (1 $\mu M \times P = 40$ mM) for the case when Pgp extracts it substrate from the plasma membrane. The logic of this assumption is that the K_m of Pgp has been determined, assuming that it pumps its substrates from the cytoplasm (Horio *et al.,* 1990; Spoelstra *et al.,* 1992). Under the same experimental conditions, the drug concentration within the membrane is much higher and the K_m for the cases that Pgp extracts its substrates from the plasma membrane has to be modified accordingly. As shown in Fig. 7, the efficacy of Pgp in removing drug from cells is similar whether Pgp extracts its substrates from the cytoplasm or from the inner leaflet of the membrane. This result holds over a wide range of drug release rates from the plasma membrane into the cytoplasm (results not shown). Thus, in terms of total drug concentration, the efficacy of Pgp is similar whether it functions as a high-affinity cytoplasmic pump or a low-affinity pump extracting its substrates directly from the inner leaflet of the membrane.

Pgp-mediated drug pumping from the outer leaflet appears futile. It has no effect on cellular drug content (Fig. 27) due to the fast binding of drugs to the membrane, as drugs extracted from the outer leaflet of the membrane are immediately replenished by drug present in the extracellular medium.

Analysis of the effect of the mechanism of Pgp pumping on drug exclusion from the various cell compartments showed that the source of drugs pumped, whether from the cytoplasm or from the plasma membrane, has no effect on the drug concentration in the cytoplasm (Fig. 28) or that trapped within the cell (data not shown). In both cases, Pgp sharply reduces drug concentration in the cytoplasm, and in highly resistant cells the drug concentration in the cytoplasm could be lowered ~500-fold. However, the

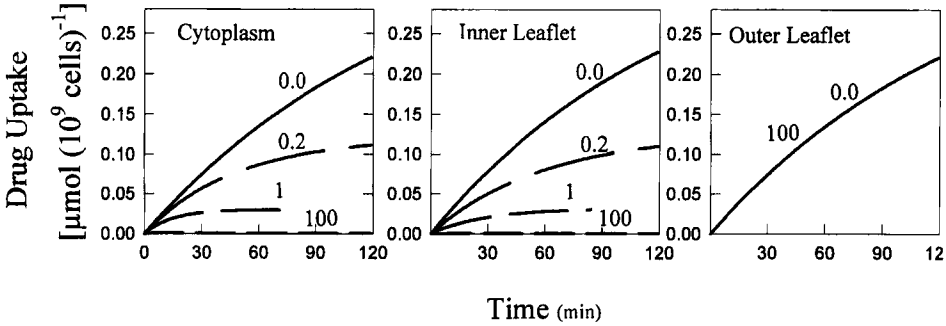

FIG. 27 Effect of the Pgp-mediated pumping mechanism on the time course of drug uptake by cells. Drug uptake into cells containing various levels of Pgp ATPase activities [numbers represent ATPase activity in nmol $(10^9$ cells$)^{-1}$ sec^{-1}] were simulated using the parameters of Figure 21 except that for the second and third panels the Pgp extracted its substrates from the inner and outer leaflets of the plasma membrane, respectively. In the case in which Pgp extracted its substrates from the plasma membrane, a K_m of 40 mM was used for Pgp.

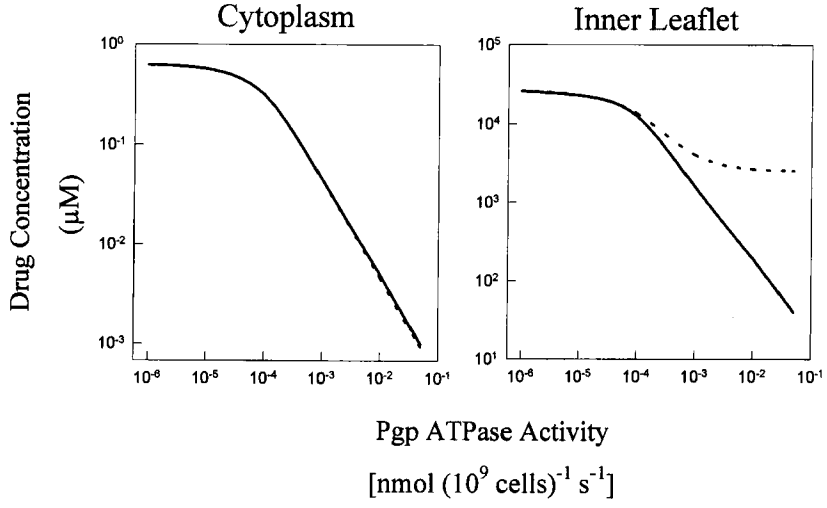

FIG. 28 Effect of the Pgp-mediated pumping mechanism on drug concentration in the cytoplasm and in the inner leaflet of the plasma membrane. Drug uptake into cells containing 5 nmol (10^9 cells)$^{-1}$ sec^{-1} Pgp ATPase activity, assuming that Pgp extracts its substrates from either the cytoplasm (solid lines) or the inner leaflet of the plasma membrane (dotted lines), was simulated using the parameters described in the legend of Figure 7. The curves show drug uptake into the cytoplasm and the inner leaflet of the plasma membrane after quasi-equilibration had been achieved.

pumping mechanism has an important role in determining the concentration within the plasma membrane. Only Pgp directly pumping drug out of the inner leaflet of the plasma membrane can effectively reduce the drug concentration in this compartment. Pgp-mediated pumping from the cytoplasm cannot effectively compete in the inner membrane leaflet with the flip-flop rate of drugs from the outer leaflet. Thus, only experiments designed to determine the effect of Pgp on drug concentrations within the plasma membrane are relevant to elucidation of this aspect of the Pgp-mediated pumping mechanism.

Pgp reduces the apparent initial uptake rate of certain drugs while not affecting the initial uptake rate of others. This apparent reduction in initial rate has been assumed to be proof of the drug pumping directly from the plasma membrane (Stein, 1997). However, the theoretical experiment described in Figure 29 shows that the effect of Pgp on the initial drug uptake could be a function of the initial intracellular trapping rate and does not constitute proof that Pgp extracts its substrates directly from the plasma membrane. The model described in Figure 29 assumes Pgp pumping from the cytoplasm and not from the plasma membrane. The intracellular drug-binding rate had no effect on either the long-term drug uptake in

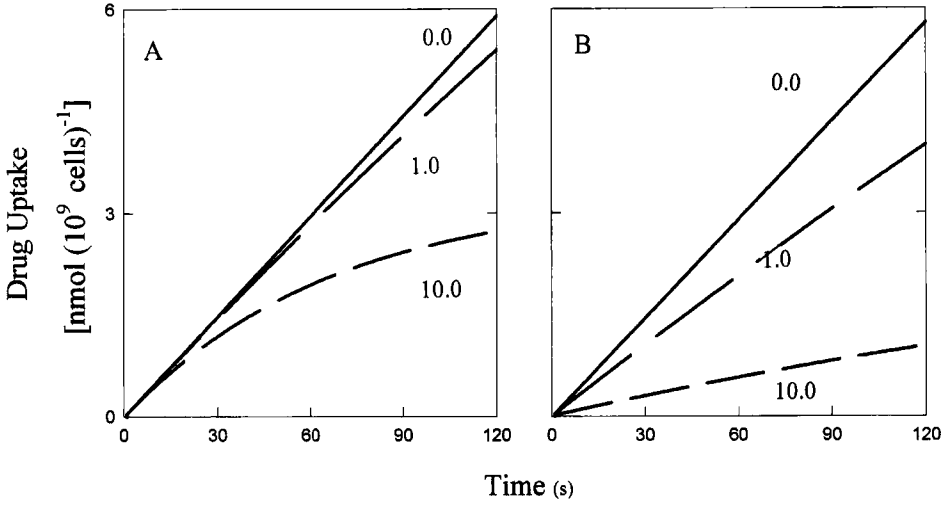

FIG. 29 Effect of the intracellular binding rate of the drug on its short-term uptake into cells. The computer simulation was run exactly as described in Figure 4. The short-term uptake kinetics of drug with either a slow initial intracellular trapping rate (A) or a fast one (B) are presented.

drug sensitive and resistant cells (data not shown, but similar to the curves shown in Fig. 24) or the initial phase of passive drug uptake into Pgp-less cells (Fig. 29). Surprisingly, however, analysis using the computer model indicated that the intracellular drug-binding rate determines the initial kinetics of drug uptake into Pgp-overexpressing cells. In cells with a fast binding rate, the initial drug uptake rate in Pgp-overexpressing cells is not affected by the Pgp pump and is identical to the rate in the absence of a pump (Fig. 29A). Presumably, the drug reaching the cytoplasm is almost immediately trapped and its concentration in the cytoplasm is too low for effective activity of the Pgp pump. However, in cells with a slow intracellular drug-binding rate, the initial uptake into Pgp-overexpressing cells appears slow relative to passive uptake into drug sensitive cells (Fig. 29B). Presumably because of the slow drug binding to the intracellular molecular sites, the free drug concentration in the cytoplasm rises rapidly and allows, within seconds, efficient Pgp pumping. The curves shown in Figure 29A compare favorably with experimental data obtained with colchicine uptake into cells (Stein *et al.*, 1994), whereas the curves shown in Figure 29B, cells with a slow intracellular drug-binding rate, compare very favorably with data obtained from daunorubicin (Cano-Gauci *et al.*, 1990), vinblastine (Cano-Gauci *et al.*, 1990; Shalinsky *et al.*, 1993), and vincristine (Sirotnak *et al.*, 1986). The shape of the curves describing initial drug uptake is a function

of the affinity of the intracellular traps, but it shows little variation with extracellular drug concentration. Reduction of extracellular drug concentration 1000 times to 1 n*M* has little effect on the shape of the initial drug uptake curves (data not shown). The present model shows that kinetic experiments, measuring the total amount of drug associated with cells, can yield an apparent Pgp-mediated reduction in initial drug uptake. This also occurs when the Pgp is presumed to extract the drug directly from the cytoplasm. As described in Figure 22, in the first few seconds most of the drugs associated with these cells are trapped within the cells, bound to molecular sinks, such as DNA.

An overall lesson from the remarkable effect that intracellular molecular sinks can have on the initial uptake of MDR drugs is that kinetic details of either drug uptake or efflux should be interpreted with extreme caution. The model presented here, containing one class of sites binding the drugs, is an oversimplification. All MDR drugs exhibit variable affinity to multiple classes of intracellular binding sites, such as DNA, cytoskeletal elements, enzymes, and intracellular membranes. A combination of the transport characteristics and binding to multiple molecular sinks with varying binding affinities and rates can produce apparently contradicting results that could lead to wrong conclusions. The difficulty in interpreting results is even more prominent in experiments where the relative rate of drug efflux has been shown to be similar in drug-sensitive and MDR cells (Sirotnak *et al.*, 1986; Bornmann and Roepe, 1994). The aspect ignored in these experiments is that the nature of the drug-trapping sinks is different in the two cell types. The initial amount of drug associated with drug-sensitive cells is much higher compared to the amount of drug associated with MDR cells. Moreover, because of the competition between Pgp and drug-binding sinks, the drug is present in MDR cells either bound to high-affinity sites or sequestered within intracellular compartments. Thus, it is not surprising that the drug efflux from MDR cells is not significantly faster compared to efflux from drug-sensitive cells.

B. Experimental Data

As described earlier the acid test for Pgp extracting its substrates directly from the membrane is demonstration that Pgp can reduce the concentration of a substrate in the inner leaflet of the plasma membrane by more than 100-fold. Regrettably, such a demonstration is still lacking, although several research approaches have shown that Pgp is capable of reducing the drug concentration in its membrane vicinity, although not by 100-fold.

The first and possibly still the best indication that Pgp extracts its substrates from the plasma membrane was obtained using the photolabeling

probe 5-[^{125}I]iodonaphthalene-1-azide (Raviv *et al.*, 1990). This probe is hydrophobic and as such is localized within the hydrophobic core of the membranes. Pgp becomes specifically labeled in MDR human KB carcinoma cells by the probe when photoactivation of the probe is triggered by the energy transfer from Pgp substrates, such as doxorubicin and rhodamine 123. In contrast, in drug-sensitive cells, drug-induced specific labeling of membrane proteins with the probe was not observed. Instead, multiple membrane proteins became labeled in a nonspecific manner. Thus, in addition to becoming labeled through bound substrates, it clears the membrane of its substrates and prevents nonspecific labeling of other proteins in the membrane. The clearing of Pgp substrates from the membranes was reversed by disruption of the cells and by Pgp modulators, such as verapamil. Results presented by Raviv *et al.*, (1990) demonstrate clearly that Pgp is capable of removing drugs from the plasma membrane, but that "regrettably" Pgp does not completely eliminate its substrates from the membrane. Thus, although it is tempting to accept these striking results as unequivocal evidence that Pgp eliminates its substrates directly from the plasma membrane, the possibility that Pgp pumps its substrates very efficiently from the cytoplasm (and only as a consequent result substrate concentration in the plasma membrane is reduced) cannot be ignored.

A completely different demonstration of Pgp-mediated elimination of substrates from the plasma membrane is the resistance exhibited by MDR cells toward the pore-forming ionophore, gramicidin D (Slovak *et al.*, 1988; Lincke *et al.*, 1990; Assaraf and Borgnia, 1994; Loe and Sharom, 1994). Gramicidin D is a hydrophobic peptide capable of forming a channel across one leaflet of a membrane bilayer. Alignment of two such channels forms a functional pore allowing for ion transport across the membrane. Gramicidin D is an MDR-type drug. Exposure of cells to gramicidin D results in the selection of Pgp-overexpressing MDR cells. Cells transfected with *mdr1* exhibited cross-resistance to gramicidin D. Presumably, gramicidin D cytotoxicity is due to the formation of functional pores across the plasma membrane. In this case, the 70- to 80-fold resistance to gramicidin D exhibited by MDR cells indicates a parallel reduction in the concentration of gramicidin D in the inner leaflet of the plasma membrane. A direct indication that Pgp is capable of reducing gramicidin D concentration within the plasma membrane was the 8-fold reduction in $^{86}Rb^{+}$ uptake into Pgp-overexpressing cells (Assaraf and Borgnia, 1994).

Certain hydrophobic fluorescent Pgp substrates allow for direct measurement of their concentration in their membrane. Such a dye, Hoechst 33342, was used to demonstrate for the first time drug transport by purified and reconstituted Pgp. Hoechst 33342 fluorescence is enhanced upon association with membrane lipids. Pgp activity reduces Hoechst fluorescence, presumably by its extraction from the lipid matrix into the aqueous medium (Sha-

piro and Ling, 1995). A puzzling feature of this system is that the Pgp-mediated dye-pumping rate is low and linear with time over hours. This is inconsistent with expected pumping kinetics in a small vesicle such as a reconstituted proteoliposome (see Section IV). A possible explanation is that Pgp extracts the dye from the membrane, but most of the extracted dye rebinds immediately to the vesicles apart from a small fraction that is lost by bleaching or some other artifact. The outcome of such a possibility is that the pumping activity measured is only a fraction of the actual Pgp-mediated dye pumping.

A striking observation that has been considered relevant to the mechanism of Pgp-mediated pumping was the efficient exclusion of fluorescent cellular indicators from Pgp-overexpressing cells (Homolya *et al.*, 1993; Hollo *et al.*, 1994). NIH3T3 mouse fibroblasts stably expressing the human multidrug transporter (MDR1 or Pgp), in contrast to control NIH3T3 cells, actively extrude the hydrophobic acetoxymethyl ester derivatives used for the cellular loading of various fluorescent calcium and pH indicators. This dye extrusion is blocked by competing substrates and inhibitors of the Pgp, e.g., by verapamil, vincristine, sodium orthovanadate, oligomycin, and a monoclonal anti-MDR1 antibody. Cytoplasmic nonspecific esterases hydrolyze these hydrophobic ester derivatives rapidly, thus trapping nonpermeant hydrophilic-free acids in the cell interior (Tsien, 1983). The Pgp-mediated reduction in the accumulation of fluorescent dyes has been interpreted as evidence that Pgp prevents the influx of the indicators into the cytoplasm, presumably by extrusion from the plasma membrane before they reach the cytoplasm. However, as detailed in Section V, the most efficient pumping from a specific cell compartment is achieved by extracting the substrates from this particular compartment. Pumping from an upstream compartment is less efficient and thus the reduction in hydrolysis of the indicators is not proof of direct pumping from the plasma membrane, but a result of a very effective competition between Pgp pumping and the esterases activity on the ester derivatives in the cytoplasm.

Thus, despite lack of final proof that Pgp extracts its substrates directly from the plasma membrane, this possibility is supported by evidence collected using the three different experimental approaches described earlier. Moreover, the hydrophobicity and wide specificity of Pgp substrates strongly support Pgp as a low-affinity pump extracting its substrates from the plasma membrane rather than as a high-affinity pump extruding substrates directly from the cytoplasm.

VII. Mechanism of Pgp Modulation

Once it has been accepted that drugs participating in the MDR phenomenon are extruded from MDR cells by active efflux, it was natural to search for

competitive inhibitors that will keep the then putative MDR efflux pump "busy," thus allowing cytotoxic MDR-type drugs to also accumulate in resistant cells. The first such inhibitor was *N*-acetyldaunorubicin (Skovsgaard, 1980), which increased daunorubicin accumulation twofold in resistant cells and has little effect in drug-sensitive cells. Hundreds of nontoxic drugs capable of reversing MDR were identified and named modulators, reversers, chemosensitizers, potentiators, or Pgp inhibitors. These drugs have diverse chemical structures and physiological functions and include calcium channel blockers such as verapamil (Tsuruo *et al.,* 1981), the antimalarial agent quinine, the cardiac depressant quinidine (Tsuruo *et al.,* 1984), detergents such as Tween 80 (Riehm and Biedler, 1972) and Cremophor EL (Woodcock *et al.,* 1992), calmodulin inhibitors such as phenothiazines (Tsuruo *et al.,* 1982), the antiestrogen tamoxifen (Ramu *et al.,* 1984), and the immunosuppressant cyclosporin A (Twentyman, 1988) and its analogue, PSC 833 (Boesch *et al.,* 1991). All these drugs reverse resistance in MDR cells *in vitro,* reinstating sensitivity to MDR-type drugs in MDR cells. Several have been tried clinically; they were administered together with MDR-type drugs in the hope of enhancing the cytotoxic effects of the drugs on resistant tumors. The initial clinical results have proven disappointing (Arceci, 1993; Fisher and Sikic, 1995; Kaye, 1995; Bates *et al.,* 1996) and more research into the modulation mechanism would benefit the future development of effective modulators capable of overcoming MDR in cancer patients.

Modulators have been identified by scanning multiple drugs, already approved for human administration by the FDA, for the inhibition of Pgp-mediated extrusion of drugs from Pgp-overexpressing cells. Without prior knowledge, we would expect the modulators to be similar to MDR-type drugs, except for being nontoxic. Thus, the administration of excess modulator compared to the cytotoxic drugs is expected to lead to effective competitive inhibition of Pgp and reversal of its capacity to exclude cytotoxic drugs from MDR cells.

Indeed, modulators behave similarly to MDR-type drugs in *in vitro* systems such as purified Pgp reconstituted into proteoliposomes or plasma membrane vesicles enriched in Pgp. Thus, Cornwell *et al.* (1987) have measured binding of the MDR-type drug vinblastine and three modulators, including verapamil, to plasma membrane vesicles from cells overexpressing Pgp and demonstrated Pgp-dependent binding of vinblastine and the modulators. The binding was specific and saturable and was inhibited by the modulators, desmethoxyverapamil and quinidine $>$ the MDR-type drug, vinblastine, and the modulators, diltiazem $\gg$ the MDR-type drug daunomycin. Horio *et al.,* 1988, 1991) have shown that ATP-dependent Pgp-mediated [^{3}H]vinblastine uptake into plasma membrane vesicles purified from MR human KB carcinoma cells can be inhibited by MDR-type drugs and modu-

lators. The inhibition is a result of an increase in apparent K_m of [^{3}H]vinblastine transport, suggesting that these agents may be competitive inhibitors of Pgp-mediated transport. A similar inhibition of intravesicular accumulation of [^{3}H]vinblastine by the modulators, verapamil, quinidine, and reserpine, was observed with plasma membrane vesicles from murine erythroleukemia cells resistant to vinca alkaloids and overexpressing MDR3 (Schlemmer and Sirotnak, 1994). Rhodamine 6G uptake into plasma membrane vesicles obtained from yeast, overexpressing the Pgp analogue, was also competitively inhibited by drugs and modulators (Kolaczkowski *et al.,* 1996).

Competition between MDR-type drugs and modulators on a Pgp common pharmacophore, as revealed by Pgp ATPase activity, has been demonstrated initially using Chinese hamster Pgp reconstituted into proteoliposomes (Borgnia *et al.,* 1996). Advantage was taken of the competitive inhibition of the basal and drug-stimulated Pgp ATPase activity by gramicidin D to show that both MDR-type drugs and modulators compete on a common drug-binding site. It was later suggested that the interaction between Pgp and its substrates, MDR-type drugs and modulators, as revealed by its ATPase activity, is more complex and possibly involves an additional site (Ferry *et al.,* 1992; Orlowski *et al.,* 1996, Ayesh *et al.,* 1996; Litman *et al.,* 1997, Urbatsch *et al.,* 1998).

A different approach to demonstrate a common binding site for MDR-type drugs and modulators is labeling Pgp with analogues of either a Pgp modulator or an MDR-type drug and inhibition of the affinity labeling by both agent types. Thus, specific photolabeling of Pgp with an analogue of the MDR-type drug vinblastine was inhibited by micromolar concentrations of the modulators, verapamil and quinidine (Cornwell *et al.,* 1986; Safa *et al.,* 1986; Cornwell *et al.,* 1987). Similarly, photoaffinity labeling of Pgp with analogues of modulators, such as verapamil, cyclosporin A, and progesterone, was inhibited by a variety of MDR-type drugs and modulators (Foxwell *et al.,* 1989; Qian and Beck, 1990). Photolabeling studies and kinetic evidence suggest that Pgp possesses a site separate from the site-binding vinca-alkaloids that is capable of binding certain modulators, such as 1,4-dihydropyridines, but not cyclosporin A, for example. Modulators binding to this site cause allosteric noncompetitive inhibition of various Pgp functions (Tamai and Safa, 1991; Ferry *et al.,* 1992; Martin *et al.,* 1997), in agreement with the noncompetitive inhibition of Pgp ATPase activity observed with certain modulators, as described earlier. This putative site also binds drugs transported by Pgp; thus azidopine capable of specifically labeling this site is extruded by Pgp from MDR cells in a modulator-sensitive mode (Tamai and Safa, 1991). In this respect, Shapiro and Ling (1997) have shown that Pgp contains two distinct sites for drug binding and transport and that, unexpectedly, these sites interact in a positively

cooperative manner. The kinetics of transport of rhodamine 123 and Hoechst 33342 in plasma membrane vesicles isolated from Pgp-overexpressing cells were followed by continuous fluorescence monitoring. Each substrate stimulated Pgp-mediated transport of the other. Colchicine and quercetin stimulated rhodamine 123 transport and inhibited Hoechst 33342 transport. In contrast, anthracyclines such as daunorubicin and doxorubicin stimulated Hoechst 33342 transport and inhibited rhodamine 123 transport. Vinblastine, actinomycin D, and etoposide inhibited the transport of both dyes. It is tempting to correlate the evidence that a Pgp molecule possesses two drug-binding sites with it being a tandemly duplicated molecule. Indeed, kinetic evidence supports the binding of two daunorubicin molecules to one Pgp molecule (Spoelstra *et al.,* 1992). Reversal of drug accumulation has been shown to occur with a Hill coefficient of close to 2, whereas other modulators have shown no evidence of cooperativity (Ayesh *et al.,* 1996). Similarly, evidence of cooperativity between modulator-binding sites in accelerating Pgp ATPase was obtained with several modulators (Orlowski *et al.,* 1996; Litman *et al.,* 1997).

MDR-type drugs and modulator behavior are indistinguishable in *in vitro* systems containing either purified Pgp or plasma membrane from MDR cells. However, in living cells where MDR-type drugs are extruded efficiently from MDR-type cells, Pgp does not reduce the cellular concentration of most modulators. Moreover, whereas modulators inhibit cellular Pgp and allow the enhanced uptake of MDR-type drugs into MDR cells, most MDR-type drugs do not inhibit cellular Pgp and have no effect on the drug uptake in MDR cells. The most extensive survey comparing MDR-type drugs and modulators was carried out by Scala *et al.* (1997). They surveyed 84 drugs and defined them prospectively as MDR-type drugs if their cytotoxicity was increased more than fourfold by the addition of cyclosporin A and as Pgp modulators if they increased rhodamine 123 accumulation in MDR cells by more than fourfold. Among the 84 drugs, only 35 met the criterion for substrates, only 42 met the criterion for antagonists, and only 7 met both criteria. The distinction between MDR-type drugs and modulators was confirmed by measuring vinblastine uptake accumulation into cells. Furthermore, the two drug subsets could not be differentiated based on the *in vitro* assays of the inhibition of azidopine labeling and the induction of ATPase activity.

A saturable Pgp-mediated transport of modulators was demonstrated with porcine kidney epithelium cells that formed a highly polarized epithelium capable of transporting MDR-type drugs and cyclosporin A (Saeki *et al.,* 1993). A transformant cell line derived by transfecting the epithelium cells with human *MDR1* cDNA expressed Pgp specifically on the apical surface and showed increased transcellular transport of the immunosuppressive modulators, cyclosporin A and FK506. It is interesting that the

Pgp-mediated transport of modulators could be best demonstrated in cells where the Pgp function is to transport drugs across the cell and not exclude them from the cell.

The main difference between Pgp function *in vitro* and in intact cells is that in the first case the ATPase and drug-binding sites are exposed to the medium, whereas in intact cells the drugs have to cross the plasma membrane to reach the Pgp drug-binding site. Pgp *in vitro* in plasma membrane vesicles and in reconstituted proteoliposomes is activated by ATP added to the medium. Thus, only Pgp molecules located in the vesicles with their ATPase site exposed mediate drug transport and ATPase activity. It has been suggested that the difference between MDR-type drugs and modulators is their rate of transbilayer movement (Eytan *et al.*, 1996b). The rate of transbilayer movement of modulators is fast compared to the transmembrane mobility of MDR-type drugs. Moreover, a prerequisite for a successful modulator is fast transbilayer movement, allowing it, once it is extruded by Pgp, to immediately reenter the cells and occupy the Pgp pharmacophore. However, MDR-type drugs, which are extruded efficiently from MDR cells by Pgp, reenter the cells slowly and their intracellular concentration is kept too low by Pgp to compete efficiently on the Pgp pharmacophore.

The role of transmembrane mobility of Pgp substrates in determining their effect on MDR in resistant cells is best exemplified with the two ionophore antibiotics, valinomycin and gramicidin. Valinomycin, a carrier-type ionophore, is a cyclic molecule made up of three repeats of L-lactate, L-valine, D-hydroxyisovalerate, and D-valine residues (Fig. 30). It is a donut-shaped molecule capable of transporting a K^+ ion chelated to its oxygen atoms projecting into the central cavity of the molecule. The hydrophobic periphery makes the molecule soluble and freely mobile in the lipid interior.

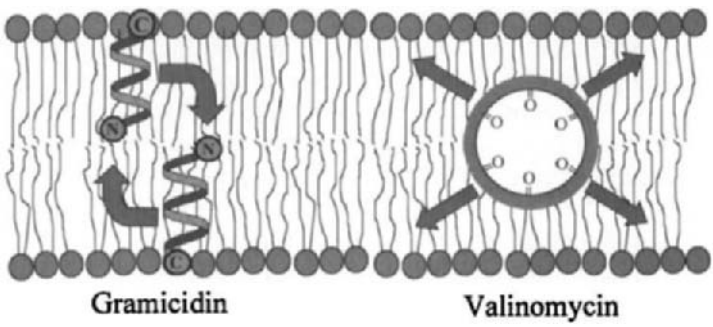

FIG. 30 The movement of gramicidin and valinomycin in membranes. Whereas gramicidin flip-flops across the membranes, valinomycin is free to diffuse within the hydrophobic core of the membrane.

However, the channel-type ionophore gramicidin is an unusual 15 residue peptide containing alternating L and D residues forming a helix spanning a membrane leaflet and anchored at the membrane surface by a C-terminal hydroxyl group (Urry, 1971; Urry *et al.,* 1971) (Fig. 30). Taking advantage of its electrical conductance capacity, it has been shown that gramicidin flip-flops across biological membranes with a lifetime of minutes (O'Connell *et al.,* 1990). Thus, although both valinomycin and gramicidin are soluble in organic solvents, valinomycin traverses the membrane at least 25×10^4 times sec^{-1}, i.e., with a lifetime of microseconds (Benz and Lauger, 1976).

The mobile carrier-type ionophore, valinomycin, behaves as an MDR modulator, whereas the channel-type ionophore, gramicidin, exhibits characteristics typical of an MDR-type drug. Carrier-type ionophores, including valinomycin, but not channel-type drugs, such as gramicidin, reverse MDR in Pgp-overexpressing cells (Assaraf and Borgnia, 1994; Borrel *et al.,* 1994; Loe and Sharom, 1994). MDR cells exhibit collateral resistance to gramicidin (Slovak *et al.,* 1988; Lincke *et al.,* 1990; Assaraf and Borgnia, 1994; Loe and Sharom, 1994), but only limited resistance to mobile ionophores, such as valinomycin (Assaraf and Borgnia, 1994; Loe and Sharom, 1994). Exposure of cells to gramicidin, but not to valinomycin, selected the cells for an MDR phenomenon concomitant with overexpression of Pgp in them (Assaraf and Borgnia, 1994). Presumably, Pgp does not afford efficient protection against valinomycin and, thus, valinomycin in contradistinction to MDR-type drugs, such as gramicidin, does not select Pgp-overexpressing cells.

In contradistinction to the situation in living cells, where valinomycin behaves as an MDR modulator whereas gramicidin exhibits characteristics of an MDR-type drug, both drugs compete on a common Pgp pharmacophore. Interestingly, gramicidin that is extruded by Pgp from MDR cells competitively inhibits its ATPase activity, whereas valinomycin that is not effectively excluded from MDR cells accelerates its ATPase activity *in vitro* (Borgnia *et al.,* 1996). Presumably, both ionophores are transported by Pgp; moreover, the Pgp-mediated transport of valinomycin is fast compared to gramicidin transport. However, due to the fast transmembrane movement of valinomycin, its Pgp-mediated extrusion from MDR cells does not lead to reduced cellular concentrations and drug resistance phenomena.

Taking advantage of quenching of doxorubicin fluorescence by DNA it has been shown that this MDR-type drug indeed traverses membranes by a slow flip-flop mechanism [(Regev and Eytan, 1997), see Section II]. A similar experimental approach was also used to show that the MDR dye probe, rhodamine 123, traverses membranes by a flip-flop mechanism, whereas the modulators, quinine and quinidine, diffuse rapidly across biological membranes. The transfer of rhodamine 123 from the aqueous phase to the bilayer of artificial lipid vesicles is accompanied by quenching of its

fluorescence. As shown in Figure 31, upon addition of low concentrations of the dye to unilamellar vesicles, quenching occurred in two steps. About 50% of the total fluorescence decrease occurred in the first step and was too fast to be recorded under our experimental conditions. The further decrease in fluorescence occurred slowly by a first-order process that could be described by a linear semilog plot with a lifetime of about 3 min (data not shown). The 50:50 ratio for the change in the fluorescence intensity in the two steps was seen only in unilamellar vesicles. In multilamellar vesicles, which are composed of multiple concentric membranes, the fast decrease in fluorescence intensity comprised ~10–15% of the total fluorescence decrease (Fig. 31). Compared to unilamellar vesicles, the decrease in fluorescence intensity during the second phase observed with multilamellar vesicles was much slower. It could not be described by a single exponential, but the final fluorescence intensity reached after approximately 3 hr was similar to the final level of fluorescence intensity observed with unilamellar vesicles.

The initial rapid decline in fluorescence observed in the first phase represented binding of the dye to the outermost leaflet, which would have constituted 50% of the lipid in unilamellar vesicles and about 8–13% in multilamellar liposomes (Schwartz and McConnell, 1978). The second slower phase

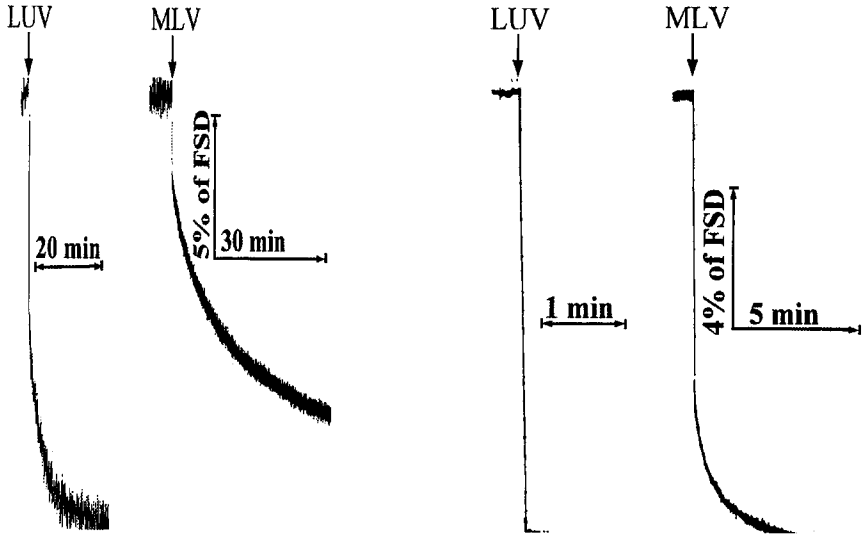

FIG. 31 Transbilayer movement rate of the MDR probe, rhodamine 123, and the modulator, quinidine, across an artificial lipid membrane. Unilamellar vesicles (LUV) or multilamellar vesicles (MLV) were added to either a rhodamine 123 or a quinidine solution. The fluorescence of the dye or the drug was monitored continuously. The full-scale deflection (FSD) was set to match the fluorescence of the dye or drug in the absence of added vesicles.

was due to the transbilayer movement of the dye from the outer leaflet to the inner leaflet of unilamellar vesicles or movement from the outermost leaflet of multilamellar vesicles to the inner leaflets. Upon transfer of the drug to the inner leaflets, more drug could be bound to the outermost leaflet, thereby resulting in further quenching of the fluorescence. Thus, the rate of the slow binding phase actually reflects the transbilayer movement of the drug.

The transbilayer movement of rhodamine 123, an established Pgp substrate, occurred in unilamellar vesicles with a lifetime of ~3 min at room temperature, and equilibration of the dye throughout multilamellar vesicles required ~1 h (Fig. 31). As shown in Fig. 31, the comparable movement of the MDR modulator, quinidine, measured under similar conditions, was much faster. The equilibration of quinidine with both leaflets of large unilamellar vesicles was too fast to be observed under our experimental conditions. The equilibration of quinidine throughout all the leaflets of multilamellar vesicles was also relatively fast and was complete within 10 min.

As noted in Section II, determination of the rate of transbilayer movement by fluorescence-quenching techniques is limited to drugs whose fluorescence is altered upon transfer from the membrane bilayer. Thus, the rate of transmembrane movement of modulators was determined using the binding methods described in Section II. As shown in Fig. 12, the removal rate from the medium by multilamellar vesicles of the modulators, verapamil, quinine, and quinidine, was much faster than the corresponding rate of the MDR-type drugs, doxorubicin and mitoxantrone. Whereas the binding of the latter proceeded for more than an hour, the modulators reached equilibrium with the vesicles within minutes. The fast transmembrane movement rate of modulators, compared to MDR-type drugs, was confirmed by studies where the binding of drugs to multilamellar vesicles was measured directly (Fig. 32). The equilibration of four modulators with the vesicles was over within minutes. Moreover, the fast transmembrane movement caused the loss of almost half the amount of the modulators, progesterone, trifluoperazine, and quinine, during the brief wash of the vesicles necessary for the removal of unbound drugs.

Thus, the rate of transbilayer movement of modulators was shown by various experimental approaches to be markedly slower than the rate of MDR-type drugs. The efficacy of modulators that are transported by Pgp depends on their fast transmembrane movement. This statement is corroborated by application of the computer-simulation model to MDR reversal by modulators. As shown in Fig. 33, exposure of Pgp-overexpressing cells to a putative modulator with a transmembrane movement rate comparable to an MDR-type drug, i.e., with a lifetime of 1 min, results in practically no reversal of resistance to drug accumulation by this modulator. However,

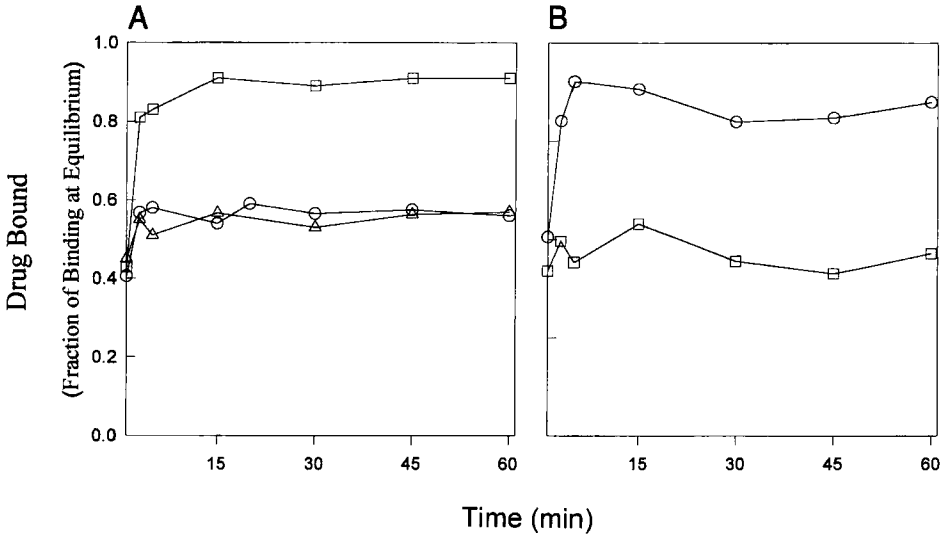

FIG. 32 Equilibration kinetics of MDR modulators with multilamellar vesicles. Multilamellar vesicles were incubated with a medium containing 60% (w/v) at room temperature. (A) The modulators verapamil (△), quinine (□), or trifluoperazine (○) were added to a 0.2 m*M* final concentration. (B) The modulators verapamil (○) and progesterone (□) were added to a concentration of 20 n*M*. After various further incubation periods, samples were withdrawn, centrifuged through a 40% (w/v) sucrose cushion into an ether fraction, and the amount of MDR-type drug or modulator in the ether fraction was determined. The amount of drug bound at equilibrium was determined by equilibrium dialysis (adapted with permission from Eytan *et al.*, 1996b).

a modulator with the same characteristics apart from a faster rate of transmembrane movement, with a lifetime of 1 sec, results in close to complete reversal.

The vast majority of, if not all, modulators capable of reversing MDR in Pgp-overexpressing cells without compromising cell viability seem to bind to the Pgp pharmacophore. As detailed earlier, kinetic evidence supports the suggestion that Pgp possesses two drug-binding sites with nonidentical binding specificity. Numerous modulators capable of competitive inhibition of Pgp ATPase at high concentrations seem to inhibit Pgp in a noncompetitive fashion (Borgnia *et al.*, 1996; Litman *et al.*, 1997). Some modulators exhibit only noncompetitive inhibition of Pgp. However, it seems that these modulators also affect Pgp only at high concentrations, which are toxic to cells. The best characterized noncompetitive inhibition of Pgp is by vanadate (Sarkadi *et al.*, 1992; al-Shawi and Senior, 1993; Borgnia *et al.*, 1996), which does not seem to interact with the Pgp drug-binding site(s). Other modulators seem to inhibit Pgp by nonspecific effects

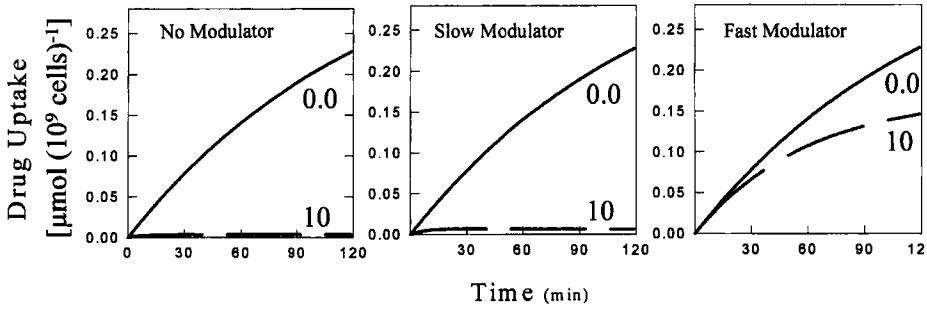

FIG. 33 Simulated time course of doxorubicin uptake into MDR cells in the presence of Pgp modulators. The time course of doxorubicin uptake into cells containing DNA was predicted as described in Figure 21. Solid lines are predictions for a drug-sensitive cell system with no pump activity. Broken lines are predictions for cells overexpressing Pgp with a pump activity of 10 nmol sec^{-1} $(10^9$ cells$)^{-1}$. The simulations were performed, assuming either the absence or the presence of a competitive modulator with characteristics similar to doxorubicin (slow modulator) or a modulator with a rate of transmembrane movement occurring with a lifetime of 1 sec.

on its microenvironment. Pgp, in contradistinction to ion-transporting ATPases, is very sensitive to its lipid microenvironment (Doige *et al.,* 1993; Urbatsch and Senior, 1995). Thus, it is not surprising that various mild, neutral detergents modulate Pgp activity; e.g., cremophor EL and Tween 80 reverse drug resistance in MDR cells and inhibit Pgp activities *in vitro* (Friche *et al.,* 1990; Spoelstra *et al.,* 1991; Woodcock *et al.,* 1992; Loe and Sharom, 1993; Syed *et al.,* 1993; Zordan-Nudo *et al.,* 1993; Ross *et al.,* 1994; Dudeja *et al.,* 1995). Because fluidizers, such as benzyl alcohol, inhibit Pgp-mediated drug transport (Sinicrope *et al.,* 1992) and the detergent modulators of Pgp fluidize membranes noncompetitively, it is possible that at least some of the MDR reversal by the modulators can be attributed to membrane fluidization. However, the concentrations of modulators required for noncompetitive modulation are, in most cases, toxic. Moreover, it has been shown that detergent modulators and the concentrations of other modulators allowing noncompetitive inhibition of Pgp ATPase interfere with membrane structure and integrity, causing disruption of liposomes and leakage of their contents (Wadkins and Houghton, 1993; Drori *et al.,* 1995).

VIII. Concluding Remarks

The ultimate "clinical" goal of research into the mechanism of Pgp is to overcome MDR in the treatment of neoplasia. In lowering the cytoplasmic

concentration of a drug, the efficacy of Pgp is dependent on its pumping capacity and its affinity toward the particular drug, the rate of transmembrane diffusion of the drug, the affinity of the pharmacological target in the cell for the drug, and the affinity of intracellular trapping sites for the drug. Thus, an ideal modulator of Pgp-mediated MDR should exhibit (1) high affinity of the active site(s) of Pgp toward it, (2) a fast rate of transmembrane exchange, (3) low toxicity and side effects, and (4) low affinity of cellular components, other than Pgp, toward it. A possible drawback of such a Pgp inhibitor, in terms of circumvention of MDR in patients with cancer, is that it is not necessarily an efficient inhibitor of alternative MDR mechanisms that are possibly present in tumors, such as MRP, LRP, or as yet undefined mechanisms.

A better approach to the circumvention of clinical MDR, which is logically based on the mechanism of Pgp function that is described in this review, is the design of anticancer drugs with a rapid rate of transmembrane exchange. Such drugs will overcome cellular MDR mechanisms, irrespective of whether they are mediated by Pgp or by alternative extrusion mechanisms. Such putative drugs would not necessarily be very hydrophobic and yet probably would not be amphipathic in nature. Combinations of characteristics of anticancer drugs, such as cytotoxicity, with the characteristics of modulators, such as the fast rate of transmembrane exchange, could yield anticancer drugs insensitive to MDR mechanisms.

Appendix

Mathematica Model of Drug Transport into and out of a Cell

A. Introduction

The formulation of a kinetic model of drug transport and binding in a cellular system follows most of the general principles used in the early computer models of enzyme systems (e.g., Garfinkel, 1966). This approach has been extended to allow comment on or predictions of the metabolic consequences of defects in the kinetics of enzymes such as those on the urea cycle (Kuchel *et al.*, 1977). These earlier models of enzyme systems involved reactions that were assumed to be taking place in homogeneous solutions. Another order of complexity is encountered when the goal is to simulate reactions in heterogeneous systems (Kuchel and Chapman, 1983); however, account must be taken of relative compartment volumes and different solubilities in the various phases of the system. This problem is

confronted when experiments are conducted on cellular suspensions and tissues in which the transmembrane exchange of solutes takes place; it is especially problematical when the solute dissolves in the phospholipid leaflets of the cell membrane and also binds to cellular macromolecules such as DNA. Nevertheless, the conceptual basis of modeling transport and metabolism is well established and those principles have been applied in the present work.

B. Fundamental Kinetic Considerations

Of central importance to formulating chemical kinetic models of cellular systems is the "principle" of mass action; it defines the rate of a chemical reaction or a transport process in terms of the product of a unitary rate constant and the activity (or concentration in dilute solution) of the reactant(s)/solute(s) (Roberts, 1977; Kuchel, 1985; Kuchel and Ralston, 1997). The next consideration is the relationship between rate constants measured experimentally and the corresponding constants expressed in terms of unitary rate constants; this is manifest in the expression of the steady-state constant, K_m, in terms of the unitary rate constants of the enzyme reaction scheme; e.g., for the simplest enzyme mechanism, $K_m = (k_{-1} + k_2)/k_1$, where k_{-1} is the off-rate constant of the enzyme–substrate complex, k_2 characterizes the rate of the catalytic breakdown step, and k_1 is the "on" rate constant for the association of the substrate and enzyme (Kuchel and Ralston, 1997).

An important check of the consistency of an experimental (and computer simulation) kinetic analysis is the conservation of mass of reactants. Also, the ratio of the product of the forward rate constants and those of the corresponding reverse constants for a reversible reaction or sequence of reactions must be a constant value; this also pertains to a cycle of reactions. The latter is often referred to as the Onsager relationship and is used in nonlinear thermodynamic analysis (Moore, 1981); it was apparently Wegscheider (1901), according to Hearon (1953), who first showed that for any chemical reaction cycle the product of the equilibrium constants around the cycle is equal to one. Thus, the Onsager principle is also referred to as the "principle of detailed volume" or the "law of microscopic reversibility" (Lam, 1981). The principle of detailed volume also appears in a less direct way in the Haldane relationships that are used in steady-state enzyme kinetic analysis (Roberts, 1977; Kuchel and Ralston, 1997).

C. The *Mathematica* Program

The first section is one in which the program is described in free text and then the values of parameters and any derived expressions are "cleared."

This is a "safety" measure as other *Mathematica* (Wolfram, 1996) programs that could be running simultaneously may have used the same parameter names and the values could be unwittingly/automatically used in the simulation. Next the main numerical integration module is encountered and the maximum simulated time and the cytocrit, or for red blood cells the hematocrit (fraction of the volume of the sample that is occupied by cells), are entered. This is followed by the entry of the fraction of the cell volume that is available to solutes inside the space occupied by the plasma membrane; the fractional volumes occupied by the inner and outer leaflets of the plasma membrane are then entered. Values of the unitary rate constants are defined and then come the values of the steady-state kinetic parameters V_{max} and K_m for the Pgp.

The principle of mass action is then used in the definition of forward and reverse reaction rates. Some of the reaction steps are unitary ones whereas others are described by compound rate constants as used in the enzyme and carrier models. The whole set is denoted by vel1[t_], . . ., vel9[t_1]. The velocities of the reactions are functions of time so that they are explicitly declared as such by the use of the appropriate *Mathematica* convention. Finally the whole kinetic scheme is described by a set of simultaneous first-order, nonlinear differential equations. Because these equations, in general, have no analytical solutions, they are solved using numerical integration; this is achieved with the command NDSolve that requires, as an argument set, the specification of the initial values of each of the solute concentrations in the respective compartments outside and inside the cell. Several other parameters that influence the numerical integrator algorithm are also set at this stage. The numerical integration algorithm that is used as a default in *Mathematica* is designed to handle "stiff" sets of differential equations; in other words, those systems for which there are large differences in the values of the various rate constants so it is ideally suited to the present task. Output from the numerical integration is then graphed using the appropriate *Mathematica* syntax. A further sophistication is to interpolate functions through the time course data and thus be able to predict solute concentrations at *any* time rather than simply at the times specified by the output format of the integrator.

D. Conclusions

The *Mathematica* numerical integration procedures are capable of handling huge arrays of simultaneous differential equations. For example, an enzyme kinetics model of metabolism in the pentose phosphate pathway of human erythrocytes has 434 differential equations and generates simulated time courses over several hours in only a few seconds (Berthon and Kuchel,

1995). Hence the present model of drug transport and binding is cells does not tax the capability of *Mathematica* and executes it in a few seconds on a medium-range Macintosh or IBM computer. This response time for the simulation implies that numerical "experiments" that can be used to guide real experimental design are conducted readily with rapid feedback to the analyst.

E. The Source Code

This code can be downloaded in a platform-independent executable form from the following URL: http://www.technion.ac.il/~eytan/.

```
(*A transmembrane transport and reaction scheme
for MDR-type drugs in cells overexpressing Pgp.
The names of the reactants are (see Fig. 20):
Dm = drug outside cells; Dom = drug
in the outer leaflet of the plasma membrane; Dim = drug
in the inner leaflet of the membrane; Dc = drug
in the cytoplasm.*)
Clear[Dm, Dom, Dim, Dc, t, Fcv, Acf, Bov,
      Biv, k1, kn1, k2, kn2, k3, kn3, k4, kn4, Vmax, Km]
Modeller=
      Module[
      {

      maxT=120., (*This is the maximum time of
            the simulated time-course, in seconds.*)


      Fcv=0.0015, Acf=0.71,

(*Fcv = cell volume as a fraction of total volume;
 Acf = fraction of cell volume accessible to solutes;
 Bov = fraction of cell volume in the outer leaflet
       of the cell membrane;
 Biv = fraction of cell volume in the inner leaflet
       of the cell membrane *)

      Bov=0.00005, Biv=0.00005,

(*Below are the intrinsic k's. . .they are
NOT the k's we would estimate experimentally from plotting
```

```
the change in extracellular CONCENTRATION versus time. . .that
experimental value (initial slope of a time-course plot) would
be divided by the compartment volume to give the intrinsic
k. The partition coefficient is related to these intrinsic
rate constants by including the relative compartment volumes.
Thus P=k1 (1-Fcv)/(kn1 Bov Fcv). *)

(*k1 to kn3 are first-order rate constants. We have used
micromolar concentrations throughout so the values of the
ki's are accordingly scaled down by a factor of 10^6. This
is very important, not only from the point of view on
maintaining a connection with experimental reality but also
to ensure that the numerical integrator behaves properly;
otherwise it could be forced to handle a very "stiff" array
of differential equations.*)

        k1=100,
        k2=0.01667,
        k3=0.25,
        k4=0.001,

(*Vmax is the pump Vmax (micro-mol/(L Cells)/s;
Km is the 'Km' of the pump. *)

        Vmax=0.0, Km=1,},

(*THIS IS A CRITICAL CONSIDERATION. . .
derive ki values so that at equilibrium the Onsager
relationship is satisfied; namely that
k1 k2 k3/(kn1 kn2 kn3)= Acf Fcv/(1-Fcv), the ratio of
the compartment volume inside and outside the cells.*)

        kn1=k1 0.000025 (1-Fcv)/(Bov Fcv)
        kn2=k2 Bov/Biv
        kn3=k3 40000 Biv/Acf
        kn4=k4 4
        Vmax=Vmax (1-Fcv)/(Biv Fcv)

        Print["Reached initialization"]

(*Now we will use CONCENTRATIONS in the rate expressions
AND the differential equations. In other words, the lower
case names denote concentrations.*)
```

```
vel1[t_]:= k1 Dm[t]

vel2[t_]:= kn1 Dom[t]

vel3[t_]:= k2 Dom[t]

vel4[t_]:= kn2 Dim[t]

vel5[t_]:= k3 Dim[t]

vel6[t_]:= kn3 Dc[t]

(*The pump-flux equation is as follows.*)

vel7[t_]:= (Vmax Dim[t])/(Km + Dim[t])

(*Note. . .the added DNA-binding reaction. The drug
(Dc) binds to DNA via a reaction characterized by the
rate constant k4, and the complex (DNADc) dissociates via
a reaction characterized by kn4. DNA and DNADc have the
units of concentration, viz., mol L^-1.*)

vel8[t_]:= k4 DNA[t] Dc[t]

vel9[t_]:= kn4 DNADc[t]

(*Note that the Dm'[t] etc terms (derivatives) are
expressed as CONCENTRATIONS s^-1 (i.e., micro-mol L^-1 s^-1)
and the corresponding terms in the veli[t_] expressions
are CONCENTRATIONS s^-1 as well! The way to "view" the
equations is that each velocity term describes the
delivery of the solute from one compartment to another;
the unidirectional velocity depends on the concentration
in the compartment of origin, but its concentration in
its destination compartment will depend on that volume
relative to the compartment of origin. Or, you could say
that you multiply the concentration term by the compartment
volume to give the number of moles (amount) transferred
per second and then divide this by the volume of the
destination compartment to give the concentration rate
in that compartment.*)

P1:= Dm'[t]==-vel1[t] + vel2[t] (Bov Fcv)/(1-Fcv) +
                vel7[t] (Fcv Bov/(1-Fcv))
```

```
P2:= Dom'[t]==vel1[t] ((1−Fcv)/(Fcv Bov)) − vel2[t] −
                vel3[t] + vel4[t] (Biv/Bov)

P3:= Dim'[t]==vel3[t] (Bov/Biv) − vel7[t] −
                vel4[t] − vel5[t] + vel6[t] (Acf/Biv)

P4:= Dc'[t]==vel5[t] (Biv/Acf) − vel6[t] − vel7[t] −
                                        vel8[t] + vel9[t]

P5:= DNA'[t]==−vel8[t] + vel9[t]

P6:= DNADc'[t]==vel8[t] − vel9[t]

      Print["Hi! equations"]


sol=NDSolve[
            {P1,P2,P3,P4,P5,P6, Dm[0.]==1.0, Dom[0.]==0.0,
                  Dim[0.]==0.0, Dc[0.]==0.0,DNA[0.]==2500.0,
                  DNADc[0.]==0.0},

      {Dm[t],Dom[t],Dim[t],Dc[t],DNA[t],DNADc[t]},

      {t, 0, maxT}, WorkingPrecision−>15,AccuracyGoal−>10,
      PrecisionGoal−>10,StartingStepSize−>1.0 10^−12,
      MaxSteps−>10000]

Print["Reached NDSolve"]


graph1=Plot[Dm[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[Dm] (moles L^−1)"}]
graph2=Plot[Dom[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[Dom] (moles L^−1)"}]
graph3=Plot[Dim[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[Dim] (moles L^−1)"}]
graph4=Plot[Dc[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[Dc] (moles L^−1)"}]
graph5=Plot[DNA[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[DNA] (micro-moles L^−1)"}]
graph6=Plot[DNADc[t]/.sol, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "[DNADc] (micro-moles L^−1)"}]
```

```
(* Check conservation of mass. . .*)

graph7=Plot[(Dm[t]/.sol) (1−Fcv) +(Dom[t]/.sol) Bov Fcv +
        (Dim[t]/.sol) Biv Fcv +(Dc[t]/.sol) Acf Fcv +
        (DNADc[t]/.sol) Acf Fcv, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "Total (micro-moles)"}]
(* Check drug amount bound excluding drug adsorbed to cells *)
graph8=Plot[(Dim[t]/.sol) Biv Fcv +(Dc[t]/.sol) Acf Fcv +
        (DNADc[t]/.sol) Acf Fcv, {t,0.,maxT}, PlotRange−>Automatic,
        AxesLabel−>{"Time (s)", "Total bound(micro-moles)"}]

(*The interpolation functions below enable the extraction
of estimates of the concentrations for the variables for
ANY time between 0, and maxT seconds. *)

IntDm=Interpolation[Table[Flatten[{t,Dm[t]/.sol}],{t, 0,maxT}]]
IntDom=Interpolation[Table[Flatten[{t,Dom[t]/.sol}],{ t,0,maxT}]]
IntDim=Interpolation[Table[Flatten[{t,Dim[t]/.sol}],{ t,0,maxT}]]
IntDc=Interpolation[Table[Flatten[{t,Dc[t]/.sol}],{t, 0,maxT}]]
IntDNA=Interpolation[Table[Flatten[{t,DNA[t]/.sol}],{ t,0,maxT}]]
IntDNADc=Interpolation[Table[Flatten[{t,DNADc[t]/.sol}],


                                                {t,0,maxT}]]
(* The following lines allow extraction of drug concentration
  at a specified time point and export of a time course *)

Print[IntDc[100]];
For[i=0,i<maxT,i=i+10,IntDc[i]>>>temp.txt];

]
```

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Plasmodesmata and Cell-to-Cell Communication in Plants

Biao Ding, Asuka Itaya, and Young-Min Woo
Department of Botany, Oklahoma State University, Stillwater, Oklahoma 74078

Plasmodesmata provide a cytoplasmic pathway for direct cell-to-cell communication in plants. They are highly dynamic in biogenesis and structural modifications, organize cells into symplasmic domains that serve as basic developmental units in a plant, and mediate intercellular trafficking of proteins and nucleic acids as well as small molecules. Intercellular macromolecular trafficking appears to be a significant mechanism of regulating gene expression and physiological functions among neighboring cells and between distant organs. Current data suggest that intercellular macromolecular trafficking is also likely a general function in animals. Evolutionarily, intercellular macromolecular trafficking may be a primitive rather than an advanced biological function.

KEY WORDS: Plasmodesmata, Protein trafficking, RNA trafficking, Double-stranded RNA, *Caenorhabditis elegans,* PKR, Cell-to-cell communication, Movement protein, Cytoskeleton, Intercellular communication, viroid.

I. Introduction

Cell-to-cell communication is vital to the coordinated gene expression and cellular processes that lead to cell differentiation, morphogenesis, development, and growth of multicellular organisms. Plasmodesmata are cytoplasmic strands that interconnect cells and form an intercellular communication network within a plant body. They are highly dynamic in terms of their biogenesis, structural modifications, and transport functions. Plasmodesmata can be formed at cytokinesis or *de novo* across existing cell walls. They can be further modified to reduce or enhance intercellular transport. Down-regulation of plasmodesmal transport at specific cellular boundaries defines the so-called symplasmic domains that conceivably function as developmental units. Some modifications of plasmodesmata enhance intercel-

lular transport or allow plasmodesmata to acquire new transport functions. Among the most significant advances in plasmodesmal biology is the recent discovery that plasmodesmata can mediate intercellular trafficking of proteins and RNAs, which is likely an important means of regulating various developmental and physiological processes. This intercellular macromolecular trafficking function appears to be shared by animals. Parallel mechanisms and functions exist between macromolecular trafficking in plants and animals.

This review presents the current status of research on cell-to-cell communication through plasmodesmata in plants. Discussions focus on the biogenesis and structure of plasmodesmata, transport functions, and mechanisms. In addition, findings from animal systems are also discussed to emphasize the potentially universal feature of intercellular macromolecular trafficking. Finally, some evolutionary aspects of intercellular communication in plants are discussed.

II. Plasmodesma, a Supramolecular Complex

A. Substructure

As shown in Figure 1, the plasmodesma consists of the plasma membrane that is continuous from one cell to another and the appressed endoplasmic reticulum (ER) cylinder in the center. Proteinaceous particles of approximately 3 nm are embedded in both the plasma membrane and the appressed ER membrane (Ding *et al.,* 1992b; Botha *et al.,* 1993). The appressed ER particles might be arranged helically or as a series of gyres (Ding *et al.,* 1992b). In a cross-sectional view, there are approximately seven to nine particles in the outer leaflet and one particle in the fused inner leaflets of the appressed ER (Ding *et al.,* 1992b). Spoke-like extensions connect the plasma membrane and the appressed ER membrane particles (Ding *et al.,* 1992b; Botha *et al.,* 1993). Spaces between these protein particles are suggested to be microchannels, 2.5 nm in diameter, for intercellular transport (Fig. 1) (Ding *et al.,* 1992b; Botha *et al.,* 1993). These microchannels may be tortuous (Ding *et al.,* 1992b). In support of a role of microchannels in intercellular transport, Schulz (1995) demonstrated elegantly that osmotic treatment that enhances the intercellular transport of sucrose results in widening of the space between the plasma membrane and the appressed ER of plasmodesmata in pea roots. The microchannels, however, are unlikely the only passageways. Transport, especially of lipids, can occur via the membrane bilayers of the appressed ER (Grabski *et al.,* 1993). A dilated ER lumen within the plasmodesma is also proposed to be a pathway for

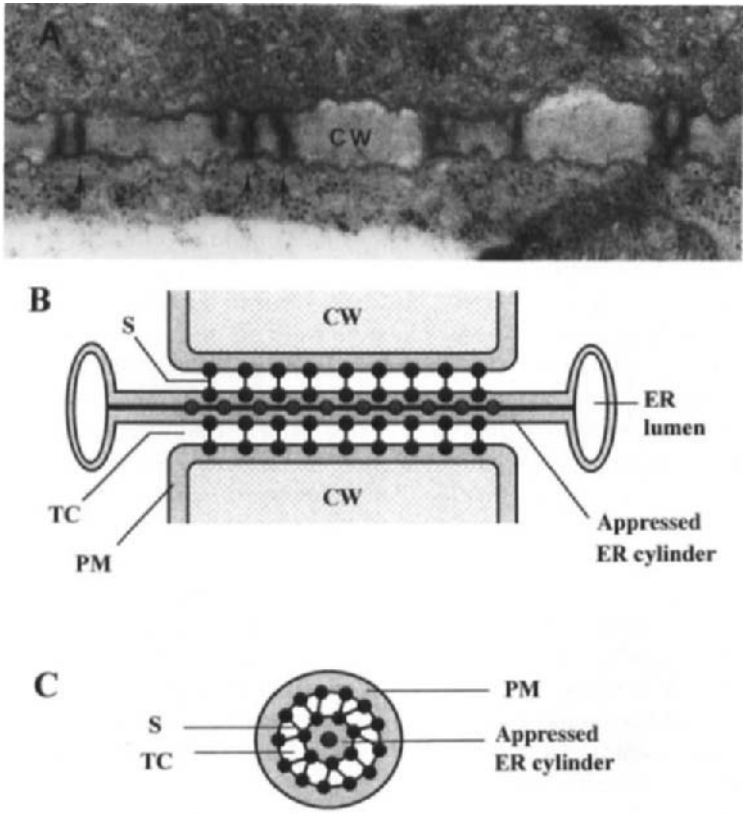

FIG. 1 (A) An electron micrograph showing plasmodesmata (arrows) between epidermal cells of a tobacco leaf. (B and C) A schematic model of the plasmodesmal substructure. (B) A median-longitudinal view and (C) a transverse view. Electron-dense spokes (S) interconnect particles embedded in the appressed ER and plasma membrane (PM). The biochemical nature of the spokes and the particles remains to be characterized. They could be composed of actin/myosin (White *et al.*, 1994; Radford and White, 1998) and/or kinases (Citovsky *et al.*, 1993). The structures are not drawn to scale. CW, cell wall; TC, transport channels. Redrawn from Ding (1998), with kind permission from Kluwer Academic Publishers.

transport, although direct evidence has yet to be provided (Gamelei *et al.*, 1994).

An open tubular structure, instead of an appressed ER cylinder, may be present in plasmodesmata of trichome cells of *Nicotiana clevelandii* (Waigmann *et al.*, 1997). Waigmann *et al.* (1997) suggest that this tubular structure might be composed of only proteins, as postulated by Tilney *et al.* (1991) for fern gametophyte plasmodesmata. As discussed in Ding (1998), the observation in the trichome cells needs to be confirmed with

alternative fixation and staining protocols that can preserve and contrast plasmodesmal structures better.

Sphincter-like structures have been observed to exist in the cell walls immediately surrounding the orifices of plasmodesmata and have been proposed to regulate plasmodesmal transport in some species (Olesen, 1979; Olesen and Robards, 1990; Badelt *et al.,* 1994; Overall and Blackman, 1996). In addition, a cell wall-localized spiral structure is also observed to circle around the entire length of a plasmodesma in several species (Badelt *et al.,* 1994). The functions of these structures have never been characterized experimentally. A recent study, however, offers a developmental perspective of thinking about and investigating the true nature and functions of the putative sphincters. Rinne and van der Schoot (1998) showed that during the induction of dormancy in birch seedlings, cells in the shoot apical meristem become symplasmically isolated from one another to some extent, and closure of plasmodesmata appears to be due to the formation of sphincters, composed at least of callose, in the plasmodesmal orifice.

It is important to point out that although the plasma membrane and the ER membrane systems are physically continuous from cell to cell through plasmodesmata, they are modified in the plasmodesmata and differ in some aspects from their cytoplasmic counterparts. Furthermore, the two plasmodesmal membrane systems exhibit some different physical properties. Hepler (1982) showed that an osmium tetroxide–potassium ferricyanide mixture, which stains cytoplasmic ER specifically, does not stain the ER in plasmodesmata. In aging moss protonemata, plasmodesmata between neighboring cells are gradually occluded. In such plasmodesmata, the appressed ER disappears, whereas the plasma membrane remains for a long period of time (Schnepf and Sawidis, 1991). The labile and resilient nature of the appressed ER and plasma membrane, respectively, is also demonstrated in tobacco leaf tissues freeze substituted by acetone alone, in which all the cytoplasmic membranes are lost, but the plasmodesmal plasma membrane (or the plasma membrane-associated proteins) appears to be retained (Ding *et al.,* 1992b). ER lipid analogues can diffuse between neighboring cells, but plasma membrane lipid analogues cannot (Grabski *et al.,* 1993). Fleurat-Lessard *et al.* (1995) demonstrated that, in the young reactive primary pulvinus of *Mimosa pudica,* the plasma membrane H^+-ATPase is distributed along the entire plasma membrane, but is absent from plasmodesmata. These data, taken together, indicate that the plasma membrane and appressed ER membrane of plasmodesmata must have been modified to perform specific functions related to plasmodesmal transport, as well as to prevent unrestrictive diffusion of proteins or even some lipid components within a membrane network between neighboring cells. This may function to ensure that plasma membrane proteins with specific functions can be localized only to specific cell types. For instance, the sucrose transporter

SUT1 is localized in the plasma membrane in the sieve elements, but not in the associated companion cells (Kühn *et al.*, 1997).

B. Regulation of Cell-to-Cell Diffusion through Plasmodesmata

1. The Size Exclusion Limit

The capacity of plasmodesmata to allow intercellular transport is usually measured in terms of their molecular size exclusion limit (SEL). A basal plasmodesmal SEL of approximately 1 kDa for the passive diffusion of molecules was established for several plant species by examining the intercellular diffusion behavior of fluorescent dyes of a range of sizes (Tucker, 1982; Barclay *et al.*, 1982; Goodwin, 1983; Terry and Robards, 1987; Wolf *et al.*, 1989) or by examining the ability of inhibitors of various sizes to enter cells to inhibit specific enzyme functions (Burnell, 1988; Weiner *et al.*, 1988).

However, some recent work suggests that plasmodesmal SEL can have more variations than previously expected, and this may be associated with specific cell types or cellular boundaries. Fluorescent dextrans of approximately 7 kDa can diffuse between leaf trichome cells of *N. clevelandii* (Waigmann and Zambryski, 1995). Dextrans of 3 and 10 kDa can diffuse through plasmodesmata between sieve elements and companion cells in the stems of *Cucurbita maxima* (Kempers *et al.*, 1993) and *Vicia faba* (Kempers and Van Bel, 1997), respectively.

A structural study by Radford *et al.* (1998) suggests that plasmodesmal microchannels may be more open than currently thought. Electron micrographs of plasmodesmata have been obtained frequently from dissected tissues fixed with glutaraldehyde, a procedure known to cause the rapid deposition of callose (β-1, 3-glucan) in the cell wall immediately surrounding the orifices of plasmodesmata (Hughes and Gunning, 1980). Even for cryofixation and freeze substitution (Ding *et al.*, 1992b), the tissues need to be dissected into small pieces and this step may have also caused callose deposition to "squeeze" the plasmodesmal orifices. Radford *et al.* (1998) contend that minor wounding of a cell during microinjection may also cause rapid callose deposition so that the measured SEL in many cases may be an underestimate. In an attempt to circumvent these potential problems, Radford *et al.* (1998) treated intact onion seedlings with 2-deoxy-D-glucose (DDG) to inhibit callose deposition and then fixed the samples with glutaraldehyde. They found that the plasmodesmal orifice in these samples is approximately twice as wide in diameter as that in untreated and glutaraldehyde-fixed samples. Unfortunately, the dimensions of microchannels were

not measured. Nevertheless, data indicate that plasmodesmal SEL under undisturbed conditions could be larger than previously reported (Radford *et al.,* 1998).

Clearly, these new data challenge our current view of plasmodesmal structure and transport capabilities. In order to further advance our understanding of the structure and functions of plasmodesmata, it is imperative that novel experimental methods be developed to study plasmodesmata under the least disruptive conditions.

2. Regulation of Size Exclusion Limit

There are perhaps multiple mechanisms to regulate plasmodesmal SEL to reduce or enhance intercellular transport. These mechanisms may operate under different physiological or developmental situations of a plant. Furthermore, plasmodesmata between different cell types, organs, or even species might also be regulated differently.

a. Phosphorylation/Dephosphorylation of Putative Plasmodesmal Components Plasmodesmal SEL can be down-regulated by injection of Ca^{2+} (Erwee and Goodwin, 1983; Baron-Epel *et al.,* 1988; Tucker, 1990; Tucker and Boss, 1996), inositol trisphosphate (IP_3) (Tucker, 1988; Tucker and Boss, 1996), and the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which can serve as a diacylglycerol (DAG) analogue stimulating the activity of protein kinase C (Baron-Epel *et al.,* 1988). The effect of IP_3 is speculated to be via the activation of Ca^{2+} release through IP_3-sensitive Ca^{2+} channels from intracellular storage sites such as vacuoles (Tucker and Boss, 1996). It has been postulated that elevated Ca^{2+} levels in a cell may have stimulated some kinase activity, and phosphorylation of some plasmodesmal components leads to closure of microchannels (Tucker, 1988; Tucker and Boss, 1996).

Cleland *et al.* (1994) demonstrated that the reduction of cellular ATP levels, by treatment with azide or anoxia, results in an increase in the plasmodesmal SEL from less than 800 Da to 7–10 kDa in epidermal and cortical cells of wheat roots. This is consistent with Tucker's (1993) observation that azide treatment of *Setcreasea purpurea* staminal hair cells enhances the rate of intercellular diffusion. Cleland *et al.* (1994) suggested that plasmodesmal microchannels may be controlled by ATP-dependent phosphorylation of some plasmodesmal components. Presumably, ATP and Ca^{2+} are all involved in the same regulatory mechanism.

A gram-negative bacterium, *Bradyrhizobium japonicum* R110d, can infect and induce nodule formation in soybean roots. Gharyal *et al.* (1989) showed that lipopolysaccharide (LPS) purified from this bacterium can inhibit cell-to-cell diffusion of fluorescein in cultured soybean root cells.

Specifically, the inhibition is conferred by the O-antigen region of the LPS. This is a highly specific function because LPS from noninfectious bacterial strains has no such inhibitory effect. Because LPS from animal cells can induce biochemical activities related to the polyphosphoinositide/protein kinase C signaling pathway, Gharyal *et al.* (1989) postulated that LPS may bind to a plant receptor that can activate a protein kinase, leading to the phosphorylation of some plasmodesmal components and thus closure of the microchannels.

The plasmodesmal components as the substrates of phosphorylation, by whatever kinase, are still a matter of speculation. Actin and myosin are perhaps among the potential candidates. Both actin (White *et al.,* 1994; Blackman and Overall, 1998) and myosin (Radford and White, 1998; Blackman and Overall, 1998) are suggested to be components of plasmodesmata based on immunolabeling studies. Furthermore, White *et al.* (1994) showed that treatment of plant cells with actin antagonists such as cytochalasins leads to an increase in the diameter of the plasmodesmal orifices. In support of this observation, Ding *et al.* (1996) demonstrated that microinjected cytochalasin D (CD) and profilin, a small actin-binding protein that can depolymerize actin filaments in *Tradescantia* staminal hairs (Staiger *et al.,* 1994), induces an increase in the plasmodesmal SEL from about 1 kDa to at least 20 kDa in tobacco mesophyll.

A protein kinase that can phosphorylate a viral protein is detected in purified cell walls, and possibly in plasmodesmata, from mature tobacco leaf mesophyll (Citovsky *et al.,* 1993) (see Section III,C,3). It will be of great interest to determine whether this kinase is indeed a genuine plasmodesmal component, and whether this or other kinases are responsible for the putative phosphorylation of plasmodesmal components to regulate transport.

b. Callose Deposition/Degradation High levels of ATP and Ca^{2+} could also stimulate the synthesis and deposition of callose at the orifices of plasmodesmata, in the cell wall region immediately below the plasma membrane, to regulate plasmodesmal microchannels (Cleland *et al.,* 1994). Involvement of callose deposition in closing plasmodesmal microchannels has been shown by dye-coupling experiments (Wolf *et al.,* 1991; Rinne and Van der Schoot, 1998).

Although callose deposition can occur rapidly, its reabsorption takes hours (Galway and McCully, 1987). Therefore, callose may not play a role in rapid closing/opening of plasmodesmal microchannels. Rather, it may be involved in the long-term regulation of plasmodesmal closure during dormancy induction (Rinne and Van der Schoot, 1998) and under various other biotic and abiotic stress conditions (Radford *et al.,* 1998).

c. Pressure Differentials Oparka and Prior (1992) generated pressure differentials between neighboring trichome cells of *N. clevelandii* leaves by

either puncturing or raising the pressure of a cell and showed that a pressure differential of approximately 200 kPa is able to block the cell-to-cell diffusion of Lucifer Yellow CH (LYCH), a 457-Da fluorescent and membrane-impermeable dye. These observations suggest that plasmodesmal SEL may be regulated by pressure differentials in some cases, especially in the phloem where solute concentrations vary greatly between cells so that a large pressure gradient can be generated (Oparka and Prior, 1992).

A pressure differential of a similar magnitude also regulates plasmodesmal transport in green algae *Chara corallina* (Côté *et al.*, 1987; Ding and Tazawa, 1989; Reid and Overall, 1992) and *Nitella flexilis* (Barclay and Fensom, 1984; Zawadzki and Fensom, 1986) and in liverwort *Conocephalum conicum* (Trebacz and Fensom, 1989).

The mechanisms of pressure-regulated transport are unclear. One possibility is that pressure differentials directly cause rearrangement of the plasma membrane and appressed ER particles to close the microchannels. Another possibility is that an intracellular signaling pathway transduces the effect of pressure differentials into chemical or physical modifications of plasmodesmal components to shut down the transport.

3. The Nature of Molecules That Can Diffuse from Cell to Cell

Cell-to-cell diffusion through plasmodesmata is not simply governed by the size of molecules. The charge, shape, and biochemical nature of a molecule also appear to be important factors. Erwee and Goodwin (1984) showed that aromatic amino acids tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Try) not only are incapable of diffusing from cell to cell, but can also inhibit the diffusion of other amino acids that can otherwise be transported in *Egeria densa* leaves. Tucker and Tucker (1993) also reported that Phe, Try, methionine (Met), and histidine (His) cannot diffuse from cell to cell in the staminal hairs of *Setcreasea purpurea.* Furthermore, plasmodesmata in this system select for molecules that are hydrophilic and have a charge of -2 to -4. Conceivably, specific interactions between a molecule and some plasmodesmal components dictate whether a molecule can pass or not.

The findings of Erwee and Goodwin (1984) and Tucker and Tucker (1993) on the selectivity of plasmodesmata for molecules may have important implications in studying what kinds of physiological molecules, including amino acids, can diffuse from cell to cell. For instance, the plant hormone auxin (IAA) cannot pass through plasmodesmata (Drake and Carr, 1978), but gibberellins can (Drake and Carr, 1979; Kwiatkowska, 1991). Tucker and Tucker (1993) suggest that this is because IAA is derived from and has a structure similar to tryptophan, and gibberellins are similar to fluorescein, which can diffuse cell to cell.

Baron-Epel *et al.* (1988) first reported that lipid analogues can be transported from cell to cell through plasmodesmata in cultured soybean root cells. This finding was followed up by Grabski *et al.* (1993) showing that ER lipid analogues can move from cell to cell, but plasma membrane lipid analogues cannot. Thus, the appressed ER may provide a pathway for lipid transport between cells. It elicits speculations as to if this constitutes an intercellular lipid signaling pathway.

III. Plasmodesmata in Intercellular Trafficking of Proteins and Nucleic Acids

A recent breakthrough in studying the plasmodesmal transport functions is the discovery of intercellular trafficking of macromolecules (Mezitt and Lucas, 1996; Ghoshroy *et al.*, 1997; Ding, 1998). These macromolecules include viral proteins, plant defense-related proteins, transcription factors, mRNAs, and others the function of which remains to be characterized. This discovery casts new light on how plant developmental and physiological processes are regulated at the transcellular and whole plant level and how plants interact with pathogens and other invaders.

A. Protein Trafficking

1. Trafficking of Transcription Factors Important for Morphogenesis in the Shoot Apex

All postembryonic organs of a plant are the products of mitotic activities of the apical meristematic tissues. As shown in Fig. 2, the shoot apical meristem (SAM) in most plants consists of layers of cells with different lineages, as defined by clonal analysis (Satina *et al.*, 1940; Sussex, 1989). Formation of an organ such as a leaf or a tissue involves coordinated division, differentiation, and growth of a group of cells from different layers. L1 cells divide anticlinally (perpendicular to the plant surface) to produce the epidermis, L2 cells divide anticlinally and periclinally (parallel to the plant surface) to produce subepidermal cells such as the mesophyll, and L3 cells divide in all planes to produce inner tissues such as the vasculature. Numerous studies have established that cells within a layer and between layers interact to coordinate gene expression and differentiation that lead to morphogenesis (Lucas, 1995; Mezitt and Lucas, 1996; Hake and Char, 1997; Jackson and Hake, 1997; and Ding, 1998). The underlying mechanisms have remained largely unknown.

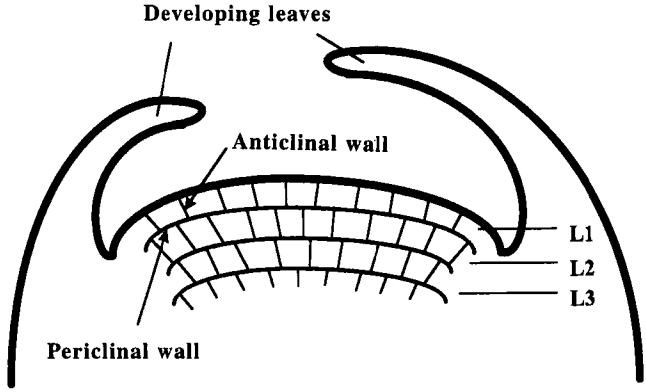

FIG. 2 Clonal layers of cells in a shoot apical meristem. Initiation of a lateral organ such as a leaf requires coordinated division, differentiation, and growth of a group of cells from all the layers. Cells in L1 divide only anticlinally, and cells in other layers can divide both anticlinally and periclinally. There are no division walls between L1 and L2 layers.

The plasmodesma-mediated intercellular trafficking of proteins, especially transcription factors, may be an important component of the mechanisms for cell-to-cell interactions. In maize plant, a transcription factor called KNOTTED 1 (KN1) is involved in maintaining the apical meristematic cells in an undifferentiated state (Sinha *et al.,* 1993). An *in situ* hybridization experiment shows that, in the maize SAM, *KN1* mRNA is detected in the L2 and inner layers, but not in the L1 layer. The KN1 protein, however, is detected in L1 as well as inner layers by immunolabeling. This implies that the KN1 protein produced in the inner layers trafficks into the L1 layer (Jackson *et al.,* 1994). Employing microinjection method, Lucas *et al.* (1995) demonstrated that a recombinant KN1 produced in an *Escherichia coli* can indeed traffic from cell to cell in tobacco and maize leaf mesophyll tissue.

Several transcription factors essential for flower formation in snapdragon (*Antirrhinum majus*) can function noncell autonomously. Their expression in one layer of cells in the floral meristem can trigger downstream expression of other genes in neighboring cell layers to initiate flower development. These proteins include FLORICAULA (FLO) (Carpenter and Coen, 1995; Hantke *et al.,* 1995), DEFFICIENS (DEF) and GLOBOSA (GLO) (Perbal *et al.,* 1996). DEF and GLO have been shown to be able to traffic from cell to cell based on their localization patterns revealed by immunolabeling and *in situ* hybridization (Perbal *et al.,* 1996) and by microinjection (Mezitt and Lucas, 1996). In particular, DEF trafficks in a polar fashion from L2 to L1 (Perbal *et al.,* 1996). The *Arabidopsis* transcription factor PISTILLA (PI) can also regulate, transcellularly, gene expression necessary for the

initiation of flower organs (Bouhidel and Irish, 1996). It will be interesting to test whether this protein can traffic from cell to cell.

What could be the specific functions of intercellular trafficking of transcription factors? First, it may be a means to ensure that cells destined to develop into a specific organ are highly coordinated in gene expression (Lucas, 1995; Mezitt and Lucas, 1996; Jackson and Hake, 1997). Second, trafficking of a transcription factor may establish a gradient of a morphogenetic signal that triggers differential gene expression among cells destined for different developmental pathways (Jackson and Hake, 1997). In this regard, some transcription factors may be the morphogens themselves. This would be functionally similar to the asymmetrical distribution, in the *Drosophila* embryo, of morphogens such as bicoid (Driever and Nüsslein-Volhard, 1988; Struhl *et al.*, 1989; St Johnston and Nüsslein-Volhard, 1992) and caudal (Mlodzik *et al.*, 1985; Macdonald and Struhl, 1986; Rivera-Pomar *et al.*, 1995) that play concentration-dependent regulatory roles in subsequent gene expression to establish the anterior and posterior patterns of *Drosophila*. Third, some transcription factors may traffic from cell to cell to interfere with the translation of mRNAs encoding other transcription factors to establish specific gene expression patterns. This possibility is suggested by the finding that bicoid protein also binds to the *caudal* mRNA to repress its translation, thereby establishing the asymmetrical distribution of caudal protein in the *Drosophila* embryo (Dubnau and Struhl, 1996; Rivera-Pomar *et al.*, 1996). Experimental testing of these and other models will be one of the most important tasks in future studies.

2. Trafficking of Proteins with Various Functions in the Phloem

The phloem tissue consists of several types of cells, including sieve elements, companion cells, and phloem parenchyma cells. During differentiation, sieve elements become enucleate and are believed to be devoid of ribosomes and mRNAs. However, they are living cells and form the conducting channels for the long-distance transport of nutrients and signals throughout a whole plant (Parthasarathy, 1975; Sjölund, 1997). There are over 200 soluble proteins in the phloem exudate, collected from the enucleate sieve elements, in several plant species studied so far, including wheat (*Triticum aestivum*) (Fisher *et al.*, 1992), casterbean (*Ricinus communis*) (Sakuth *et al.*, 1993; Schobert *et al.*, 1995), and rice (*Oryza sativa*) (Nakamura *et al.*, 1993; Ishiwatari *et al.*, 1995). These proteins range in size from 10 to 200 kDa. The identities of some of these proteins have been characterized. These include thioredoxin h (RPP13-1) (Ishiwatari *et al.*, 1995, 1998) and a protein kinase (Nakamura *et al.*, 1995) from rice, and ubiquitin, HSP70, Rubisco-subunit-binding protein (Schobert *et al.*, 1995) and glutaredoxin (Szeder-

kényi *et al.*, 1997) from casterbean. Molecular chaperones related to Rubisco-subunit-binding protein and cyclophilin, as well as ubiquitin and the redox proteins, thioredoxin h and glutaredoxin, actin, and profilin are detected in the phloem exudate of wheat, rice, *Yucca filamentosa, C. maxima, Robinia pseudoacacia,* and *Tilia platyphyllos* (Schobert *et al.*, 1998).

Most, if not all, of the proteins are likely synthesized in the companion cells and are transported into the sieve elements via plasmodesmata connecting the two cell types (Fisher *et al.*, 1992). This is demonstrated by examining the localization patterns of some phloem proteins and their mRNAs. The phloem lectin PP2 and phloem protein PP1 are found in the phloem of many plants. *In situ* hybridization shows that the mRNAs encoding these proteins are localized only in companion cells, and immunocytochemistry shows localization of the proteins in both companion cells and sieve elements (Bostwick *et al.*, 1992; Clark *et al.*, 1997, Dannenhoffer *et al.*, 1997). The RPP13-1 mRNA is also expressed exclusively in the companion cells of rice (Ishiwatari *et al.*, 1998). Using microinjection as an alternative approach, Balachandran *et al.* (1997) and Ishiwatari *et al.* (1998) provided direct evidence that some phloem exudate proteins can traffic from cell to cell in leaf mesophyll.

To fully understand the functions of protein trafficking in the phloem, their compositions need to be identified continuously. Furthermore, the synthetic sites of these proteins need to be determined. A full spectra of possibilities should be considered at this stage. First, is every protein transported in the sieve elements synthesized in the companion cells? Second, are some proteins synthesized in the companion cells and some synthesized in other cells such as leaf epidermal cells, mesophyll cells, and even bundle sheath cells? Third, are some proteins also synthesized in the sieve elements using mRNAs imported from other cells? A knowledge of the composition and their cell or tissue origins could then lead to a full characterization of the functions of these proteins.

3. Intercellular and Phloem Trafficking of Viral and Plant Defense-Related Proteins

Intercellular protein trafficking is important not only for plant growth and development, but also for plant–pathogen interactions and for plant defense. In fact, studies on viral cell-to-cell movement provided some of the initial experimental evidence for macromolecular trafficking through plasmodesmata.

During systemic infection of a whole plant, a plant virus needs to move from an infected cell into neighboring cells and then from an infected organ to another. Because cell walls impose a physical barrier for movement, plant viruses have evolved mechanisms to utilize plasmodesmata to move

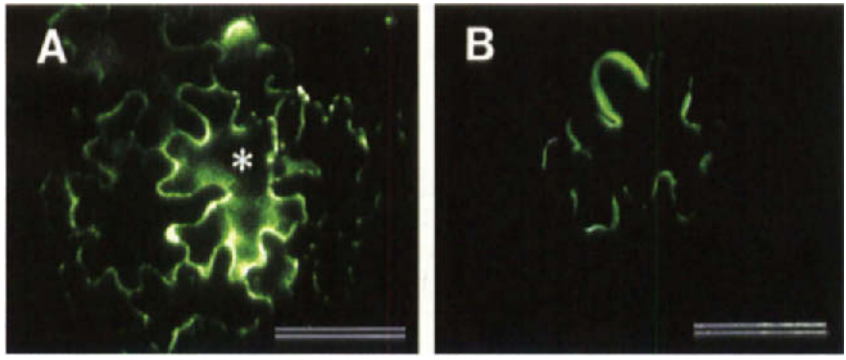

FIG. 3 Intercellular trafficking of cucumber mosaic virus (CMV) 3a movement protein (3a MP) fused to green fluorescent protein (GFP) in tobacco leaf epidermis (A). The star indicates the fusion protein-expressing cell. Spreading of GFP fluorescence into neighboring cells indicates trafficking. A mutant form of 3a MP:GFP does not traffic from cell to cell (B). The fusion proteins are produced by biolistic bombardment of DNA encoding the respective fusion genes into tobacco leaf epidermis. Bar = 40 μm. (From Itaya, A., Hickman, H., Bao, Y., Nelson, R., and Ding, B. (1997). Cell-to-cell trafficking of cucumber mosaic virus movement protein:green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. *Plant J.* **12**, 1223–1230, with permission.)

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from cell to cell, and the phloem to move from organ to organ. The smallest known plant viruses, even free and folded viral nucleic acids, are at least 10 nm in diameter (Gibbs, 1976). This is much larger than the 2.5 nm of microchannels of plasmodesmata. Therefore, viruses cannot simply diffuse through plasmodesmata and must employ active mechanisms to traffic through these small tunnels. It is now well established that viruses encode nonstructural proteins, collectively called movement proteins (MPs), that assist viral particles or viral genomes to move through plasmodesmata (Atabekov and Taliansky, 1990; Deom *et al.,* 1992; Lucas and Gilbertson, 1994; Carrington *et al.,* 1996; Ding, 1998). In addition, some structural proteins such as the coat protein of some viruses are also involved in movement (Forster *et al.,* 1992; Chapman *et al.,* 1992a,b; Oparka *et al.,* 1996; Canto *et al.,* 1997). The MPs of some viruses, typified by that of the cowpea mosaic virus (CPMV), are able to form tubular structures across the cell walls, presumably in place of plasmodesmata in an infected tissue, so that viral particles can move intercellularly within these tubules or the tubules containing viral particles move from cell to cell (Carrington *et al.,* 1996; Ding, 1998).

Many viruses use different mechanisms for movement. As typified by tobacco mosaic virus (TMV) (Citovsky *et al.,* 1990; 1992), cucumber mosaic virus (CMV) (Ding *et al.,* 1995; Nguyen *et al.,* 1996; Blackman *et al.,* 1998), and red clover necrotic mosaic virus (RCNMV) (Fujiwara *et al.,* 1993), they appear to move intercellularly as ribonucleoprotein complexes. In this case, the MPs do not modify the plasmodesmal structure permanently. Rather, they interact with plasmodesmata to increase their SEL and to modify the viral genome to fit the enlarged plasmodesmal microchannels.

An important finding from studying the functions of these viral MPs is that some of them can traffic from cell to cell through plasmodesmata, as shown by microinjection and other means. This has been established for the MPs of RCNMV (Fujiwara *et al.,* 1993), bean dwarf mosaic virus (BDMV) (Noueiry *et al.,* 1994), TMV (Waigmann and Zambryski, 1995), and CMV (Ding *et al.,* 1995; Canto *et al.,* 1997; Itaya *et al.,* 1997) (Fig. 3, see color plate) and for the capsid protein (CP) and helper component-proteinase (HC-Pro) of bean common mosaic necrosis potyvirus (BCMNV) and lettuce mosaic potyvirus (LMV) (Rojas *et al.,* 1997). The functions of these MPs in promoting viral RNA trafficking will be discussed later.

Plants synthesize defense chemicals or proteins in reaction to wounding caused by attacking herbivores and all sorts of pathogens (Bowles, 1990; Schaller and Ryan, 1995; Bergy *et al.,* 1996). Among the inducible defense proteins are proteinase inhibitors that will interfere with the metabolism of the invaders (Schaller and Ryan, 1995; Bergy *et al.,* 1996). An important feature of this defense reaction is that the proteinase inhibitors accumulate rapidly in both wounded and distant, unwounded leaves. Thus, a systemic

signal must have been transmitted from the wounded leaf throughout the whole plant to turn on the expression of defense genes. A polypeptide of 18 amino acids called systemin is such a signal (Pearce *et al.*, 1991; Schaller and Ryan, 1995; Bergy *et al.*, 1996), which is transported through the phloem (Pearce *et al.*, 1993; Narváez-Vásquez *et al.*, 1995). It is likely that systemin is produced in the phloem parenchyma or companion cells and is then targeted to sieve elements via plasmodesmata for long-distance transport (Jacinto *et al.*, 1997; Ding, 1998). Systemin may be only an example of a myriad of polypeptides that traffic throughout a whole plant as signals to orchestrate plant growth and developmental processes (Schaller and Ryan, 1995; Ding, 1988).

Plants also synthesize other proteins called pathogenesis-related proteins (PR proteins) during pathogen invasion (Bowles, 1990; Van Loon *et al.*, 1994). In analyzing the expression pattern of the maize *PRms* gene induced by infection of the fungus *Fusarium moniliforme*, Murillo *et al.* (1997) showed that the PRms protein is produced in the root protoxylem cells and is then transported via plasmodesmata into the pith cells. The specific function of this protein trafficking is still unclear. In a general sense, however, trafficking of some PR proteins may result in the blockage of plasmodesmata to confine a pathogen in an infected cell, to mobilize defense chemical reactions, or to orchestrate gene expression leading to defense (Ding, 1998).

B. RNA Trafficking

The concept of intercellular RNA trafficking has been discussed for decades. In reviewing early literature on the functions of plasmodesmata in plant growth and development, Carr (1976) commented on the unlikely possibility of intercellular mRNA trafficking in plants and some fungi. In discussing the evolutionary origins of viroids and viroid-like RNA molecules, Zimmern (1982) postulated that these RNA molecules might have been derived from endogenous RNA molecules that traffic from cell to cell to function as signaling molecules in both plants and animals. In contrast to early electron microscopic studies showing the presence of viral particles in the structurally modified plasmodesmata of plant tissues infected by some viruses, Dorokhov *et al.* (1984) proposed that tobacco mosaic virus (TMV) may traffic from cell to cell as a ribonucleoprotein complex during infection. These pioneering concepts are now gaining experimental support from a variety of systems.

1. Intercellular Trafficking of Viral RNAs

In studying the intercellular movement mechanisms of RCNMV, Fujiwara *et al.* (1993) showed by microinjection that the RCNMV MP not only

trafficks from cell to cell by itself, but also potentiates cell-to-cell trafficking of *in vitro* transcripts of RCNMV RNA in tobacco and cowpea leaf mesophyll tissues. This study provided the first concrete experimental evidence for RNA trafficking through plasmodesmata. The ability of a viral protein to potentiate RNA trafficking was later demonstrated also for CMV (Ding *et al.*, 1995; Nguyen *et al.*, 1996), TMV (Nguyen *et al.*, 1996), and the CP and HC-Pro of BCMNV and LMV (Rojas *et al.*, 1997). Therefore, an viral RNA can traffic from cell to cell in the presence of a viral protein. It is generally thought that the viral RNA and protein form a complex for trafficking (Citovsky *et al.*, 1990, 1992; Lucas and Gilbertson, 1994; Carrington *et al.*, 1996; Mezitt and Lucas, 1996; Ghoshroy *et al.*, 1997; Ding, 1998). However, direct evidence that a ribonucleoprotein complex, rather than a naked RNA, trafficks cell to cell remains to be provided.

2. Intercellular Trafficking of Endogenous Plant RNAs

That viral nucleic acids in conjunction with specific viral proteins can traffic from cell to cell within seconds of microinjection implies that cellular mechanisms exist for the trafficking of plant RNAs. It is unlikely that the injected viral proteins and RNAs elicit new reactions in a cell to generate a special trafficking system within such a short period of time.

Lucas *et al.* (1995) showed that the KN1 protein was able to potentiate cell-to-cell trafficking of its sense mRNA, but not antisense mRNA, when these mRNAs were labeled fluorescently and each coinjected with the protein into tobacco and maize mesophyll cells. The significance of this finding remains to be understood, because *in situ* hybridization studies by Jackson *et al.* (1994) showed that *KN1* mRNA is absent from the L1 layer of maize SAM where the KN1 protein is present. Thus, if KN1 trafficking from the L2 layer into the L1 layer is accompanied by *KN1* mRNA trafficking, one would expect presence of this mRNA in the L1 as well, albeit at a low level. Further studies are needed to resolve whether the absence of *KN1* mRNA from L1 is becasue the level of this mRNA is below the detection sensitivity of the *in situ* hybridization method used or is because mesophyll cells as used in microinjection function differently from apical meristematic cells.

Studies on the localization patterns of the leaf sucrose transporter SUT1 and its mRNA in the phloem tissue of tobacco, potato, and tomato provided additional evidence for plant RNA trafficking. Kühn *et al.* (1997) detected both SUT1 protein and its mRNA in enucleate sieve elements. Significantly, the *SUT1* mRNA was found to be concentrated in or near plasmodesmata connecting the sieve elements and companion cells. These data suggest that *SUT1* mRNA trafficks from companion cells, where it is synthesized, into the enucleate sieve elements via plasmodesmata.

Systemic RNA trafficking from cell to cell via plasmodesmata and from organ to organ via phloem may provide a means of controlling gene expression at the whole plant level. This possibility is suggested by studies on the systemic propagation of signals causing gene silencing in plants. The introduction of certain transgenes into a plant can lead to suppression of the expression of homologous endogenous genes. This is generally called "homology-dependent gene silencing." When both the transgene and the homologous endogenous gene are silenced, it is called "cosuppression." Gene silencing or cosuppression can occur at both transcription and post-transcription (mRNA turnover) levels (Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe, 1996; Jorgensen *et al.,* 1998; Van den Boogaart *et al.,* 1998). Importantly, gene silencing in some cases has been shown to be noncell autonomous and involves cell-to-cell communication (Kunz *et al.,* 1996; Que *et al.,* 1998).

Direct evidence for cell-to-cell and systemic propagation of signals triggering gene silencing in a plant came from two studies. Palauqui *et al.* (1997) used grafting procedures to study the systemic transmission of cosuppression of endogenous and transgenes encoding nitrate reductase and nitrite reductase as well as posttranscriptional silencing of a *uidA* transgene in tobacco. They demonstrated that gene silencing could be transmitted unidirectionally from a silenced stock to the nonsilenced scion expressing a corresponding transgene. Their results suggest that a nonmetabolic, transgene specific messenger is involved in the transmission of the silencing signal.

In another system, Voinnet and Baulcombe (1997) used transgenic *N. benthamiana* plants that constitutively produce GFP to investigate systemic transmission of gene silencing. Inoculation of a leaf of the transgenic plant with *Agrobacterium tumefaciens* carrying a *GFP* gene in its T-DNA results in DNA integration into the plant genome to express the *GFP* gene. Expression of this *GFP* gene caused silencing of the *GFP* transgene expression throughout the whole plant after certain days of inoculation, even though the *Agrobacterium* and T-DNA remained in the inoculated leaf. Furthermore, such cosuppression is sequence specific because inoculation of a transgenic leaf with potato virus X (PVX):GFP fusion led to no accumulation of PVX, whereas inoculation with PVX:GUS resulted in viral accumulation. Thus, *GFP* expression from the chimeric PVX:GFP construct specifically triggered the systemic silencing of the *GFP* transgene.

These data suggest that a signal is transmitted from cell to cell and from one leaf to another to cause specific gene silencing. Although candidates for the trafficking signals have not been identified, they are postulated to be aberrant RNAs transcribed from a transgene (probably methylated) or small copy RNAs (cRNAs) made by an RNA-dependent RNA polymerase using the target mRNA as a template (Lindbo *et al.,* 1993; Dougherty and

Parks, 1995; Voinnet and Baulcombe, 1997; Palauqui *et al.,* 1997; Jorgensen *et al.,* 1998). The possibility that some small double-stranded (ds) RNAs also function as such signals cannot be excluded in the light of the finding that a dsRNA, but not an antisense or sense RNA, is a specific and potent inhibitor of gene expression and can traffic from cell to cell to elicit gene silencing in nematodes (Fire *et al.,* 1998; Montgomery and Fire, 1998).

3. Systemic Trafficking of Viroid RNAs

Viroids are small, covalently closed circular, and autonomously replicating RNA molecules that infect many economically important crop species (Diener, 1979, 1987; Garnsey and Randles, 1987). Most known viroids replicate in the nucleus (Semancik *et al.,* 1976; Harders *et al.,* 1989; Bonfiglioli *et al.,* 1994, 1996). Thus, for such a viroid to move within a plant to cause systemic infection, movement consists of the following distinct, but related steps (Fig. 4): (1) import into the nucleus (for replication), (2) export (after replication) out of the nucleus, (3) cell-to-cell movement, (4) entry into the phloem, (5) long-distance transport via phloem, (6) exit from the

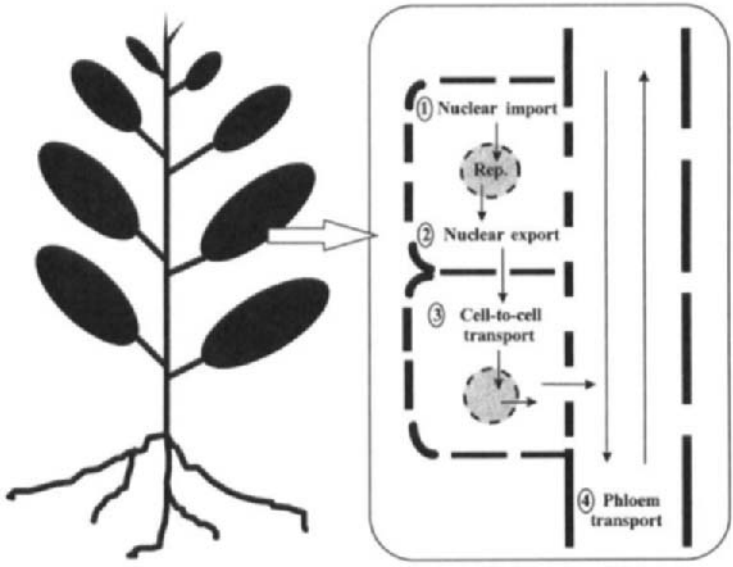

FIG. 4 Systemic trafficking of a viroid RNA in a plant. For a viroid that replicates in the nucleus, it is imported into the nucleus for replication. Afterward, it is exported out of the nucleus and is then transported from cell to cell to initiate new infection. Then it is transported from an infected organ to another through the phloem to cause systemic infection. Although not illustrated, exit from the phloem is required to infect a new organ.

phloem, and (7) reentry into nonvascular tissues to establish new infection at a remote site.

An outstanding feature of viroids is that they do not encode any proteins. Consequently, these RNA molecules must interact with cellular factors directly for replication and movement. Viroids are the only known example of nontranslatable RNAs to date that are capable of nuclear transport, cell-to-cell transport, and long-distance transport. Future research, however, may provide other examples. This fact makes viroids an attractive model system to investigate the mechanisms and functions of RNA trafficking in plants.

Ding *et al.* (1997) showed, by microinjection, that fluorescently labeled and infectious *in vitro* transcripts of potato spindle tuber viroid (PSTVd) traffic from cell to cell via plasmodesmata, and such trafficking might involve a viroid structural or sequence motif. Such a putative motif remains to be defined. At present, a number of scenarios can be envisaged for such a motif to interact with cellular factors. PSTVd may piggy-back on cellular proteins that traffic between cells; alternatively, PSTVd may interact directly with certain plasmodesmal components for transport. Whatever mechanism is involved, the ability of PSTVd to mediate plasmodesmal trafficking of nucleic acids provides strong support for the hypothesis that an endogenous mechanism exists for cell-to-cell trafficking of RNA in plants (Ding *et al.*, 1997).

Woo *et al.* (1999) used an *in vitro* system to study the nuclear import of PSTVd in permeabilized protoplasts of tobacco BY2 cells. They showed that the nuclear import of fluorescently labeled and infectious PSTVd transcripts is a carrier-mediated and specific process, as it can be effectively blocked by the addition of unlabeled PSTVd, but not by an unlabeled control RNA of the same size. Data suggest that PSTVd possesses a structure or sequence for nuclear import.

Systemic movement of a viroid was first studied by Palukaitis (1987), who showed that the systemic spread of PSTVd apparently follows the photoassimilate transport pattern, suggesting that this viroid moves long distance via the phloem. *In situ* hybridization work has provided direct evidence that PSTVd moves within the phloem during systemic infection (Y. Zhu, Y. M. Woo, and B. Ding unpublished results).

It is unlikely that plants have evolved mechanisms for a coordinated nuclear, cell-to-cell, and phloem RNA trafficking just for the sake of viroid infection. We favor the hypothesis that these mechanisms exist for the systemic trafficking of endogenous RNA molecules for signaling purposes. Viroids have simply pirated these mechanisms for their own trafficking. Under such a scenario, further investigations on viroid nuclear, cell-to-cell, and long-distance transport should provide important insight into the mechanisms and functions of systemic trafficking of endogenous RNAs in

a plant. Identification of the PSTVd motifs involved in each transport process and the interacting cellular factors may eventually facilitate the isolation of endogenous plant trafficking RNAs sharing similar structural and functional features.

4. Functions of Intercellular RNA Trafficking in Plant Growth and Development

RNA trafficking and gene silencing possibly provide a powerful surveillance mechanism of detecting and eliminating unwanted (overproduced or aberrant) cellular RNAs and viral RNAs (Matzke and Matzke, 1995; Dougherty and Parks, 1995; Jorgensen *et al.*, 1998). The potential significance of RNA trafficking as a means of coordinating gene expression for plant growth and development has also been considered (Jorgensen *et al.*, 1998). The following discussions attempt to present a number of scenarios that RNA trafficking may function to regulate cellular processes underlying plant growth and development (Fig. 5). Some of the scenarios will be necessarily speculative for the sake of discussion.

a. Interacting with DNA to Regulate Transcription An example of transcription regulation by RNA is RNA-dependent DNA methylation. In transgenic tobacco plants expressing PSTVd cDNAs, autonomous viroid RNA–RNA replication leads to the specific methylation of the PSTVd cDNAs, but not plant genomic DNA or the flanking T-DNA (Wassenegger *et al.*, 1994). Data suggest that the methylation of specific DNA sequences mediated by its mRNA, or by a special class of RNA molecules yet to be identified, may be a mechanism of regulating transcription (Wassenegger *et al.*, 1994). Presumably, the RNA serves to guide the methylase to target DNA sequences by hybridizing with the DNA (Wassenegger *et al.*, 1994).

Dickson and Robertson (1976) hypothesized that a special class of RNA molecules may be able to regulate gene expression by binding to specific DNA sequences to prime transcription. This would serve to initiate or enhance transcription. Whether such a mechanism exists deserves investigation. If this mechanism exists, then RNA trafficking could also be involved in the up-regulation of gene expression by interacting directly with DNA.

Thus, an RNA can possibly regulate gene expression by interacting with DNA directly, alone or together with other factors such as proteins. If such an RNA trafficks from cell to cell, it can then modulate gene expression patterns in a cluster of cells.

b. Regulation of mRNA Turnover A trafficking RNA may interact with an mRNA directly to regulate translation of the latter. This would serve as a means of posttranscriptional regulation of gene expression at the supracellular level. Numerous examples of posttranscriptional gene silenc-

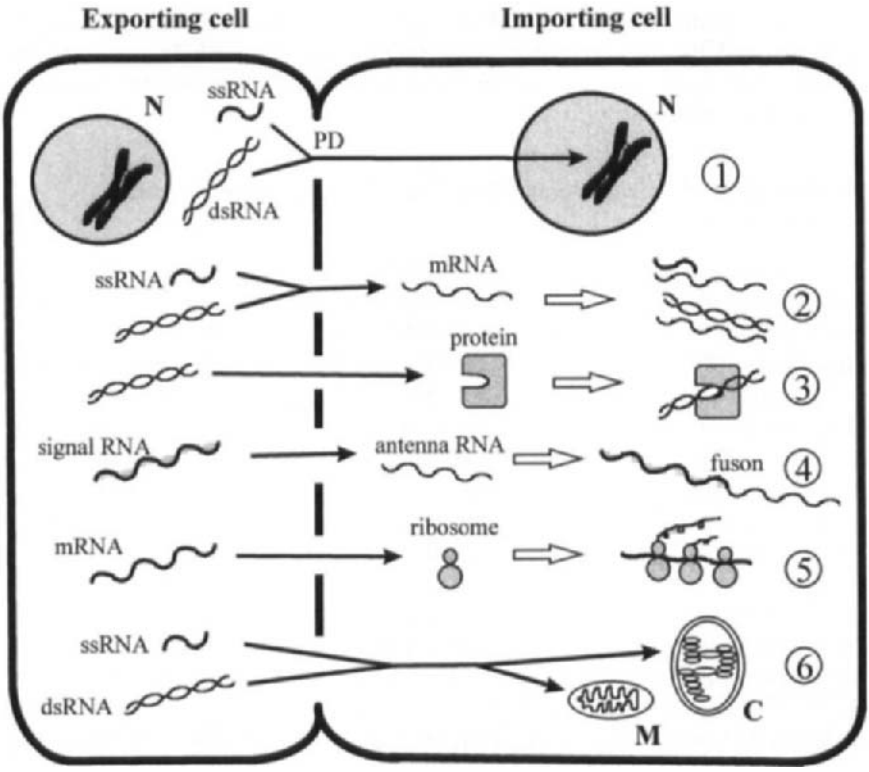

FIG. 5 Potential mechanisms and functions of intercellular RNA trafficking in a plant. (1) A single-stranded (ss) RNA or dsRNA trafficks from one cell to another and enters the nucleus (N) to interact with DNA directly to regulate transcription. (2) A ssRNA or dsRNA trafficks into a cell and interacts with an mRNA to regulate turnover of the latter. (3) A dsRNA trafficks into a cell to interact with a protein to regulate its activity. (4) A signal RNA, single stranded, trafficks into a cell to recombine with an antenna RNA to form a fuson to be translated. (5) An mRNA trafficks from a cell into another to be translated. (6) A ssRNA or dsRNA trafficks into a cell and enters either a mitochondrion (M) or a chloroplast (C) to coordinate gene expression between the nucleus and the organelles.

ing involve RNA-mediated degradation of overproduced or aberrant mRNAs (Lindbo *et al.,* 1993; Dougherty and Parks, 1995; Baulcombe, 1996; Metzlaff *et al.,* 1997; Angell and Baulcombe, 1997; Ratcliff *et al.,* 1997; Van den Boogaart *et al.,* 1998). RNA molecules, whether aberrant RNA, dsRNA, or cRNA, can presumably traffic from cell to cell to orchestrate mRNA turnover in a cluster of cells. This mechanism likely functions in defense against viruses. It could also function under normal plant growth and developmental conditions to maintain a balanced level of mRNA in a group of cells (e.g., perhaps cells within a symplasmic domain?) or to

establish an mRNA gradient among groups of cells (e.g., across symplasmic domains) for morphogenesis.

c. Interacting with Proteins to Regulate Cellular Processes The mammalian interferon-induced and dsRNA-activated protein kinase (P68 or PKR) plays a critical role in the regulation of gene transcription (Kumar *et al.,* 1994), protein synthesis (Maran and Mathews, 1988; Edery *et al.,* 1989), cell differentiation, and cell growth (Koromilas *et al.,* 1992; Meurs *et al.,* 1993). In particular, this protein has a unique function as an antiviral agent. It can phosphorylate itself and the translation initiation factor eIE-2α, resulting in the inhibition of protein synthesis. The replication of many viruses involves the production of dsRNA, which can activate the PKR, leading to the suppression of viral proliferation. To counteract this effect, viruses have evolved various strategies to down-regulate the activities of PKR (Katze, 1992; Proud, 1995).

A plant analogue of the mammalian PKR (pPKR) has been identified (Hiddinga *et al.,* 1988; Langland *et al.,* 1995). pPKR is a Ser/Thr kinase of 68–70 kDa present in the cytosol and also in association with ribosomes. It can be activated by a dsRNA, phosphorylate the plant eukaryotic initiation factor eIF-2α, and inhibit protein synthesis *in vitro* (Langland *et al.,* 1996).

PSTVd can specifically induce phosphorylation of pPKR from tomato (Hiddinga *et al.,* 1988). Diener *et al.* (1993) showed that PSTVd strains that cause severe or mild symptoms can differentially activate the mammalian PKR *in vitro.* Furthermore, PSTVd specifically binds to the PKR. TMV infection also stimulates phosphorylation of a pPKR from infected tobacco tissues. The phosphorylation is enhanced by the addition of dsRNA (Crum *et al.,* 1988).

It is attractive to think of the regulation of pPKR activity by PSTVd and TMV (perhaps a double-stranded replicative form of the virus) as an example of regulation of protein functions by dsRNA in plants. Thus, it will be extremely useful to find out if endogenous dsRNAs exist in plants that regulate the activities of pPKR or pPKR-like proteins. Furthermore, it will be important to determine if such putative dsRNAs can traffic from cell to cell to regulate pPKR functions. The activated pPKR could be involved in the regulation of gene regulation, cell differentiation, growth, and viral or viroid replication.

d. Recombination with RNA in an Importing Cell to Create a New mRNA Zimmern (1982) proposed that the intercellular trafficking of RNA between plant and even between animal cells may lead to the creation of new mRNAs encoding novel proteins. Based on this hypothesis, some RNAs function as antenna molecules that are localized in the cytoplasm in an inactive form. Cleavage of some heterogeneous nuclear RNAs

(hnRNAs) in a cell produces the so-called signal RNAs that traffic into neighboring cells to combine with antenna RNAs to create new RNAs, which Zimmern (1982) termed fusons. These fusons are then translated. Zimmern (1982) further postulated that viroids and viroid-like RNAs may have evolved from such signal RNAs by retaining the essential functions of autonomous replication and trafficking.

RNA recombination occurs widely in viruses and is an important means of virus evolution (Roossinck, 1997). Numerous examples of recombination between viroid RNAs to produce new species or variants have also been provided (Hammond *et al.,* 1989; Keese and Symons, 1985; Rezaian, 1990; Puchta *et al.,* 1991; Semancik *et al.,* 1994; Stasys *et al.,* 1995; Kofalvi *et al.,* 1997).

e. Trafficking of mRNA to New Cells for Translation In several biological systems, some mRNAs exported from the nucleus are not translated immediately. Rather, these mRNAs are transported to specific locations within a cell for protein synthesis. This is particularly well documented in polarized cells. There are a number of advantages for this trafficking and localization. First, it ensures localized, need-based, and high-fidelity translation of an mRNA into a protein. Second, this is presumably economical in terms of saving energy for trafficking proteins to such locations for function because a single transcript translocated to a specific site can be translated many times to produce the amount of protein molecules needed (Wilhelm and Vale, 1993; St Johnston, 1995; Okita *et al.,* 1998).

Because of the capability of plasmodesmata to traffic RNA, such mRNA transport may be extended beyond a single cell level. Thus, an mRNA produced in one cell can be transported into a neighboring cell to be translated. The translational product could be involved in basic cell metabolism, regulation of protein function in neighboring cells, or even regulation of gene expression patterns. It will of great interest to determine if such mRNA transport is limited to cells within a symplasmic domain or if it can occur between domains.

Current examples of mRNA trafficking include the *KN1* mRNA (Lucas *et al.,* 1995), *SUT1* mRNA (Kühn *et al.,* 1997), and viral RNAs (Ding, 1998). The meaning of *SUT1* mRNA trafficking into sieve elements, however, remains an enigma because mature sieve elements do not seem to have a protein translation machinery. Of course, such a machinery may indeed exist but it has not been detected by current techniques.

f. Coordination of Nuclear and Chloroplast and/or Mitochondrion Functions *In situ* hybridization localizes avocado sunblotch viroid (ASBVd) predominantly in the chloroplasts of infected leaves (Bonfiglioli *et al.,* 1994; Lima *et al.,* 1994). Whether this viroid replicates in the chloroplasts is still

unclear. Irrespective of site of replication, the chloroplast localization of ASBVd suggests that there is an RNA trafficking pathway at least between the cytoplasm and the chloroplasts. If such a pathway is utilized for the trafficking of endogenous plant RNA molecules, the pathway clearly extends from the nucleus to the chloroplasts. Two potential functions of this trafficking can be envisaged. First, some nuclear RNAs traffic into chloroplasts and are translated locally. Second, some signaling RNA molecules shuttle between the nucleus, the cytoplasm, and the chloroplasts to coordinate gene expressions in the two organelles and other cytoplasmic processes. Zimmern (1982) has also speculated on the possibility of this trafficking. This speculation could also be extended to RNA trafficking between the nucleus and mitochondria.

C. Mechanisms of Intercellular Protein and RNA Trafficking

The cellular mechanisms underlying intercellular protein and RNA trafficking are potentially complex. Available data suggest that this trafficking process consists of several distinct steps that require specific interactions between a trafficking molecule and cellular factors. The cytoskeleton and other factors are likely involved. Many detailed aspects of the models described remain to be experimentally characterized.

1. Intercellular Protein and RNA Trafficking Is a Multistep Process

Intercellular protein trafficking involves several major steps, including targeting to a plasmodesma, translocation through the microchannels, release from the plasmodesma, and sorting to the site of biological action (Fig. 6; Ding, 1998). Conceivably, each step requires specific macromolecular interactions between the trafficking protein and cellular factors. A plasmodesmal targeting signal (PTS), whether being a specific protein sequence(s) or a three-dimensional structure, is presumably recognized by a cellular receptor for targeting to plasmodesmata (Ding, 1998). Evidence for the existence of a PTS came from studies showing that the TMV MP (Waigmann and Zambryski, 1995) and CMV 3a MP (Itaya *et al.*, 1997) can mediate cell-to-cell trafficking of a fused reported protein such as β-glucuronidase (GUS) and green fluorescent protein (GFP) that cannot otherwise traffic by themselves. Mutagenesis and crystal structure predictions suggest that the N-terminal amino acids of rice RPP13-1 contain structural motifs that are necessary, but insufficient, for trafficking (Ishiwatari *et al.*, 1998). Both a PTS and the corresponding receptor remain to be identified for any trafficking proteins studied to date.

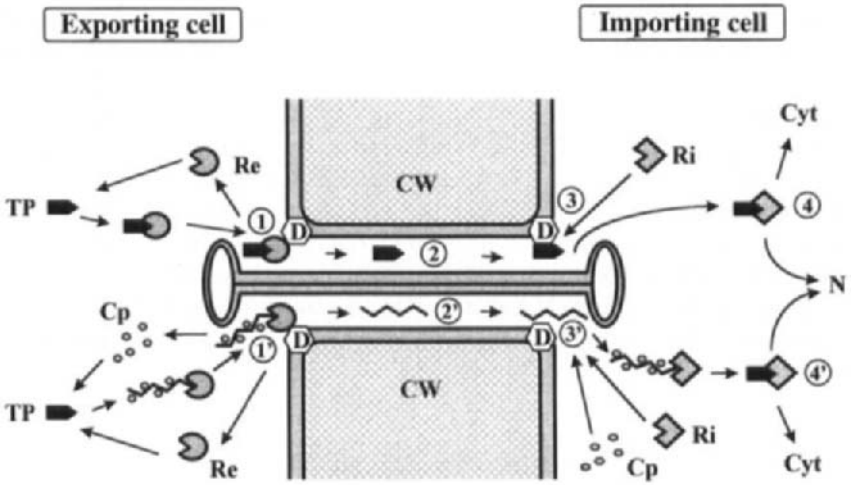

FIG. 6 Multistep intercellular protein trafficking through a plasmodesma. In steps 1 through 4, a protein trafficks in its native conformation. In the exporting cell, a cytosolic receptor (Re) recognizes and binds to the trafficking protein (TP), and the protein complex is targeted to the plasmodesma, presumably along the cytoskeleton (not illustrated) (1). The complex interacts with a docking protein (D) at the plasmodesmal orifice, which triggers translocation of the TP through microchannels (2). This step may require dilation of the microchannels, depending on the size of the TP and structural features of the plasmodesma. The receptor is recycled. On the importing cell side, the TP interacts with a docking protein at the plasmodesmal orifice and is recognized and released by another cytosolic receptor (Ri) (3). The TP is finally sorted to a specific cytoplasmic site (Cyt) or the nucleus (N) (4). It is possible that other factors, such as the cytoskeleton, are also involved in this final step of trafficking. In steps 1′ through 4′, a TP is recognized by chaperones (Cp) and a cytosolic receptor (Re) in the cytoplasm of the importing cell. The chaperones unfold the TP. This protein complex is then targeted to a plasmodesmal orifice. Interactions between the complex and a docking protein (D) result in the release of the chaperones and receptor and subsequent translocation of the unfolded TP (2′). It is also possible that a chaperone–TP complex is translocated. This step again may require the dilation of plasmodesmal microchannels. The chaperones and the receptor are recycled. On the importing cell side, the TP is recognized and released by a cytosolic receptor (Ri). Chaperones in the importing cell bind and refold the TP (3′). The TP complexed with the receptor and/or other cytosolic factors is finally sorted to a specific cytoplasmic site (Cyt) or the nucleus (N) (4′). The key feature of this model is that plasmodesma-mediated intercellular trafficking of a protein involves a number of distinct steps that each require specific protein–protein interactions. This ensures precise regulation of the trafficking of a protein. An RNA may traffic in the form of a ribonucleoprotein complex and follow similar steps. Redrawn from Ding (1998), with kind permission from Kluwer Academic Publishers.

Translocation of a protein through plasmodesmata may require an increase in the plasmodesmal SEL, as well as unfolding of a protein. Conceivably, translocation of proteins of different sizes has different requirements (Ding, 1998). Small proteins may be able to traffic through plasmodesmata

with an increase in the plasmodesmal SEL but not protein unfolding. Larger protein trafficking may need both an increase in plasmodesmal SEL and protein unfolding.

Release from plasmodesmata is postulated to be mediated by a plasmodesmal releasing signal (PRS) residing on the protein (Ding, 1998). This putative signal is recognized by a cellular receptor that releases the protein from the plasmodesmal orifice. The receptor may then direct the protein to the cytoplasmic or nuclear site where the protein exerts its functions.

Itaya *et al.* (1998) found that the CMV 3a MP:GFP fusion protein is targeted to truncated plasmodesmata between epidermal and mature guard cells in a tobacco transgenic plant. This is unexpected as these plasmodesmata do not have intercellular transport functions (Erwee *et al.*, 1985; Palevitz and Hepler, 1985; Ding *et al.*, 1997). Nevertheless, this fact indicates that protein targeting to plasmodesmata is not necessarily coupled to its translocation through or release from plasmodesmata for import into a neighboring cell. This supports the hypothesis that targeting to and release from plasmodesmata are separate steps in intercellular protein trafficking that require specific interactions between the trafficking protein and dedicated cellular factors (Ding, 1998).

RNA trafficking presumably follows similar steps. Based on studies on viral genome trafficking, an RNA may not traffic as a free molecule. More likely, it interacts with a protein(s) to form a ribonucleoprotein complex that trafficks from cell to cell. If this were indeed the case, the signal for trafficking may reside on the RNA or on a protein complexed with it.

2. A Potential Role of the Cytoskeleton

How a macromolecule finds its way from the cellular site where it is synthesized or assembled to a plasmodesma in an exporting cell, how it is mechanistically translocated through the plasmodesmal microchannels, and how it is then sorted to site of biological function in an importing cell are still outstanding issues. There are two possibilities: either a macromolecule diffuses between the cytoplasm and plasmodesmata or it follows specific trafficking routes.

Given the highly structured cytoplasm, it is reasonable to assume that a macromolecular complex trafficks between the cytoplasm and plasmodesma following specific patterns. Many studies in various eukaryotic cells have shown that actin filaments and microtubules play essential roles in the trafficking and localization of proteins and mRNAs. For instance, microtubules are involved in the transport of myelin basic protein (MBP) mRNA in mouse oligodendrocytes (Ainger *et al.*, 1993), targeting of the isoform-2 of glucose transporter to the plasma membrane of the cell body in the protozoan *Leishmania enriettii* (Snapp and Landfear, 1997), and transport

of *bicoid* mRNA to the anterior pole of the developing oocyte of *Drosophila* (Wang and Hazelrigg, 1994). In the budding yeast (*Saccharomyces cerevisiae*), actin and myosin are used for the targeting of chitin synthase 3 to cortical sites (Santos and Snyder, 1997) and transport of *ASH1* mRNA to the daughter bud (Long *et al.*, 1997; Takizawa *et al.*, 1997).

The TMV MP interacts with microtubules (Heinlein *et al.*, 1995; McLean *et al.*, 1995; Padgett *et al.*, 1996; Heinlein *et al.*, 1998a) and actin filaments (McLean *et al.*, 1995) in transgenic as well as infected tobacco cells, suggesting that the cytoskeleton may play a role in shuttling macromolecules from the cytoplasm to plasmodesmata for transport (Heinlein *et al.*, 1995; McLean *et al.*, 1995; Gilbertson and Lucas, 1996; Carrington *et al.*, 1996; Ding, 1997; McLean *et al.*, 1997; Heinlein *et al.*, 1998a). Furthermore, the cytoskeleton is also speculated to be used in sorting an imported macromolecule to the site of biological function (Ding, 1998). In addition to free and membrane-bound polysomes, actin-bound polysomes have also been demonstrated in plants (Okita *et al.*, 1998). More recently, the actin cytoskeleton is implicated in the localization of prolamine mRNA to the ER in rice endosperm (Muench *et al.*, 1998). Perhaps some proteins destined for intercellular trafficking can be synthesized on the cytoskeleton and then targeted to plasmodesmata directly along the cytoskeleton.

Plasmodesmata may contain actin (White *et al.*, 1994; Blackman and Overall, 1998) and myosin (Radford and White, 1998; Blackman and Overall, 1998). The actomyosin system may serve to actively maintain the integrity and opening and closure of plasmodesmal microchannels, using energy supplied by the consumption of ATP and by cross-linking the plasma membrane and appressed ER particles (see Fig. 1). This would be consistent with the observations that plasmodesmal SEL can be increased by the depletion of ATP (Cleland *et al.*, 1994) and by the disruption of actin filaments (Ding *et al.*, 1996). A trafficking macromolecule may interact with the actomyosin system directly or indirectly to induce transient and localized depolymerization of actin filaments, thereby leading to opening of the plasmodesmal microchannels (Ding, 1997). Alternatively, the actomyosin system may function to transport molecules actively through the plasmodesmal microchannels, as suggested by McLean *et al.* (1995) and Overall and Blackman (1996).

Future work should focus on experimental tests of the cytoskeleton function in the plasmodesmal trafficking of macromolecules. Work is also needed to determine precisely how the cytoskeleton interacts with plasmodesmata physically.

3. The Role of Other Cellular Factors

A full understanding of the regulation of macromolecular trafficking awaits isolation and functional characterization of plasmodesmal components and

cytosolic factors that are involved. In addition to actin and myosin, several other proteins, the functions of which are completely unknown, appear to be plasmodesmal components (Epel *et al.,* 1996; Waigmann *et al.,* 1997).

Of particular interest is that tobacco mesophyll cell walls contain a putative protein kinase that can phosphorylate the TMV MP. The activity of this kinase is absent from young leaves and is present in more mature leaves (Citovsky *et al.,* 1993). There is a possibility that this kinase is a component of complex secondary plasmodesmata that predominantly connect the cells in mature leaf tissues (Citovsky *et al.,* 1993; for a definition of complex secondary plasmodesmata, see Section VI,B,2). Citovsky *et al.* (1993) speculated that phosphorylation of TMV MP may lead to its inactivation and subsequent accumulation in complex secondary plasmodesmata. Based on the observation that cell-to-cell trafficking of the CMV 3a MP:GFP fusion protein is mediated by complex secondary plasmodesmata formed during tobacco leaf development, Itaya *et al.* (1998) suggested that phosphorylation of a MP such as CMV 3a MP, and perhaps even TMV MP, may be required for intercellular trafficking, contrary to the speculation of Citovsky *et al.* (1993). Further experimental studies in this direction should provide important insight into the mechanisms that regulate intercellular protein trafficking through plasmodesmata.

IV. Intercellular Macromolecular Trafficking in Animals: Parallel Mechanisms and Functions with Trafficking in Plants

Although intercellular trafficking of macromolecules has so far been most extensively characterized in plants, this function does not seem to be unique to plants. Many observations suggest that this is also likely a general function of animal cells. For the sake of discussion, the authors classify operationally the pathways for intercellular macromolecular trafficking between animal cells into two broad categories: the cytoplasmic pathway through the intercellular bridges and the transmembrane pathway across cell membranes.

A. Cytoplasmic Transport through Intercellular Bridges

Various intercellular bridges, formed due to incomplete cytokinesis, exist between animal cells to permit direct cytoplasmic transport between cells (Robinson and Cooley, 1996). The best studied is the ovarian ring canals in *Drosophila,* which function to selectively transport the cytoplasm from

the nurse cells to the developing embryo, via cytoskeleton-dependent mechanisms (Mahajan-Miklos and Cooley, 1994; Robinson and Cooley, 1996).

B. Transmembrane Intercellular Trafficking of Transcription Factors

Some transcription factors important for animal development may traffic from cell to cell, by crossing cell membranes, to convey positional information and regulate gene expression. The homeodomain of transcription factor Antennapedia of *Drosophila,* when supplied in a nerve cell culture, can be internalized and transported into the nucleus to induce morphological differentiation of the nerve cells (Joliot *et al.,* 1991). The internalization is energy independent and does not follow an endocytotic pathway (Perez *et al.,* 1992; Derossi *et al.,* 1994). This homeodomain is able to mediate internalization and nuclear import of fusion peptides of up to 100 amino acid long (Perez *et al.,* 1992). There is no signal peptide to direct such internalization, but the third helix of the homeodomain is important for both internalization and nuclear import (Derossi *et al.,* 1994; Le Roux *et al.,* 1993). Chatelin *et al.* (1996) extended these observations by showing that the entire length of the transcription factor Hoxa-5 is internalized and transported into the nucleus by cultured cells. These data led to the hypothesis that some transcription factors may be able to translocate between cells to confer positional information and allow cell-to-cell interactions (Prochiantz and Théodore, 1995). If intercellular protein trafficking does occur, a protein also needs to be secreted by a cell. Joliot *et al.* (1997) showed that the transcription factors Engrailed-1 and Engrailed-2 are associated with caveolae-like vesicles that may enable them to gain access to a secretion pathway in transfected COS-7 cells.

If confirmed experimentally, cell-to-cell trafficking of transcription factors could explain the observed noncell autonomous function of Antennapedia in *Drosophila* (Struhl, 1981). It could also explain how a gradient of the *hairy* gene product can be established in the short-germ embryo of flour beetle *Tribolium castaneum* to initiate the metameric segmentation process (Sommer and Tautz, 1993; Tautz and Sommer, 1995).

C. Transmembrane Intercellular Trafficking of a Viral Protein

Direct evidence for intercellular protein trafficking between animal cells came from the study of Elliott and O'Hare (1997) showing that VP22, a structural protein of herpes simplex virus type 1 (HSV-1), has the ability to

traffic between COS-1 cells. This intercellular trafficking was demonstrated when the DNA encoding the protein was delivered into the cells by either microinjection or transfection or when the protein was produced during viral infection. VP22 is exported from a cell via a Golgi-independent pathway called nonclassical secretion. Remarkably, the protein entry into a cell does not involve endocytosis, similar to the internalization of transcription factors as just described. Integrity of actin filaments, but not microtubules, is required for VP22 trafficking. VP22 is also able to mediate intercellular trafficking of fused proteins (Elliott and O'Hare, 1997; Phelan *et al.,* 1998). This finding echos the observation that vaccinia virus utilizes actin filaments to spread from cell to cell (Cudmore *et al.,* 1995; Wolffe *et al.,* 1998).

D. Intercellular Trafficking of dsRNAs in *Caenorhabditis elegans*

Fire *et al.* (1998) demonstrated that microinjected dsRNAs confer highly potent and specific inhibition of gene expression in *C. elegans.* Microinjection of highly purified antisense RNA or sense RNA has very little effect on gene expression. Very significantly, the dsRNA injected into the body cavity can inhibit gene expression in distant tissues and F1 progeny, suggesting strongly that the injected dsRNA trafficks intercellularly to down-regulate target gene expression. Thus, intercellular RNA and protein trafficking could be a biological function shared by many, if not all, multicellular organisms.

E. Parallels between Trafficking in Plants and Animals

The transmembrane intercellular trafficking in animals certainly involves a pathway, which remains to be clearly defined, different from the direct cytoplasmic transport via plasmodesmata in plants. However, some common mechanistic characteristics can be noted. First, plasmodesma-mediated intercellular trafficking of the CMV 3a MP:GFP fusion protein in tobacco epidermis occurs at 4°C (Itaya *et al.,* 1997), similar to trafficking of VP22 (Elliott and O'Hare, 1997), Antennapedia homeodomain (Perez *et al.,* 1992; Derossi *et al.,* 1994), and Hoxa-5 (Chatelin *et al.,* 1996) between animal cells. Second, a fusion protein can traffic from cell to cell through plasmodesmata in plants (Waigmann and Zambryski, 1995; Itaya *et al.,* 1997; Canto *et al.,* 1997) as well as across membranes in animal cells (Perez *et al.,* 1992; Elliott and O'Hare, 1997; Phelan *et al.,* 1998). Third, there are no signal peptides contained in proteins that can traffic via plasmodesmata in plants or across membranes in animals. The signal for trafficking may be a particular

protein sequence or structure (Derossi *et al.*, 1994; Le Roux *et al.*, 1993; Waigmann and Zambryski, 1995; Itaya *et al.*, 1997; Ishiwatari *et al.*, 1998). Fourth, the actin cytoskeleton is required for trafficking of VP22 between animal cells (Elliot and O'Hare, 1997), and perhaps also for trafficking of some proteins in plants (McLean *et al.*, 1995; Heinlein *et al.*, 1998). Finally, an important form of trafficking RNA may be dsRNA or dsRNA-like RNA. Here a circular covalently closed viroid can be visualized functionally as a "dsRNA," which trafficks from cell to cell through plasmodesmata (Ding *et al.*, 1997). In *C. elegans,* it is likely that the dsRNA injected trafficks from cell to cell (Fire *et al.*, 1998).

V. Plasmodesmata and Plant Developmental Domains

Although plasmodesmata interconnect plant cells to form a communication network, this network is never static and simple. Rather, it undergoes constant reorganization throughout the course of plant growth and development. This reorganization involves quantitative and qualitative changes in plasmodesmal numbers, structure, and transport functions. Plasmodesmal frequency has commonly been used to quantify plasmodesmata at various cellular interfaces, and such data are used to speculate possible changes in the capacity of intercellular transport (Robards and Lucas, 1990; Van Bel and Oparka, 1995). Qualitative changes in plasmodesmal transport refer to the nature of molecules that are transported through plasmodesmata between specific cells and at specific developmental stages.

All cells in an embryo are interconnected by plasmodesmata, as shown in *Arabidopsis thaliana* (Mansfield and Briarty, 1991). More importantly, dye-coupling experiments, with the same plant, indicate that all of the cells in an embryo are completely coupled symplasmically (McLean *et al.*, 1997). During the course of postembryonic growth and development of a plant, groups of cells become more or less isolated symplasmically from other cells. This divides a whole plant into the so-called symplasmic domains, as shown in *Ergeria densa* (Erwee and Goodwin, 1985). Establishment of symplasmic domains is generally considered to be essential for groups of cells to pursue specific developmental pathways (Robards and Lucas, 1990; Lucas *et al.*, 1993; McLean *et al.*, 1997). This section discusses selected examples of symplasmic domains to highlight the potential significance of domain formation in plant morphogenesis. Other discussions on various aspects of symplasmic domains in plant development and function can be found in Lucas *et al.* (1993), Fisher and Oparka (1996), McLean *et al.* (1997), Kragler *et al.* (1998), and Nelson and Van Bel (1998). Section VI addresses another type of plasmodesmal network reorganization: the ability

of a plant to generate secondary plasmodesmata or modify existing plasmodesmata to modulate cell-to-cell communication.

A. Symplasmic Domains in Embryogenesis

Schulz and Jensen (1968) showed that, in *Capsella pastoris,* the egg cell is connected to the synergids and central cell via plasmodesmata. Such connections remain after fertilization. Before zygote division, however, all plasmodesmata connecting the zygote to the surrounding cells are eliminated. As a result, the zygote forms a symplasmic domain. The functional significance of this requires further characterization. Presumably, symplasmic isolation of the zygote prevents information flow between the zygote and surrounding cells and triggers programmed cell division leading to embryogenesis.

B. Symplasmic Dynamics and Morphogenesis in Apical Meristems

1. Symplasmic Domains and Morphogenesis in the Shoot Apical Meristem of Higher Plants

As discussed in Section III,A,1, all postembryonic organs of a plant are the products of apical meristematic activities. In addition to clonal cell layers defined by lineages, a SAM can also be divided into histological zones with distinct cytological and mitotic features and specific contributions to tissue and organ formation (Fig. 7) (Steeves and Sussex, 1989; Medford, 1992). Cells in the central zone are mitotically inactive and serve as the source of other meristematic cells. Lateral organs initiate from the peripheral zone. The rib zone produces inner tissues of the stem. Because of the complex organization of the SAM, cellular interactions during morphogenesis include those between clonal layers of cells and those between histological zones of cells.

Rinne and Van der Schoot (1998) investigated the potential roles of the plasmodesmal network in establishing developmental domains in the SAM of birch seedlings. They found that the SAM is divided into two concentric symplasmic domains (or morphogenetic fields in terms of animal development), based on dye coupling with LYCH. The central zone forms one domain, and the peripheral zone forms the other. LYCH does not diffuse across the domains. The plasmodesmata nevertheless interconnect all the cells. Hence, differences in the transport capacities of plasmodesmata between defined cellular boundaries may have contributed to the establish-

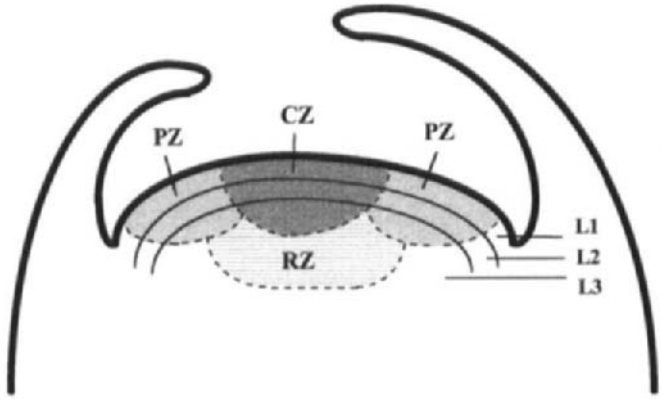

FIG. 7 Histological zonation of a shoot apical meristem and its relationship with clonal cell layers (L1–L3). The central zone (CZ) is the source of all apical meristematic cells. The peripheral zone (PZ) gives rise to lateral organs. The rib zone (RZ) produces internal tissues of the stem. Note that CZ and PZ include cells from the three clonal layers. Morphogenesis requires cell-to-cell interactions between and within the clonal layers and histological zones.

ment of symplasmic domains that would follow different developmental paths. What remains to be addressed further is how far these domains penetrate into the SAM vertically. In other words, do the central domain and/or peripheral domain cover all three clonal layers of cells? When the birch was treated with short photoperiods to induce dormancy, all cells in the SAM became symplasmically isolated. Structural and immunolabeling studies indicated that this appears to result from formation of sphincters, one component of which is callose, around the orifices of plasmodesmata (Rinne and Van der Schoot, 1998). The latter observation supports an earlier study showing constriction and blockage of plasmodesmata during dormancy induction in the poplar apical buds (Jian *et al.,* 1997).

2. Reorganization of the Symplasmic Network during Flower Development

A dramatic developmental event in a plant is the transition from vegetative to reproductive growth. Again, organization of cells to form specific developmental domains must be a critical component of this developmental transition, so that groups of cells will develop into specific flower organs such as sepals, petals, stamens, and carpels.

Structural approaches have been taken to investigate changes in the plasmodesmal network during the transition in the SAMs of several species. Milyaeva and Nikiforova (1996) found that, in a long-day plant (LDP) *Rudbeckia bicolor* and short-day plant (SDP) *Perilla nankinensis,* plasmo-

desmal frequencies increase by approximately 10% between L1 cells, 300% between L2 cells, between L2 and L3 cells, and between L3 cells. However, the frequency decreases by approximately 30% between L1 and L2 cells. During transition in *Iris xiphium,* plasmodesmal frequency decreases by 25% between L1 and L2 cells, similar to that in *R. bicolor* and *P. nankinensis* (Bergmans *et al.,* 1997). However, in contrast to those in the latter two species, plasmodesmal frequencies in *I. xiphium* decrease by 25% between L2 cells and 40% between L2 and L3 cells. They remain unchanged between L1 cells and between L3 cells (Bergmans *et al.,* 1997). Some of the differences between the two studies could be due to different species studied or to different criteria used for plasmodesmal counting. Nevertheless, both studies demonstrate changes in plasmodesma distribution, especially a decrease in plasmodesmal connections between L1 and L2 cells during flower initiation in all three species.

Dye-coupling experiments demonstrated directly formation of symplasmic domains during flower development. In the shoot apex of *Silene coelirosa,* plasmodesmal SEL decreases on flower induction by long-day treatment. Whereas a fluorescent probe of about 750 Da can diffuse freely between cells in both uninduced and induced apices, a probe of 850 Da can diffuse between cells in the uninduced apices but not in induced apices (Goodwin and Lyndon, 1983). This reduction in symplasmic transport is associated with the high mitotic index of the apical cells, a major cellular process associated with evocation (Santiago and Goodwin, 1988). In Dutch *Iris,* all apical cells are connected symplasmically, as shown by LYCH coupling, in the inflorescence meristem. During further development of the meristem toward flower initiation, apical cells form several symplasmic domains (Bergmans *et al.,* 1993).

Thus, combined structural and dye-coupling studies have provided evidence for the reorganization of the plasmodesmal network during the vegetative-to-reproductive growth transition in an SAM. This reorganization, in particular the establishment of symplasmic domains, is likely important for cell differentiation and development to form flower parts. Future challenges are to develop experimental protocols to map such domains more precisely and relate the formation of domains to specific gene expression patterns. Some outstanding issues include the following. First, because formation of an organ involves the participation of cells from the three clonal layers, analysis of plasmodesmal structure and numbers should focus on determining if groups of cells from L1–L3 layers form a symplasmic subdomain at specific stages of transition. For instance, it will be interesting to investigate plasmodesmata at boundaries that would demarcate future sepals, petals, stamens, and carpels. This could be more meaningful than a generalized comparison of plasmodesmata within and between layers. Second, although dye-coupling studies should continue to provide vital

information on the reorganization of the symplasmic network during the transition, future studies should include analysis of the macromolecular trafficking capabilities of plasmodesmata within and between putative domains. This is particularly important considering the finding that transcription factors key to flower formation, such as GLO, FLO, and DEF, are able to traffic intercellularly. Third, although structural analysis shows dynamics of plasmodesmal distribution, what are the functional implications of these data? Do quantitative changes in plasmodesmal numbers such as a two- to threefold increase or decrease reflect only changes in the amount of information or nutrient flow between cells, or do such changes reflect qualitative changes in the transport functions or both? One possibility is that these changes reduce or enhance the transport of morphogens so that concentration gradients of the morphogens are established across domains to trigger differential gene expression.

3. Plasmodesmal Transport Regulates Developmental Patterns of Fern Gametophyte

The complex nature of higher plant meristems makes it a formidable task to tackle the problem of symplasmic transport and morphogenesis. Tilney *et al.* (1990) approached the problem by taking advantage of the simple organization and developmental pattern of the gametophyte of sensitive fern (*Onoclea sensibilis*). Here, all the gametophytic cells are ultimately derived from the activity of one apical, meristematic cell formed at spore germination. The gametophyte grows as a linear file of cells, called protonema, in the dark. The protonema switches to grow two-dimensionally when exposed to light, forming a one cell-thick and heart-shaped prothallus, making it feasible to map the plasmodesmal distribution precisely at each cell division. Tilney *et al.* (1990) determined that the number of plasmodesmata produced with each division of the apical cell increases as the prothallus grows, reaching a maximum of 50-fold, and that the number of plasmodesmata between neighboring cells is determined precisely during the gametophyte development. The older a cell, the less the plasmodesmal connection with surrounding cells. Consequently, there is a gradient of plasmodesmal number and frequency, from high to low, established from the apical to the basal parts of the gametophyte.

The importance of plasmodesmata in fern morphogenesis is demonstrated in some earlier experimental studies. Nagai (1914) and Nakazawa (1963) reported that temporary plasmolysis, which breaks plasmodesmal connections, can inhibit normal prothallial development and trigger a symplasmically isolated cell to differentiate into a new prothallus in several ferns. Presumably, the breakage of plasmodesmal connections prevents intercellular transport of substances that would normally inhibit the differ-

entiation of individual prothallial cells. This inhibitor probably comes from the apical cell, as suggested by cell ablation experiments. Using a glass needle to ablate cells in the prothallus, Ito (1962) was able to demonstrate that a turgid cell surrounded by ablated cells could develop into a new prothallus in *Pteris vittata.* Furthermore, the farther a cell is away from the apical cell, the shorter the time it takes for a cell to regenerate into a prothallus, implying that the apical cell has an influence on the ability of a cell to regenerate. This is further supported by Albaum's (1938a, b) experiments showing that removal of the apical half of a prothallus permits cells in the basal half to differentiate into new prothalli in two *Pteris* species. Data are consistent with the aforementioned gradient of plasmodesmal number and density between apical and basal cells in a developing gametophyte (Tilney *et al.,* 1990). The putative role of the apical cell in controlling fern gametophyte development could perhaps be tested more directly with laser ablation of the apical cell itself. The nature of the inhibitor remains to be identified. It could be a growth hormone such as an auxin as implied from Albaum's (1938b) experiments showing the polar transport of auxin and inhibition of *de novo* prothallial regeneration by auxin. It could also be a macromolecule that functions alone or together with a hormone.

Taken together, these data suggest that the plasmodesmal gradient from the apical cell to the rest of the gametophyte may establish a morphogen gradient by differentially transporting this morphogen. This morphogen gradient governs orderly development of the fern gametophyte.

4. Symplasmic Isolation of the Apical Cell of *Azolla* Root May Contribute to Determinate Growth

In the root of water fern *Azolla pinnata,* all cells are derived from an apical, meristematic cell (Gunning *et al.,* 1978). The root growth in *Azolla* is determinate, with the apical cell dividing approximately 55 times (Gunning *et al.,* 1978). In a comprehensive analysis of the plasmodesmal distribution between various cells during root growth, Gunning (1978) showed that the apical cell gradually loses the ability to produce sufficient plasmodesmata with each cell division, starting from the 35th division. Eventually the apical cell becomes rarely connected to neighboring cells. This is correlated with a gradual decrease in electrical coupling between the apical cell and its derivatives during development (Overall and Gunning, 1982). Gunning (1978) suggested that the symplasmic isolation of the apical cell may have contributed to the determinate growth of *Azolla.* Interestingly, this is a situation opposite to that observed in the gametophyte of *Onoclea* just described, where the apical cell produces more plasmodesmata with each successive division.

C. Symplasmic Isolation of Epidermal Cells during Development in *Arabidopsis*

In an *Arabidopsis* root, epidermal cells arise from a ring of meristematic cells in the root tip (Dolan *et al.*, 1993; Scheres *et al.*, 1994). The farther away from the root tip, the more mature an epidermal cell is developmentally. After the meristem region there is an elongation zone where all epidermal cells remain the same type. Then specific epidermal cells differentiate into root hair cells in the mature region. Ducket *et al.* (1994) found that all epidermal cells in the meristematic and elongation zones are connected symplasmically as revealed by dye coupling. In the mature region, epidermal cells become isolated symplasmically as shown by the retention of fluorescent dyes in individual cells. Electron microscopic examination shows plasmodesmata connecting epidermal cells in the mature as well as the younger regions of the root. Data indicate that the symplasmic isolation of cells is important for cell differentiation. In this particular case, such isolation may allow some cells to develop into root hairs whereas some cells remain regular epidermal. This, however, may be achieved only after the exchange of positional information between cells.

D. Plasmodesmata and Programmed Cell Death in Xylem Differentiation

Plasmodesmata may also play a role in the coordination of differentiation of xylem, one of the components of the vascular tissue that transports water and minerals throughout the whole plant. The most dramatic developmental consequence of the xylem is that some xylem cells lose their cytoplasm completely to become dead cells to perform transport functions. These are tracheids found in gymnosperms and some primitive angiosperms and vessels in advanced angiosperms. The formation of tracheids and vessels can be considered an example of programmed cell death (PCD). Living parenchyma cells are always associated with tracheids or vessels. Lachaud and Maurousset (1996) showed that plasmodesmata exist in the primary membranes (or primary cell walls) of the bordered pits connecting vessels to tracheids and those connecting the vessels to the xylem parenchyma cells until a very late stage of xylem development. Those authors suggest that the xylem can be considered to be a symplasmic domain, and plasmodesmata may function to coordinate xylem differentiation and, at a later stage, to release hydrolytic enzymes to cells destined to become tracheids or vessels. This hypothesis can perhaps be extended to a general discussion of plasmodesmata in PCD. Questions that can be asked include: (1) do plasmodesmata transmit signals to trigger and coordinate PCD in neigh-

boring cells and (2) do plasmodesmata change structures and functions between cells destined for PCD and other cells?

E. Plasmodesmata and Developmental Domains in *Chara*

Some of the most insightful information on the functions of plasmodesmata in plant development was obtained by studying dynamic changes in plasmodesmal structures in relation to antheridial development and spermatogenesis in green alga *Chara vulgaris*. The plant hormone gibberellin is implicated in regulating antheridial development (Kwiatkowska and Godlewski, 1988). This hormone is most likely transported intercellularly through plasmodesmata (Kwiatkowska, 1991). Disruption of plasmodesmata developmentally between the antheridium and the supporting thallus cells leads to symplasmic isolation of the antheridium and initiation of spermatozoid. Premature symplasmic isolation of the antheridium by plasmolysis leads to a reduction in cell cycles necessary for spermatogenesis and in the length of the antheridium (Kwiatkowska, 1988).

Electron microscopic studies revealed that plasmodesmata are generally open channels between cells with synchronized cell division and are plugged by an osmiophilic substance between synchronized and asynchronized cells or between different cell types (Kwiatkowska and Maszewski, 1976, 1985, 1986). The plugging can be reversed. Obviously, plugging of plasmodesmata results in the formation of symplasmic domains that integrate cells at the same phases of the cell cycle or cells of the same type. Dye-coupling studies by Shepherd and Goodwin (1992) showed that the structural blockage of plasmodesmata is correlated with the restricted intercellular diffusion of fluorescent probes. These data, collectively, suggest strongly that changes in plasmodesmal structure and transport function serve to regulate cell-to-cell communication critical to *Chara* morphogenesis.

F. Function of the Symplasmic Domains: From Descriptive to Experimental Investigation

The examples just discussed indicate that establishment of symplasmic domains is a fundamental plant function. This presumably allows groups of cells to achieve a certain degree of autonomy and pursue specific developmental pathways. Thus, symplasmic domains may be considered as basic developmental units in a plant. Different domains must interact with one another to ensure the coordination of development at the organismal level. Based on this concept, it can be envisaged that the modes of cell-to-cell

communication within a domain and across domains may exhibit common as well as unique features.

To test these concepts and substantiate our knowledge of the functions of symplasmic domains, experimental studies are needed to answer the following questions: (1) What molecular mechanisms control the establishment of the symplasmic domains during plant development? (2) How does gene expression pattern change during the establishment of symplasmic domains? (3) What types of molecules can be transported between cells within a domain but not across domains? (4) How do cells in different domains communicate with one another?

Many fluorescent probes that are used in microinjection experiments to establish symplasmic domains have no biological functions. Thus, when differences in the SEL of plasmodesmata are detected between certain cell types, it is unclear how much these differences are related to the cell-to-cell transport of physiological molecules in terms of the nature of the molecules being transported and the efficiency of transport. It would appear that further advances in understanding the functions of symplasmic domains should come from studies on the identification and characterization of transport mechanisms of physiological molecules, including proteins and nucleic acids.

VI. Secondary Plasmodesmata and Modification of Existing Plasmodesmata

As just discussed, down-regulation of plasmodesmal number, structure, and transport capacity at specific cellular boundaries establishes plant developmental domains. This section discusses the formation of secondary plasmodesmata as another major means of reorganizing the plasmodesmal network to establish new or to enhance existing intercellular communication. Secondary plasmodesmata are defined as those that are formed postcytokinetically across established cell walls (Jones, 1976; Lucas *et al.*, 1993; Ding and Lucas, 1996). A third mode of reorganization of plasmodesmal network is also discussed: modification of existing plasmodesmata to enhance transport capacity or to achieve new macromolecular trafficking functions. Presumably, the formation of secondary plasmodesmata and the modification of existing plasmodesmata are powerful means used by a plant to modulate its cell-to-cell communication network so as to shape its developmental patterns. This will not only enable a plant to unfold correctly its prescribed developmental programs, but also enable a plant to reprogram its developmental patterns to better cope with various biotic and abiotic conditions.

A. *De Novo* Formation of Secondary Plasmodesmata

1. Formation of Secondary Plasmodesmata during Cell Fusion

When two cells fuse, secondary plasmodesmata can be produced across the fusion walls. This has been demonstrated in regenerated protoplasts of *Solanum nigrum* (Monzer, 1990, 1991; Ehlers and Kollmann, 1996) and in interspecific cell fusion (Wu *et al.*, 1995). Monzer (1990, 1991) showed that during regeneration of cell walls and before cell-to-cell contact is established, branched half plasmodesmata are formed in individual cells by entrapment of the ER membrane cisternae between fusing Golgi vesicles along the cell surface. When two cells are associated secondarily with each other, continuous secondary plasmodesmata are formed between the two cells by coalescencing of the half plasmodesmata. This membrane/vesicle fusion mechanism is essentially the same as used for primary plasmodesma formation.

Secondary plasmodesmata are also formed during the establishment of graft unions, which involves the fusion of cells from the stock and scion (Jeffree and Yeoman, 1983; Kollmann and Glockmann, 1985, 1991; Kollmann *et al.*, 1985). Based on their meticulous electron microscopic studies, Kollmann and Glockmann (1991) were able to reconstruct the major cellular events that lead to the formation of secondary plasmodesmata in this system (Fig. 8). When two cells from the scion and stock approach each other, some ER cisternae become anchored, by electron-dense structures of approximately 4–6 nm in diameter, to certain areas of the plasma membrane. The cell walls undergo thinning. Finally, the two walls meet each other and the ER cisternae from the two cells merge to form a continuous endomembrane system. Subsequent rebuilding of the cell walls is accompanied by entrapment of the ER cisternae, leading to the formation of branched secondary plasmodesmata. Mechanistically, this process mimics that in the regenerating protoplasts (Monzer, 1990, 1991).

The nature of the electron-dense structures that link the plasma membrane and ER membrane remains to be determined. Whether these structures are the same as or similar to those detected between the plasma membrane and the appressed ER membrane in tobacco mesophyll plasmodesmata (Ding *et al.*, 1992b) is worth further investigation. They may function to bridge the two membrane systems as the initial cellular event for plasmodesmal formation and later for maintaining the spacing between the plasma membrane and ER to create plasmodesmal microchannels.

2. Formation of Secondary Plasmodesmata during Plant Development

In the SAM, cells in the L1 layer usually divide anticlinally to propagate themselves and to produce the epidermis (Sussex, 1989). There are no

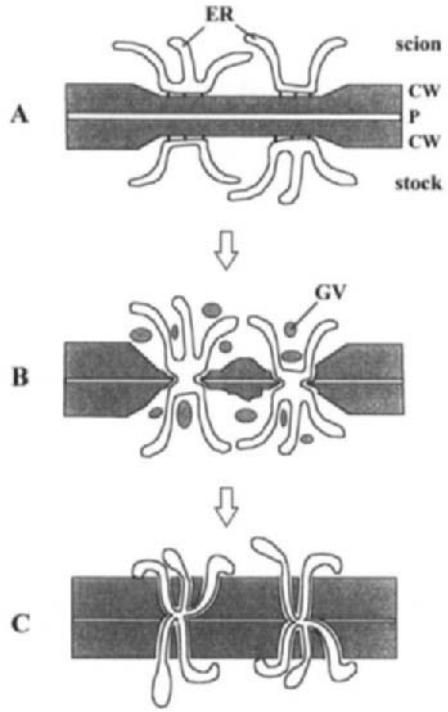

FIG. 8 Mechanisms of formation of secondary plasmodesmata at a graft interface, based on Kollmann and Glockmann (1991). (A) Cells from the scion and stock approach each other. Some endoplasmic reticulum (ER) strands are anchored to the plasma membrane via electron-dense structures. Cell walls in this area undergo thinning. (B) Thinning of the cell walls eventually leads to fusion of the ER from the two cells. The walls are subsequently rebuilt via Golgi vesicles (GV). (C) Highly branched secondary plasmodesmata are formed in the fully rebuilt cell walls between the scion and stock. CW, cell wall; P, pectic material.

division walls between L1 and L2 layers. Plasmodesmata are nevertheless present in the walls connecting L1 and L2 cells (Steinberg and Kollmann, 1994; Ding and Lucas, 1996). These plasmodesmata must therefore be formed secondarily. The underlying mechanisms remain to be characterized. There is currently no evidence for either membrane–vesicle fusion as observed in the regenerating protoplast culture (Monzer, 1990, 1991; Ehlers and Kollmann, 1996) and graft unions (Kollmann and Glockmann, 1991) or for direct enzymatic digestion of existing cell walls to facilitate the insertion of new cytoplasmic strands as proposed by Jones (1976) and Ding and Lucas (1996). In studying the intercellular trafficking patterns of DEF and GLO in the floral meristem of *Antirrhinum,* it should be noted that Perbal *et al.* (1996) detected directional trafficking of these proteins

between cell layers, but rarely between cells in the same layer. It is speculated that these proteins can traffic through the secondary plasmodesmata formed between the layers, but not through primary plasmodesmata between cells in the same layer (Perbal *et al.,* 1996).

During flower development in Madagascar periwinkle (*Catharanthus roseus* L.), carpel primordia grow and make contact with each other. Diffusible factors are transmitted between the contacting cells, resulting in rapid dedifferentiation of the carpel epidermal cells (Siegel and Verbeke, 1989). The latter eventually fuse so that the two carpels form the gynoecium (Verbeke and Walker, 1985). Van der Schoot *et al.* (1995) demonstrated that functional secondary plasmodesmata are formed soon after carpel fusion, presumably establishing intercellular communication pathways to facilitate the coordination of subsequent developmental processes between the two carpels.

The importance of secondary plasmodesmal formation to the growth of a plant is also illustrated elegantly in the case of lateral root development in *Arabidopsis* (Oparka *et al.,* 1995). Lateral roots are derived from mitotic activities of the pericycle cells surrounding the vascular cylinder. The phloem of the primary root functions as an isolated domain before lateral root initiation. The differentiation of phloem connector elements in the dividing pericycle, however, leads to the rapid establishment of cell-to-cell communication, as demonstrated by dye coupling, between the phloem of the primary root and the lateral root primordia. This communication is most likely via plasmodesmata that are formed secondarily between the phloem and the newly differentiated connector elements (Oparka *et al.,* 1995). Interestingly, the newly formed phloem in the lateral root also becomes progressively isolated from the surrounding cells.

In studying the role of positional signaling in cell differentiation in the *Arabidopsis* root, Van den Berg (1995) used a laser beam to ablate specific meristematic cells that are known to give rise to specific files of cells along the longitudinal axis of the root. When a cell is ablated, it can be replaced by a new cell derived from the division of a neighboring cell of a different lineage. Although clonally different, this cell will switch its developmental fate in the new position. This is most likely directed by a positional signal received from more mature cells in the same longitudinal file (Van den Berg *et al.,* 1995). The nature of the signaling molecule(s) remains to be identified. This signal could be transmitted apoplastically via cell walls, as shown in *Fucus* embryo development (Bouget *et al.,* 1998), or symplasmically via plasmodesmata. If the latter were the case, it must involve plasmodesmata that are formed secondarily as the new cell grows into its new position.

Secondary plasmodesmal formation is a general phenomenon associated with plant growth and development. Seagull (1983) demonstrated that plas-

modesmal frequencies between root cells remain constant despite cell expansion during root elongation in several monocot and dicot species. This suggests that secondary plasmodesmata must have been formed to compensate for the dilution of primary plasmodesmata during cell expansion. On sections glancing through the radial longitudinal cell walls, the author also observed a transition from dispersed to clustered patterns for plasmodesmal distribution during root elongation. Because longitudinal views of these plasmodesmata were not provided, how these secondary plasmodesmata were generated remains unknown. One possibility is that they are formed completely *de novo* across existing cell walls. Another possibility is that they are formed via the modification of existing primary plasmodesmata by branching, as documented in tobacco mesophyll (Ding *et al.,* 1992a, 1993) and epidermal cells (Itaya *et al.,* 1998) (see later for more discussion). An analysis of plasmodesmal distribution patterns in developing leaflets of the moss *Sphagnum* also suggests the secondary formation of plasmodesmata (Schnepf and Sych, 1983).

3. Regulation of Secondary Formation of Plasmodesmata

How a cell determines when, where, and how many secondary plasmodesmata need to be formed is unknown. Based on data from regenerating protoplasts (Monzer, 1990, 1991; Ehlers and Kollmann, 1996; Ehlers *et al.,* 1996) and graft unions (Kollmann and Glockmann, 1991) showing the formation of half plasmodesmata in individual cells before any cell-to-cell contact, it would appear that forming secondary plasmodesmata is a basic and autonomous function of a cell that does not require cell-to-cell contact or communication in other ways. Cell-to-cell contact is, however, needed to join the half plasmodesmata from two cells to form a continuous, functional cytoplasmic network. In the absence of such a contact, a cell somehow "decides" that there is not a need to keep the half plasmodesmata and eliminates them possibly via a ubiquitin-mediated process (Ehlers *et al.,* 1996).

B. Modifications of Existing Plasmodesmata

A plasmodesma, whether formed primarily at cytokinesis or secondarily across cell walls, can be further modified during cell growth. The mechanisms of modification appear to be diverse. Studies have started to cast new light on the potential significance of the modifications.

1. Branched Primary Plasmodesmata

In regenerating protoplasts, primary plasmodesmata formed during cytokinesis may become branched during the further growth of cells (Ehlers and

Kollmann, 1996). Specifically, cell walls adjoining two cells thicken by the fusion of Golgi vesicles. During this process, cytoplasmic ER strands connected to the appressed ER of a plasmodesma are entrapped. Further cell wall development and ER modification produce new branches associated with the primary plasmodesma (Fig. 9). The formation of these new cytoplasmic branches is very similar to the formation of a primary plasmodesma. Based on this consideration, such a branched plasmodesma can be considered a "branched primary plasmodesma" (Ding, 1998). A distinct feature of these plasmodesmata is a lack of central cavities, as in contrast to the branched secondary plasmodesmata formed at the graft interface or between fusion cells.

Branching of primary plasmodesmata may be simply a means to enhance the capacity of cell-to-cell transport of nutrients between two cells. It is also possible that such modifications permit plasmodesmata to acquire new functions, if the branches contain new protein components.

2. Complex Secondary Plasmodesmata with New Macromolecular Trafficking Functions

In tobacco leaf development, primary plasmodesmata can be modified into highly branched ones in the epidermis, mesophyll, and phloem (Ding *et al.,* 1992a, 1993; Itaya *et al.,* 1998). There is no evidence that branching in this case is through cell wall thinning and membrane/vesicle fusion as observed in graft unions (Kollmann and Glockmann, 1991) or through entrapment of ER cisternae between fusion Golgi vesicles as detected in regenerating *S. nigrum* protoplasts (Ehlers and Kollmann, 1996). Evidence from electron microscopic studies suggests that the majority of these primary plasmodesmata are modified by the lateral fusion of neighboring plasmodesmata at the middle lamella region of the cell walls and the subsequent addition of new cytoplasmic strands containing the ER and the plasma membrane across the existing cell walls (Ding *et al.,* 1992a, 1993; Itaya *et al.,* 1998) (Fig. 9). In some cases, new cytoplasmic strands seem to be added to a primary plasmodesma without fusion of neighboring primary plasmodesmata (Ding *et al.,* 1992a, 1993; Itaya *et al.,* 1998). Volk *et al.* (1996) have also demonstrated the formation of branched plasmodesmata via the modification of primary plasmodesmata as a function of leaf development between several cell types in *C. melo* and *C. pepo.* Because these modifications involve the *de novo* addition of cytoplasmic strands containing ER across the existing cell walls, they have been considered as a type of secondary plasmodesmata (Ding *et al.,* 1992a, 1993; Ding and Lucas, 1996). More specifically, they are referred to as "complex secondary plasmodesmata" (Ding, 1998). Some workers (Ehlers and Kollmann, 1996) have raised concerns about the use of "secondary plasmodesmata" to describe these

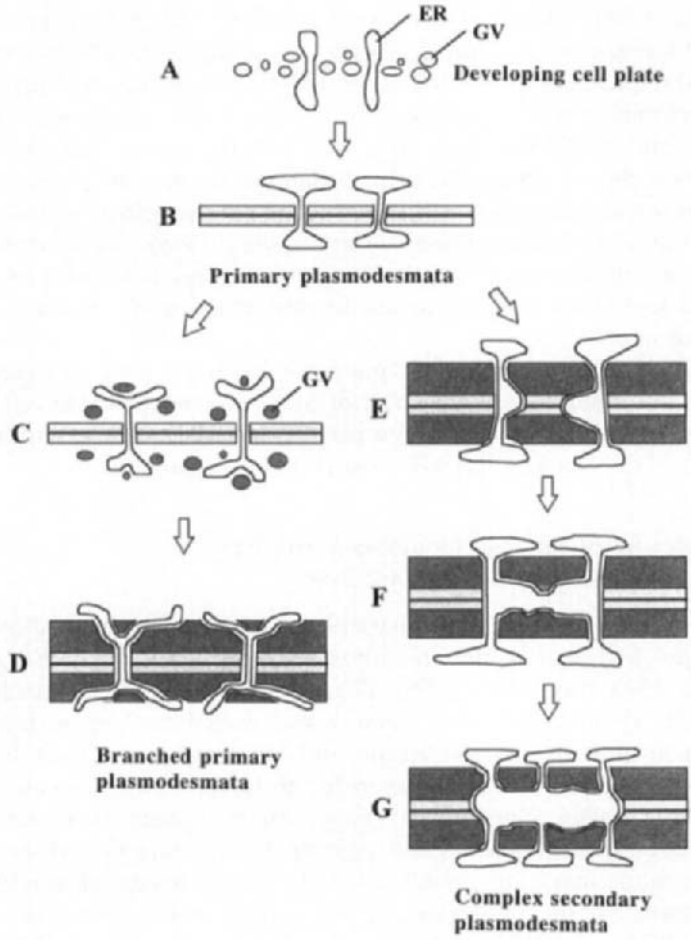

FIG. 9 Formation of primary plasmodesmata and different mechanisms of subsequent modifications. (A) Entrapment of the endoplasmic reticulum (ER) between Golgi vesicles (GV) in a developing cell plate between two daughter cells leads to the formation of primary plasmodesmata (B). (C and D) Entrapment of cytoplasmic ER branches connected to primary plasmodesmata during thickening of the cell walls gives rise to branched primary plasmodesmata, based on Ehlers and Kollmann (1996). This mechanism of generating plasmodesmal branches is essentially the same as for the primary formation of plasmodesmata. (E–G) Neighboring primary plasmodesmata fuse in the middle portion of the cell walls and new cytoplasmic strands containing the ER are inserted *de novo* across the cell walls to form complex secondary plasmodesmata, based on Ding and Lucas (1996).

"branched" plasmodesmata, as they are not formed entirely *de novo* across existing cell walls. Ehlers and Kollmann (1996) suggested that these plasmodesmata are still primary in nature and should be called modified or branched plasmodesmata. However, as just discussed, primary plasmodesmata can branch by different mechanisms, and the term "branched plasmodesmata" can hardly account for the differences. The use of "complex secondary plasmodesmata" to describe a specific class of "branched" plasmodesmata, the developmental origin of which is known, represents an extension of the classical definition of secondary plasmodesmata necessary to describe new findings (Ding, 1998).

Previous studies on the interactions between the TMV MP and plasmodesmata suggest that primary and complex secondary plasmodesmata differ in some functional aspects. In TMV MP-transgenic tobacco plants, the MP is detected by immunolabeling in complex secondary plasmodesmata in mesophyll and bundle sheath of mature leaves (Ding *et al.,* 1992a; Moore *et al.,* 1992), but not in any primary plasmodesmata in young and mature leaves (Ding *et al.*, 1992a). Furthermore, this MP is able to increase the SEL of complex secondary plasmodesmata in the mesophyll and bundle sheath from 1 kDa to greater than 10 kDa in mature leaves, but not that of primary plasmodesmata in young leaves, even though the MP is produced in such leaves (Deom *et al.,* 1990; Ding *et al.,* 1992a).

Itaya *et al.* (1998) provided experimental evidence that primary and complex secondary plasmodesmata in tobacco leaf epidermis have different protein trafficking functions. They showed that the CMV 3a MP:GFP fusion protein is not targeted to primary plasmodesmata in epidermis of young or mature leaves in transgenic tobacco plants constitutively expressing the *3a:GFP* fusion gene. Furthermore, the 3a MP:GFP fusion protein produced *in planta* by biolistic bombardment of the *3a:GFP* fusion gene does not traffic between cells interconnected by primary plasmodesmata in the epidermis of a young leaf. In contrast, the 3a MP:GFP is targeted to complex secondary plasmodesmata and trafficks from cell to cell when a leaf reaches a certain developmental stage.

Clearly, primary and complex secondary plasmodesmata have some different protein trafficking functions. Such differences may be significant not merely for the intercellular trafficking of a viral protein. It is suggested that these plasmodesmata can differentially traffic endogenous macromolecules critical for specific stages of plant development (Itaya *et al.,* 1998). This might explain the correlation between the arrested development of complex secondary plasmodesmata in the mesophyll and the accelerated leaf senescence in a transgenic tobacco plant expressing an acid invertase gene (Ding *et al.,* 1993). Because modification of primary plasmodesmata into complex secondary plasmodesmata appears to be a general phenomenon associated with plant development (Jones, 1976; Ding *et al.,* 1992a, 1993; Ding and

Lucas, 1996; Volk *et al.,* 1996; Seagull, 1983), elucidating the specific functions of such modifications should enhance greatly our understanding of the roles of plasmodesma-mediated intercellular communication in plant development.

VII. Some Evolutionary Aspects of Cell-to-Cell Communication

Studies on the functions of plasmodesmata in plant growth and development can be facilitated if we have knowledge of how plasmodesmata have evolved, how the transport functions of plasmodesmata have evolved, and how evolutionary changes in the patterns of intercellular transport have influenced the evolution of plant morphological patterns. Detailed analysis of literature related to plasmodesmal evolution is available in Lucas *et al.* (1993), Franceschi *et al.* (1994), Raven (1997), and Cook *et al.* (1997). This is still an area that has not received much attention in the past, and literature available is insufficient, and often even contradictory, to allow us to draw a general picture. Therefore, this review attempts to highlight only some key issues important to understanding the evolution of plasmodesma-mediated intercellular communication, with the hope of stimulating further interest in these fundamental biological issues.

A. Evolution of Plasmodesmata

The evolutionary origin of higher plant plasmodesmata remains an outstanding issue. Because green algae Charales are considered to be the most likely ancestors of land plants (Graham and Kaneko, 1991; Graham *et al.,* 1991; Kenrick and Crane, 1997), an examination of the plasmodesmal structure in this group of algae would provide a clue to the evolution of plasmodesmata. Existence of plasmodesmata in Charales is well established. However, the substructural details have not been resolved clearly. A major issue concerning algal plasmodesmal substructure is whether an appressed ER is present. Early studies provided conflicting images, in a large part due to inferior fixation quality of the cell structures (Lucas *et al.,* 1993).

Two groups of workers reinvestigated the issue using improved fixation protocols. Franceschi *et al.* (1994) showed that plasmodesmata of *Chara corallina* are formed postcytokinetically across an established cell plate. Furthermore, they found no evidence for the existence of a central structure such as the appressed ER in the plasmodesmata. Results suggest that the

secondary formation of plasmodesmata is a rather primitive feature and that the appressed ER arose at a later time during evolution.

Cook *et al.* (1997) expanded the structural analysis of plasmodesmata to *Chara zeylanica* and bryophytes *Monoclea gottschei* (liverwort), *Notothylas orbicularis* (hornwort), and *Sphagnum fimbriatum* (moss). Liverworts are believed to be basal to all land plants, with hornworts or mosses as living sister groups to vascular plants (Kenrick and Crane, 1997). Thus, the approach of Cook *et al.* (1997) is of significant value. In contrast to the situation in *C. corallina,* plasmodesmata in *C. zeylanica* appear to be formed at cytokinesis and contain an appressed ER-like structure. This structure, however, is not a consistent feature. Plasmodesmata in the three bryophytes invariably contain the appressed ER. Fine variations, however, exist among the three species. Branched plasmodesmata, perhaps formed via secondary branching as documented in tobacco (Ding *et al.,* 1992a, 1993; Itaya *et al.,* 1998), are observed in *Monoclea,* but not in the other two bryophytes. Plasmodesmata of *Sphagnum* have dense staining pattern in the orifice region, similar to the sphincters observed in a few vascular plants (Olesen, 1979; Badelt *et al.,* 1994). Cook *et al.* (1997) suggest that *Chara* plasmodesmata are less specialized than the bryophyte counterparts and that complex plasmodesmata evolved in the ancestor of land plants before extant lineages of bryophytes diverged.

The work of Cook *et al.* (1997) clearly established that the appressed ER had already existed in the plasmodesmata of (probably) the earliest land plants. The question again is whether it had truly evolved in Charales. The differences between the observations of Franceschi *et al.* (1994) and Cook *et al.* (1997) with regard to the absence or presence of an appressed ER-like structure could be interpreted in a number of ways. First, such differences might be species dependent. If this were true, then it becomes very significant that the appressed ER evolved in some *Chara* species, but not in others. Second, the differences may be due to the different fixation protocols used. In general, the high water content of these cells makes it very difficult to preserve the cell ultrastructure by any fixation procedures. Thus, either the appressed ER is lost during fixation and subsequent tissue processing (Franceschi *et al.,* 1994) or the observed appressed ER-like structure represents the entrapment of ER or even some membrane fragments during fixation (Cook *et al.,* 1997). This might explain why such a structure is observed in only some, but not all, plasmodesmata in a given cell wall section. Third, perhaps the appressed ER had evolved in *Chara,* but has not become a stable and consistent structure. Therefore, it will appear in some plasmodesmata but not in others and is also sensitive to fixation procedures. The labile nature of the appressed ER has been discussed in Section II,A. Finally, whether the presence of the appressed ER in plasmodesmata is developmental or cell specific in *Chara* is not

understood, although there are indications of plasmodesmal modifications during development (Kwiatkowska and Maszewski, 1986).

To provide further insight into the evolutionary origin of plasmodesmata, fixation protocols that can preserve algal cell structure well need to be developed to reinvestigate plasmodesmata in green algal species that have been studied and in those that have not been studied. For instance, plasmodesmata in *Coleochaetales,* another group of green algae considered to be directly related to land plants (Kenrick and Crane, 1997), deserve a detailed analysis.

It is interesting to note that while *Chara* species as well as higher plants have mechanisms to produce secondary plasmodesmata (Franceschi *et al.,* 1994) or to modify existing plasmodesmata secondarily by branching (Franceschi *et al.,* 1994; Kwiatkowska and Maszewski, 1986; Ding *et al.,* 1992a, 1993; Itaya *et al.,* 1998), ferns do not appear to have evolved such mechanisms (Gunning, 1978; Tilney *et al.,* 1990). Ultrastructurally, however, fern plasmodesmata (Overall *et al.,* 1982; Tilney *et al.,* 1990) are much more similar to higher plant plasmodesmata by indisputably having the appressed ER (Ding *et al.,* 1992b; Botha *et al.,* 1993). So have ferns lost the mechanism to form secondary plasmodesmata or have ferns evolved plasmodesmata independently? Equally puzzling is the finding that branching of plasmodesmata, as observed frequently in higher plants and *Chara* species, is a feature of plasmodesmata in liverworts, but not of plasmodesmata in the more advanced bryophytes hornworts and mosses (Cook *et al.,* 1997). Finally, a few fungal species have plasmodesmata that are ultrastructurally similar to higher plant plasmodesmata (Lucas *et al.,* 1993). Resolving these puzzles is not only of value to understanding the evolution pattern of plasmodesmata, but should also be of value to understanding the detailed aspects of the evolution of plants.

In addition to structural analysis, comparative studies of the biochemical compositions of plasmodesmata in green algae, bryophytes, and higher plants are indispensable. A significant step has been taken by Blackman and Overall (1998) to characterize the biochemical compositions of *Chara* plasmodesmata. They have shown, by immunolabeling, the presence of actin and myosin in *Chara* plasmodesmata. This mirrors the detection by immunolabeling of actin (White *et al.,* 1994) and myosin (Radford and White, 1998) in fern and higher plant plasmodesmata, respectively. If actin and myosin are confirmed by further studies to be bona fide components of *Chara,* fern, and higher plant plasmodesmata, how they function in plasmodesmal transport requires careful studies. Ding *et al.* (1996) showed that treatment of tobacco mesophyll cells injected with cytochalasin D and profilin results in an increase in the plasmodesmal SEL to at least 20 kDa, whereas Ding and Tazawa (1989) demonstrated that treatment of *Chara* cells with cytochalasin E has no effect on intercellular transport. Isolation

of protein compositions and the corresponding genes of plasmodesmata from green algae, bryophytes, ferns, and higher plants should complement structural approaches in painting an evolutionary picture of plasmodesmata.

Comparative functional studies on plasmodesmata in various groups of plants are also required to complement the structural and biochemical approaches. These include characterization of molecules that can be transported through plasmodesmata, when, during evolution, plasmodesmata became competent for such transport and the mechanisms of transport. In this regard, it is significant to note that TMV MP interacts with and modifies the functions of the intercellular communication system in cyanobacteria. These photosynthetic prokaryotes grow as filamentous and multicellular organisms. Microplasmodesmata of approximately 8–20 nm in diameter play an important role in cell-to-cell communication that regulates cell differentiation and growth (Wolk, 1996). These microplasmodesmata are probably composed of mainly proteins (Giddings and Staehelin, 1978, 1981). Expression of the TMV MP in *Anabaena* sp. strain PCC 7120 leads to cell wall accumulation of the MP and perturbation of cell differentiation and growth (Zahalak *et al.*, 1995). Further analysis revealed that the MP induces the formation of filamentous bundles of approximately 150 nm that traverse the septa (Heinlein *et al.*, 1998b). It is possible that individual filaments within a bundle traverse a group of microplasmodesmata. Alternatively, a bundle may traverse a septal pore that is derived from modification of a microplasmodesma or formed *de novo* in the presence of MP (Heinlein *et al.*, 1998b). These data suggest that some components of the intercellular communication system may be conserved in organisms ranging from photosynthetic prokaryotes to higher plants.

B. Is Intercellular Trafficking of Macromolecules an Ancient Biological Function?

Intercellular trafficking of macromolecules is a newly discovered function of plasmodesmata, which raises the question as to whether this is a primitive or advanced plant function. Protein trafficking through plasmodesmata in alga *Nitella* has been demonstrated (Kikuyama *et al.*, 1992). Proteins up to 45 kDa injected into one cell can move into a neighboring cell within 24 hr and retain their integrity and/or biological function. Whether this movement is via passive diffusion or active transport is not known. Perhaps the slow rate of movement, as compared to a time frame of a few seconds for macromolecular trafficking through higher plant plasmodesmata (Fujiwara *et al.*, 1993; Noueiry *et al.*, 1994; Ding *et al.*, 1995), suggests that an efficient and active trafficking system has not evolved yet in the green algae.

Despite this, the ability of algal plasmodesmata to traffic macromolecules is clearly established (Kikuyama *et al.,* 1992).

The authors propose that intercellular macromolecular trafficking is a primitive rather than an advanced function of plants. They reason that, in a primitive unicellular organism, macromolecules could move around in the cytoplasm easily (Fig. 10). In the earliest multicellular organisms evolved, mechanisms to partition cells were so imperfect that the partition could restrict, but still allow generous macromolecular flow among the cells. During further evolution, mechanisms for partitioning cells and for the regulation of intercellular macromolecular transport became more complex. As a result, intercellular macromolecular trafficking evolved as a highly selective and active process. This in turn could drive the evolution of more complex morphological and physiological patterns. Based on this hypothesis, the predicted evolutionary trend is that macromolecular transport would become more and more restricted during the evolutionary advancement of higher plants. This hypothetical scheme may also apply to the evolution of intercellular macromolecular trafficking in animals.

If we accept the hypothesis that intercellular macromolecular trafficking is an ancient function, then perhaps RNA trafficking is the earliest, preceding protein trafficking. This would be in parallel with RNA to have evolved as the earliest macromolecule to possess catalytic and replicating functions (Jeffares *et al.,* 1998; Poole *et al.,* 1998). In a phylogenetic study of viroids, some plant satellite RNAs, and the viroid-like domain of human hepatitis δ virus RNA, Elena *et al.* (1991) suggested a monophyletic origin for these RNAs. Furthermore, they suggested that these RNAs may be living fossils of a precellular RNA world. Thus, like ribozymes that may have been the earliest macromolecules evolved to perform enzymatic functions, special

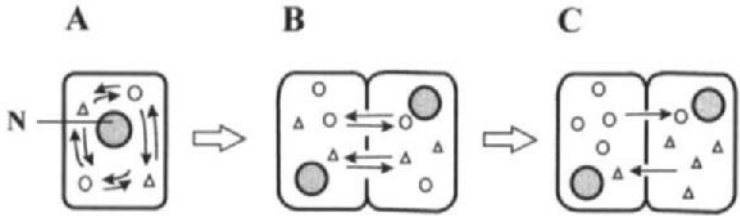

FIG. 10 A hypothetical evolution pattern of intercellular macromolecular trafficking. (A) A unicellular organism that gives rise to a multicellular organism. Macromolecules (open circles and triangles) can flow around easily in the cytoplasm. (B) A primitive multicellular organism. Partition between the cells can restrict, but still allow generous macromolecular flow between cells. (C) A more advanced multicellular organism. Partition between cells is more complex and allows only selective and, in many cases, unidirectional intercellular trafficking of macromolecules. Based on this hypothesis, intercellular macromolecular trafficking is a primitive biological function.

RNAs may have also been the earliest macromolecules evolved to perform signaling functions by trafficking from cell to cell and throughout the whole plant. Viroids may be modern derivatives of such ancient RNA molecules.

VIII. Concluding Remarks

The importance of cell-to-cell communication in regulating plant growth and development is generally accepted. Plasmodesmata play a significant role in this communication. However, it should be pointed out that cell walls also play indispensable roles in cell-to-cell interactions, as has been shown elegantly in *Fucus* embryo development (Berger *et al.,* 1994; Bouget *et al.,* 1998) and in other systems (Hake and Char, 1997).

Although the plasmodesma is clearly a supramolecular complex, our knowledge of the biochemical compositions of this complex is still very limited. In addition to biochemical approaches, genetic approaches will likely play an increasingly important role in dissecting these compositions. The identification of mutant maize (Russin *et al.,* 1996) and *Arabidopsis* (Lartley *et al.,* 1998), as well as an *Arabidopsis* ecotype (Mahajan *et al.,* 1998) defective in certain aspects of plasmodesmal transport functions, has demonstrated the tremendous potential of this approach.

A symplasmic domain can perhaps be considered as a basic developmental unit in a plant. A major challenge for future research is to elucidate how cells within a domain and between domains "talk" to each other and how such dialogues serve to regulate gene expression patterns to potentiate specific developmental processes. It will also be of great interest to elucidate how environmental factors, biotic or abiotic, influence the symplasmic dynamics and gene expression patterns. Research on gene regulation has focused mainly on genome structure, transcription, posttranscription, translation, and posttranslation levels. It would appear that it is now time to expand this focus to include mechanisms of transcellular regulation of gene expression.

Mechanisms for the formation of secondary plasmodesmata and for the modification of plasmodesmata give a plant great flexibility to modulate its intercellular communication network to suit specific plant developmental and growth needs. The importance of such mechanisms can be well illustrated by the ability of plants to form graft unions and to form a continuous communication network between the phloem of a primary root and the lateral root phloem that are formed secondarily from the pericycle. As such, characterization of the mechanisms of secondary plasmodesmal formation and function will provide critical information on the functions of

plasmodesma-mediated intercellular communication in regulating plant developmental processes.

How plasmodesmal structures and trafficking functions have evolved remains a big mystery. Resolving this mystery should provide enlightening insights into the evolutionary patterns of plants. It should also provide unique clues as to how intercellular communication shapes the developmental course of a plant. Of particular interest is the evolution of intercellular macromolecular trafficking. Its existence in both plants and animals suggests that it is likely to be of general biological significance. If we understand how this trafficking function has arisen in plants and animals, we will understand better its modern roles in regulating organismal development.

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