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Evolution of Vertebrate Voltage-Gated Ion Channel α Chains by Sequential Gene Duplication

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Abstract. Phylogenetic analysis of α chains of voltage-gated ion channels revealed that extensive gene duplication has occurred among both Ca^{2+} and Na^{+}-channels since the origin of vertebrates. Rather than showing a pattern of gene duplication consistent with the hypothesis of polyploidization early in vertebrate history, both Ca^{2+} and Na^{+} channels showed patterns of sequential gene duplication associated with specialization of the gene products. In the case of Na^{+} channels, the phylogeny supported the hypothesis that the ancestral vertebrate gene had an expression pattern including both central and peripheral nervous system cells and that duplication of vertebrate Na^{+} channel genes has repeatedly been followed by specialization for the central nervous system, the peripheral nervous system, or muscle cells. Thus, cephalization in vertebrate evolution has been accompanied by specialization of this important family of neuromuscular proteins along the central–peripheral axis.

Key words: Calcium channels — Sodium channels — Voltage-gated ion channels — Genome duplication — Nervous system evolution — Phylogeny

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Introduction

A commonly observed trend in the evolutionary history of animals has been toward increased complexity of nervous, sensory, and locomotor mechanisms (Jollie 1977). This trend has occurred independently in different taxonomic groups, reaching independent peaks of complexity in chordates, in arthropods, and in cephalopods. How complex nervous and muscular systems have evolved remains poorly understood. There is evidence that gene duplication followed by specialization of the duplicated genes has played an important role in adaptive evolution (Hughes 1994, 1999a). The fact that extensive duplications that have occurred in a number of gene families expressed in vertebrate nervous and/or muscular systems supports the hypothesis that this mechanism has been important in the evolution of complexity in these systems. A widely cited hypothesis to account for extensive gene duplication early in vertebrate history is the hypothesis that ancestral vertebrates underwent two rounds of complete genome duplication by polyploidization (the 2R hypothesis) (Lundin 1993; Sidow 1996; Meyer and Schartl 1999). The 2R hypothesis has been invoked to account for duplications of vertebrate genes important for nervous and muscular systems, particularly sodium channel genes (Plummer and Meisler 1999; Lopreato et al. 2001).

However, rigorous tests of the predictions of the 2R hypothesis have failed to support this hypothesis

Table 1. Sequences used in analyses

Scientific name	Gene name	Accession No.
	Calcium-dependent channels (Fig. 1)	
Blattella germanica	Cockroach	BAA22091
Bos taurus	Cow	AAF24229
Caenorhabditis elegans	C.elegans	S58883
Cavia porcellus	Guinea pig	BAA34185
Cyanea capillata	Cyanea	T43048
Cyprinus carpio	Carp	P22316
Drosophila melanogaster	Drosophila.1	Q24270
	Drosophila.2	P91645
Gallus gallus	Chicken.1	O73700
	Chicken.2	AAD51818
Halocynthia roretzi	Halocynthia	BAA34927
Homo sapiens	Human.1	L29534
	Human.2	NP_005174
	Human.3	NP_000711
	Human.4	NP_000060
	Human.5	NP_000712
	Human.6	NP_000059
	Human.7	NP_000709
Mesocricetus auratus	Hamster	Q99244
Mus musculus	Mouse.1	AAA62612
	Mouse.2	NP_033911
	Mouse.3	NP_062528
	Mouse.4	NP_055008
	Mouse.5	NP_033912
	Mouse.6	NP_031604
	Mouse.7	NP_031605
	Mouse.8	O55017
Oryctolagus cuniculus	Rabbit.1	S11339
	Rabbit.2	S05011
	Rabbit.3	P15381
	Rabbit.4	AAF13708
	Rabbit.5	P07293
	Rabbit.6	S29237
	Rabbit.7	I46477
	Rabbit.8	Q05152
Rana catesbeiana	Bullfrog	AAC36126
Rattus norvegicus	Rat.l	P22002
	Rat.2	AAK72959
	Rat.3	AAK71987
	Rat.4	NP_058994
	Rat.5	Q02485
	Rat.6	Q07652
	Rat.7	NP_037050
	Rat.8	AAC29043
Takifugu rubripes	Pufferfish	T30535
Sodium channel outgroup		
Homo sapiens	Human type I	P35498
Musca domestica	Housefly	CAA65448
	Sodium-dependent channels (Fig. 2)	
Aplysia californica	Aplysia	T30902
Canis familiaris	Dog	AAC39164
Cynops pyrrhogaster	Newt	AAD17315
Danio rerio	Zebrafish	AAG18440
Electrophorus electricus	Eel	CAA25587
Equus caballus	Horse	AAA67366
Halocynthia roretzi	Halocynthia.1	BAA04133
	Halocynthia.2	BAA95896
Homo sapiens	Human type I	P35498
	Human type II	AF327246
	Human type III	AF225987
	Human type IV	NP_000325
	Human type V	NP_000326
	Human type VI[a]	NP_002967

Table 1. Continued

Scientific name	Gene name	Accession No.
	Human type VIII	NP_055006
	Human type IX	NP_002968
	Human type X	NP_006505
	Human type XI	AAF17480
	Human type XII	NP_054858
Loligo opalescens	Squid	T43167
Mus musculus	Mouse.1	AAC52242
	Mouse.2	NP_033160
	Mouse.3	A55138
Musca domestica	Housefly	CAA65448
Oryctolagus cuniculus	Rabbit	AAA89159
Rattus norvegicus	Rat.1	X03638
	Rat.2	X03639
	Rat.3	Y00766
	Rat.4	M27902
	Rat.5	Y09164
	Rat.6	U79568
	Rat.7	AF059030
	Rat.8	CAA76659
	Rat.9	NP_058943
Sternopygus macrurus	Knifefish.1	AAK55437
	Knifefish.2	AAK55438
	Knifefish.3	AAK55439
	Knifefish.4	AAK55440
	Knifefish.5	AAK55441
	Knifefish.6	AAK55442
Takifugu rubripes	Pufferfish	BAA07195
Calcium channel outgroup		
Drosophila melanogaster	Drosophila.1	Q24270
Homo sapiens	Human.1	L29534

[a] Sodium channel human type VI is the same as human type VII.

(Hughes 1999b; International Human Genome Sequencing Consortium 2001; Hughes et al. 2001; Friedman and Hughes 2001). If, as current evidence suggests (Friedman and Hughes 2001), vertebrates did not undergo polyploidization early in their history, the pattern of gene duplication that diversified major gene families in the vertebrate nervous and muscular systems must be explained by other mechanisms. To understand these mechanisms, we here apply phylogenetic analysis to the voltage-gated ion channel gene family, encoding the pore-forming subunits of voltage-gated sodium and calcium channels of vertebrates.

Ion channels play a crucial role in the activity of living cells by controlling the ion movements in and out of the cell (Aidley and Stanfield 1996). Ion channels are involved in many physiological processes, including the maintenance of membrane potential, regulation of electrical excitability, and modulation of hormone and neurotransmitter secretion (Strong et al. 1993). Voltage-gated (i.e., voltage-sensitive) ion channels are those channels that induce transmembrane ionic flow in response to sensed changes in transmembrane potential. Voltage-gated (dependent) Na^+-channels are responsible for the rapid membrane depolarization that occurs during the initial "upstroke" phase of the action potential in nerve and muscle. Unlike Na^+ channels, Ca^{2+} channels are able to maintain inward currents for longer depolarization responses, since they are not rapidly inactivating. Ca^{2+} channels play an important role in secretory glands and endocrine organs, as well as in cardiac and smooth muscle (Conley and Brammar 1999).

Both Ca^{2+} and Na^+ channels are composed of multiple subunits. In mammals, the Na^+ channel is a heterotrimer, in which the α subunit forms the voltage-gated Na^+ pore, while the $\beta 1$ and $\beta 2$ subunits regulate the channel kinetics and facilitate the membrane localization (Isom et al. 1994, 1995). The Ca^{2+} channel is also multimeric and consists of four or five subunits, such as the $\alpha 1$, $\alpha 2/\delta$, β, and γ subunits (Conley and Brammar 1999; Ertel et al. 2000). Based on the amino acid sequence similarity, the pore-forming α subunit of Na^+ channels and the $\alpha 1$ subunit of Ca^{2+} channels are believed to be the members of a larger gene superfamily. All other subunits perform regulatory functions and appear to be unrelated between different channels, as well as among different subunits of the same channel. Therefore, we concentrate here on the pore-forming subunits only and refer to the pore-forming α and $\alpha 1$ proteins simply as Na^+ and Ca^{2+} channels, respectively. Such pore-forming subunits of Ca^{2+} and Na^+ channels consist

of four homologous domains, each containing six transmembrane segments (Conley and Brammar 1999; Jan and Jan 1990). Their common ancestry is assumed to go back before the metazoan divergence (Strong et al. 1993; Anderson and Greenberg 2001), but in mammals there is known to be a substantial diversity of Ca^{2+} and Na^{+} channel genes.

A number of phylogenetic analyses of Ca^{2+} and Na^{+} channels have been published recently, but these have included small numbers of sequences and/or have provided unrooted trees including vertebrate sequences only (Anderson and Greenberg 2001; Goldin 2001; Lopreato et al. 2001; Plummer and Meiseler 1999). In the present analyses, although we focus on the diversification of Ca^{2+} and Na^{+} channels in vertebrates, we reconstruct rooted trees including representative invertebrate sequences. This strategy enables us to infer the timing of gene duplication events relative to major cladogenetic events such as the divergence of protostome and deuterostome animal phyla.

Materials and Methods

Sequences were aligned at the amino acid level using the CLUSTAL X program (Thompson et al. 1997). The alignments are available from the authors upon request. In preliminary analyses, the four individual homologous domains of the pore-forming unit were aligned, and phylogenetic trees were constructed for these individual domains. In subsequent analyses, we included only sequences for which these preliminary analyses showed evidence that each domain was homologous to the corresponding domain in other molecules, i.e., where there was no evidence of concerted evolution of repeated domains (Hughes 1999c).

Sequences used in phylogenetic analyses included representatives of all major groups of vertebrate classes of Ca^{2+} and Na^{+} channel α chains, as well as representative invertebrate sequences (see Table 1). Designation of types for human genes was based on the OMIM database (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM). Phylogenetic trees were reconstructed by the following methods: (1) the maximum parsimony (MP) method (Swofford 2000), (2) the quartet-puzzling (QP) maximum likelihood method (Strimmer and von Haeseler 1996), and (3) the neighbor-joining (NJ) method (Saitou and Nei 1987) using both the uncorrected proportion of differences and the Poisson-corrected amino acid distance (Nei and Kumar 2000). All methods produced similar results, and here we present only NJ trees based on the Poisson-corrected amino acid distance. The reliability of clustering patterns in trees was tested by z tests of the equality of internal branch lengths to zero, using standard errors of branch lengths estimated by the bootstrap method (Nei and Kumar 2000). NJ trees were constructed using the computer program MEGA version 2.1 (Kumar et al. 2001). The phylogenetic tree of Ca^{2+} channels was rooted with selected Na^{+} channels, while that of Na^{+} channels was rooted with selected Ca^{2+} channels.

Results

Ca^{2+} Channels

In the phylogenetic tree of Ca^{2+} channels, there were two major subfamilies, each supporting significant internal branches: (1) the 1,4-dihydropyridine (DHP)-sensitive and (2) the DHP-insensitive Ca^{2+} channels (Fig. 1). Since each subfamily included both insect and vertebrate sequences (Fig. 1), the phylogenetic tree supported the hypothesis that the two subfamilies arose by a gene duplication that took place prior to the divergence of deuterostomes (including vertebrates) and protostomes (including insects).

Within the DHP-sensitive subfamily, invertebrate sequences clustered according to generally accepted phylogenetic relationships. The sequence from jellyfish (a diploblast) clustered outside all sequences from triploblast phyla (Fig. 1). Insect sequences clustered outside the sequences from the invertebrate chordate *Halocynthia* (a urochordate) and from vertebrates (Fig. 1). The branches supporting these clustering patterns all received significant support (Fig. 1). Within the vertebrate DHP-sensitive Ca^{2+} channels, there were four major clades, corresponding to classes S, C, D, and F, each supported by a significant internal branch (Fig. 1). This topology suggested that class S diverged first, followed by class C, and that the duplication giving rise to classes D and F was the most recent (Fig. 1). However, the internal branches establishing this pattern were not significantly supported except for the branch establishing classes D and F as sister groups (Fig. 1).

Sequences from bony fish and amphibians, as well as from mammals, were found in class S, but the other three classes of DHP-sensitive Ca^{2+} channels included only mammalian sequences, along with one avian member of class D (Fig. 1). The presence of both bird and mammal sequences in class D indicates that the gene duplication giving rise to classes D and F must have occurred prior to the divergence of birds and mammals, which took place about 310 million years ago (Kumar and Hedges 1998).

Within the DHP-insensitive subfamily, invertebrate sequences clustered outside those of vertebrates, a topology that received significant support (Fig. 1). Among the vertebrate members of this family, there were three significantly supported clades, corresponding to classes E, A, and B (Fig. 1). The tree topology indicated that class E arose first, followed by the duplication giving rise to classes A and B; and the internal branch establishing this topology was significantly supported (Fig. 1). The presence of one avian sequence in the class B cluster (Fig. 1) supported the hypothesis that the gene duplication giving rise to classes A and B occurred before the bird–mammal divergence.

Na^{+} Channels

In the phylogenetic tree of Na^{+} channels, a sequence from the urochordate *Halocynthia* clustered outside all other sequences from both chordates and nonchor-

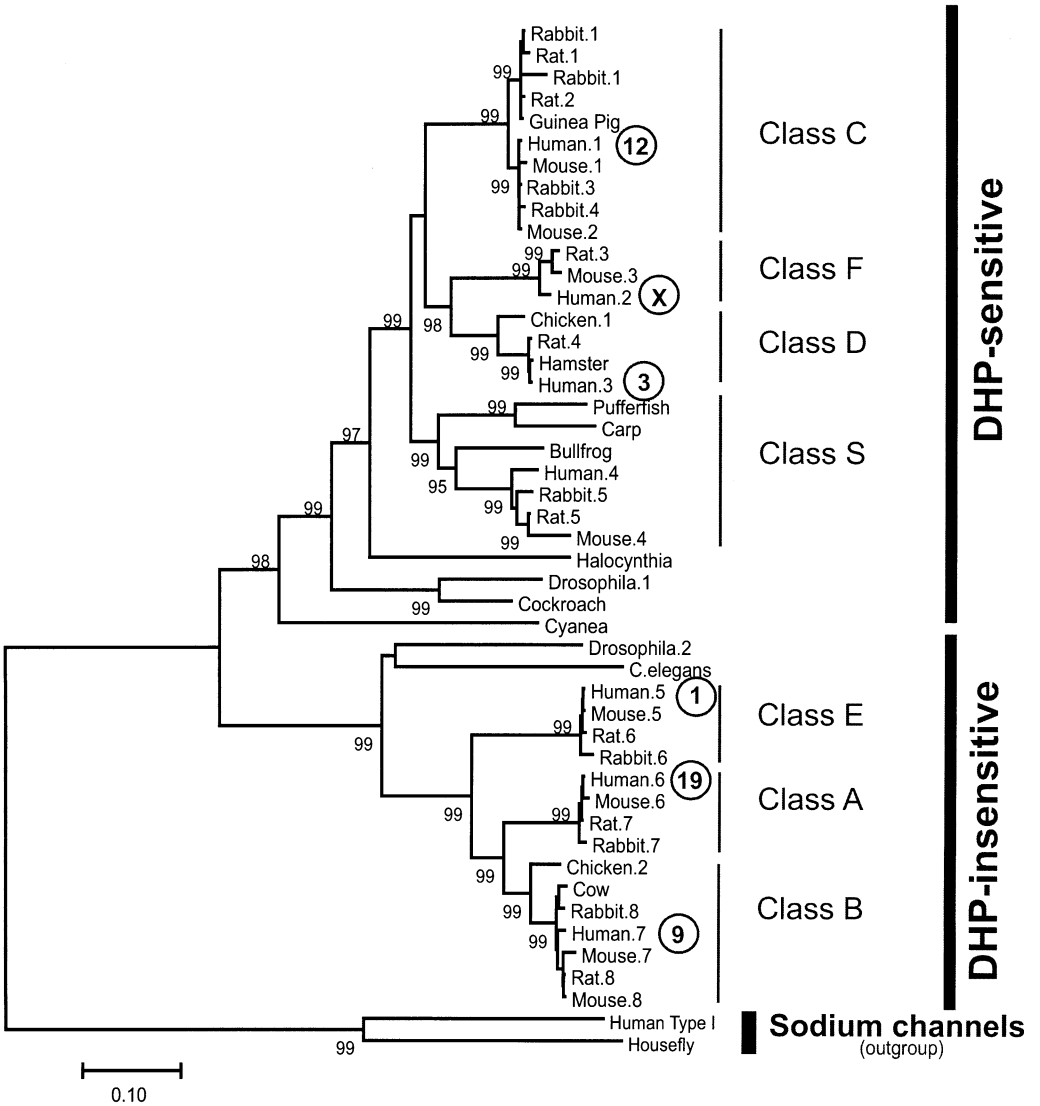

Fig. 1. Phylogenetic tree of the α subunit of voltage-gated calcium channels, constructed by the neighbor-joining method, on the basis of the Poisson-correction distance. The reliability of clusters was estimated using the internal branch length test, and only values above 95% are shown. For human genes the chromosomal locations are shown by *circled numbers*. The tree was rooted by the α subunit of voltage-gated sodium channels.

dates, and the internal branch supporting this topology received highly significant support (Fig. 2). In contrast, all other chordate Na^+ channels, including an additional *Halocynthia* sequence along with all vertebrate sequences, clustered together apart from those of nonchordates (Fig. 2). Thus, the topology supported the hypothesis that the two *Halocynthia* Na^+ channel genes duplicated prior to the deuterostome–protostome divergence. In contrast, because all vertebrate genes clustered together, the phylogeny indicated that extensive diversification of vertebrate Na^+ channels has taken place after the origin of vertebrates.

Among the vertebrate Na^+ channels, the phylogeny indicated that a group including human type XI and type XII branched off first, and this topology was supported by a highly significant internal branch (Fig. 2). Next to branch off was a group including human type X; again this topology was supported by a highly significant internal branch (Fig. 2). Interestingly, these two early-branching clades of vertebrate Na^+ channels both include genes expressed in cells of the peripheral nervous system (Fig. 2).

Next to branch in the Na^+ channel tree was a clade including vertebrate type V. The position of this clade relative to knifefish 2 was not well resolved, but a highly significant internal branch placed these two groups outside the remaining vertebrate Na^+ channels. The human members of the three earliest-branching clades among the vertebrate Na^+ channels all map to chromosome 3 (Fig. 2). However, human type V differs from the other molecules encoded on chromosome 3 in being expressed in muscle cells (Fig. 2). The fact that a ray-finned fish (Actinopterygii) sequence branched along with these three early-

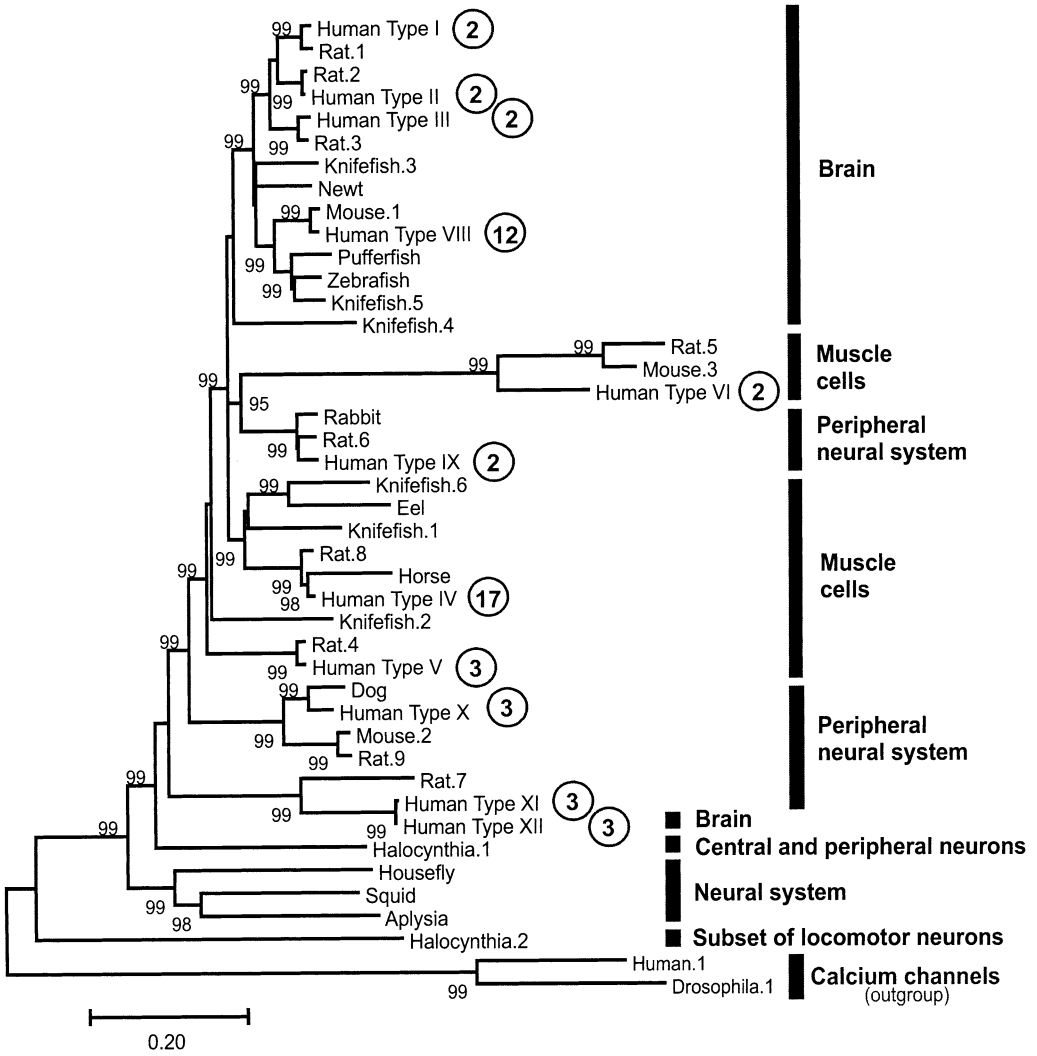

Fig. 2. Phylogenetic tree of the α subunit of voltage-gated sodium channels, constructed by the neighbor-joining method, on the basis of the Poisson-correction distance. The reliability of clusters was estimated using the internal branch length test, and only values above 95% are shown. Chromosomal locations for human genes are shown by *circled numbers*. *Filled bars* indicate the tissues where particular genes are expressed. The tree was rooted by the α subunit of voltage-gated calcium channels.

branching clades outside the remaining Na^+ channels, including sequences from both ray-finned fish and tetrapods, indicates that these clades diverged prior to the divergence of ray-finned fish and tetrapods, which occurred about 450 million years ago (Kumar and Hedges 1998).

The remaining Na^+ channels formed three significantly supported clades, each including one or more human sequence: (1) a clade characterized by expression in muscle tissue, including human type IV, as well as other sequences from both mammals and fish; (2) a clade including human types VI (expressed in muscle tissue) and IX (expressed in the peripheral nervous system), along with related mammalian sequences; and (3) a clade characterized by expression in brain, including human types I, II, III, and VIII, along with related sequences from mammals and fish (Fig. 2). The position of knifefish 4, also expressed in brain, relative to these three clades was not well resolved; it clustered nearest to other brain-expressed molecules, but the internal branch supporting this pattern was not significant (Fig. 2). Within the clade of brain-expressed channels, there were two major subgroups, each supported by a significant internal branch: (1) a group containing human type VIII and related genes from mouse and fish and (2) a group containing three human genes (types I, II, and III) all mapping to human chromosome 2, along with their rodent homologues (Fig. 2).

Discussion

Recent discussion of the evolution of vertebrate genomes has been dominated by the hypothesis that there were two rounds of genome duplication by polyploidization early in vertebrate history (the 2R

hypothesis) (Lundin 1993; Sidow 1996; Meyer and Schartl 1999). However, recent tests of the explicit predictions of this hypothesis cast considerable doubt on the ability of the 2R hypothesis to explain the evolution of the vertebrate genome (Hughes 1999b; Hughes et al. 2001; International Human Genome Sequencing Consortium 2001; Friedman and Hughes 2001). The fact that vertebrates possess four *Hox* clusters, in contrast to one in *Drosophila*, is often taken as evidence in favor of the 2R hypothesis (Sidow 1996). Similarly, the presence of members of numerous other gene families on the two or more of the chromosomes bearing the *Hox* clusters has been taken as evidence that these genes duplicated along with the *Hox* clusters (Lundin 1993). However, phylogenetic analyses of 42 of these families revealed that they duplicated at widely different times over the evolution of life, and not all at the same time as the *Hox* clusters (Hughes et al. 2001). Moreover, even many of the families that duplicated early in vertebrate history as did the *Hox* clusters could not have duplicated along with the *Hox* clusters because their phylogenies showed topologies inconsistent with those of the *Hox* clusters (Hughes et al. 2001). On the other hand, there are certain genes located on the human *Hox*-bearing chromosomes that very likely did duplicate along with the *Hox* clusters; for example, certain integrin α-chain genes (Hughes 2001).

The human Na^+ channels are encoded by genes located on three of the four human *Hox*-bearing chromosomes (chromosomes 2, 7, 12, and 17) (Fig. 2). Na^+ channel genes on chromosomes 2 (human types I, II, and II) and 12 (human type VIII) clustered together in the phylogenetic analysis (Fig. 2). This pattern is consistent with the hypothesis that the duplication separating the ancestor of human types I, II, and III from the ancestor of human type VIII occurred along with the duplication of *HOXC* (chromosome 2) and *HOXD* (chromosome 12). Likewise, it is possible that the ancestor of human type IV (chromosome 17) duplicated from the common ancestor of human types I, II, III, and VIII along with duplication of *HOXB* (chromosome 17) from the common ancestor of *HOXC* and *HOXD*. Indeed, a phylogenetic analysis of the *Hox* clusters supported the hypothesis that *HOXC* and *HOXD* are more closely related to each other than either is to *HOXB* (Zhang and Nei 1996).

On the other hand, the Na^+ channel phylogeny revealed other features inconsistent with the duplication of these genes at the same time as the *Hox* clusters. For example, human type VI and type IX are encoded on chromosome 2 but did not cluster with other chromosome 2 genes (Fig. 2). Therefore, the phylogeny does not support the hypothesis that these genes duplicated at the same time as the *Hox* clusters. Furthermore, the two basal clades within the vertebrate Na^+ channel phylogeny include human genes mapping to chromosome 3. Thus, although certain Na^+ channel genes may have duplicated along with *Hox* clusters, such duplications account for only a portion of the duplication events that have diversified this gene family in vertebrates. Furthermore, the topology of the Na^+ channel tree is not consistent with the 2R hypothesis, under which we expect a phylogeny consisting of two clusters of two genes (Hughes 1999b).

In the case of Ca^{2+} channels, neither of the two subfamilies of vertebrate genes showed a topology consistent with the 2R hypothesis. In the DHP-sensitive subfamily, although the vertebrate genes formed four clusters, the relationship among these clusters did not fit the pattern expected under the 2R hypothesis (Fig. 1). The DHP-insensitive Ca^{2+} channel genes of humans map to chromosomes 1, 9, and 19; certain gene families mapping to these chromosomes and to human chromosome 6 have been alleged to have been duplicated by ancient polyploidization events (Kasahara 1999). However, the hypothesis that these genes duplicated simultaneously is not supported. Kasahara (1999) proposes a hypothesis according to which paralogues on chromosomes 1 and 9 are more closely related to each other than to those on chromosomes 6 and 19. However, of three phylogenetic trees presented in Kasahara's (1999) paper, only one actually shows a topology consistent with his hypothesis. Furthermore, extensive phylogenetic analyses of paralogues in the allegedly duplicated regions of human chromosomes 1, 6, and 9 showed that these genes did not in fact duplicate simultaneously but rather at widely different times over the evolution of life (Hughes 1998). The phylogeny of the DHP-insensitive Ca^{2+} channels (Fig. 1) was also inconsistent with those of several other families mapping to chromosomes 1, 9, and 19 and allegedly duplicated simultaneously (Kasahara 1999; Hughes 1998).

In contrast to the "big bang" of gene duplication hypothesized by polyploidization hypotheses, the phylogenies of voltage-gated ion channels show patterns of numerous, repeated duplications occurring in sequential fashion. This sequential pattern of duplication is strikingly different from the pattern expected under the 2R hypothesis. It is also different from the "birth-and-death" pattern of gene duplication and gene loss observed in several immune system gene families (Hughes and Nei 1989; Ota and Nei 1994; Sitnikova and Nei 1998; Su and Nei 2001).

Particularly in the case of Na^+ channels, the pattern of sequential duplication is associated with functional specialization. Note that the molecule from the urochordate *Halocynthia* that groups just outside the cluster of vertebrate Na^+ channels (Fig. 2) has both central and peripheral nervous system expres-

sion. Thus, the phylogeny supports the hypothesis that the ancestral vertebrate Na^+ channel was expressed in both the central and the peripheral nervous systems. In contrast, the two basal clades of Na^+ channels include genes expressed in the peripheral nervous system (Fig. 2), suggesting that specialization between the central and the peripheral nervous system cells occurred early in vertebrate evolution.

Within the vertebrate Na^+ channels, tissue specialization has evidently repeatedly followed gene duplication, with the same tissue specialization arising independently more than once. For example, there are three separate clades (the clade including human type V, the clade including human type I, and the clade including human type VI) that have independently specialized for muscle tissue expression (Fig. 2). Likewise, three separate clades (the two basal clades and the clade including human type IX) are specialized for peripheral nervous system expression, while brain expression is found not only in the large clades including human types I, II, III, and VIII but also in one member of the most basal clade (human type XII) (Fig. 2).

The development of specialized tissue types, particularly along the peripheral–central axis in the nervous system, has been a key feature of vertebrate cephalization: that is, the evolution of an increasingly complex nervous, sensory, and locomotor apparatus directed by an anteriorly located brain. Our phylogenetic analysis of voltage-gated ion channels suggests that a distinctive pattern of sequential gene duplication has characterized the evolution of this family accompanying cephalization. Because numerous other gene families expressed in the nervous system underwent duplication during the evolutionary process, it will be interesting to determine whether a similar pattern of sequential gene duplication is a widely shared feature of nervous system gene families.

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