Molecular Evolution of Voltage-sensitive Ion Channel Genes: On the Origins of Electrical Excitability[1]

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We have analyzed nucleic acid and amino acid sequence alignments of a variety of voltage-sensitive ion channels, using several methods for phylogenetic tree reconstruction. Ancient duplications within this family gave rise to three distantly related groups, one consisting of the Na^+ and Ca^{++} channels, another the K^+ channels, and a third including the cyclic nucleotide-binding channels. A series of gene duplications produced at least seven mammalian homologues of the *Drosophila Shaker* K^+ channel; clones of only three of these genes are available from all three mammalian species examined (mouse, rat, and human), pointing to specific genes that have yet to be recovered in one or another of these species. The *Shaw*-related K^+ channels and the Na^+ channel family have also undergone considerable expansion in mammals, relative to flies. These expansions presumably reflect the needs of the high degree of physiological and neuronal complexity of mammals. Analysis of the separate domains of the four-domain channels (Ca^{++} and Na^+) supports their having evolved by two sequential gene duplications and implies the historical existence of a functional two-domain channel.

Introduction

Ion channels are membrane proteins that allow the rapid, passive passage of ions through a pore across an otherwise poorly permeable lipid bilayer. Ion transport through open channels is at least three orders of magnitude faster than that effected by pumps and carrier proteins and is 11 orders of magnitude faster than diffusion across a lipid bilayer (Moczydlowski 1986). Ion channels are involved in many physiological processes, including maintenance of membrane potential, regulation of electrical excitability, and modulation of hormone and neurotransmitter secretion (for a recent review, see Hille 1992). Electrophysiological studies have revealed the existence of a wide variety of ion channels that can be distinguished on the basis of their ion selectivity, voltage dependence, ionic conductance, inactivation, and sensitivity to blocking agents. Ion channels can generally be grouped into two major classes, ligand-gated and voltage-gated channels. Ligand-gated channels may be regulated by Ca^{++}, by nucleotides, or by neurotransmitters, whereas voltage-gated channels respond to changes in the membrane potential. Since this distinction is not absolute (e.g., ligand-gated channels may also be sensitive to voltage), we use "voltage-sensitive" as a more inclusive term.

In recent years, genes and transcripts for a large variety of ion channel proteins have been isolated and characterized. The first channel to be sequenced was the Na

1. Key words: ion channels, multigene family, K^+, Na^+, Ca^{++}, cGMP and cAMP channels, parsimony, weighted parsimony, distance matrix, likelihood, neighbor joining, phylogeny.

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Mol. Biol. Evol. 10(1):221–242. 1993.

0737-4038/93/1001-0013$02.00

channel from the electric organ of the electric eel *Electrophorus electroplax* (Noda et al. 1984), followed by the Ca^{++} channel from rabbit skeletal muscle (Tanabe et al. 1987). Soon thereafter, the first K^+ channel was characterized from the *Shaker* behavioral mutant in *Drosophila* (Kamb et al. 1987; Papazian et al. 1987; Pongs et al. 1988; Schwarz et al. 1988). Analysis of these primary sequences revealed organizational and structural similarities suggesting evolutionary relatedness (Catterall 1988; Jan and Jan 1990).

The Na^+ and Ca^{++} channels are thought to consist of four homologous domains, each domain containing six transmembrane segments, whereas the K^+ channel contains a single such domain (Noda et al. 1984; Catterall 1986, 1988; Baumann et al. 1988; Tempel et al. 1988; Jan and Jan 1990). A schematic diagram of the putative structures of K^+ and Na^+ channels is presented at the top of figure 1, which shows the six membrane-spanning domains of the K^+ (and cNMP) channels, and it incorporates the recent evidence that the S5/S6 extracellular loop of K^+ channels may participate in the formation of the channel pore (Hartmann et al. 1991; Yellen et al. 1991; Yool and Schwarz 1991). The Na^+ channel (and the related Ca^{++} channel) is shown at the bottom of the figure, as a tetramer of this basic structure.

One highly conserved feature of all these proteins is the presence of a highly charged region in the fourth putative transmembrane segment (S4), every third residue having a positively charged side chain. Substitution of neutral residues for these charged amino acids in Na^+ and K^+ channels has been shown to result in an alteration in voltage dependence (Stuhmer et al. 1989*a;* Lopez et al. 1991; Papazian et al. 1991); by extension, these mutational experiments suggest that the S4 segment is involved in sensing the membrane voltage in *all* voltage-sensitive channels.

There has been considerable interest both in the distribution of ion channels in prokaryotes, protozoa (*Paramecium* and algae), yeast, vascular plants, coelenterates,

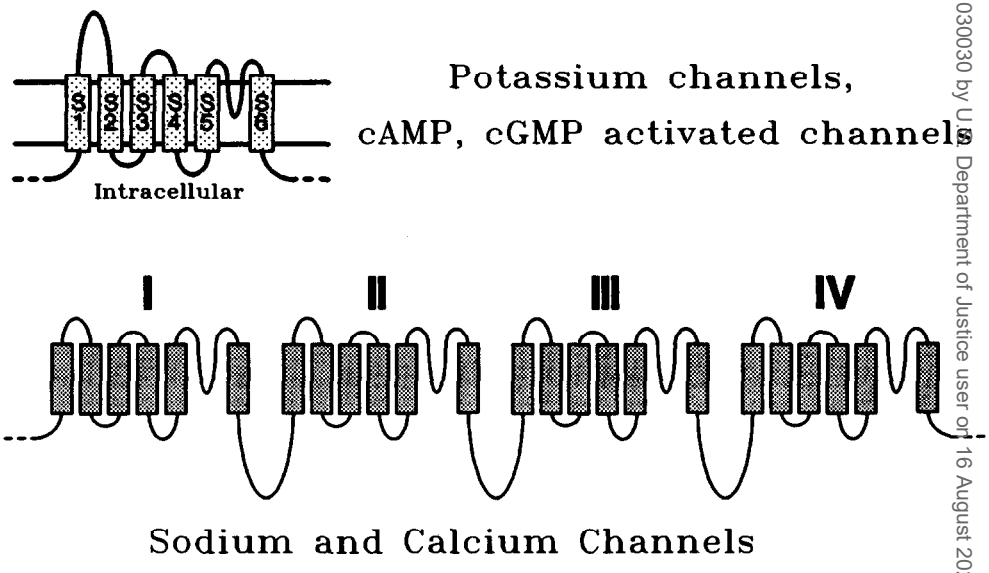

FIG. 1.—Schematic diagram of the structure of ion channels. The K^+ and cyclic nucleotide gated channels are thought to consist of six transmembrane segments constituting a single domain. Na^+ and Ca^{++} channels have four such domains linked together in a single large polypeptide. The six putative membrane-spanning segments are indicated as S1–S6. Recent evidence that the extracellular loop between S5 and S6 of K^+ channels reenters the cell membrane is reflected in this diagram.

nematodes, arthropods, mollusks, and a variety of vertebrates (Hagiwara 1983, passim; Hille 1984, pp. 371–383, 1987, 1988, 1989, 1992, pp. 525–544; Franciolini and Petris 1989; Mackie 1990; Arbas et al. 1991; Walker and Holden-Dye 1991) and in its evolutionary implications. In the present work, we analyze the molecular evolutionary relationships between various members of the multigene family of voltage-sensitive ion channels, deduced from comparisons of nucleotide and amino acid sequences by applying a variety of tree reconstruction techniques. With these results we discuss the patterns of ion channel diversity in the context of genetic and electrophysiological data.

Material and Methods

Sequences and Alignments

Table 1 lists the sequences used in this study, as well as their sources; we use the recently proposed standard nomenclature for the voltage-gated K^+ channels (see Chandy et al. 1991). Sequences were aligned manually with the aid of the editing and dot-matrix capabilities of the SnAP (Sequence Analysis Programs) package of computer programs. The aligned sequences were then used as input by the program MAKDAT, which creates output files suitable for input to various phylogenetic analysis programs (see below), optionally removing identical or uninformative positions. Some tree reconstruction methods allow the user to provide a value for the ratio of transition to transversion substitutions. Since the true value tends to be obscured with increasing time of divergence (Brown et al. 1982), we determined this ratio to be close to 2.0 (by using the program TRANS) for the most closely related homologues of K^+ channel genes available, those of rat and mouse, and we used this value when it was required. The programs SnAP, MAKDAT, and TRANS, all written for PC-compatible systems, are available from the authors on request. The sequence alignments described below have been submitted to the EMBL data base and can be obtained by sending an electronic mail message to the Internet address NETSERV@EMBL-HEIDELBERG.DE, with the message line(s) GET ALIGN:⟨submission number⟩.

S4 Segments (Submission Number DS10831)

We aligned the nucleotide sequences of 30 S4 transmembrane segments, on the basis of the amino acid sequence alignments published by Jan and Jan (1990). These consisted of the S4 segments of each of the four domains of three Na^+ (NaI, NaII, and NaIII) and three Ca^{++} (CaHt, CaSk, and CaBr) channels and of the single S4 segment of two cyclic nucleotide-binding channels (cAMP and cGMP) and four *Drosophila* K^+ channels (*Shaker, Shab, Shaw,* and *Shal*).

K^+ Channel Family (Submission Number DS10819)

We aligned portions of the amino acid sequences of four *Drosophila* K^+ channel genes (*Shaker, Shab, Shaw,* and *Shal*), together with four of their mammalian homologues (mKv1.1, rKv2.1, rKv3.1, and rKv4.2) and used these to create an alignment of the corresponding nucleotide sequences. We included the region beginning 120 codons upstream of the S1 transmembrane segment and continuing through the hydrophobic core region to 8 codons past the end of the last putative transmembrane segment, S6. The extracellular loops between S1 and S2 and between S3 and S4 were essentially nonalignable and were omitted, as was a region of 25–58 residues in the region 5′ of S1.

Table 1
Sequences Used in Present Study

Name	Comment[a]	GenBank/EMBL Accession Number	Reference
K^+ channels:[b]			
Shaker	*Drosophila*	M17211	Papazian et al. 1987
Shab	*Drosophila*	M32659	Wei et al. 1990
Shal	*Drosophila*	M32660	Wei et al. 1990
Shaw	*Drosophila*	M32661	Wei et al. 1990
XSha2	*Xenopus*	M35664	Ribera 1990
mKv1.1	Mouse, MK1	M30439	Chandy et al. 1990*b*
rKv1.1	Rat, RBK1	M26161	Christie et al. 1989
hKv1.1	Human, HK1	L02750	Ramashwami et al. 1990
mKv1.2	Mouse, MK2	M30440	Chandy et al. 1990*b*
rKv1.2	Rat, RCK5	X16003	Stuhmer et al. 1989*b*
hKv1.2	Human, HK4	L02752	Ramashwami et al. 1990
mKv1.3	Mouse, MK3	M30441	Chandy et al. 1990*b*
rKv1.3	Rat, RCK3	X16001	Stuhmer et al. 1989*b*
hKv1.3	Human, HGK5	M38217	Cai et al. 1992
rKv1.4	Rat, RCK4	X16002	Stuhmer et al. 1989*b*
hKv1.4	Human, HK2	L02751	Ramashwami et al. 1990
rKv1.5	Rat, KV1	M27158	Swanson et al. 1990
hKv1.5	Human, HPCN1	M55513	Philipson et al. 1991
rKv1.6	Rat, RCK2	X17621	Grupe et al. 1990
hKv1.6	Human, HBK2	X17622	Grupe et al. 1990
mKv1.7	Mouse, MK4	. . .	Chandy et al. 1990*a*
rKv2.1	Rat, DRK1	X16476	Frech et al. 1989
mKv3.1	Mouse, NGK2	Y07521	Yokoyama et al. 1989
rKv3.2	Rat, RKSHIIIA	M34052	McCormack et al. 1990
mKv3.3	Mouse, MK5	X60796-7	Ghanshani et al. 1992
mKv3.4	Mouse, MK6	M81253	Ghanshani et al. 1992
rKv4.2	Rat, RK5	M59980	Roberds and Tamkun 1991
Na^+ channels:			
NaI	Rat brain	X03638	Noda et al. 1986
NaII	Rat brain	X03639	Noda et al. 1986
NaIII	Rat brain	Y00766	Kayano et al. 1988
NaeeII	Eel electroplax	X01119	Noda et al. 1984
NaSkI	Rat skeletal muscle	M26643	Trimmer et al. 1989
NaHtI	Rat heart	M27902	Rogart et al. 1989
Para	*Drosophila*	M32078	Loughney et al. 1989
DSC1	*Drosophila*	Y00461	Salkoff et al. 1987
Ca^{++} channels:			
CaHt	Rabbit heart	X15539	Mikami et al. 1989
CaSk	Rabbit skeletal muscle	X05921	Tanabe et al. 1987
CaBr	Rabbit brain	X57476	Mori et al. 1991
Cyclic nucleotide-binding channels:			
cAMP	Bovine olfactory epithelium	. . .	Ludwig et al. 1990
cGMP	Bovine rod photoreceptor	X51604	Kaupp et al. 1989

[a] Source and/or synonym.
[b] Names of the mammalian channels follow the proposed standard nomenclature (Chandy et al. 1991).

Mammalian Shaker- *and* Shaw-*related Genes*

Our alignment of portions of the nucleotide sequences of the *Drosophila Shaker* gene with its vertebrate homologues (16 mammalian genes, one from *Xenopus;* see table 1; submission number DS10832) begins 138 codons upstream of the putative S1 transmembrane segment and continues through the core region to 16 codons past the end of S6; the extracellular loop between S1 and S2, as well as a portion of the S3/S4 loop, were omitted. Our nucleotide sequence alignment of five *Shaw*-related genes (*Shaw,* mKv3.1, rKv3.2, mKv3.3, and mKv3.4; submission number DS10830) begins 169 codons upstream of the S1 segment and continues through the core region to include 60 codons past the S6 segment.

Na^+ Channel Family (Submission Number DS10833)

We aligned the amino acid sequences of each of the four domains of Na^+ channel genes from *Drosophila* (Para1 and DSC1), eel (NaeelI), and mammals (NaI, NaII, NaIII, NaSk, and NaHt), beginning 149 residues upstream of the S1 segment of domain I, omitting portions of the domain I/II and domain II/III loops, and ending 154 residues past the S6 segment of domain IV.

Domains of the Ca^{++} and Na^+ Channels

We used the published amino acid alignment (Jan and Jan 1990) of the seven conserved segments, S1–S6 and H5, of each of the four domains of three Na^+ channels (NaI, NaII, and NaIII) and of each of two Ca^{++} channels (CaSk and CaHt).

Phylogeny Reconstruction

A variety of tree reconstruction methods exist for analysis of molecular sequence data, including ones based on pairwise distances, parsimony, evolutionary invariants, and maximum likelihood (for a recent review, see Swofford and Olsen 1990). If all methods yield the same topology with a given data set and with its subsets, then the choice of the "best" tree is clear. Since this situation is not always the case, however, we adopted two principles to choose among competing topologies. First, a topology preferred by several different methods was chosen over one preferred by only one or another method. Second, a given topology was regarded with greater confidence if it was supported by analysis of an increasingly larger data set (i.e., on addition of more homologous sequences).

Parsimony

We used version 3.0 of PAUP (Swofford 1990) running on a Macintosh computer to carry out both nucleic acid and protein parsimony analyses. Although some limitations of the parsimony approach have been described (Felsenstein 1978), its simplicity is intuitively attractive, alternative trees can be directly compared with one another, and existing computer implementations (including PAUP) are capable of determining the optimum tree even for fairly large data sets (Swofford and Olsen 1990). Whenever possible we have used PAUP's "branch and bound" option (B&B), which is guaranteed to discover the shortest tree, but, in some data sets for which B&B was not feasible because of time constraints, a number of options were used to explore as many trees as possible by heuristic searching, including randomizing the order of taxon joining and the use of different branch-swapping routines. PAUP was

also used to carry out parsimony "bootstrapping" and to carry out analyses using the method of evolutionary invariants (EI; Lake 1987).

Weighted Parsimony (Williams and Fitch 1989, 1990)

The WTDPARS program, provided and modified for our use by Walter Fitch (University of California, Irvine), evaluates trees by a parsimony-based analysis of nucleotide sequences but carries out a dynamic weighting process that takes into account the well-known fact that different positions within homologous sequences may accumulate changes at different rates (substitutions in the third position of codons, as one example, generally accumulate at higher rates than do those in the first and second positions). While the lengths associated with competing topologies can still be directly compared, the increased computational demands mean that an exhaustive search of the best topology can rarely be executed. Our analyses were carried out with a variety of initial user-input trees, and searches as exhaustive as possible were done. Direct comparisons of particular trees of interest were generated using an option that disables branch swapping.

Maximum Likelihood (Felsenstein 1989)

The PHYLIP program, version 3.41, running under MS/DOS, was provided by Joe Felsenstein (University of Washington). A major advantage of maximum-likelihood methods is that there exists a statistical basis for comparison of competing topologies; however, the extensive computation required greatly limits the number of trees that may be examined. We used the DNAML program of PHYLIP, having modified it to accommodate larger data sets. As required by the assumptions of this method, we did not remove identical or uninformative positions from the data sets.

Neighbor Joining (Saitou and Nei 1987)

A program that implements the neighbor-joining (NJ) method, written by L. Jin and J. W. H. Ferguson (University of Texas Health Sciences Center at Houston), was provided by S. Kumar (The Pennsylvania State University). This method, which is based on a pairwise distance matrix, can provide a rapid analysis of even very large data sets. A major limitation, however, is that its algorithm generates only a single tree for a given data set and does not provide a comparison of competing topologies. We used MAKDAT to generate distance matrices for input to NJ in which transversions were assigned twice the weight of transitions.

Displayed Trees

All the trees presented in this study (shown in figs. 2–7) were drawn using the graphics capabilities of PAUP, followed by manual editing; thus, all branch lengths (under "accelerated transformation" optimization), as well as bootstrap values, reflect PAUP's parsimony values, even though other methods (discussed in the text) will have contributed to the choice of a favored topology. In those cases in which uninformative positions had been removed for analysis by PAUP (figs. 3–5), these positions were restored before determination of the branch lengths.

Results

A Voltage-sensitive Ion Channel Superfamily

To examine the ancient divisions of the voltage-sensitive ion channel multigene superfamily (including K^+, Na^+, Ca^{++}, and cNMP channels), we have analyzed the

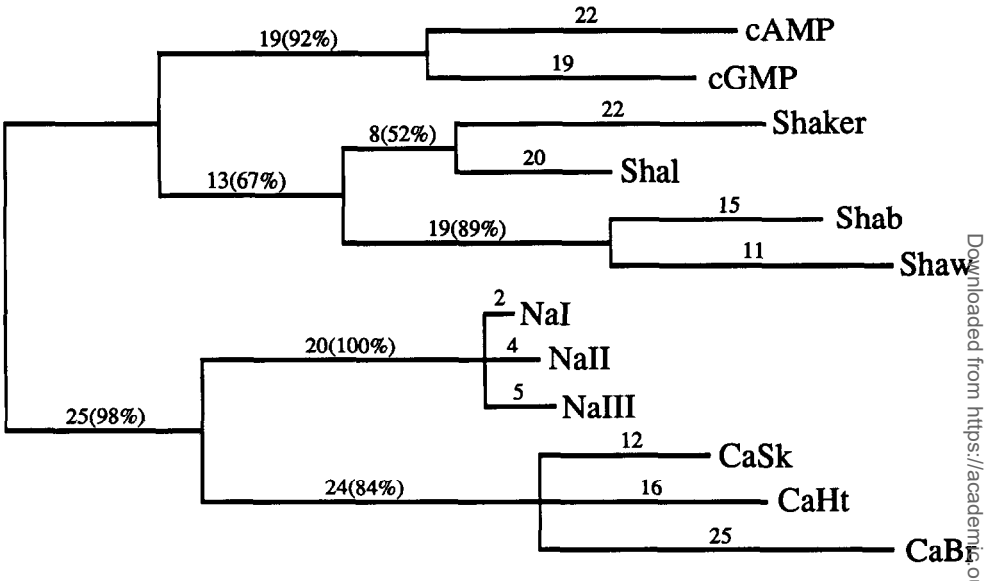

FIG. 2.—Voltage-sensitive ion channel superfamily: phylogenetic tree based on comparison of nucleotide sequences of S4 segments only. Included are two bovine cNMP-binding channels (cAMP and cGMP), four *Drosophila* K^+ channels (Shaker, Shal, Shab, and Shaw), three rat brain Na^+ channels (NaI, NaII, and NaIII), and three rabbit Ca^+ channels, one each from skeletal muscle (CaSk), heart muscle (CaHt), and brain (CaBr). The indicated branch lengths (and bootstrap values in parentheses) were generated by PAUP using domain II of the Na^+ and Ca^{++} channels, although analyses by other methods also supported this topology (see text). The total tree length is 301 substitutions.

only region for which we felt we could make a satisfactory alignment for all these genes—namely, the S4 segments. We recognize that using such a small region may not yield very robust results, because it represents such a small proportion of the entire length of these genes.

The tree presented in figure 2 shows three monophyletic groups, one consisting of the K^+ channels, another of the Na^+ and Ca^{++} channels, and the third including the two cNMP-gated channels; within the Na^+/Ca^{++} group, the Na^+ channels and Ca^{++} channels each cluster into their own groups. We favor this topology, following analysis by the several methods discussed below. The relationships within the Na^+ channel group are discussed later, as are the relationships between the four major K channels and the problem of establishing their rooting.

Since the Na^+ and Ca^{++} channels each have four different S4 segments, we analyzed separate trees by using each one in turn, as well as combinations of them. In each case PAUP (heuristic search) found a single most parsimonious tree (of length 293–308) and a total of four to seven near-parsimonious trees (i.e., within two substitutions of the best tree). The relationships within the Na^+ and Ca^{++} groups were not well resolved, and each group is therefore shown as trifurcating. For domain II, the most parsimonious tree (length 301) has the topology shown in figure 2; this same topology was included among the near-parsimonious trees by using domains I and III but not by using domain IV. Other near-parsimonious trees break up or vary the rooting of the K^+ group, but *all* keep the Na^+ and Ca^{++} channels together, as well as the two cNMP-binding channels.

The best tree found by WTDPARS using domains I, II, or IV also had the topology

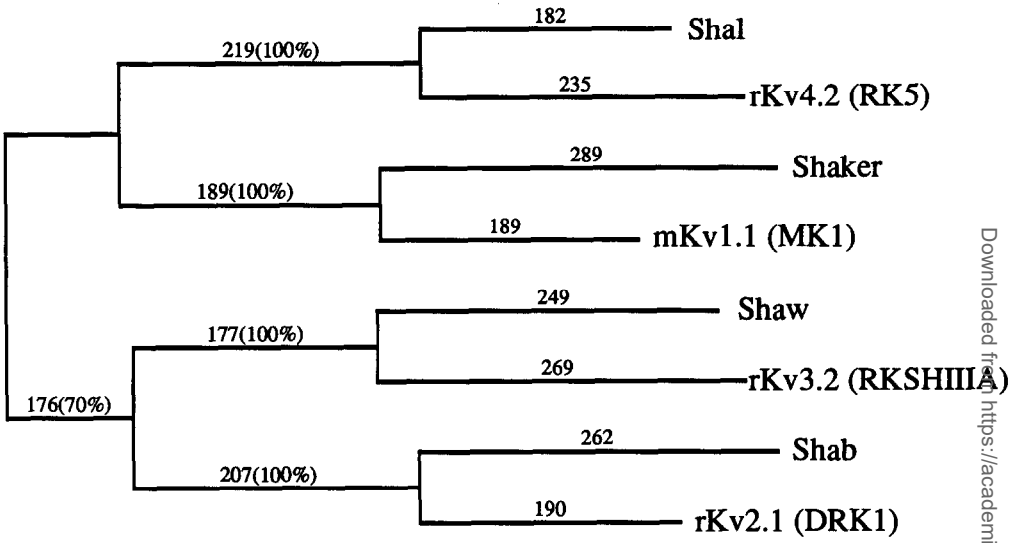

FIG. 3.—Four major K^+ channel lineages: phylogenetic tree based on comparison of nucleotide sequences of four *Drosophila* genes (Shaker, Shal, Shab, and Shaw) and four of their mammalian homologues. The indicated branch lengths (and bootstrap values in parentheses) were generated by PAUP, although analyses by other methods also supported this topology (see text). The total tree length is 2,833 substitutions.

shown in figure 2; several near-best trees differed only in the rooting of the K^+ channel group. However, using domain III alone resulted in a tree in which the Na^+ and Ca^{++} channels are broken up by cNMP and K^+ channels. With the exception of the rooting of the K^+ channel group, NJ yielded the topology shown when domains I or II (but not III or IV) were used.

Thus, despite the exceptions generated by one or another of the four domains analyzed separately, the several methods tend to converge on the topology shown in figure 2. The weakness of the indicated rooting of the K^+ channel group, shown on its central branch, is discussed further below.

Ancient Divisions within the K^+ Channel Family

We analyzed the nucleotide sequences of the *Shaker, Shal, Shab,* and *Shaw* genes of *Drosophila,* together with four of their mammalian homologues, aligned over an extensive region within the hydrophobic core of these proteins (see Material and Methods). This analysis yielded the unrooted tree shown in figure 3, which places each pair of fly and mammal homologues together and makes neighbors of *Shab* and *Shaw,* on the one hand, and of *Shaker* and *Shal,* on the other hand. WTDPARS, NJ, DNAML, and PAUP (B&B) *all* prefer this topology over the two alternative arrangements of the four groups (an unrooted tree with four taxa has only three possible topologies).

Using *only* the four fly genes or *only* the mammalian genes yielded a variety of preferred trees different from that shown with WTDPARS, DNAML, and NJ; PAUP still preferred this topology with the mammals alone, but not with the flies. EI applied to the fly sequences alone preferred the same tree, although it did not achieve statistical significance ($P = 0.08$ for this tree compared with 0.34 and 0.75 for the two alternatives); EI applied to the mammalian sequences alone did not distinguish between the three possibilities ($P > 0.25$ for all three).

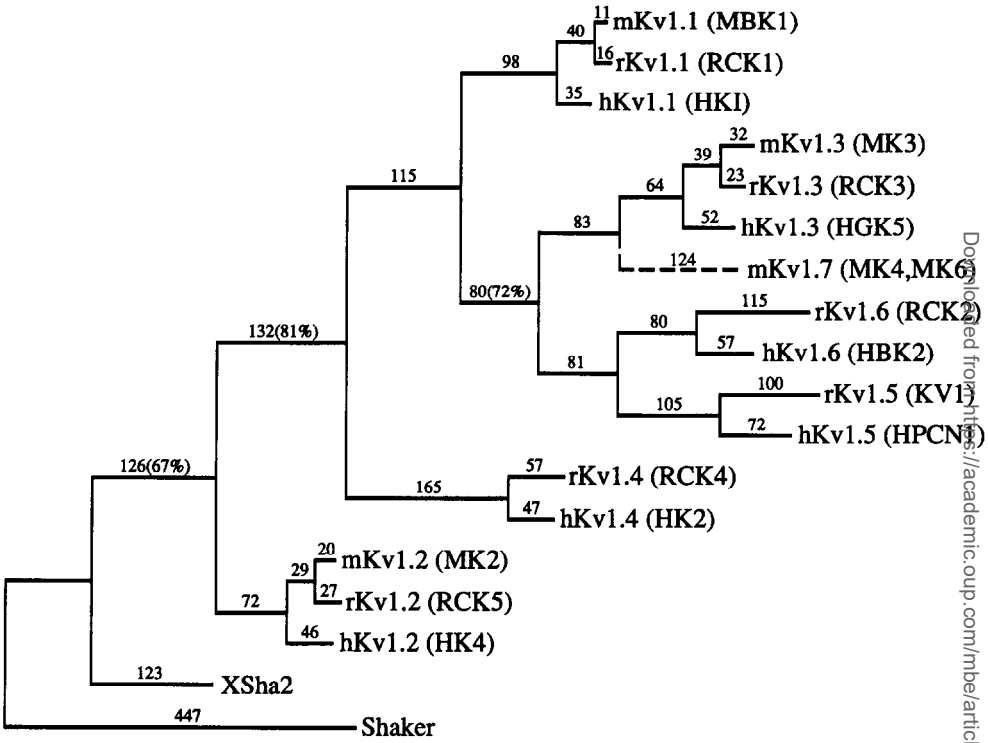

FIG. 4.—Expansion of the mammalian *Shaker*-related gene family: phylogenetic tree based on comparison of nucleotide sequences of the *Drosophila Shaker* gene with 17 of its vertebrate homologues. Human, rat, and mouse genes are identified by the first letter of their names, which conform to the proposed standard nomenclature (see Chandy et al. 1991), while names in parentheses are older synonyms; XSha2-*Xenopus* K^+ channel gene. The indicated branch lengths were generated by PAUP, and the dotted line leading to mKv1.7 (MK4,MK6) indicates that its position in this tree was not strongly established by any method. Bootstrap analyses by PAUP (with mKv1.7 omitted) showed most branchings present in 96%–100% of trials, except for those three in which bootstrap values are given in parentheses; other methods, however, also support this topology. The total tree length is 2,713 substitutions.

That the topology shown in figure 3 was uniformly preferred with the larger number of sequences strengthens our confidence in its reliability. It should also be noted that *all* of the preferred trees generated by our analyses of the S4 segments (discussed above) made neighbors of *Shab* and *Shaw,* even those trees in which the *Shaker*/*Shal* pair was broken up by other channel genes.

We have not established a satisfactory rooting for the K^+ channel family. The S4 tree in figure 2 shows the root on the central branch of the K^+ channel group, but making *Shaker* a sister taxon of *Shab* and *Shaw* (in effect rooting this group on the *Shal* branch) yields an only marginally longer tree. Even with the combination of fly and mammalian genes, our attempts to establish a root by using S4 segments from any of the other channel genes failed to give compelling results, and the indicated root must be considered tentative.

Expansion of the Mammalian *Shaker*-related Gene Subfamily

Analyzing the nucleotide sequence of *Shaker* together with those of 17 of its known homologues in rat, mouse, human, and frog yielded the unrooted tree shown

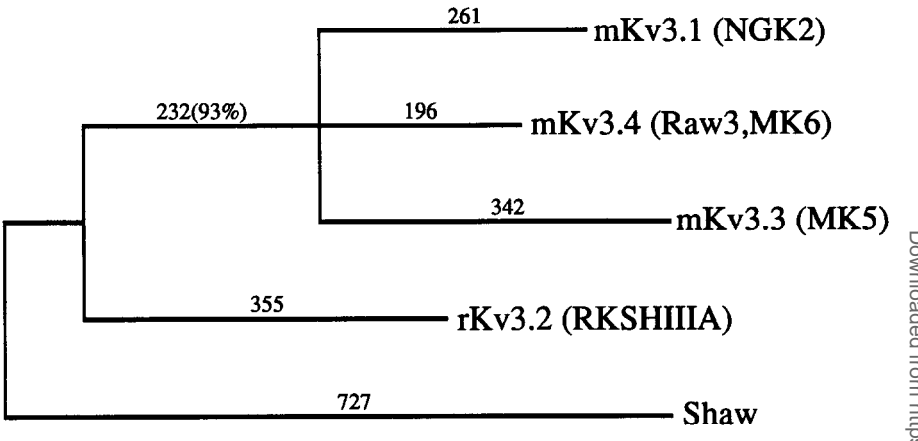

FIG. 5.—Mammalian *Shaw*-related gene family: phylogenetic tree based on comparison of nucleotide sequences of the *Drosophila Shaw* gene with four of its mammalian homologues. The indicated branch lengths (and the bootstrap value) were generated by PAUP. The total tree length is 2,113 substitutions.

in figure 4. The topology shown was the best one found by WTDPARS, PAUP (heuristic), and DNAML. Analysis using smaller numbers of mammalian homologues (*Shaker* and XSha2, with rat, mouse, or human sequences alone) supported this topology more weakly (as determined by bootstrap analysis), and addition of the multiple homologues made the displayed topology progressively more robust. If Kv1.7 is excluded from consideration (see below), parsimony bootstrap analysis (PAUP) supports all bifurcations at the 96% level or greater, except for the three branches with indicated bootstrap values (67%–81%); all branches, it should be emphasized, are also supported by the other methods of analysis mentioned above. Although the major features of this tree (discussed below) are clearly robust, the Kv1.7 gene can be placed at any one of several alternative positions, with few additional substitutions required, when it is analyzed by any of the methods; the weakness of its position in this tree is indicated by the dotted line.

From this unrooted tree it is apparent that the *Shaker*-related gene family in mammals is the result of an extensive set of gene duplications that have occurred since their divergence from flies. The single *Shaker* gene in *Drosophila* is represented by at least seven distinct mammalian homologues (Kv1.1–Kv1.7).

A Mammalian *Shaw*-related K^+ Channel Subfamily

Figure 5 shows the result of a parsimony bootstrap analysis (using PAUP) of the *Drosophila Shaw* gene and four mammalian *Shaw*-related sequences. The fact that the rat gene rKv3.2 fails to cluster with any one of the three mouse genes, which is also a feature of the trees preferred by WTDPARS, DNAML, and NJ, indicates the existence of at least four distinct *Shaw* homologues in mammals. The limited data set did not allow us to satisfactorily resolve the branching pattern of the three mouse genes that are shown trifurcating, with WTDPARS, DNAML, and PAUP (B&B) yielding only weak and conflicting results in this regard; the tree produced by NJ showed mKv3.4 branching off before the other two mouse genes. Analysis of multiple mammalian homologues of each of these genes may help to resolve their relationships.

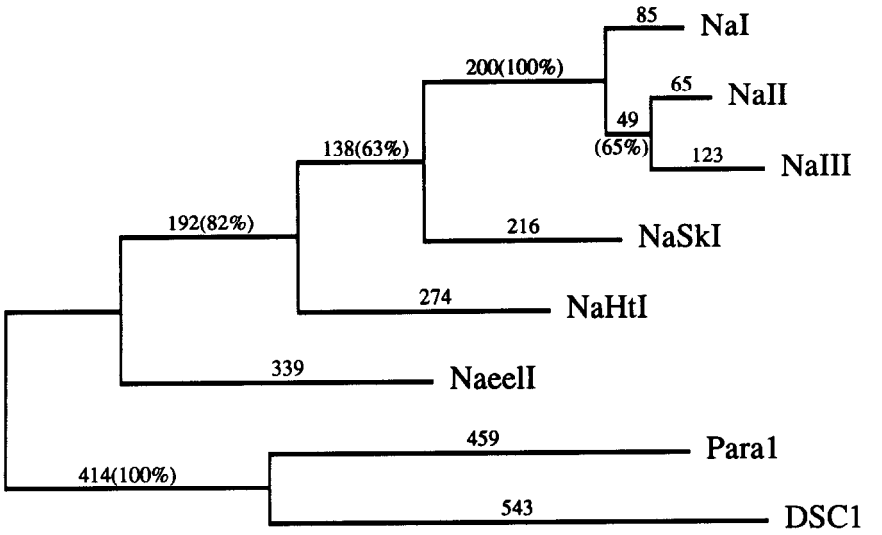

FIG. 6.—Na^+ channel family: most parsimonious tree based on amino acid sequence alignment of two Na^+ channel proteins from *Drosophila* (Para1 and DSC1), one from eel (NaeelI), three expressed in mammalian brain (NaI, NaII, and NaIII), and one each in mammalian skeletal muscle (NaSkI) and mammalian heart muscle (NaHtI). Branch lengths (and bootstrap values in parentheses) generated by PAUP are indicated. The total tree length is 3,097 substitutions.

The Na^+ Channel Family

Several Na^+ channel genes have been characterized in vertebrates as well as in *Drosophila,* and we analyzed an alignment of the amino acid sequences of eight of these channels. Using the protein parsimony method implemented in PAUP (heuristic search), we generated the unrooted tree shown in figure 6, which shows the five mammalian genes clustered in a single group. While this is the preferred topology, this tree is shorter by only five substitutions than the next best tree, which transposes the heart and skeletal genes. The monophyletic grouping of the five mammalian genes is fairly robust (82% bootstrap), and the clustering of the three brain channels (NaI, NaII, and NaIII) is even more so (100% bootstrap). This branching pattern suggests that one series of duplications generated the five Na^+ channel genes known in mammals, while an independent duplication yielded the two *Drosophila* genes. The eel channel branches off outside the mammalian cluster, suggesting that the duplication of the mammalian genes occurred after the mammal/fish split. A tunicate Na^+ channel gene has recently been characterized (Okamura et al. 1991), which the authors showed to branch off their tree between fly and eel.

Domains of Ca^{++} and Na^+ Channels

While the cyclic nucleotide and K^+ channel genes each encode a single domain that contains the S1–S6 segments, the Na^+ and Ca^{++} channel genes have four such domains repeated in tandem. To examine the relationship between these four domains, we analyzed alignments of their amino acid sequences by using PAUP protein parsimony (heuristic search). The unrooted topology shown in figure 7 was favored with several different combinations of Na^+ and Ca^{++} channel genes (NaI/NaII/CaSk, NaI/CaSk, NaII/CaSk, NaI/CaHt, and NaII/CaHt), although only the NaI and CaSk

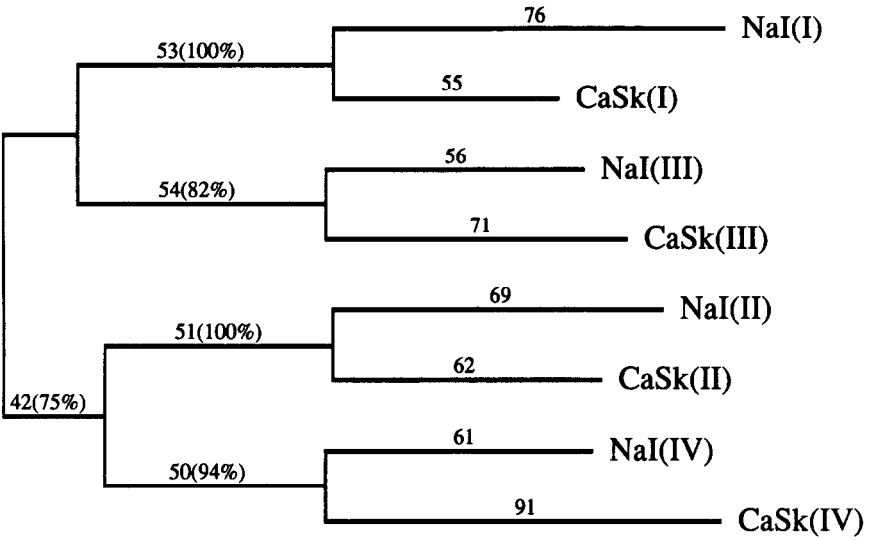

FIG. 7.—Relationships between the four domains of Ca^{++} and Na^{+} channels: most parsimonious tree based on amino acid sequence alignment. Roman numerals in parenthesis indicate each of the four domains within each channel protein. The total tree length is 791 substitutions.

genes are shown. This topology places domains I and III, on the one hand, and domains II and IV, on the other hand, into monophyletic groups, and the tree is drawn as if it were rooted centrally to these monophyletic groups; the implications of this pattern are discussed below. However, *no* pair of channels resulted in a best tree shorter than its nearest competitor by more than a few substitutions. Nevertheless, a parsimony bootstrap analysis (PAUP) using NaI and CaSk supports this topology at the 75% level.

Discussion

A Voltage-sensitive Ion Channel Superfamily

The tree in figure 2 shows the putative phylogenetic relationships of an ion channel gene superfamily. The Na^{+} and Ca^{++} channels appear to have arisen as a separate lineage from that of the cyclic nucleotide channels, on the one hand, and the voltage-gated K^{+} channels, on the other hand. While this tree was the result of analysis of aligned sequences of the S4 regions only, this grouping is consistent with the known sequence similarities that exist within each group outside the S4 region, as well as with structural features (one- vs. four-domain organization) and functional aspects (ion selectivity and cyclic nucleotide binding) of these ion channels.

Additional major lineages of the voltage-sensitive ion channel family have recently been characterized. The *Drosophila* genes encoding the behavioral mutants "slowpoke" (*slo*) (Atkinson et al. 1991) and "ether-a-go-go" (*eag*) (Warmke et al. 1991) and two Arabidopsis genes, KAT1 (Anderson et al. 1992) and AKT1 (Sentenac et al. 1992), appear only distantly related to *Shaker, Shab, Shal,* and *Shaw* (Guy et al. 1991, and personal communication). Two other mammalian genes, K13 and IK8 (Drewe et al. 1991), only distantly related to each other and to the four *Drosophila* genes, clearly extend the family of voltage-sensitive K^{+} channels. Thus it appears that at least two additional families of K^{+} channel genes exist in mammals, although no *Drosophila* homologues have yet been found.

Duplication of an Ancestral Single-Domain Channel Gene

Na^+ and Ca^{++} channels each consist of four homologous domains, while K^+ and cyclic nucleotide-binding channel genes each consist of a single such domain. On the basis of electrophysiological data and the sequence similarities of each domain of the Na^+ channel to the corresponding domain of the Ca^{++} channel, Hille (1989) hypothesized that a common ancestral single-domain channel gene gave rise to the primordial Ca^{++} channel by two intragenic duplications and then gave rise to the Na^+ channel by further divergence following gene duplication. The tree presented in figure 7 supports this scheme. As diagramed in figure 8, a primordial single-domain gene underwent an internal duplication to create a two-domain structure; following sequence divergence of the two domains, a second internal duplication gave rise to the four-domain structure known today. Hence, domains I and III, as well as domains II and IV, remain more similar to each other as a result of their evolutionary history, and these relationships are reflected in the topology of the tree in figure 7. One implication of this scheme is that an ancestral two-domain peptide was capable of producing a functional channel protein, since it existed long enough both for substantial sequence divergence to occur and to give rise to the four-domain structure by an additional duplication. In fact, Isacoff et al. (1990) showed that head-to-tail dimers of fly and mammalian K^+ channel proteins are capable of forming functional and diverse channels.

What Was the Nature of the Na^+ and Ca^{++} Channel Ancestor?

Voltage clamp experiments on cells of protozoa (*Paramecium*) and plants have revealed the presence of both Ca^{++} and K^+ channels (Kung 1989; Tester 1990), while channels selective for Na^+ appear to be absent in those organisms that diverged early in the eukaryotic radiation. It is interesting that Na^+ channels with pharmacological

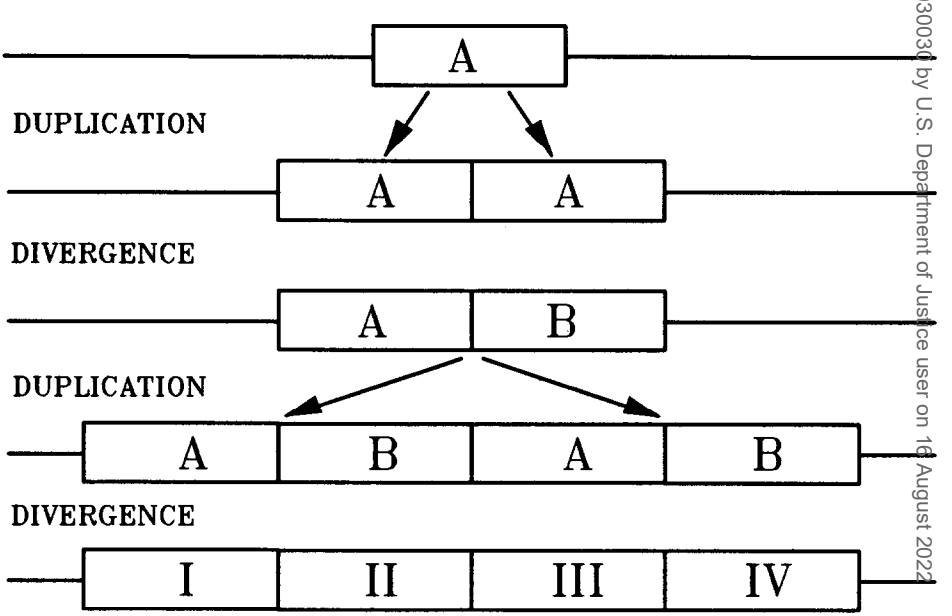

FIG. 8.—Schematic diagram of the intragenic duplications presumed to have given rise to the modern Ca^{++} and Na^+ channel genes (see text).

properties similar to those of Ca^{++} channels can be found in the cells of a coelenterate (Anderson 1989). Collectively, these data suggest that the first four-domain channel was a Ca^{++} channel and that the Na^{+} channel subsequently evolved from the Ca^{++} channel after gene duplication.

What Was the Nature of the Single-Domain Ancestor of Na^{+}/Ca^{++} and K^{+} Channels?

Escherichia coli expresses a voltage-sensitive cation-selective ion channel (Delcour et al. 1989; Kung 1989), while plants, as well as the ciliated protozoa, the most primitive eukaryotes studied, possess both voltage-sensitive Ca^{++} and K^{+} channels (Kung 1989; Tester 1990). The modern single-domain channels, which include the cyclic nucleotide-binding channels and the voltage-gated K^{+} channels, are all permeable to monovalent cations. Thus one might assert that the ancestral channel was permeable to K^{+} and/or Na^{+} and that Ca^{++} permeability was achieved only in the eukaryotes and only after the single domain duplicated to the four-domain (or the earlier two-domain?) structure. In contrast to this view, Franciolini and Petris (1989) have argued that a Ca^{++} channel would have evolved first, to mediate the rapid effects of intracellular Ca^{++} signaling. However, such a signaling process is not known in prokaryotes (Cavalier-Smith 1991), and this argument fails to take into account the critical importance of ion channels in cell volume regulation and maintenance of membrane potential, two evolutionary developments that must have been required very early in cellular evolution, to avoid the fate alluded to by Kung (1989, p. 203): "A room without a door is but a tomb."

Prokaryotic cells are known to have ionic gradients similar to those known in eukaryotes—namely, high intracellular K^{+} and low intracellular Na^{+} and Ca^{++}, relative to the outside (Lynn and Rosen 1987; Skulachev 1987; Walderhaug et al. 1987). It seems reasonable, therefore, to suppose either that the earliest ion channels of living organisms evolved in connection with the development or maintenance of such ionic gradients or that the existence of such gradients, produced by ion pumps, may have altogether predated the earliest ion channels. In either case, the modern involvement of ion channels in regulation of membrane potential and intracellular signaling would have to be regarded as a more recent evolutionary development.

Relationships between the Four K^{+} Channel Gene Lineages

We propose the tree shown in figure 3 as representing the most likely history of the K^{+} channel gene family. A single ancestral K^{+} channel gene underwent a duplication generating the *Shaker/Shal* and *Shaw/Shab* precursors, and each of these subsequently duplicated to produce the modern K^{+} channel multigene family represented by the four *Drosophila* genes *Shaker, Shal, Shaw,* and *Shab.*

Molecular studies have revealed *Shaker, Shab,* and *Shaw* homologues in *Aplysia,* phylum Mollusca (Pfaffinger et al. 1990, 1991); a *Shaker*-like gene in the leech, phylum Annelida (Johansen et al. 1990); and a *Shaw*-like gene in the flatworm *Caenorhabditis elegans,* phylum Platyhelminthes (Wei et al. 1991). Electrophysiological studies have also shown the existence of at least two classes of K^{+} channels in the protozoan *Paramecium* (Kung 1989) and three classes in coelenterates (Holman and Anderson 1991), although their sequence relationships to the four *Drosophila* genes are not yet known. Thus, the four lineages of the K^{+} channel family are clearly ancient with respect to the divergence of animals. The recent identification of genes encoding plant

K^+ channel proteins (Anderson et al. 1992; Sentenac et al. 1992) may open the door to broader molecular comparisons between eukaryotic K^+ channels.

Multiple Shaker Homologues in Vertebrates

The phylogenetic tree in figure 4 shows the existence of at least seven distinct mammalian homologues of the *Drosophila Shaker* gene—namely, Kv1.1–Kv1.7. The indicated phylogeny predicts that there exist several genes of this family that remain to be found—namely, the mouse homologues of Kv1.4, Kv1.5, and Kv1.6 and the rat and human homologues of Kv1.7. Although full-length clones are not yet available, short PCR (polymerase chain reaction) fragments have been reported that derive from mouse Kv1.4 and Kv1.6 and from rat Kv1.7 (Betsholtz et al. 1990). In addition, a genomic clone that represents the mouse homologue of Kv1.4 is currently being characterized (authors' unpublished results).

The Kv1.7 gene, the full sequence of which is known only from its mouse homologue, presents a puzzle. First, it differs from the other *Shaker*-related genes (see below) in having an intron within its coding region. Second, its evolutionary relationship to the other *Shaker*-related genes remains unclear, since its placement in the three is ambiguous at best.

The single nonmammalian vertebrate gene represented on this tree, the *Xenopus* XSha2 gene, originally derived its name from the fact that it shows greater sequence similarity to mouse Kv1.2 than to Kv1.1 or Kv1.3. Its placement outside the mammalian group, however, suggests that it is not the amphibian homologue of Kv1.2 but that the gene duplications in the mammalian K^+ channel genes occurred *after* the mammal/amphibian split. Its similarity with Kv1.2 would therefore simply be the consequence of these genes having diverged more slowly from their common ancestor than did other members of this family. If this is the case, then other *Shaker*-like genes that may exist in *Xenopus* should be found to cluster with XSha2 and not with any specific mammalian gene. Alternatively, additional *Xenopus* sequences may change the topology of this tree and move the duplication events farther back in the vertebrate lineage. It is worth noting that the phylogeny of Na^+ channels shown in figure 6 also supports their expansion by a series of duplications within the mammalian lineage, to the exclusion of eels.

No Evidence for Concerted Evolution

Genes within closely related families may evolve in a coordinated fashion by the mechanisms of unequal crossing-over and gene conversion (Dover 1982; Arnheim 1983). Such "concerted evolution" violates the basic assumptions of most phylogenetic reconstruction methods—namely, that sequences evolve independently of one another. It could be argued that the multiple closely related members of the mammalian *Shaker*-related family provide a fertile ground for concerted evolution. However, if such processes were important in the divergence of this subfamily, one might have expected the rat, mouse, and human homologues of the seven genes to have been homogenized within each species. The fact that this has not happened (note the clustering of genes into rat/mouse/human groups seen in fig. 4) increases our confidence in this tree as representing the correct evolutionary history of these genes. The ambiguous affinities of the mKv1.7 gene within this family, however, remain a puzzle. There is little rationale for invoking local conversion events in this case, as neither half of the gene shows any more robust placement on the tree than does the entire sequence.

Intronless K^+ Channel Gene Coding Regions in Mammals

The *Shaker* gene in *Drosophila* is composed of 21 exons that can be alternatively spliced to generate at least five distinct transcripts (Schwarz et al. 1988). In contrast, six of the vertebrate *Shaker* subfamily genes (Kv1.1–Kv1.6) in mice, rats, and humans, as well as the one known *Shaker*-like gene in *Xenopus* (XSha2), apparently lack introns in their coding regions, although there may exist introns in some of their 5′ untranslated sequences (Tempel et al. 1988; Chandy et al. 1990*b;* Ribera 1990; Swanson et al. 1990). If one accepts the presence of introns as an ancient character, then the precursor of the *Shaker*-like genes in vertebrates must have contained introns and must have lost them before the divergence of amphibians and mammals.

Intronless genes may be generated by integration into the genome of reverse-transcribed DNA copies of spliced messenger RNAs. A common result of such an event is a "processed pseudogene" or "retroposon" (Weiner et al. 1986; Brosius 1991; Li and Graur 1991, pp. 184–190), which lacks introns and often displays the remnants of its poly-A tail. Most such "retrogenes," however, are inactive pseudogenes. To produce a *functional* gene, the retrotransposition event must fortuitously insert the gene next to a functional promotor, or else the transcript itself must contain sequences with promotor activity.

While the vast majority of vertebrate protein-coding regions contain intervening sequences, a number of mammalian genes with intronless coding regions are known, including genes encoding the preproinsulin I gene (Soares et al. 1985), testis-specific phosphoglycerate kinase (McCarrey and Thomas 1987), muscle-specific calmodulin (Gruskin et al. 1987), and the immunoglobulin "recombinase" genes RAG-1 and RAG-2 (Oettinger et al. 1990). Also known to have intronless coding regions are numerous mammalian members of the GTP-binding protein receptor superfamily (Kobilka et al. 1987; Fargin et al. 1988; Ruat et al. 1991; Tiberi et al. 1991), including the extensive multigene family represented by the recently identified olfactory receptors (Buck and Axel 1991). However, the significance of the *absence* of introns in some mammalian genes is understood no better than the significance of their *presence* in most others [although possible functional roles of intervening sequences have been suggested, e.g., in developmentally regulated transcription (Brinster et al. 1988; Palmiter et al. 1991) or in maintaining appropriate nucleosome positioning (Csordas 1989)]. The absence of introns in the mammalian *Shaker*-family genes, therefore, may either reflect a fortuitous event in their evolutionary history (e.g., retroposition) or be the consequence of selection related to some unknown aspect of their expression or regulation.

Ion Channels and the Evolution of Complexity

Ohta (1991) has discussed the theoretical basis for the evolution of complex organisms in the context of multigene families. Through positive Darwinian selection of the products of gene duplication and divergence, a process of division and specialization can form increasingly complex systems. The multiplicity of ion channel genes may therefore be a reflection of the demands of increasing organismal complexity.

The trees in figures 4–6 illustrate substantial expansions of the numbers of K^+ and Na^+ channel genes in vertebrates relative to *Drosophila;* the single fly *Shaker* gene, for example, has at least seven known mammalian homologues. Such expansion may have allowed the evolution of more highly specialized and developmentally regulated channel genes, giving rise to channels selectively expressed in heart, kidney,

etc. However, nearly every ion channel gene now known, even those also found in other cell types, is expressed in brain cells (Beckh and Pongs 1990; Drewe et al. 1991). The evolutionary expansion and divergence of voltage-sensitive ion channel genes may therefore have been central to the increased complexity of the vertebrate nervous system.

Note added in proof—The recent report by Malouf et al. (1992) of a rabbit Ca^{++} channel protein with a two-domain structure, produced by alternate splicing of a four-domain transcript, supports our expectation of the historical existence of a physiologically functional two-domain channel gene. In addition, it has been proposed that the single-domain polypeptides encoded by the *trp* and *trpl* genes of *Drosophila* produce light-sensitive Ca^{++} channels (Hardie and Minke 1992; Phillips et al. 1992); this supports the notion that the acquisition of Ca^{++} selectivity may have preceded *both* of the intragenic duplication events by which the modern four-domain Ca^{++}- and Na^{+}-channel genes evolved from their single-domain ancestor.

Acknowledgments

We thank Walter Fitch and David Swofford for generous assistance with tree reconstruction methods and their programs; Michael Rose and Dick Hudson for thoughtful discussions of evolutionary mechanism and their significance; Aguan Wei, Tim Jegla, and Michael Pak for discussions of channel evolution and for providing unpublished sequences; Angie Ribera for helpful discussions and for providing unpublished sequences; Mark Tanouye and Gail Mandel for providing unpublished sequences; Jayashree Aiyar, Brent Dethlefs, Jim Hall, Gina Ruiz, Chris Schoonover, Robert Spencer, Stephen White, and Randy Wymore for comments on the manuscript and stimulating discussions; Alan Goldin and Diane O'Dowd for information on the Na^{+} channel world; Larry Vickery for computer use; and Alan Goldin for support of the SnAP programs. This study was supported in part by the Department of Physiology and Biophysics (support to M.S.), by USPHS grants AI24783 (to G.A.G.), and AI21366 (to K.G.C.), by Juvenile Diabetes Foundation International grant 188209 (to G.A.G.), and by Pfizer, Inc. (support to K.G.C.).

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