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EVOLUTION AND ISOFORMS OF V-ATPase SUBUNITS

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Summary

The structure of V- and F-ATPases/ATP synthases is remarkably conserved throughout evolution. Sequence analyses show that the V- and F-ATPases evolved from the same enzyme that was already present in the last common ancestor of all known extant life forms. The catalytic and non-catalytic subunits found in the dissociable head groups of both V-ATPases and F-ATPases are paralogous subunits, i.e. these two types of subunits evolved from a common ancestral gene. The gene duplication giving rise to these two genes (i.e. those encoding the catalytic and non-catalytic subunits) pre-dates the time of the last common ancestor. Similarities between the V- and F-ATPase subunits and an ATPase-like protein that is implicated in flagellar assembly are evaluated with regard to the early evolution of ATPases. Mapping of gene duplication events that occurred in the evolution of the proteolipid, the non-catalytic and the catalytic subunits onto the tree of life leads to a prediction of the likely quaternary structure of the encoded ATPases. The phylogenetic implications of V-ATPases found in eubacteria are discussed.

Different V-ATPase isoforms have been detected in some higher eukaryotes, whereas others were shown to have only a single gene encoding the catalytic V-ATPase subunit. These data are analyzed with respect to the possible function of the different isoforms (tissue-specific, organelle-specific). The point in evolution at which the different isoforms arose is mapped by phylogenetic analysis.

The evolution of the catalytic and non-catalytic subunits

The V-ATPases that energize the endomembranes of eukaryotes have evolved from the archaebacterial coupling factor ATPases (Gogarten *et al.* 1989*a*; Kibak *et al.* 1992). These ATPases show a high degree of sequence similarity; in particular the *A* and *B* subunits are surprisingly conserved between the eukaryotic and the archaebacterial enzyme (about 50% identical amino acid residues), whereas both ATPase types show a much lower degree of similarity to the homologous F-ATPase subunits (α and β subunits, only about 25% identity). Based on this high degree of similarity one might be tempted to classify the archaebacterial coupling factor ATPase as a V-type ATPase. However, with regard to function, tertiary and quaternary structure, the archaebacterial ATPases appear to be more similar to the eubacterial F-ATPases. Therefore, in the following discussion

Key words: ATPase, progenote, archaebacteria, origin of life, membrane transport.

the term V-ATPase will be used to denote the eukaryotic V-ATPases only; the archaebacterial ATPases will be abbreviated as A-ATPases. The whole group of homologous ATPases will be denoted as VFA-ATPases.

The catalytic subunits of VFA-ATPases are homologous (orthologous) to each other. In addition, the catalytic and non-catalytic subunits of these ATPases were also derived from a common ancestral gene, i.e. these subunits are also homologous to each other; however, their relatedness does not reflect the evolution of the species, but the ancient gene duplication event. This type of homology is termed paralogy (see Fig. 1).

A flagellar assembly peptide is paralogous to the VFA-ATPase subunits

An ATPase-like peptide has been found to be encoded as part of the *Fla* operon (open reading frame no. 4) of *Bacillus subtilis* (Galizzi *et al.* 1991). A thermosensitive mutant of the homologous *flil* gene in *Salmonella typhimurium* exhibits normal flagellar usage at the restrictive temperature; only the assembly of the flagella is interrupted in the mutant (Vogler *et al.* 1991). The two gene products from *B. subtilis* and *S. typhimurium* are 47% identical to each other and about 23% identical each to the catalytic and non-catalytic subunits of the VFA-ATPases; the ATPase-like flagellar proteins have about the same degree of similarity to the catalytic and non-catalytic subunits as the paralogous VFA-ATPase subunits have among themselves (see Fig. 3). On the basis of this similarity, it is appealing to assume that the ATPase-like flagellar protein is involved in the generation of

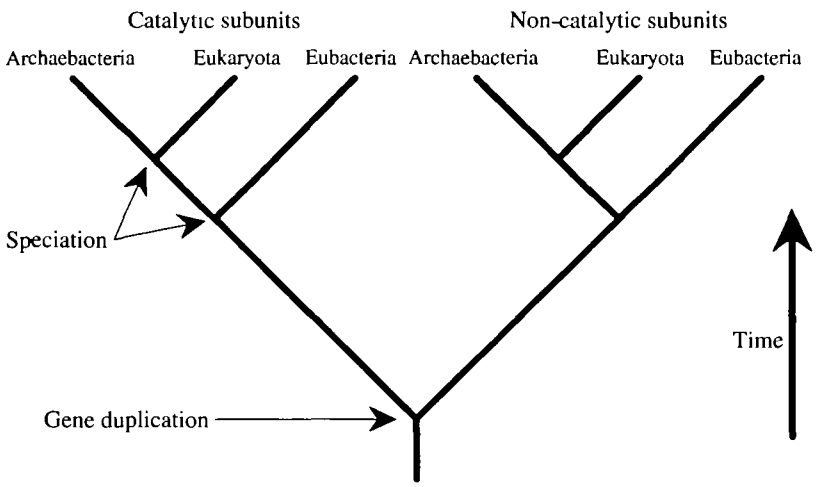

Fig. 1. Schematic diagram depicting the evolution of orthologous (catalytic subunits compared with each other or non-catalytic subunits compared among themselves) and paralogous VFA-ATPase subunits (catalytic *vs* non-catalytic subunits). The orthologous subunits can be used to trace the evolution of species, whereas comparison of paralogous subunits points to the much earlier gene duplication event. The gene duplication in the case of the VFA-ATPases pre-dated the speciation events that led to the known extant species (archaebacteria, eubacteria and eukaryotes). Therefore, the paralogous subunits can be used as an outgroup to root the universal tree of life (Gogarten *et al.* 1989*a*).

local proton gradients. However, the phenotype of the thermosensitive mutants (see the above description) is more compatible with the role of this ATPase in the export of flagellum-specific proteins. A phylogenetic tree based on pairwise comparisons of the protein sequences is depicted in Fig. 2. The flagellar ATPase-like peptide joins the VFA-ATPase subunits at the innermost branch before the lines leading to the ATPases of the extant species separate from each other.

The three domains in the tree of life

The ancient gene duplication that gave rise to the paralogous catalytic and non-catalytic subunits had already occurred in the last common ancestor of eubacteria,

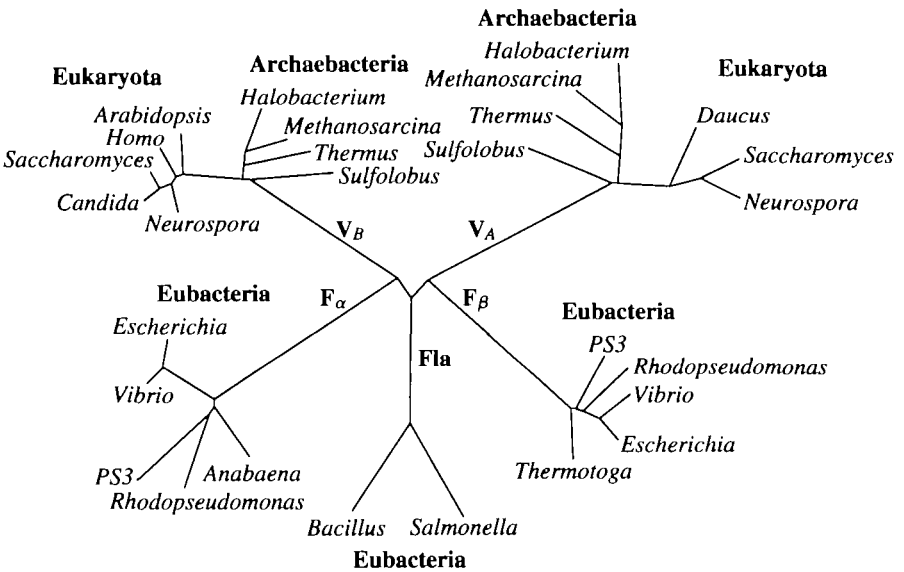

Fig. 2. Evolution of catalytic and non-catalytic VFA-ATPase subunits and the ATPase-like flagellar protein. The depicted unrooted tree represents the minimum-length tree calculated from a distance matrix. The tree is preliminary in the sense of Feng and Doolittle (1987) in that it utilizes only pairwise comparisons. This procedure sacrifices accuracy in favor of a less-biased alignment procedure. The tree was calculated using pairwise comparisons between the amino acid sequences. Optimum alignment scores were calculated using Needleman and Wunsch's (1970) algorithm and the unitary matrix. Distances between two sequences were calculated as described by Feng and Doolittle (1987); however, the alignment scores for the randomized sequences were actually calculated (mean of 20 randomizations each) instead of estimated. Topology and branch lengths of the depicted tree were calculated using the algorithm of Fitch and Margoliash (1967) as implemented by Felsenstein (1988). The sequence for *Thermotoga maritima* was kindly provided by K. H. Schleifer, Technische Universität Munich, FRG. The sequences for *Halobacterium* were taken from Ihara and Mukohata (1991). The *Thermus* sequence was taken from Tsutsumi *et al.* (1991). All other sequences were retrieved from GenBank. V_A and V_B represent the *A* and *B* subunits of the V- and A-ATPases; F_α and F_β, the α and β subunits of the F-ATPase; and Fla, the flagellar ATPase-like peptide.

archaebacteria and eukaryotes (Gogarten *et al.* 1989*a*). Rooting the tree of life by means of this ancient gene duplication (see Fig. 1) shows the archaebacteria to be more closely related to the eukaryotes than to the eubacteria. The root is placed between eubacteria on one side and eukaryotes and archaebacteria on the other. Sequences of other archaebacterial and eukaryotic ATPase subunits have since been obtained (see Fig. 2). When these sequences were subjected to phylogenetic analyses, the results confirmed the above conclusion (Gogarten *et al.* 1989*b*; Ihara and Mukohata, 1991; Linkkila and Gogarten, 1991). Analyses of other duplicated genes (t-RNAmet, dehydrogenases, elongation factors; Iwabe *et al.* 1989; Cammarano *et al.* 1992) agree with the conclusion obtained for the ATPases: namely, all archaebacteria branch off from the line leading from the root to the eukaryotes (see Fig. 2).

Origin of the eukaryotic endomembrane system

The analysis of the ATPase sequences results in phylogenetic trees of the same topology as the ones that are obtained using other markers for the nuclear/cytoplasmic component (e.g. Iwabe *et al.* 1989; Cammarano *et al.* 1992). This confirms the usefulness of the V-ATPase subunits as a marker for the nuclear cytoplasmic component of the eukaryotic cell. Furthermore, it suggests that the ATPases energizing the eukaryotic endomembranes and the endomembrane system itself evolved within the cell that functioned as the host for the symbioses that evolved into the present-day eukaryotes, i.e. the endomembrane system itself is *not* due to another symbiotic event similar to the ones that gave rise to plastids and mitochondria. The evolution of V-ATPases from the coupling factor ATPase of archaebacterial plasma membrane furthermore suggests that the endomembrane system evolved from invaginations of the plasma membrane of the cell that contributed the bulk of the nuclear genome.

Lateral gene transfer between kingdoms

It was reported recently that the proton-pumping ATPase from *Thermus aquaticus* (=*Thermus thermophilus* strain HB8; Yokoyama *et al.* 1990) and the Na^+-pumping ATPase from *Enterococcus hirae* (Kakinuma *et al.* 1991) are vacuolar-type ATPases. Based on rRNAs and other biochemical characters (cell wall, lipids, antibiotic resistance), the genus *Thermus* is classified as eubacterial, with closest similarities to the *Deinococci* (Woese, 1987; Hensel *et al.* 1986). However, note that the malate dehydrogenase from a *Thermus* species groups together with its eukaryotic, rather than its eubacterial, counterparts (Iwabe *et al.* 1989).

The recently published sequence of the *A* subunit of this enzyme (Tsutsumi *et al.* 1991) has 50–55% identical amino acid residues when aligned to the *A* subunits of the eukaryotic V-ATPase and 53–60% identical residues when compared to the various archaebacterial subunits. If this sequence is included in a phylogenetic reconstruction (Fig. 2), the *Thermus* sequence groups *within* the archaebacterial domain together with the subunits of *Halobacterium* and *Methanosarcina*. The same grouping is found for the *B* subunit (Fig. 2). The *Thermus* ATPase shares a long history of evolution with the archaebacterial ATPases, and it does not represent an 'earlier' V-ATPase branch. This

grouping of the *Thermus* sequence within the archaebacterial domain contradicts scenarios that assume that both F- and V-type ATPases were already present in the last common ancestor and that one or the other was lost in the lines leading to the extant species. The grouping of the *Thermus* ATPase within the archaebacteria indicates a lateral gene transfer across domain boundaries between an archaebacterial-like organism and a eubacterium.

The evolution of the proteolipid

With respect to function and with respect to quaternary structure the archaebacterial coupling factor ATPase appears to be more similar to F- than to V-ATPases (Schafer *et al.* 1990). The switch in function from ATP synthase/ATPase (F-type and archaebacterial-type) to an exclusively proton-pumping ATPase (V-type) appears to be correlated with a change in subunit stoichiometry and an increased proteolipid size (Cross and Taiz, 1990; Gogarten and Taiz, 1992). The V-ATPase proteolipid is thought to have evolved by a gene duplication and fusion event from a smaller F-ATPase-like proteolipid (Mandel *et al.* 1988). The first two and the last two membrane-spanning helices are each similar to the F-ATPase proteolipid (Mandel *et al.* 1988). Using these front and back halves of the V-ATPase proteolipid as separate entries for phylogenetic reconstruction, the gene duplication that gave rise to these two parts is mapped to the early evolution of the eukaryotes (Fig. 3). The duplication occurred after the bifurcation of the *Sulfolobus*

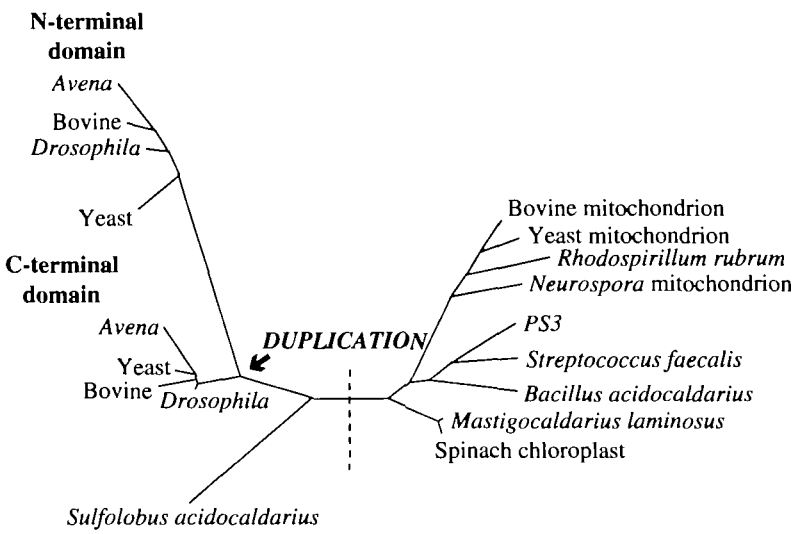

Fig. 3. Evolution of the proteolipids of VFA-ATPases depicted as an unrooted tree. The two amino terminal and the two carboxy terminal membrane-spanning helices were treated as separate entries. The duplication event giving rise to these two similar halves is indicated. Distance matrix and tree calculation were performed as described for Fig. 2, except that Dayhoff's mutation matrix (Dayhoff *et al.* 1978) and more than 10 randomizations each were used to calculate the distances. Trees with the same topology (except for a minor rearrangement within the eubacterial domain) were obtained using the neighbor-joining method as implemented by Felsenstein (1988). The sequences used were retrieved from GenBank or were taken from Hoppe and Sebald (1984). For further discussion, see the text.

sequence and before the radiation of the eukaryotes (Fig. 3). In accordance with the finding that the carboxy terminal helix of the V-ATPase proteolipid is more intimately involved with proton translocation (the carboxylic acid residue thought to participate in the proton passage through the membrane, Glu139, is part of the fourth membrane-spanning helix; Mandel *et al.* 1988), the two amino terminal helices have changed much faster since the duplication event occurred. This high rate of change might reflect the changed, and probably relaxed, selection pressure acting on this portion of the proteolipid.

V-ATPase isoforms

As discussed elsewhere in this volume, V-type ATPases were found to energize a variety of endomembrane-enclosed organelles in eukaryotes (Nelson, 1992), and in some cases these enzymes are also found to be responsible for proton secretion through the plasma membrane (Gluck, 1992; Chatterjee *et al.* 1992). Gene disruption experiments in yeast that led to a complete loss of V-ATPase activity gave no indications for multiple isoforms in *Saccharomyces cerevisiae* (e.g. Umemoto *et al.* 1990; Nuomi *et al.* 1991; Foury, 1990); also, in other fungi only one gene per subunit and per genome has been identified.

In the case of humans and in higher plants, different genes encoding the same subunit type have been reported. Sudhoff *et al.* (1989) reported a cDNA from human kidney encoding the V-ATPase *B* subunit. A different, partial cDNA encoding the same subunit type has been obtained from hippocampal mRNA (Bernasconi *et al.* 1990). The part of the encoded proteins for which both sequences have been determined is 91% identical on the amino acid level. The two isoforms are much more similar to each other than either is to the homologous subunit from plants or fungi. 75–80% identical amino acid residues are found for the comparisons between each of the two human isoforms and the sequences from *Arabidopsis, Neurospora, Candida* and *Saccharomyces*. This suggests that the two isoforms observed in humans evolved during the evolution of the metazoans but that these, probably tissue-specific, isoforms are not characteristic of multicellular eukaryotes in general. In contrast to these reports demonstrating different isoforms for the non-catalytic subunit, Puopolo *et al.* (1991) reported that only a single gene encodes the catalytic *A* subunit in cattle. From these findings it appears that, if different V-ATPase isoforms exist in higher animals, then some subunits can be shared between the different isoforms.

Organelle-specific isoforms in plants

In carrots at least some of the different endomembranes appear to be energized by different V-ATPase isoforms. Carrot cells were transformed *via Agrobacterium*-mediated gene transfer with genes that expressed an antisense mRNA to the *A* subunit of the vacuolar ATPase (Gogarten *et al.* 1992). Constructs utilizing either the coding or the 5′ noncoding region gave the same surprising result: in contrast to the various yeast mutants lacking V-ATPase activity (Nuomi *et al.* 1991), the transformed plant cells grew

without noticeable impairment as callus cultures; plants expressing the antisense constructs were readily regenerated from these cultures.

These transgenic carrot cells still contain vacuolar-type ATPase activity (measured as bafilomycin-sensitive ATPase activity and immuno-detectable *A* subunit), but the level of V-ATPase at the tonoplast (the membrane surrounding the central vacuole) is reduced by more than 70%. The reason that the cells and plants regenerated from these cells grow without noticeable impairment is probably because of the presence of a proton-pumping pyrophosphatase that can provide at least some energization of the tonoplast. However, hexose transport into the vacuoles of these transgenic plant cells, a process that apparently depends on an acidified vacuole (Rausch *et al.* 1987), is greatly impaired (Gogarten *et al.* 1992; J. P. Gogarten, unpublished results). Table 1 depicts results from 3-*O*-methyl [U-^{14}C]glucose (3-OMG) efflux experiments. In the control transformants the bulk of 3-OMG is located inside the vacuole after 20 h of incubation, whereas in the V-ATPase-deficient line most of the 3-OMG remains in the cytoplasm. The higher unidirectional flux from the cytoplasm to the outside in the latter case is due to *trans*-stimulation of this flux by the higher external 3-OMG concentration (Gogarten and Bentrup, 1989). Transport across the tonoplast is greatly reduced, whereas the cytoplasmic concentrations remain nearly unchanged. Note that in both cases the main accumulation occurs across the plasmalemma; the amount of 3-OMG in the vacuole of the controls is larger than the amount in the cytoplasm, but the vacuole has about 10 times the volume of the cytoplasm.

Surprisingly, the V-ATPase activity in Golgi-enriched microsomal fractions was not inhibited but stimulated in both types of antisense-expressing cells. In both cases, the bafilomycin-inhibitable ATPase activity increased by about 30% in the Golgi-enriched fraction.

Western blots of two-dimensional gels probed with a monospecific polyclonal antiserum directed against the *A* subunit revealed two cross-reacting *A* subunit types with different isoelectric points. The concentration of the more acidic isoform did not change in microsomes isolated from the antisense-expressing cell lines, whereas the

Table 1. *3-O-methyl glucose (3-OMG) fluxes and quantities in transgenic carrot suspension cells*

	Q_{cyt} (nmol)	Q_{vac} (nmol)	F_{vc} (pmol min^{-1})	F_{co} (pmol min^{-1})	$C_{outside}$ (μmol l^{-1})
GUS	5.1	15.2	55	278	4
CR	6.3	3.3	11	420	14

Efflux experiments and evaluations were performed as described by Gogarten and Bentrup (1983) after an uptake period of approx. 20 h using 1 g (fresh mass) of cell suspension and an initial external concentration of 25 μmol l^{-1} 3-OMG.

CR, cells expressing an antisense mRNA to the coding region of the *A* subunit; GUS, control transformants expressing the *GUS* gene; Q_{vac}, Q_{cyt}, 3-OMG contents in the vacuoles and cytoplasm, respectively; F_{vc} and F_{co}, unidirectional flux from the vacuole into the cytoplasm and from the cytoplasm to the outside medium; $C_{outside}$, 3-OMG concentration in the external medium at the end of the uptake period and in the efflux medium.

concentration of the more alkaline form was greatly reduced. This more alkaline isoform was the only isoform that was detectable in vacuole preparations from control plants.

In higher plants, two genes encoding the *A* subunit can be characterized by the size of an intervening sequence (Starke *et al.* 1991; T. Starke and J. P. Gogarten, unpublished results). This intron is surprisingly conserved and its unequal size in the two isoform-encoding genes allows their easy detection using the polymerase chain reaction (PCR) across the exon–intron boundaries. This intron and 90 bp of the surrounding exon region

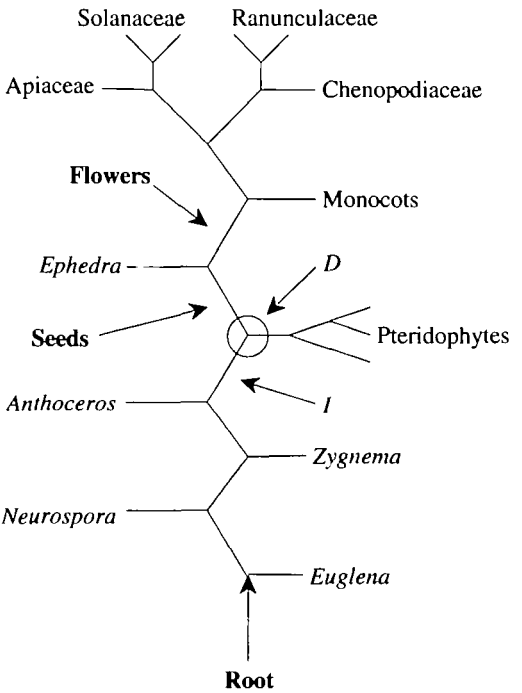

Fig. 4. Schematic representation of the evolution of land plants deduced from partial sequences of the genes encoding the V-ATPase *A* subunit. These gene fragments were amplified using the polymerase chain reaction. Analyses of the sequences showed the insertion of a non-coding sequence (intron) into the coding regions of these particular fragments. The sequence information was employed to construct phylogenetic trees utilizing the maximum likelihood method as implemented by Felsenstein (1988). The root of the tree is inferred by using two archaebacterial sequences (*Sulfolobus acidocaldarius* and *Methanosarcina barkeri*) as outgroup. The arrow labelled *I* indicates the point of the invasion of the gene by the intervening sequence. The circle marked by the arrow *D* locates the duplication event that gave rise to the two *A*-subunit-encoding genes; these two genes are present in the pteridophytes (*Cyathea*, *Psilotum* and *Equisetum*) and in all higher land plants depicted in the tree. The two genes differ in the number of nucleotides contained in the intervening sequence. For further discussion, see the text. The transition to seed plants is indicated. The invention of flowers occurs on the lineage leading to the bifurcation separating the monocotyledonous (*Avena*) and dicotyledonous angiosperms. Within the dicotyledonous lineage, a fork marks the separation of the Apiaceae (*Daucus*) and Solanaceae (*Lycopersicon* and *Nicotiana*) on one side and the Chenopodiaceae (*Chenopodium*) and Ranunculaceae (*Hydrastis* and *Clematis*) on the other.

were used to investigate the origin and evolution of land plants. The analysis of the PCR product revealed that the *A* subunit is encoded by at least two genes in all vascular plants except in *Arabidopsis thalliana*. In addition, all fragments amplified from 13 different land plants (with the exception of the horn moss *Anthoceros*) show the presence of the intervening sequence. This intron is surprisingly conserved throughout at least 400 million years of evolution, suggesting the presence of a functional constraint on the intron sequence. This intervening sequence is also found in *Coleochaete scutata*, a green alga that is considered to be closely related to the higher plants, whereas other green algae (e.g. *Zygnema*), various fungi and *Euglena* have no intron in the corresponding position. These findings suggest that this intron was already present in the ancestor of the land plants. Phylogenies inferred from these intron/exon sequences (see Fig. 4) are in general agreement with phylogenies based on other characters. The duplication that gave rise to the two isoforms, distinguished by the differently sized introns, occurred early in the evolution of land plants. Analyses using parsimony, maximum likelihood and evolutionary parsimony methods point to two duplication events that occurred independently in the lineages leading to the ferns and to the seed plants. However, it is likely that the greater similarity of the two isoforms within these two lineages is due to a gene conversion event that occurred in one of the lineages shortly after a unique duplication event that gave rise to the two isoforms.

These two isoforms are not a characteristic of higher eukaryotes in general; their presence appears to be restricted to higher land plants.

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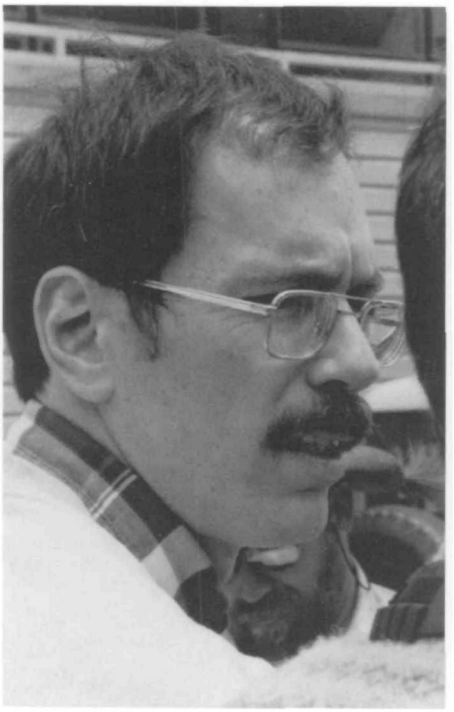
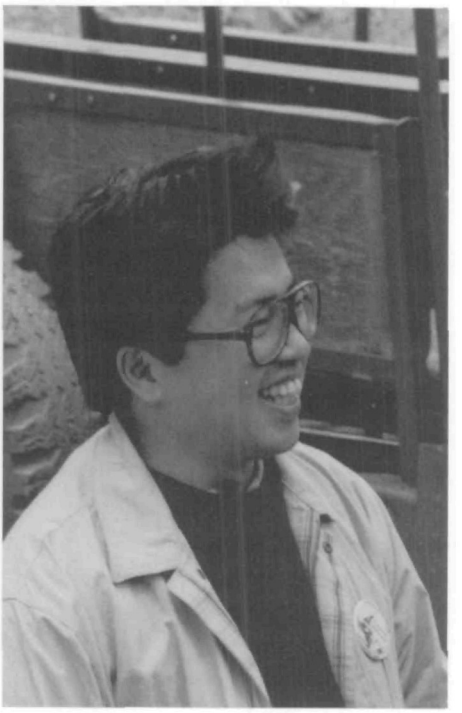
CHAPTER 4. Biochemistry of mammalian endomembranes



M. Forgac



Y. Moriyama