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RESEARCH

Multiple Members of a Third Subfamily of P-Type ATPases Identified by Genomic Sequences and ESTs

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The *Saccharomyces cerevisiae* genome contains five P-type ATPases divergent from both of the well-known subfamilies of these membrane ion transporters. This newly recognized third subfamily can be further divided into four classes of genes with nearly equal relatedness to each other. Genes of this new subfamily are also present and expressed in multicellular organisms such as *Caenorhabditis elegans* and mammals; some, but not all, can be assigned to the classes identified in yeast. Different classes of genes and different genes within a class are expressed differentially in tissues of the mouse. The recently cloned gene for the mammalian aminophospholipid translocase belongs to this new subfamily, suggesting that other subfamily members may transport other lipids or lipid-like molecules from one leaflet of the membrane bilayer to the other.

The P-type ATPases are among the best known and most extensively studied of the membrane transporters (Møller et al. 1996). They derive their name from their mechanism of transport. A high energy phosphate is alternately added to or removed from an aspartate residue to effect transport of a metal ion. This aspartate is contained in a conserved sequence of amino acids (DKTGTLT) diagnostic of the P-type ATPase family. Characterized members of the family have been categorized into two well-recognized subfamilies. The larger of these subfamilies includes a variety of transporters of non-heavy metal ions (NHMI); these enzymes belong to several classes that differ in the ion transported or the membrane across which the transport occurs. For example, one class transports Ca^{2+} across the plasma membrane, another transports Ca^{2+} across the sarcoplasmic reticulum membrane, and another transports Na^+/K^+ across the plasma membrane. Each class of transporter is traceable in organisms as disparate as yeast and humans, and within a given organism each class may be represented by several recognizably related variants. The second and smaller subfamily of P-type ATPases catalyzes transport of heavy metal ions (HMI), such as Cu^{2+}; disruptions of genes in this subfamily cause Menkes and Wilson's diseases (DiDonato and Sarkar 1997). This subfamily may also contain multiple classes of proteins; in addition to the several mammalian and yeast Cu^{2+} transporters, P-type ATPases transport Cd^{2+}, although thus far they have been identified only in bacteria (Lutsenko and Kaplan 1995).

When any of the NHMI transporters is compared with any of the HMI transporters, there is only ~20% identity in amino acid sequence. Among the NHMI transporters, there is 30%–35% identity between proteins in different classes, and within a class, proteins are identical at a majority of residues. These sequence relationships imply that the NHMI and HMI transporter subfamilies diverged from each other before the differentiation of classes of transporters with different ion specificities or locations within the cell. Moreover, there appear to be members of each of the two subfamilies in prokaryotes (Møller et al. 1996), implying that the two subfamilies diverged in prokaryotes before the emergence of eukaryotes.

RESULTS

A New Subfamily of Yeast P-Type ATPases

The availability of the complete genomic sequence of the yeast *Saccharomyces cerevisiae* allows an exact determination of the number of P-type ATPases nec-

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essary to sustain this eukaryotic organism. Sixteen yeast genes contain DKTGTLT and other sequences diagnostic of P-type ATPases. Eleven of these genes are clearly members of the two well-known subfamilies, on the basis of their greater sequence similarity to members of one subfamily or the other. However, five are no more similar to members of either of the two subfamilies of enzymes than those two subfamilies are similar to each other, thus identifying a third subfamily clearly distinct from the HMI and NHMI transporters (Andre 1995; Catty et al. 1997).

The extent of sequence similarity among the five *S. cerevisiae* P-type ATPases in the new subfamily is shown in Figure 1. With one exception, any two of these proteins are ~30%–35% identical and 50%–60% similar in amino acid sequence. This divergence is similar to that between different classes of NHMI transporters, such as the Ca^{2+}- and Na^+/K^+-ATPases. On this basis, the new subfamily can be divided into four different classes in yeast, arbitrarily assigned the numbers in the last column of Figure 1. The one exception to this pattern are two genes, referred to here as ATC5 and YD8557.1, which are 70% identical and 85% similar. This level of similarity is similar to that seen between the Na^+/K^+- and H^+/K^+-ATPases, and on that basis these two genes are members of the same or closely related classes. It should be noted that several genes in this subfamily were designated initially as putative Ca^{2+} transporters (Ripmaster et al. 1993; Andre 1995) and are still labeled as such in some database entries. However, the subfamily is not significantly more related to that particular class of NHMI transporters than to any other class in that large subfamily, and there is now good reason, in addition to sequence divergence, to believe they transport an entirely different sort of substrate (see below).

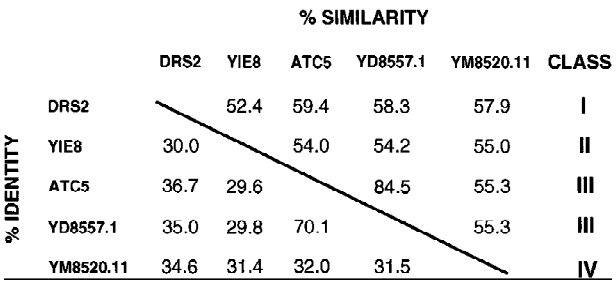

Figure 1 Relatedness at the amino acid level and classification of the five *S. cerevisiae* subfamily members, DRS2 (GenBank accession no. U1298x41, Swiss-Prot accession no. P39524), YIE8 (Z47047x130, P40527), ATC5 (U18922x2, P32660), YD8557.01 (Z47746x1), YM8520.11 (Z49700x11).

Hydrophobicity analysis performed on members of the new subfamily identifies 10 putative transmembrane helices, just as are found in the majority of transporters in the other two subfamilies. The arrangement of helices in the new subfamily suggests a large central extramembrane domain containing the DKTGTLT sequence flanked by two pairs of transmembrane helices toward the amino terminus and three closely spaced pairs of transmembrane helices toward the carboxyl terminus of the protein (Fig. 2); this arrangement is much more similar to that of the NHMI than to that of the HMI transporters (Lutsenko and Kaplan 1995). This relationship suggests either that (1) this arrangement is the more primitive one, from which the HMI transporter pattern diverged; or (2) the ancestral protein of the new subfamily separated from the ancestral NHMI transporter after the latter diverged from the original HMI transporter. Although members of the metal ion transporting subfamilies are represented in prokaryotes, a search of the several available prokaryote genomes failed to reveal any recognizable representative of the new subfamily. This finding suggests that the latter is the case, and that the ancestral protein of the new subfamily diverged from the ancestor of the NHMI transporters. This branching probably occurred early in the eukaryote lineage, as the extent of sequence divergence between all three subfamilies is about the same.

ESTs and Genomic Sequences Reveal Members of the Third Subfamily in Higher Eukaryotes

If this group of transporters arose in an early precursor to the eukaryotes, representatives of the subfamily and perhaps of the various classes observed in yeast should be identifiable in multicellular organisms, including mammals. This possibility was borne out by examination of available genomic sequences and ESTs. In the nematode *Caenorhabditis elegans*, five inferred ORFs from genomic sequences can be identified unambiguously as coding for subfamily members by the presence of subfamily-specific amino acid substitutions in three sequences diagnostic of all P-type ATPases as well as in a fourth well-conserved region of the family (Tang et al. 1996; Fig. 3, below, identified by numbers). The structure of these ORFs is shown in Figure 2A.

One of the genomic sequences (CEY49E10) is most similar to the yeast class I gene (42% identity, 62% similarity). In addition, there are six ESTs derived from the gene. These sequences thus imply that class I genes have persisted and are expressed in nematodes. Two genomic sequences (CEF36H2 and

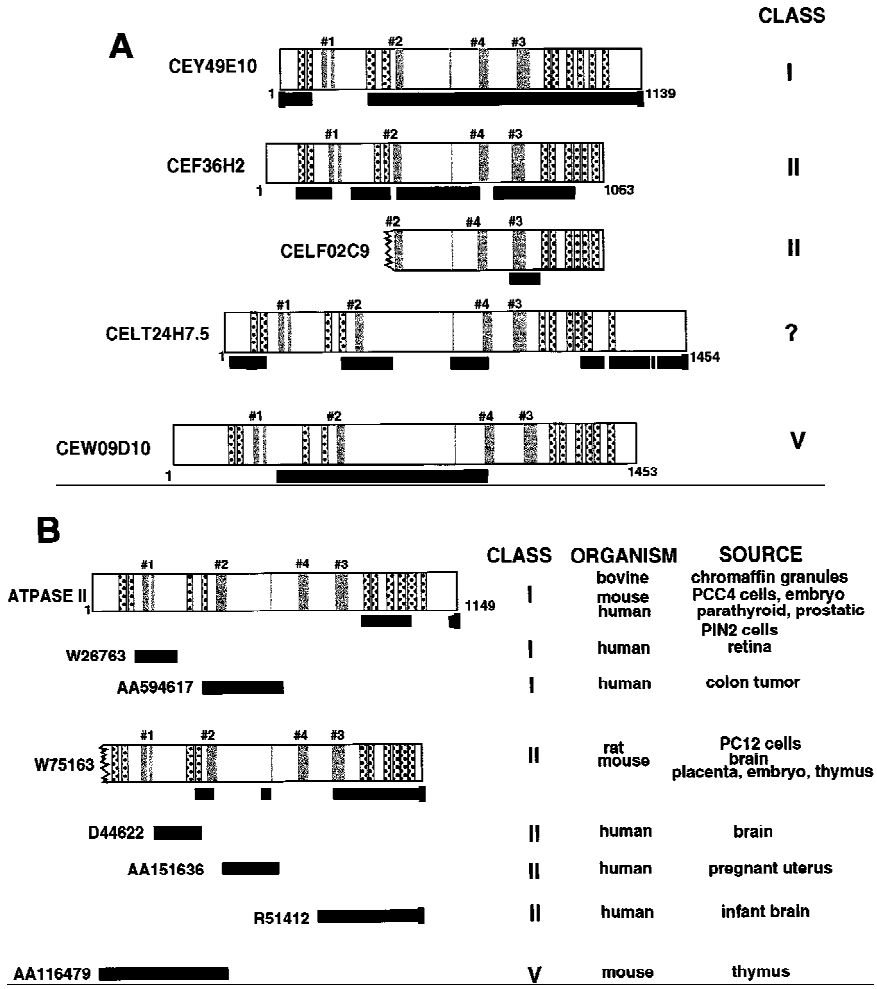

Figure 2 *C. elegans* and mammalian subfamily members. (*A*) Proteins translated from five *C. elegans* genomic sequences. Black bars underneath the annotated diagrams represent EST cDNA amino acid sequences identical to those derived from the corresponding genomic sequences. Translated proteins from genomic sequences CEY49E10, CEF36H2.1, CELF02C9.3, CELT24H7.5, and CEW09D10 were predicted by Genefinder, as described in Methods. EST cDNAs D34857, D68551, D34761, D36536, C46723, and C12815 are derived from genomic sequence CEY49E10; C62504, C61119, C61257, C64739, C63236, C62558, C64835, D37065, D35631, D34828, and D35545 from CEF36H2.1; C71645 from CELF02C9; C60978, C60796, D33790, D36710, D64692, D64829, D66617, D68066, D70457, D72885, D75837, and M89042 from CELT24H7.5; and C64755, C63577, C63707, C63104, C65800, C65500, C65912, C64572, C65850, D67512, and D27733 from CEW09D10. (*B*) Proteins translated from mammalian cDNAs. Annoted diagrams represent the complete mammalian class I ATPase II (U75321) sequence and the nearly complete class II EST cDNA sequence W75163 (AF011336). Black bars directly below these sequences represent EST cDNA sequences encoding proteins identical with those diagrammed (AA387574, W39181, AA225031, AA224982, AA652796 with class I ATPase II; AA510705, W50374, AA016798, AA061973, AA039159, AA039035, AA200493, AA200511, AA198017, W55124, H35595, and H32606 with class II W75163). Other black bars represent EST cDNA sequences not identical with those diagrammed, which are identified by their dbest accession numbers. For both *A* and *B,* class designations are based on sequence homology with yeast subfamily members using BLAST. Putative transmembrane domains are patterned; P-type ATPase consensus regions, with numbers corresponding to those in Fig. 3, are in gray.

CELF02C9) are most similar to the yeast class II gene. In the region where the two *C. elegans* genes overlap, they are 84% identical and 92% similar. Eleven ESTs are derived from one of the genes and one EST from the other, indicating that they, too, are expressed in the animal. Two other genes for which complete genomic sequences are available (CELT24H7.5 and CEW09D10) are no more related at the sequence level to one class defined in yeast than to another, suggesting that the yeast classes are not an exhaustive list of the types of enzymes in the subfamily. At least 22 ESTs are derived from these two genes. This combined set of *C. elegans* genomic and EST sequences indicates that representatives of at least two of the yeast classes of the new subfamily of transporters are present and expressed in nematodes, that at least one of these classes has diversified in this lineage, and that additional subfamily classes have either evolved or been retained in the worm lineage since its divergence from the fungi.

In mammals, genomic sequences representing subfamily members are unavailable, but analyses of expressed sequences confirm and extend the conclusions drawn from *C. elegans*. The ATPase II from adrenal chromaffin granules in cattle (Tang et al. 1996) and its mouse homolog (84% identical at the nucleotide level and >95% identical at the amino acid level) contain sequences diagnostic of the third subfamily. These complete coding sequences are most homologous to the yeast class I gene (47% identical, 67% similar) and the *C. el-*

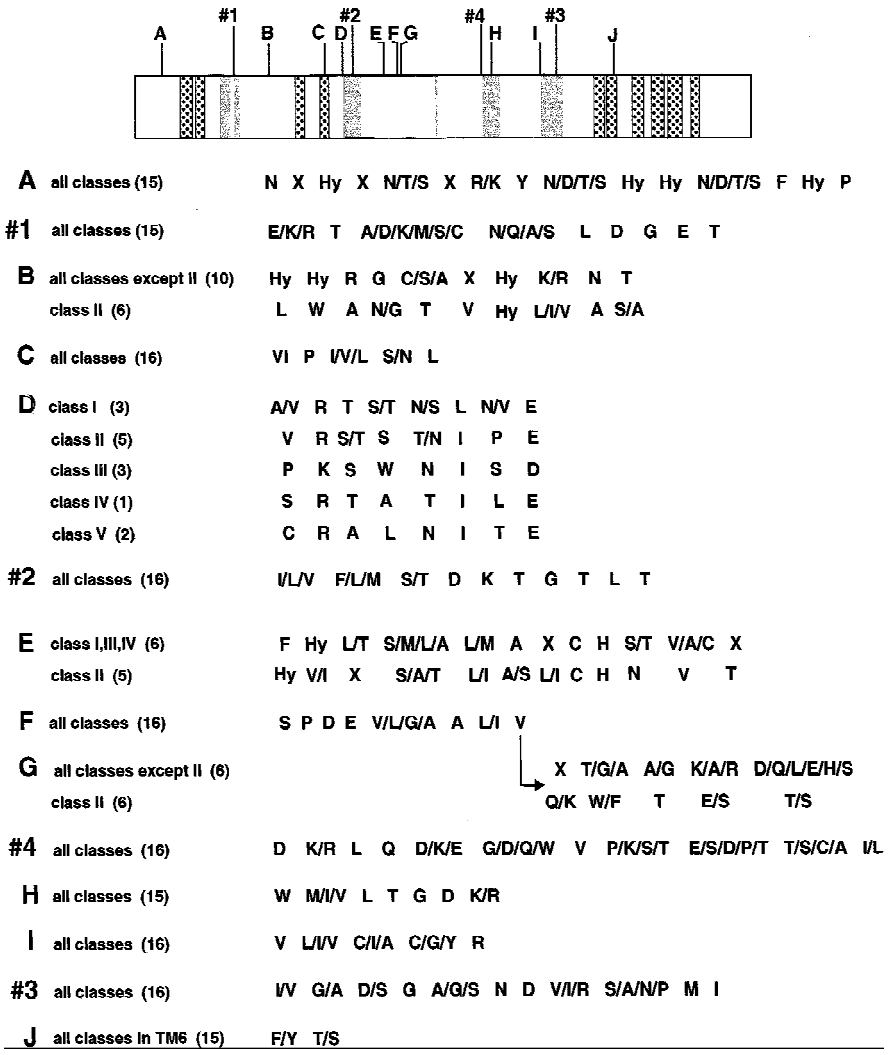

Figure 3 Sequences diagnostic of the new subfamily. Diagnostic sequences were determined from alignments of all inferred protein sequences from ESTs, complete cDNAs, and genomic sequences of subfamily members from *S. cerevisiae, C. elegans,* and mammals, as well as from *Schizosaccharomyces pombe, Plasmodium falciparum,* and *Drosophila melanogaster* (see footnote in Discussion). Numbered sequences are P-type ATPase consensus sequences; lettered sequences are other diagnostic sequences. Numbers in parenthesis are the number of different gene sequences used to determine the diagnostic sequences; total numbers vary because incomplete sequences may not overlap all diagnostic regions.

egans class I gene (48% identical, 67% similar). In addition, there are several human and rodent ESTs that are most homologous to class I genes (Fig. 2B). Four of these human ESTs (W39181, AA225031, AA224982, AA652796) are clearly derived from the human homolog of the ATPase II. Two others (W26763, AA594617) differ from the ATPase II; whether they are derived from a single gene cannot be determined, as they do not overlap with one another. In addition to these class I sequences, there are several ESTs representing at least two, and possibly as many as four, different class II genes. EST W75163 represents nearly the entire open reading frame of one of these genes and contains the sequences of several ESTs from other species and tissues. In combination, these ESTs confirm that the subfamily is well represented in multicellular animals and imply that classes I and II identified in yeast have not only persisted in mammals as well as other eukaryotes, but also proliferated, giving rise to multiple, expressed variants.

As in *C. elegans,* there are ESTs that cannot be assigned confidently to any one of the yeast classes. One of these (AA116479) appears to be most related (47% identity, 68% similarity) to one of the *C. elegans* genomic sequences (CEW09D10), also unclassifiable by the yeast scheme. Therefore, this class is not specific to worms and thereby is designated as class V in recognition of its generality.

These numerous coding sequences of subfamily members from a variety of organisms provide a database for identifying specific sequences that are potentially diagnostic of subfamily or even class members. The most obvious of these diagnostic sequences were identified in P-type ATPase consensus sequences when only a few members of the subfamily were known (Tang et al. 1996). With the numerous sequences now available, diagnostic sequences outside these consensus domains can also be identified (Fig. 3). The bulk of the sequences are characteristic of all members of the subfamily, but some are useful for identification of members of specific classes (sequences B, D, E, and G). Of the latter, however, only those for class I and II can be used with any confidence, as these are the only classes for which a rea-

sonable diversity of class members is currently available for analysis.

Genomic Structure

The genomic structure of P-type ATPases in animals is generally complex. For example, the coding regions of the mammalian plasma membrane Ca^{2+}- and Na^+/K^+-ATPase genes are interrupted ~20 times, dispersing the exons over >50 kb of DNA (Maeda et al. 1990; Hilfiker et al. 1993). The region coding for the main DKTGTLT-containing cytosolic loop contains seven to eight introns. The known *C. elegans* versions of these genes are much less dispersed, with <10 relatively small introns fragmenting the coding sequence, and only one interruption in the DKTGTLT loop (Fig. 4A). Although less is known about the HMI transporting subfamily, the mammalian Menkes gene is also heavily fragmented, with 21 interruptions including 6 in the DKTGTLT loop, resulting in dispersal of the gene over >80 kb of DNA (Tümer et al. 1995).

The genomic structure of the five *C. elegans* genes of the new subfamily is shown in Figure 4B. Although the introns are relatively small, the highly interrupted character of the mammalian NHMI and HMI transporter genes is reproduced in the full-length *C. elegans* genes, which contain 16, 18, and 13 introns, respectively, including four to seven in the region coding for the main cytosolic loop. The intron patterns of the two class II *C. elegans* genes, although different, are closely related to one another, in keeping with their high degree of sequence similarity. These also appear to be less complex, more in keeping with the less dispersed structure of the *C. elegans* NHMI transporters than the other members of the subfamily.

Tissue-Specific Expression of the Subfamily in Mouse

The existence in multicellular organisms of multiple members within a class raises the question of whether they represent tissue-specific variants. To investigate this possibility, Northern blot analysis of the mRNA isolated from various mouse tissues was used to compare the expression of class I and II genes, and of two different class II genes with each other. As shown in Figure 5, the mouse class I gene is expressed as two very large mRNAs of different size between 7.5 and 9.5 kb, implying that they include large untranslated regions. These two forms are expressed in roughly equivalent amounts and at very high levels in lung; a similar pattern, but at lower expression levels, is seen in brain and muscle. A different pattern, with the smaller form predominant, is observed in heart, spleen, and kidney. Finally, much lower levels of expression of both forms are observed in liver and testis.

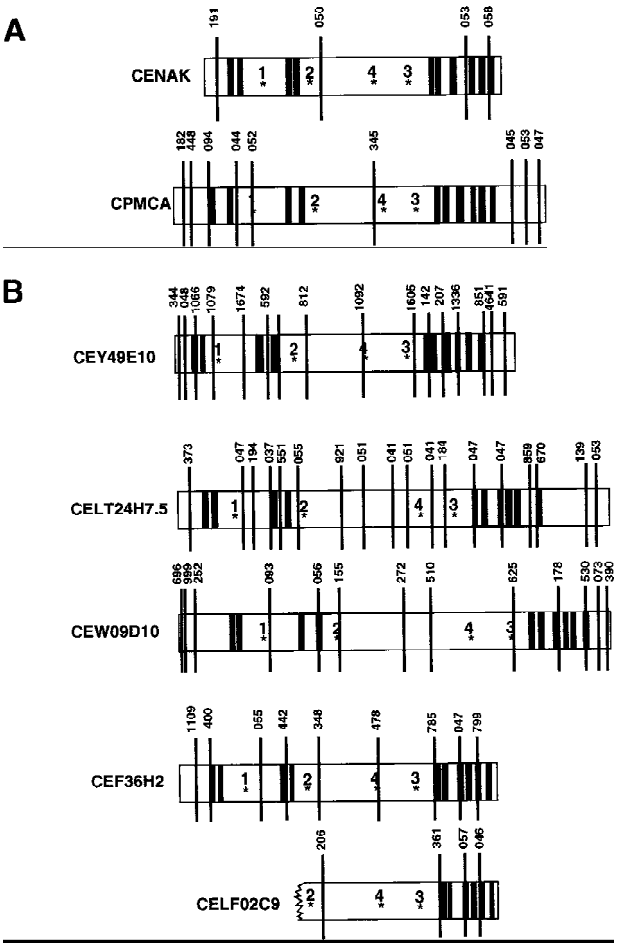

Figure 4 Genomic structure of *C. elegans* P-type ATPases. Coding regions are shown interrupted by introns (black vertical lines with intron length in base pairs at the top). Putative transmembrane domains are black bars; P-type ATPase consensus regions, with numbers corresponding to those in Fig. 3, are marked by asterisks. Intron/exon information for the Na^+/K^+–ATPase α-subunit CENAK was translated from *eat-6* gene (GenBank accession no. U18546). Coding regions for the plasma membrane Ca^{2+} transporter CPMCA and for the five new subfamily members were predicted by Genefinder from the cosmids W09C2 (GenBank accession no. Z68221), Y49E10 (Z98866), T24H7.5 (U28940), W09D10 (Z93785), F36H2 (Z81078), VF36H2L (AL021466), and F02C9 (U80025), respectively, as described in Methods.

In contrast to this rather complex expression pattern, the class II genes generate mRNAs that are not much larger than the size expected from the

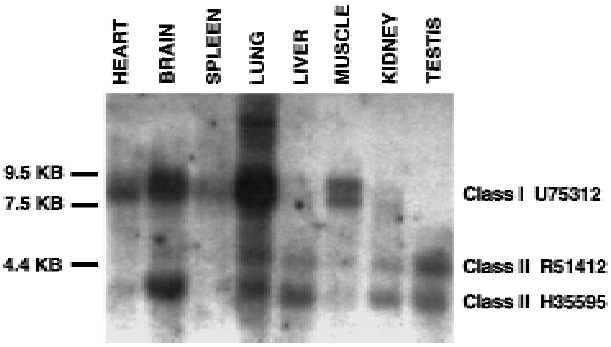

Figure 5 Northern blot analysis of expression of three different mammalian subfamily members in mouse tissue. Radiolabeled oligonucleotides derived from murine ATPase II (class I, U75312), rat EST H35595 (class II, same as rat W75163), and human EST R51412 (class II) were hybridized separately to a Northern blot of mouse tissues. As each probe hybridized to a transcript of different mobility, the three autoradiographs have been superimposed.

coding sequence, and of just one size per gene in all mouse tissues. However, one generates an mRNA that is slightly smaller than 4.4 kb and is most heavily expressed in brain followed by lung, liver, and testis. The other class II gene sequence is expressed as a transcript larger than 4.4 kb and, in contrast to the first class II sequence, is not highly expressed in brain, but is most abundant in testis, followed by lung, liver, and kidney.

DISCUSSION

The well-known P-type ATPase transporters of NHMIs constitute a highly diverse group of enzymes with different transport specificities, several of which further diversified early in the evolution of the group into varieties that are expressed in different tissues or subcellular localizations. The results presented here indicate that in the earliest eukaryote, the ancestral protein of that group gave rise to a separate line, which underwent independently a similar process of diversification. The profusion, persistence, and diversification of the various classes of this new subfamily in a wide variety of organisms[1] suggest that they mediate an important general class of transport reactions, which in turn poses the question of the nature of the molecular species that these enzymes transport. The one complete mammalian gene from the new subfamily provides a clue, as its function is known.

In animal cells, the phospholipids of the plasma membrane are not distributed randomly across the bilayer; the charged or partially charged aminophospholipids, phosphatidylserine (PS), and phosphatidylethanolamine are concentrated in the inner leaflet and the choline phospholipids, phosphatidylcholine, and sphingomyelin are concentrated in the outer leaflet (Williamson and Schlegel 1994). Because phospholipids diffuse across the bilayer over the course of several hours in cells such as erythrocytes, which maintain lipid asymmetry over a lifespan of several months, some mechanism must operate against diffusion to prevent randomization of the lipid distribution. That mechanism is the aminophospholipid translocase, an ATP-dependent membrane transporter that translocates the aminophospholipids from the outer to the inner leaflet of the membrane bilayer against a concentration gradient (Seigneuret and Devaux 1984). Originally identified in erythrocytes, the same activity has been demonstrated in the plasma membrane of every mammalian cell type thus far examined, from fibroblasts to sperm, and in intracellular membranes including chromaffin granules (Williamson and Schlegel 1994). When the presumed translocase from chromaffin granules, called ATPase II, was cloned (Tang et al. 1996), it was found to be a member of the new family, and in particular was a mammalian homolog of the previously identified yeast class I gene, *DRS2*. Analysis of the internalization of a fluorescent form of PS in wild-type yeast and a *drs2* null mutant strain of yeast indicated that wild-type yeast were capable of specifically transporting PS, whereas the mutant was not (Tang et al. 1996).

If class I genes code for aminophospholipid transporters, what are the functions of the other classes of ATPases in the new subfamily? The transmembrane helices of the metal ion transporters have several conserved, charged residues implicated in substrate binding and transport (Clarke et al. 1989). In the new subfamily, these residues are replaced by conserved hydrophobic amino acids, consistent with the possibility that the entire subfamily transports amphipathic molecules (Tang et al. 1996). The substantial evolutionary distance between the classes and the profound effect of *DRS2* disruption on transport of aminophospholipids by yeast cells make it unlikely that all the classes code for plasma membrane aminophospholipid translocators. It is more likely that each of the classes of

[1]In addition to the organisms whose subfamily members are analyzed here, *Plasmodium falciparum* (U16955), *Toxoplasma gondii* (N61749), *Schizosaccharomyces pombe* (Z67757, Z69731), *Drosophila melanogaster* (AA441439), and *Arabidopsis thaliana* (B10989, B11481) also contain sequences recognizable as subfamily members.

transporters in the new subfamily catalyzes the transbilayer movement of a different class of substrates, such as different phospholipids or perhaps related molecules such as glycolipids or other lipid-like molecules. If such is the case, different members within a class may represent enzymes with the same substrate specifity, but with different cellular locations. For instance, the second class I gene identified in mammals may represent the plasma membrane form of the aminophospholipid translocase, a possibility that emphasizes the need for obtaining and analyzing the sequence of that enzyme.

To date, information is available on the chromosomal localization of only one mammalian subfamily member. The human class II gene from which EST R51412 is derived has been mapped to human chromosome 18q22.3-qter using the STS markers A004F30 and WI-11906 (Unigene, as described in Methods). Among the some 25 human diseases that have been localized to chromosome 18 (Overhauser et al. 1995), susceptibility loci have been identified for bipolar disorder (manic depressiveness). In a study designed to identify possible candidate genes for this disease, Yoshikawa et al. (1997) isolated, sequenced, and mapped brain transcripts specific for chromosome 18 and found transcripts containing sequences encoding the carboxy-terminal end (42 amino acids) of the class II R51412 protein whose sequence is reported here, as well as 3′-untranslated sequence from this gene. These were mapped to 18q22-23, a region proposed by Freimer et al. (1996) as a predisposing locus for severe bipolar disorder.

Our analyses suggest that the new subfamily arose very early in the evolution of eukaryotes, and diversified to roughly its current complexity in a common ancestor of yeast and mammals. This diversification may reflect the elaboration of cellular compartments and corresponding separation of functions across membrane bilayers, which occurred during the evolution of eukaryotes. This development provided many new potential venues for transport molecules that manipulate specifically the transbilayer distribution of phospholipids and related molecules, and thereby regulate cellular functions. In the case of the mammalian aminophospholipid translocase, for example, its removal of PS from the cell surface is the basis of a signaling system by which display of this phospholipid on the cell surface triggers recognition and phagocytosis of apoptotic cells (Pradhan et al. 1997) and catalyzes the coagulation cascade (Williamson and Schlegel 1994). Other examples of the functional importance of compositional differences between the two sides of a bilayer may be hidden in our ignorance of the lipid distributions characteristic of internal membranes.

METHODS

Sequence Comparisons and Analyses

TBLASTN (Altschul et al. 1990) was used to search the databases. The inferred ORFs encoding *C. elegans* proteins were deduced from cosmid sequences using the FGENEN program for prediction of gene structure in nematode DNA sequences available from the Baylor College of Medicine at http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html (Solovyev et al. 1994) and confirmed where possible by comparison with EST sequence data. The GAP procedure of the GCG Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI) was used for pairwise amino acid sequence comparisons. The NALIGN program of the PCGene package, Intelligenetics Inc., was used to compare the murine and bovine ATPase II cDNA sequences. Localization of the gene represented by EST R51412 on human chromosomes was obtained in part from The Human Genome Map and UniGene at NCBI, http://www.ncbi.nlm.nih.gov/UniGene/index.html. Putative transmembrane domains were identified using the HELIXMEM program of the PCGene package, Intelligenetics, Inc., based on the method of Eisenberg et al. (1984).

Cloning the Mouse Homolog of ATPase II

Two overlapping clones representing the entire murine ATPase II coding sequence (U75321) were isolated from a PCC4 mouse teratocarcinoma cDNA library constructed in Lambda ZapII (Stratagene) using an oligonucleotide probe derived from the bovine ATPase II gene (U51100; Tang et al. 1996). The clones were sequenced manually using a Sequenase version 2.0 [α-^{35}S]dATP DNA sequencing kit (Amersham). A 39-bp insert, located between nucleotides 3078 and 3118 of the bovine gene, contained several stop codons and characteristics of a partial intronic sequence. After several RT–PCR products from a murine myeloid precusor cell line (FDC WEHI2) were found not to contain it, the insert was removed from the sequence submitted to the database.

Sequencing EST Clones

Rat H35595 and H32606 were obtained from The Institute for Genomic Research. Rat W75163, human R51412 and AA594617, and mouse AA116479 EST cDNAs were obtained from Genome Systems. *C. elegans* EST cDNA D36536 (yk34c11) was the kind gift of Yuji Kohara at the DNA Data Bank of Japan. EST cDNAs were sequenced in their entirety using fluorescent terminators and an Applied Biosystems ABI 377 Prism sequencer, and re-entered into the database (GenBank accession nos. U78977, AF011336, U78978, AF032442, AF011337, and AF034078).

Northern Blot Analysis

A Northern blot containing poly(A)$^+$-enriched RNA from murine heart, brain, spleen, lung, liver, skeletal muscle, kidney,

and testis was purchased from Clontech. Random-primed oligonucleotide probes (a 1.4-kb *Eco*RV–*Bam*HI sequence from mouse ATPase II, a 1.1-kb *Xho*I–*Eco*RI sequence from rat H35595, and a 727-bp *Hin*dIII–*Bal*I sequence from human R51412) were labeled with ^{32}P by the method of Feinberg and Vogelstein (1983). Each probe was hybridized (Church and Gilbert 1984) separately to the same stripped Northern blot.

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