Genomic Organization and Evolution of Alternative Exons in a Drosophila Calcium Channel Gene

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ABSTRACT

The genomic organization of a gene coding for an $\alpha 1$ subunit of a voltage-gated calcium channel of *Drosophila melanogaster* (*Dmca1A*) was determined. Thirty-four exons, distributed over 45 kb of genomic sequence, have been identified and mapped, including exons in three regions involved in alternative splicing and new sites potentially involved in RNA editing. The comparison of the intron/exon boundaries of this channel with a mammalian counterpart shows that the genomic structure of these two genes has remained fairly similar during evolution, with more than half of the Drosophila intron positions being perfectly conserved compared to the human channel. Phylogenetic analysis of the mutually exclusive alternative exons revealed that they have diverged considerably. It is suggested that this divergence, rather than reflecting evolutionary age, is the likely result of accelerated rates of evolution following duplication.

MUTATIONS involving voltage-sensitive ion channels in *Drosophila melanogaster* have been implicated in phenotypes as diverse as paralysis, olfactory defects and learning (COWAN and SIEGEL 1986; WU and GANETZKY 1992; LILLY *et al.* 1994). This variety of phenotypic effects seems to correlate with the diversity and functional importance of these channels with respect to different cellular phenomena (HILLE 1992).

Courtship and vision appear to be further phenotypes influenced specifically by certain ion-channel variants. In the former behavior, *D. melanogaster* males vibrate the wings, producing a "lovesong." A few genetic variants have been isolated that affect distinct features of this courtship song (HALL 1994). One of these mutants, *cacophony* (*cac*), is characterized by longer interpulse intervals (IPIs) and pulses that contain more cycles than normal (SCHILCHER 1977). This mutation maps to a locus on the *X* chromosome that is also the site of *night-blind-A* (*nbA*) visual mutations and the lethal mutations at a locus called *l(1)L13*. However, while the *l(1)L13* mutations fail to complement the song and visual defects of *cac* and *nbA*, respectively, *cac/nbA* flies are apparently normal (KULKARNI and HALL 1987). The complex complementation pattern among these genetic variants suggests a similar complexity at the molecular level. Recently, we have cloned cDNAs that map to this locus and encode a new $\alpha 1$ subunit of a voltage-sensitive calcium channel, named *Dmca1A* (SMITH *et al.* 1996).

Voltage-gated calcium channels are usually formed by four or five proteins; they are involved in a number of important processes, such as neurotransmitter release and muscular contraction (MCCLESKEY 1994; DUNLAP *et al.* 1995). The $\alpha 1$ subunit is the calcium-conducting pore; it is usually formed by four homologous domains or repeats, each one composed of six putative α-helical transmembrane domains (TANABE *et al.* 1987). The fourth transmembrane domain in each repeat has positively charged residues every three to four amino acids and is thought to be the voltage sensor of the channel; the link between the fifth and sixth transmembrane domains in each repeat forms part of the pore of the channel (TANABE *et al.* 1987).

Six different classes (A, B, C, D, E and S) of voltage-gated calcium channels have been identified so far, based on sequence similarities, tissue distribution and electrophysiological and pharmacological characteristics (HOFMANN *et al.* 1994; STEA *et al.* 1995). Preliminary analysis of the predicted amino-acid sequence of Drosophila's *Dmca1A* indicated that this channel is more related to the non-L-type, dihydropyridine-insensitive mammalian calcium channels compared to other vertebrate subtypes (SMITH *et al.* 1996). Previously only one other voltage-sensitive calcium channel was known in Drosophila (ZHENG *et al.* 1995). A *Musca domestica* homologue has also been cloned (GRABNER *et al.* 1994). However, the amino-acid sequence encoded by these two genes show stronger sequence similarity to dihydropyridine-sensitive, L-type calcium channels from mammals.

In this paper we present a detailed analysis of the genomic organization of the Drosophila gene (*Dmca1A*) encoding this new calcium-channel $\alpha 1$ subunit. We have also examined the evolution of two pairs of mutually exclusive exons involved in alternative splicing. The data not only establish the necessary basis for further experi-

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ments and evolutionary analysis involving genomic DNA from the *cac/nbA/l(1)L13* locus but also reveal the extent to which the genomic structure of calcium channels is conserved throughout evolution.

MATERIALS AND METHODS

Isolation of cosmid clones: Library screens and other molecular techniques followed SAMBROOK *et al.* (1989) unless stated otherwise. For the isolation of cosmid 3.2a, the Tamkun library (TAMKUN *et al.* 1992) was screened with a fragment from a phage clone called 320, which had been derived from a chromosomal walk through the *gastrulation defective* locus (GORALSKI 1985; SMITH *et al.* 1996), which neighbors *cac* and interacting mutations. The cosmid LST-1 was also isolated from the Tamkun library, using a PCR-generated probe derived from the cDNA cS9a (SMITH *et al.* 1996), which encodes part of the intracellular C-terminal tail of the *Dmca1A* channel (residues 1556–1802).

PCR and DNA sequencing: PCR was carried out in a PTC-100 (MJ Research) for 30 cycles (95° for 1 min, 65° or 60° for 1 min and 70° for 1–5 min). Taq DNA polymerase from Promega or the TaqPlus Long PCR System (Stratagene) were used. Reactions were performed in 50 μl using 200 ng of genomic DNA as template, primers at 1 μM, and dNTP at 0.25 mM each (each of these being final concentrations). In most cases primers used were based on the cDNA sequence (SMITH *et al.* 1996). PCR fragments were purified using either the QIAEX II Gel Extraction kit (QIAGEN) or the QIAQUICK Spin PCR Purification kit (QIAGEN). Sequencing reactions were also carried out on a PTC-100 for 25 cycles (96° for 30 sec, 50° for 20 sec and 60° for 4 min) using the PRISM Dye-Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems). The reactions were run on a Perkin-Elmer/Applied Biosystems model 373A DNA sequencer. PCR fragments were sequenced directly or were cloned first, using the pGEM-T Vector System I (Promega) or the TA cloning kit (Invitrogen). Additional sequencing reactions were carried out using subcloned fragments from cosmids or using the cosmid clones directly as template; both vector-based and gene-specific primers were used in these sequencings.

Analysis of DNA and protein sequences: The GCG Package Version 7 (GENETICS COMPUTER GROUP 1991) was used for the assembling and analysis of the DNA sequences. BLAST (ALTSCHUL *et al.* 1990) was used for database searches. The CLUSTAL W software (THOMPSON *et al.* 1994) was used for protein and DNA sequence alignments and phylogenetic analysis. Alignments were performed using the BLOSUM matrices and trees were constructed using the neighbor-joining method (SAITOU and NEI 1987), excluding positions with gaps and not correcting for multiple hits.

RESULTS

Genomic organization of *Dmca1A*: One of the first steps in the molecular analysis of a genomic region known to contain *cacophony* (*cac*), *night-blind-A* (*nbA*), and the *l(1)L13* lethals was to isolate the cosmid 3.2a (see MATERIALS AND METHODS and TAMKUN *et al.* 1992) using as a probe a fragment from a phage clone; this material (called 320) had been derived from a chromosome walk through the nearby locus *gastrulation defective* (GORALSKI 1985) and had been shown to recognize restriction fragment length polymorphisms associated with inversion breakpoints in this region (GORALSKI 1985; SMITH *et al.* 1996). Extensive sequence analysis of this cosmid was carried out showing that the insert in 3.2a contains a genomic fragment that includes *Dmca1A* exons from number 2 to 23 (see Figure 1). A second cosmid (LST-1) was isolated from the same library using as a probe a cDNA fragment containing part of the intracellular C-terminal tail (see Figure 1, bottom left). Sequence analysis revealed that the insert in this case did not overlap with the previous one but contained sequences including exons 26–34 (Figure 1). Genomic sequences containing the missing exons were then obtained by PCR (see MATERIALS AND METHODS).

Figure 1 shows a diagrammatic representation of the channel with its four homologous domains or repeats (called I, II, III and IV), each one composed of six membrane spanning regions (IS1, IS2, IS3, . . . to IVS6). The figure also shows a schematic representation of the genomic organization of the gene with the corresponding positions of all these channel domains. A total of 34 exons, of which 32 contain coding sequences, distributed over 45 kb of genomic sequence have been identified and mapped. In two of the homologous repeats, domains I and IV, the six transmembrane domains are usually found in different exons. That is not the case for the II and III repeats, for which single exons encode, respectively, four and five transmembrane domains (see also Figure 2).

Two mutually exclusive alternative exons (nos. 5 and 6) that code for the IS3/IS4 extracellular loop plus the IS4 transmembrane domain were found. They were called, respectively, IS4a and IS4b. Two other mutually exclusive alternative exons coding for part of the loop between homologous domains I and II (exons 10 and 11 called, respectively, I/IIa and I/IIb) have also been identified.

Intron/exon boundaries in *Dmca1A*: Table 1 shows the length of each exon, the sequences at the 5′ splice donor and 3′ splice acceptor sites, and the approximate intron sizes. The consensus sequences at the intron/exon boundaries and the frequency of occurrence of each base are shown at the bottom of the table. The amino-acid positions at which introns are found in the *Dmca1A* gene are also shown. "Intron phase" in the last column of Table 1 refers to the base position within the coding sequence in which a given intron falls (phase 0 → before first base, phase 1 → after first base and phase 2 → after second base).

The calcium-channel encoding exons vary in length between 6 to >500 bp (Table 1), whereas intron sizes range from 62 bp to ~6 kb. The consensus sequences for the 5′ donor and 3′ acceptor sites agree well with the previously reported Drosophila consensus sequences (MOUNT *et al.* 1992). Note that exon 23 has only six bases. However, eight of the flanking intronic bases (cagCACGATgtaag) show a perfect match to the Drosophila consensus sequences (MOUNT *et al.* 1992) as well as to the consensus shown in Table 1. Analysis

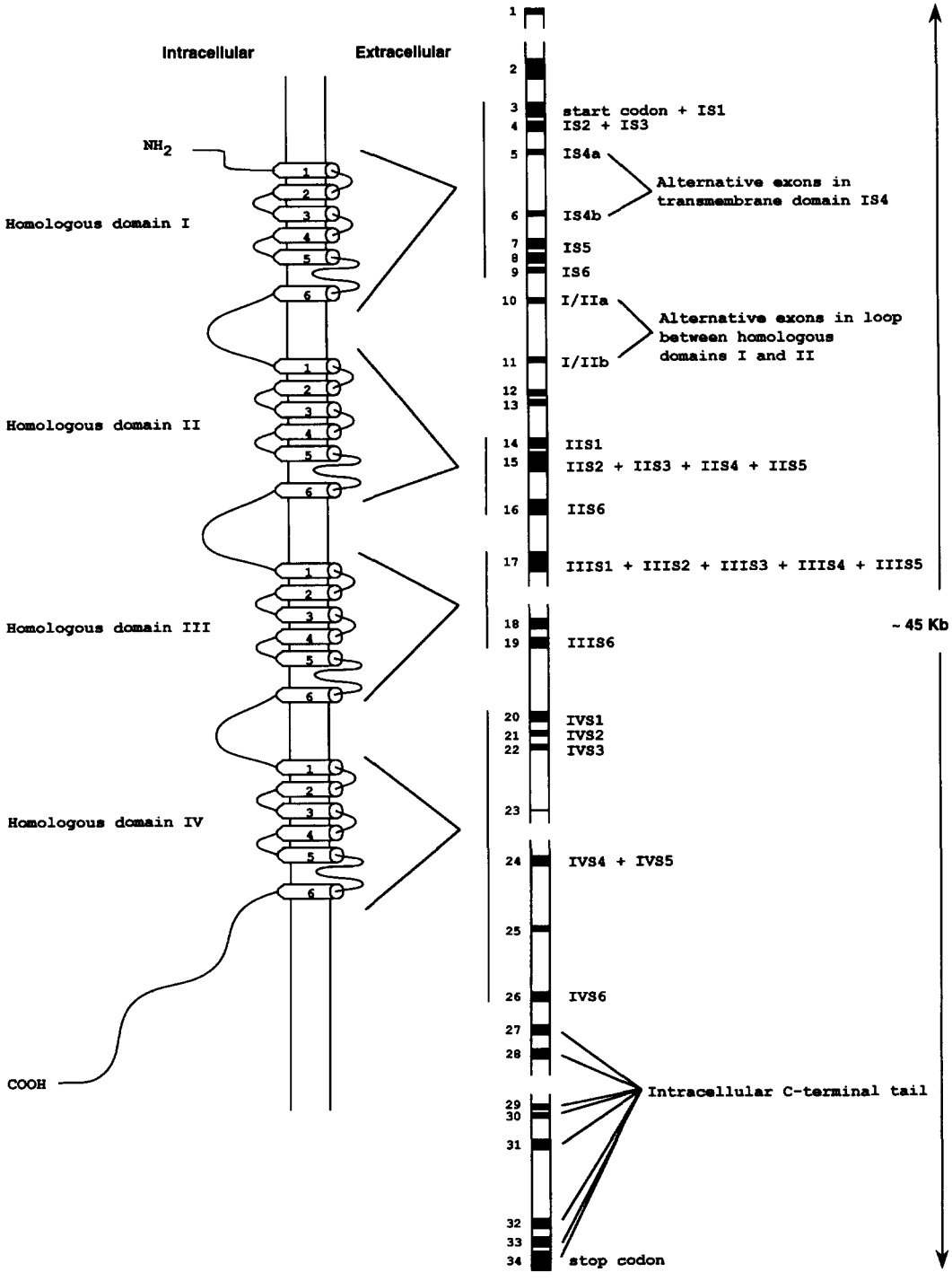

FIGURE 1.—Genomic organization of *Dmca1A*. The left side of the figure shows a diagram of the putative topology of the channel. There are four homologous domains, or repeats, called I, II, III, and IV. Each one is composed of six transmembrane domains called, respectively, S1, S2, S3, S4, S5, and S6. Therefore IS1, for example, is the first transmembrane domain from the homologous repeat I. The right side of the figure shows a schematic representation of the genomic organization of the DNA cloned from the relevant *X*-chromosomal locus. ■, the 34 exons so far identified; □, intervening introns. The approximate position of the transmembrane domains is indicated. The exons containing the start and stop codons as well as the intracellular C-terminal tail are also indicated. Note that there are two alternative exons coding for the IS4 domain (called IS4a and IS4b) and two for part of the loop between homologous domains I and II (called I/IIa and I/IIb). The length of introns and exons is not to scale. Boxes representing introns >2 kb are shown with broken lines.

of cDNAs (SMITH *et al.* 1996) indicated that this exon is an optional insertion in the IVS3/IVS4 loop not present in all transcripts.

Among the 29 introns that interrupt the coding sequence (thus excluding the introns between mutually exclusive alternative exons) the three phases occur at similar frequencies (10 for phase 0, 9 for phase 1 and 10 for phase 2). However, an excess of exons that are "symmetric," that is, which start and end in the same phase, seems to occur (14 out of 28, therefore 50%, compared to an expectation of ~33%; yet this difference failed to reach statistical significance with this small sample size, $\chi^2 = 3.39$, d.f. = 1, $0.10 > P > 0.05$). They also tend to cluster together. For example, exons 20–24 start and end in phase 0, exons 12–14 start and end in phase 1, and exons 15–18 start and end in phase 2.

Position and conservation of intron/exon boundaries: In Figure 2 the predicted protein sequence of *Dmca1A* is aligned to the human calcium channel CACNL1A1 (SOLDATOV 1992, 1994). The approximate locations of the transmembrane domains are highlighted above the sequences, and intron positions are marked

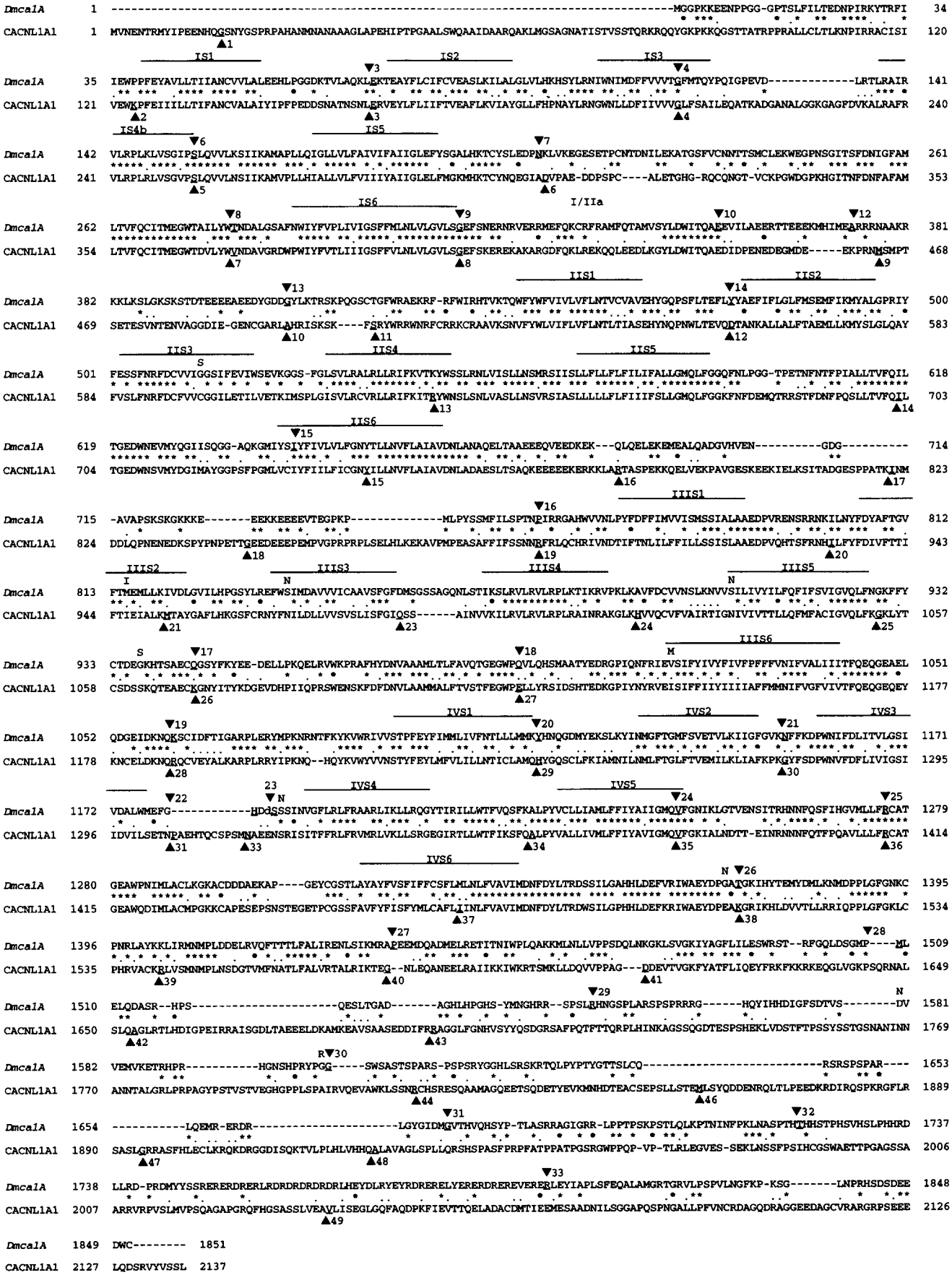
DmcaIA
CACNL1A1
IS1
IS2
IS3
IS4b
IS5
IS6
I/IIa
IIS1
IIS2
IIS3
IIS4
IIS5
IIS6
IIIS1
IIIS2
IIIS3
IIIS4
IIIS5
IVS1
IVS2
IVS3
IVS4
IVS5
IVS6

TABLE 1

Intron/exon boundaries in *Dmca1A*

Exon	Length (bp)	5′ donor site −3	−2	−1	1	2	3	4	5	Intron size (kb)	3′ acceptor site −3	−2	−1	1	2	3	Amino acid	Intron phase
1	>38	C	T	G	g	t	g	a	g	3	c	a	g	T	T	C		
2	414	A	C	G	g	t	a	c	g	0.61	c	a	g	G	T	T		
3	319	T	T	G	g	t	a	a	g	0.074	t	a	g	[G	A	A]	Glu74	(0)
4	140	G	[G	G	g	t	a	a	a	0.47	c	a	g	A}	G	C	Gly120*	(2)
5	101	C	A	{A	g	t	a	a	g	1.5	c	a	g	A}	T	T	Gly120*	(2)
6	101	C	T	{A	g	t	g	a	g	0.42	c	a	g	G	T]	T	Ser154	(1)
7	156	C	A	[A	g	t	a	a	t	0.064	c	a	g	A	C]	A	Asn206	(1)
8	221	T	G	G	g	t	a	a	g	0.099	c	a	g	[A	C	C]	Thr280	(0)
9	104	T	[G	G	g	t	a	a	g	0.55	c	a	g	A}	G	A	Gly314*	(2)
10	116	C	A	{G	g	t	t	t	a	1.4	t	a	g	T}	G	A	Gly314*	(2)
11	116	C	T	{G	g	t	a	t	g	0.74	c	a	g	A	G]	G	Glu353	(1)
12	60	A	G	[G	g	t	a	a	g	0.063	c	a	g	C	T]	C	Ala373	(1)
13	105	A	T	[G	g	t	t	a	g	1.0	t	a	g	G	T]	T	Gly408	(1)
14	198	T	G	[T	g	t	a	c	g	0.073	c	a	g	A	C]	T	Tyr474	(1)
15	514	T	[A	T	g	t	a	a	g	0.79	c	a	g	A]	T	A	Ile645	(2)
16	336	T	[C	C	g	t	a	a	a	1.1	c	a	g	C]	A	T	Pro757	(2)
17	564	C	[C	A	g	t	a	a	g	5	a	a	g	G]	G	G	Gln945	(2)
18	144	A	[C	A	g	t	a	a	g	0.37	t	a	g	G]	G	T	Gln993	(2)
19	202	C	A	G	g	t	g	a	g	1.5	t	a	g	[A	A	A]	Lys1061	(0)
20	165	A	A	G	g	t	a	a	g	0.11	c	a	g	[T	A	T]	Tyr1116	(0)
21	111	A	A	G	g	t	g	a	g	0.126	t	a	g	[A	A	C]	Asn1153	(0)
22	84	G	G	G	g	t	a	a	t	2	c	a	g	[C	A	C]	His1181	(0)
23	6	G	A	T	g	t	a	a	g	6	c	a	g	[T	C	G]	Ser1184	(0)
24	183	C	A	G	g	t	a	a	g	2	t	a	g	[G	T	G]	Val1245	(0)
25	95	C	[A	G	g	t	a	c	g	2	c	a	g	G]	T	G	Arg1276	(2)
26	282	G	[A	C	g	t	a	a	t	0.62	t	a	g	T]	G	G	Thr1370	(2)
27	203	C	G	[C	g	t	a	t	g	0.21	c	a	g	C	T]	G	Pro1438	(1)
28	209	C	C	G	g	t	a	a	g	5	t	a	g	[A	T	G]	Met1508	(0)
29	122	G	[A	G	g	t	a	a	g	0.062	c	a	g	G]	C	A	Arg1548	(2)
30	164	G	A	[G	g	t	a	t	t	0.57	t	a	g	G	T]	T	Gly1603	(1)
31	201	T	G	[G	g	t	a	c	a	2	c	a	g	G	T]	G	Gly1670	(1)
32	152	C	A	T	g	t	a	a	g	0.19	c	a	g	[A	C	A]	Thr1721	(0)
33	245	A	[A	G	g	t	g	a	g	0.065	c	a	g	A]	C	T	Arg1802	(2)
34	>557																	
Consensus		C	A	G	g	t	a	a	g		c	a	g	A	T	T		
		0.4	0.5	0.6	1	1	0.8	0.8	0.8		0.7	1	1	0.4	0.4	0.3		

The length of exons are exact, whereas intron sizes are in most cases only approximations. Intron sequences are shown in lower case. The amino acids at the intron/exon boundaries are shown in the sixth column. The bases coding for these amino acids are shown between brackets, [ ], except for those from mutually exclusive alternative exons (5 and 6; 10 and 11), which are shown between braces, { }; note also that the relevant amino acids are marked with asterisks. "Intron phase" refers to the base position in the coding sequence for which the intron occurs (phase 0 → before first base, phase 1 → after first base, and phase 2 → after second base). The consensus sequence and the frequency of occurrence of each base for the intron/exon boundaries are shown at the bottom.

with triangles in CACNL1A1 and inverted triangles in *Dmca1A*. Of the 29 introns that interrupt the coding sequence of the Drosophila channel, 16 (nos. 3, 4, 6, 8, 9, 14, 16, 17, 18, 19, 20, 21, 22, 24, 25, and 26) seem perfectly conserved compared to the human channel. Not only the positions but also the phases in which the

FIGURE 2.—Position and conservation of intron/exon boundaries. The predicted protein sequence of *Dmca1A* (*cf.* SMITH *et al.* 1996) is aligned to the human calcium channel CACNL1A1 (SOLDATOV 1992, 1994). Asterisks between the sequences indicate positions with identical amino-acids, while the dots indicate similar ones. The approximate locations of the transmembrane domains are highlighted above the sequences. Intron positions are marked with ▲ in CACNL1A1 and ▼ in *Dmca1A*. The introns are numbered according to the preceding exons (see also Table 1, and Table 1 of SOLDATOV 1994). The amino acids at the intron-exon boundaries are underlined. The 10 differences observed between genomic and cDNA sequences, which might reflect RNA editing, are shown above the *Dmca1A* sequence (see text and Table 2). The putative 3-bp exon encoding the aspartic acid (D) at position 1183 (see SMITH *et al.* 1996) has not been mapped yet and for that reason this amino acid is shown in lower case.

TABLE 2

Putative RNA editing sites in *Dmca1A*

Amino acid position	Genomic sequence	cDNA sequence
514	**A**GT (S)	**G**GT (G)
815	AT**A** (I)	AT**G** (M)
839	**A**AT (N)	**A**GT (S)
906	**A**AC (N)	**A**GC (S)
937	**A**GT (S)	**G**GT (G)
1016	**A**TG (M)	**G**TG (V)
1185	**A**AC (N)	**A**GC (S)
1368	**AA**T (N)	**GG**T (G)[a]
1580	**A**AT (N)	**G**AT (D)
1602	**A**GA (R)	**G**GA (G)

Base differences between genomic and cDNA codons are shown in bold, whereas the encoded amino acids are shown in parentheses.

[a] Two nucleotide differences at this codon.

intron occurs were taken into account in specifying this level of conservation. Three others (nos. 7, 13 and 27) map to very similar positions and are in the same phase. Note that in both genes the introns usually fall between rather than within transmembrane domains. The putative 3-bp exon encoding the aspartic acid (D) at position 1183, which was found in some cDNAs (SMITH *et al.* 1996), has not been mapped yet. For that reason this amino-acid is shown in lower case.

Figure 2 also shows 10 amino-acid positions for which differences were found between the predicted protein used in the alignment, which is based on cDNAs sequences (SMITH *et al.* 1996), and the predicted protein based on the genomic sequences. Six of those differences have been observed previously among cDNAs. In all 10 cases the differences are due to A → G changes in which the adenosine is always found in the genomic sequence. Therefore, these differences might be caused by RNA editing similar to what is found in mammalian glutamate receptors (see DISCUSSION). In one case two adjacent nucleotides from the same codon were changed (see Table 2).

Sequence analysis of mutually exclusive alternative exons: As a result of the extensive sequence analysis of cosmid 3.2a we found two regions in which the same protein domain seem to be encoded by two different exons. In one case, alternative exons coding for the IS3/IS4 extracellular loop and the IS4 transmembrane domain (called IS4a and IS4b) were identified within 2.5 kb of intronic sequences separating domains IS3 and IS5. These exons were easily recognized by BLAST searches of databases, due to the high conservation of the transmembrane domain IS4. Later a cDNA containing the form IS4b was found (SMITH *et al.* 1996); one that would encode form IS4a has yet to be isolated. However, the precise intron/exon boundaries of IS4a were confirmed by RT-PCR and sequencing. The other pair of alternative exons (deduced from cDNAs) codes for part of the loop between homologous domains I and II (called I/IIa and I/IIb).

To gain additional insight on the origin and rate of divergence of these alternative exons and on the ones coding the IS3/IS4 extracellular loop and the IS4 transmembrane domain, a phylogenetic analysis of the regions encoded by these exons was carried out. One gene representing each one of the six classes (A, B, C, D, E and S) of mammalian calcium channels was included in the analysis as well as the only other Drosophila calcium channel known so far (*Dmca1D*).

Figure 3a shows a gene tree based on the amino-acid sequences encoded by the IS4a and IS4b alternative exons and the mammalian sequences for the region. Figure 3b shows a similar tree for the I/II loop region. The alignments for the two regions with alternative exons that were used in the tree constructions are shown in Figure 4, a and b. The overall topology of both trees is similar to a preliminary analysis using whole sequences (SMITH *et al.* 1986). Based on this previous analysis the roots of these trees fall between the non-L-type, dihydropyridine-insensitive channels classes A, B and E—plus the Drosophila gene *Dmca1A*—on one side; and the L-type, dihydropyridine-sensitive channel classes C, D and S—plus the Drosophila gene *Dmca1D*—on the other. An outgroup such as a voltage-sensitive sodium channel was not used to construct the trees in Figure 3, a and b, because the alignments in the regions encoded by the alternative exons were found to be very poor (not shown).

The results obtained for the IS3/IS4 extracellular loop and the IS4 transmembrane domain (Figure 3a) show that the isoforms IS4a and IS4b appear as independent splits from the lineage that gave rise to classes A, B and E. This could be interpreted as meaning that the duplication giving rise to these alternative exons was before the arthropod/vertebrate separation. However, because the region compared is very small and the bootstrap value low (55%), these results should be viewed with caution. Moreover, as can be seen in Figure 4a, the alignment in the IS3/IS4 loop (see also Figure 2) is very difficult with obvious past deletion and insertion events. In fact, the position of the gaps in the alignment clearly suggests that IS4a and IS4b are more closely related to each other than to any other channel. Other possible alignments are obtained if different gap penalties are used or if the conserved IS3 domain is used to "anchor" the alignment. However the trees obtained from these other alignments are rather similar to Figure 3a, and even when IS4a and IS4b are placed on the same branch, the node is very deep. Note that the protein divergence between the two mutually exclusive alternative exons for the whole region (including the gaps in the multiple alignment) is 39% (13/33). This value is similar to, for example, the divergence of the mammalian genes D and S (18/47 = 38%) and much higher than the divergence between channel classes A

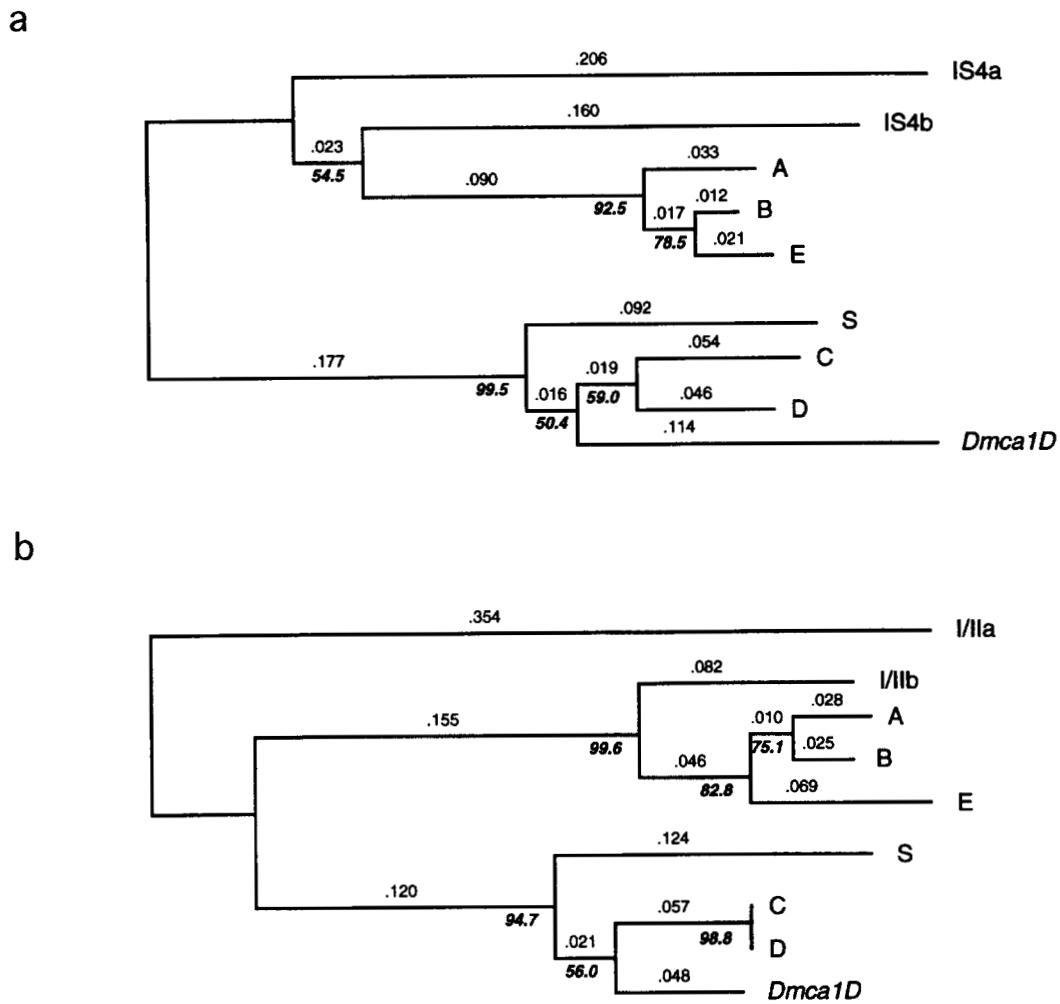

FIGURE 3.—Phylogenetic analysis of alternative exons. Representatives of the six classes of mammalian channels and the Drosophila gene *Dmca1D* (ZHENG *et al.* 1995) were compared to *Dmca1A*. The following mammalian genes were used: rabbit classes A (MORI *et al.* 1991), B (FUJITA *et al.* 1993), E (NIIDOME *et al.* 1992), and S (TANABE *et al.* 1987); and human classes C (SOLDATOV 1992) and D (WILLIAMS *et al.* 1992). The trees were constructed with the neighbor-joining method (SAITOU and NEI 1987) using CLUSTAL W software (THOMPSON *et al.* 1994). The numbers on the trees represent the branch lengths (placed upon branches) and the percentage bootstrap values (in italics) based on 1000 replicates (FELSENSTEIN 1985). (a) A tree for the protein sequences encoded by the alternative exons IS4a and IS4b and the mammalian sequences for the same region. (b) A similar tree for the protein sequences encoded by the alternative exons I/IIa and I/IIb. The alignments for the two regions with alternative exons that were used in the tree constructions are shown in Figure 4, a and b.

and B (2/30 = 7%). The phylogenetic analysis, therefore, does indicate that the divergence between the two alternative exons is very high.

Figure 3b shows an even more striking result. The alternative exon I/IIb seems much closer to channel classes A, B and E than to exon I/IIa. While, for example, the protein divergence between I/IIb and the mammalian channel A is only 16% (6/38), the divergence between the two alternative exons is 61% (23/38). That is the same value for the divergence between I/IIa and channel class A, and higher than the value for the divergence between I/IIa and channel classes C or D (20/38 = 53%). In fact, taken at face value, the result of Figure 3b suggests that I/IIa separated very early from the lineage that gave rise to I/IIb and the non-L-type, dihydropyridine-insensitive channels classes A, B and E, perhaps about the same time the separation between L and non-L type channels occurred. Once again, in spite of the associated high bootstrap value in this case (99%), due to the short sequences involved, this result must be regarded with caution. Yet it does point to a very high divergence between those two isoforms.

DISCUSSION

The α1-subunits of voltage-gated calcium channels in vertebrates are encoded by an extensive gene family, broken down into six different classes (HOFMANN *et al.* 1994; STEA *et al.* 1995). These channels mediate a number of functions in many different tissues (MCCLESKEY 1994; STEA *et al.* 1995). In insects only three genes coding for α1-subunits have been cloned so far (GRABNER

a

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IS4a      -AMTIFAEANIDV-------------DLRMLRSFRVLRPLKLVSRIP
IS4b      -FMTQYPQIGPEV-------------DLRTLRAIRVLRPLKLVSGIP
A         ----ILATVGTEF-------------DLRTLRAVRVLRPLKLVSGIP
B         ----ILATAGTDF-------------DLRTLRAVRVLRPLKLVSGIP
E         ----ILATAGTHFN---------THVDLRTLRAVRVLRPLKLVSGIP
C         LFSAILEQATKADGANALG-GKGAGFDVKALRAFRVLRPLRLVSGVP
D         LFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLVSGVP
S         VFTAILEQVNVIQSNTAPMSSKGAGLDVKALRAFRVLRPLRLVSGVP
Dmca1D    AISTALSQLMKDA------------FDVKALRAFRVLRPLRLVSGVP
                                    *.. **. ******.*** .*
```

b

```
I/IIa     EFSNERNRVERRMEFQKCRFRAMFQTAMVSYLDWITQA
I/IIb     EFAKEREKVENRQEFLKLRRQQQLERELNGYVEWICKA
A         EFAKERERVENRRAFLKLRRQQQIERELNGYMEWISKA
B         EFAKERERVENRRAFLKLRRQQQIERELNGYLEWIFKA
E         EFAKERERVENRRAFMKLRRQQQIERELNGYRAWIDKA
C         EFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQA
D         EFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQA
S         EFTKEREKAKSRGTFQKLREKQQLEEDLRGYMSWITQG
Dmca1D    EFSKERNKAKNRGDFQKLREKQQIEEDLRGYLDWITQA
          **. ** .  *  * * * .  .  .  *  ** .
```

FIGURE 4.—Protein alignments of the regions encoded by the mutually exclusive alternative exons. Asterisks indicate positions with identical amino acids, while the dots indicate similiar ones. (a) Alignment of the protein sequences encoded by the alternative exons IS4a and IS4b and the mammalian sequences for the same region. A gap penalty equal to 3 was used. (b) Similar alignment for the region encoded by the alternative exons I/IIa and I/IIb.

et al. 1994; ZHENG *et al.* 1995; SMITH *et al.* 1996). Analysis of the evolution of many gene families (IWABE *et al.* 1996) suggests that most gene duplications giving rise to different functions occurred before the vertebrates-arthropods separation, whereas duplications concerning genes with similar functions but different tissue distributions usually occurred later. Phylogenetic analysis indicates that the separation of the lineages giving rise to the dihydropyridine-insensitive mammalian calcium channel classes A, B and E, and the dihydropyridine-sensitive L-type mammalian calcium channels classes C, D and S, occurred before the vertebrate-arthropod separation. *Dmca1A* seems more related to the dihydropyridine-insensitive mammalian calcium channels (SMITH *et al.* 1996), whereas the other two insect genes (GRABNER *et al.* 1994; ZHENG *et al.* 1995) seem more related to the L-type channels. That implies that the duplications that putatively gave rise, for example, to the gene classes A, B and E occurred after the vertebrate-arthropod separation; it remains to be seen whether similar duplications occurred in insects.

It is possible that, as in the case of the voltage-gated potassium channels (STRONG *et al.* 1993; CHANDY and GUTMAN 1995), Drosophila has fewer calcium-channel genes than vertebrates. In this respect, at least within the *Shaker* (*Sh*) subfamily, this apparent lack of diversity in terms of gene number in Drosophila is compensated by more complex patterns of alternative splicing compared to the mammalian counterparts of *Sh* (see below). Comparison of *Shaker* with its mammalian relatives shows that they have quite different genomic structures. Whereas *Shaker* itself contains multiple exons (KAMB *et al.* 1988; SCHWARTZ *et al.* 1988), the coding sequence of most known mammalian homologues are intronless (CHANDY and GUTMAN 1995). In contrast, the comparison of the intron/exon boundaries of *Dmca1A* with a human counterpart (CACNL1A1) revealed a much higher degree of conservation in the genomic structure of these two calcium-channel genes during evolution. Because the human calcium channel used here for the comparison with *Dmca1A* is an L-type class C channel (SOLDATOV 1994), the separation between the two genes is actually older than the split of the lineages that gave rise to arthropods and vertebrates. In spite of that, 16 out of 29 intron/exon boundaries seem perfectly conserved between the two genes, with three additional ones occupying very similar relative positions within the Drosophila and the mammalian loci. With this level of conservation it is very likely that the genomic organization will prove to be very conserved within the Drosophila genus, as observed in a number of other genes (*e.g.*, SWIMMER *et al.* 1990; HOOPER *et al.* 1992; LEICHT *et al.* 1993; THACKERAY and GANETZKY 1995).

Comparison of the genomic structure of a human sodium-channel gene and a Drosophila locus encoding that kind of function (*para*) shows that many of the intron/exon boundaries are also conserved (LOUGHNEY *et al.* 1989; GEORGE *et al.* 1993). For these sodium-channel genes (in Drosophila and in a mammal), little correlation between the genomic and protein structure seems to exist. This correlation is stronger in the case of the calcium-channel genes—as exemplified by *Dmca1A* and the human calcium channel CACNL1A1—where the intron/exon boundaries tend to fall between rather than within calcium-channel transmembrane domains. The families of voltage-gated calcium and sodium channel genes are thought to have diverged from a common branch within a superfamily of ion channels that also includes potassium and cyclic nucleotide-binding channels (JAN and JAN 1990; STRONG *et al.* 1993). In fact, our closer examination of the genomic structure of both types of ion channel genes, calcium and sodium, suggests that some of the intron/exon boundaries might have remained conserved between then. Whether exons correspond to discrete protein domains has been an important issue on the theories dealing with the age and origin of introns (STOLTZFUS *et al.* 1994). Also relevant is the apparent excess of symmetric exons and the fact that they tend to occur in clusters; this seems to occur in *Dmca1A* and is common to many genes (LONG *et al.* 1995). The phenomena has been argued to be evidence for the importance of exon shuffling during evolution (LONG *et al.* 1995).

Alternative splicing is a major source of genetic variability in voltage-sensitive ion channels. As noted, *Shaker* shows extensive diversity due to alternative splicing (KAMB *et al.* 1988; SCHWARTZ *et al.* 1988), with the various isoforms showing differences in expression patterns and in electrophysiological properties (reviewed in PER-

NEY and KACZMAREK 1991). The *para* gene also undergoes alternative splicing, which is developmentally regulated (THACKERAY and GANETZKY 1994). In mammalian calcium channels there are a number of examples of alternative transcripts that involve either mutually exclusive exons coding for the same domain or optional inclusion/exclusion of exons (HUI *et al.* 1991; SNUTCH *et al.* 1991; DIEBOLD *et al.* 1992; SOLDATOV 1992, 1994; WILLIAMS *et al.* 1992).

So far, three regions have been identified in which alternative splicing seems to occur in *DmcaA1*. One of these is the IVS3/IVS4 extracellular loop where cDNAs, differing for one to three optional amino-acid insertions in the region in question have been identified (SMITH *et al.* 1996). We have now found a genomic sequence that seems to correspond to a 6-bp exon; this would explain the origin of part of this variability. Although unusual, examples of such small exons exist (COLEMAN *et al.* 1987; SANTONI *et al.* 1989; REYES *et al.* 1991), and alternative splicing involving a three-amino acid insert in the loop between domains II and III has been reported for a class-C calcium channel from rat (SNUTCH *et al.* 1991). The possible significance of the variability in the IVS3/IVS4 loop is unclear. However, it is interesting to note that alternative inclusion/exclusion of a 33-bp exon, encoding a central part of this loop, occurs in some mammalian calcium channels (SOLDATOV 1994).

The second region of *DmcaA1* giving rise to alternative exons encode the IS3/IS4 loop and the IS4 voltage sensor. There, the two isoforms, differing with respect to a 33-amino acid region of the polypeptide, vary in the number of positively charged residues in the voltage sensor (six in IS4a and five in IS4b); this could affect the voltage sensitivity of the channel. The two forms are also quite different in the IS3/IS4 loop, which has been shown to have a critical role in the activation kinetics in mammalian L-type calcium channels (NAKAI *et al.* 1994). Therefore, this alternative-splice is likely to affect the electrophysiological properties of the two channel forms in question.

The third region that undergoes alternative splicing is the loop between homologous domains I and II, where two 116-bp mutually exclusive alternative exons exist. Interestingly, the region of the loop encoded by these exons has been implicated in the binding of the calcium-channel β-subunit, which modulates channel activity (PRAGNELL *et al.* 1994). Because the two isoforms—I/IIa and I/IIb—show many amino-acid differences, including some at putatively important positions (SMITH *et al.* 1996), it possible that they interact with different β-subunits.

In the two regions where mutually exclusive alternative exons exist, sequence analysis showed that in both cases the isoforms are considerably different (Figures 3 and 4). If this simply reflects the age of the putative (intragenic) duplication events that created the additional forms, one would expect to find perhaps the same exons duplicated in some related vertebrate channel genes or in distantly related arthropod species. That remains to be seen. Among the large number of vertebrate calcium channels that have been cloned (reviewed in HOFMANN *et al.* 1994; STEA *et al.* 1995), to our knowledge no alternative exons have been reported on these regions.

Another possible, and perhaps more likely, explanation for the high level of protein divergence between the mutually exclusive alternative exons is that, instead of reflecting evolutionary age, those differences resulted from accelerated rates of divergence. This could be the result of relaxation of constrains due to the "redundancy" following duplication, positive selection that took advantage of new variants to perform new functions, or more likely both (OHTA 1991, 1994; THOMAS 1993). The fact that this gene is internally repetitive could mean that not only point mutations but also other mechanisms, such as gene conversion and slippage (reviewed in DOVER 1993), also played a role in the rapid evolution of these alternative exons.

Whereas gene duplications and alternative splicing are a rich source of complexity in ion channels, we have preliminary evidence for an extra level of variability in *Dmca1A*, putatively caused by RNA editing. A number of sites were identified at which an adenosine is found in the genomic sequence (Table 2), whereas a guanosine is found in at least one of the cDNAs analyzed (SMITH *et al.* 1996). This finding indicates that a mechanism of RNA editing, like that known for glutamate receptors of vertebrates (HIGUCHI *et al.* 1993; RUETER *et al.* 1995; HERB *et al.* 1996), might occur in this Drosophila calcium channel. Note that all edits in the glutamate receptors are A → G, and that this is a particular subcategory of this kind of posttranscriptional modification (reviewed in BASS 1993).

In the mammalian ligand-gated channels, RNA editing has an important effect on kinetic properties (LOMELI *et al.* 1994); it occurs via adenosine deamination, which requires a double-stranded RNA structure formed by exonic and intronic sequences. Recently, a candidate gene encoding the enzyme involved in this editing was cloned in mammals (MELCHER *et al.* 1996). We examined the RNA secondary structure of exonic and intronic sequences around the edited sites and found possibly analogous sequences (*i.e.*, related to secondary structure of the *Dmca1A* transcript). However, important differences regarding the double-stranded RNA structure exist between the examples of RNA editing reported for the glutamate receptor (HIGUCHI *et al.* 1993; RUETER *et al.* 1995; HERB *et al.* 1996), so the meaning of sequences flanking the edited sites for *Dmca1A* is highly speculative. One way to examine these editing issues further in Drosophila would be to carry out an evolutionary analysis of the edited sites and neighboring sequences in relatives of *D. melanogaster*. Allied

to the analysis (via RT-PCR) of the frequency of the different editing events in different species, this would give insights on the importance of these flanking sequences and on how conserved is the editing. A similar approach was used, for example, to show the conservation of alternative splicing patterns of *para* (THACKERAY and GANETZKY 1995).

An interesting point raised by the putative RNA edits in *Dmca1A*'s primary transcript is its evolutionary significance. Assuming that the frequency of editing is not 100% per site, as suggested by cDNA analysis (SMITH *et al.* 1996), and that the editing of one site is independent of others, there are >1000 possible combinations for the 10 different editing events that cause amino-acid changes. Although the RNA editing might be developmentally and/or tissue regulated, and many of the combinations might never occur, it is conceivable that at least part of this variability might be not functionally meaningful and, as a corollary, selectively neutral.

As mentioned before, the *Dmca1A* calcium-channel α1-subunit was cloned during the molecular characterization of a genomic region originally identified by the *cacophony* song mutation, the *night-blind-A* visual mutations, and *l(1)L13* lethals. Inasmuch as *cac* and *l(1)L13s* are allelic and the latter do map with the *Dmca1A* transcription unit (SMITH *et al.* 1996), *cac* is very likely also to be mutated there. This large gene indeed presents a large target for changes that could affect the song, perhaps by altering patterned neuronal (or muscle) output (*cf.* HILLE 1992) via protein-structural variations. In this respect, molecular analysis of this gene in different species could be evolutionarily interesting from the reproductive behavioral perspective. The song produced by males of different Drosophila species usually have distinct features, and these are thought to be one of the factors in the reproductive isolation (*e.g.*, KYRIACOU and HALL 1982, 1986). Thus, analysis of the species differences at the molecular level and how those affect the lovesong will determine whether variation at this ion-channel locus could have played a role in speciation (COYNE 1992), in addition to the matter of its function underlying the basics of song control.

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LITERATURE CITED

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

BASS, B. L., 1993 RNA editing: new uses for old players in the RNA world, pp. 383–418 in *The RNA World,* edited by R. F. GESTELAND and J. F. ATKINS. Cold Spring Harbor Laboratory Press, NY.

CHANDY, K. G., and G. A. GUTMAN, 1995 Voltage-gated potassium channels, pp. 1–71 in *Ligand- and Voltage-Gated Ion Channels,* edited by R. A. NORTH. CRC Press, Inc., Boca Raton, FL.

COLEMAN, K. G., S. J. POOLE, M. P. WEIR, W. C. SOELLER and T. KORNBERG, 1987 The *invect* gene of Drosophila: sequence analysis and express studies reveal a close kinship to the *engrailed* gene. Genes Dev. **1:** 19–28.

COWAN, T. M., and R. W. SIEGEL, 1986 Drosophila mutations that alter ion conduction disrupt acquisition and retention of a conditioned odor avoidance response. J. Neurogenet. **3:** 187–201.

COYNE, J. A., 1992 Genetics and speciation. Nature **355:** 511–515.

DIEBOLD, R. J., W. J. KOCH, P. T. ELLINOR, J-J. WANG, M. MUTHUCHAMY *et al.,* 1992 Mutually exclusive exon splicing of the cardiac channel α1 subunit gene generates developmentally regulated isoforms in the rat heart. Proc. Natl. Acad. Sci. USA **89:** 1497–1501.

DOVER, G., 1993 Evolution of genetic redundancy for advanced players. Curr. Opin. Genet. Dev. **3:** 902–910.

DUNLAP, K., J. I. LUEBKE and T. J. TURNER, 1995 Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neuro. Sci. **18:** 89–98.

FELSENSTEIN, J., 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:** 783–791.

FUJITA, Y., M. MYNLIEFF, R. T. DIRKSEN, M-S. KIM, T. NIIDOME *et al.,* 1993 Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rabbit brain. Neuron **10:** 585–598.

GENETICS COMPUTER GROUP, 1991 Program manual for the GCG package, Version 7, April 1991.

GEORGE, A. L., G. S. IYER, R. KLEINFIELD, R. G. KALLEN and R. L. BARCHI, 1993 Genomic organization of the human skeletal muscle sodium channel gene. Genomics **15:** 598–606.

GORALSKI, T. J., 1985 A molecular analysis of the female sterile locus *gastrulation defective* (*gd*) of *Drosophila melanogaster,* Ph.D. thesis. Indiana University, Bloomington, IN.

GRABNER, M., A. BACHMANN, F. ROSENTHAL, J. STRIESSNIG, C. SCHULTZ *et al.,* 1994 Insect calcium channels. Molecular cloning of an α1-subunit from housefly (*Musca domestica*) muscle. FEBS Lett. **339:** 189–194.

HALL, J. C., 1994 The mating of a fly. Science **264:** 1702–1714.

HERB, A., M. HIGUSHI, R. SPRENGEL and P. H. SEEBURG, 1996 Q/R site editing in kainate receptor GluR5 and GluR6 pre-mRNAs require distant intronic sequences. Proc. Nat. Acad. Sci. USA **93:** 1875–1880.

HIGUCHI, M., F. N. SINGLE, M. KOHLER, B. SOMMER, R. SPRENGEL *et al.,* 1993 RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. Cell **75:** 1361–1370.

HILLE, B., 1992 *Ionic Channels of Excitable Membranes.* Sinauer, Sunderland, MA.

HOFMANN, F., M. BIEL and V. FLOCKERZI, 1994 Molecular basis for Ca^{2+} channel diversity. Annu. Rev. Neurosci. **17:** 399–418.

HOOPER, J. E., M. PEREZ-ALONSO, J. R. BERMINGHAM, M. PROUT, B. A. ROCKLEIN *et al.,* 1992 Comparative studies of Drosophila *Antennapedia* genes. Genetics **132:** 453–469.

HUI, A., P. T. ELLINOR, O. KRIZANOVA, J-J. WANG, R. J. DIEBOLD *et al.,* 1991 Molecular cloning of multiple subtypes of a novel rat brain isoform of the α1 subunit of the voltage-dependent calcium channel. Neuron **7:** 35–44.

IWABE, N., K. KUMA and T. MIYATA, 1996 Evolution of gene families and relationship with organismal evolution: rapid divergence of tissue-specific genes in the early evolution of chordates. Mol. Biol. Evol. **13:** 483–493.

JAN, L. Y., and Y. N. JAN, 1990 A superfamily of ion channels. Nature **345:** 672.

KAMB, A., J. TSENG-CRANK and M. A. TANOUYE, 1988 Multiple products of the Drosophila *Shaker* gene may contribute to potassium channel diversity. Neuron **1:** 421–430.

KULKARNI, S. J., and J. C. HALL, 1987 Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster.* Genetics **115:** 461–475.

KYRIACOU, C. P., and J. C. HALL, 1982 The function of courtship song rhythms in Drosophila. Anim. Behav. **30:** 784–801.

KYRIACOU, C. P., and J. C. HALL, 1986 Interspecific genetic control of courtship song production and reception in Drosophila. Science **232:** 494–497.

LEICHT, B. G., E. M. LYCKEGAARD, C. M. BENEDICT and A. G. CLARK, 1993 Conservation of alternative splicing and genomic organization of the myosin alkali light-chain (*Mlc1*) gene among Drosophila species. Mol. Biol. Evol. **10:** 769–790.

LILLY, M., R. KREBER, B. GANETZKY and J. R. CARLSON, 1994 Evidence that the Drosophila olfactory mutant *smellblind* defines a

novel class of sodium channel mutation. Genetics **136:** 1087–1096.

LOMELI, H., J. MOSBACHER, T. MELCHER, T. HOGER and J. R. GEIGER, 1994 Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. Science **266:** 1709–1713.

LONG, M., C. ROSENBERG and W. GILBERT, 1995 Intron phase correlation and the evolution of the intron/exon structure of genes. Proc. Natl. Acad. USA **92:** 12495–12499.

LOUGHNEY, K., R. KREBER and B. GANETZKY, 1989 Molecular analysis of the *para* locus, a sodium channel gene in Drosophila. Cell **58:** 1143–1154.

MCCLESKEY, E. W., 1994 Calcium channels: cellular roles and molecular mechanisms. Curr. Opin. Neurobiol. **4:** 304–312.

MELCHER, T., S. MAAS, A. HERB, R. SPRENGEL, P. H. SEEBURG *et al.*, 1996 A mammalian RNA editing enzyme. Nature **379:** 460–463.

MORI, Y., T. FRIEDRICH, M-S. KIM, A. MIKAMI, J. NAKAI *et al.*, 1991 Primary structure and functional expression from complementary DNA of a brain calcium channel. Nature **350:** 398–402.

MOUNT, S. M., C. BURKS, G. HERTZ, G. D. STORMO, O. WHITE *et al.*, 1992 Splicing signals in Drosophila: intron size, information content, and consensus sequences. Nucleic Acids Res. **20:** 4255–4262.

NAKAI, J., B. A. ADAMS, K. IMOTO and K. G. BEAM, 1994 Critical roles of the S3 segment and S3-S4 linker of repeat I in activation of L-type calcium channels. Proc. Natl. Acad. Sci. USA **91:** 1014–1018.

NIIDOME, T., M-S. KIM, T. FRIEDRICH and Y. MORI, 1992 Molecular cloning and characterization of a novel calcium channel from rabbit brain. FEBS Lett. **308:** 7–13.

OHTA, T., 1991 Multi gene families and the evolution of complexity. J. Mol. Evol. **33:** 34–41.

OHTA, T., 1994 Further examples of evolution by gene duplication revealed through DNA sequences comparisons. Genetics **138:** 1331–1337.

PERNEY, P. T., and L. K. KACZMAREK, 1991 The molecular biology of K^+ channels. Curr. Opin. Cell Biol. **3:** 663–670.

PRAGNELL, M., M. D. WAARD, Y. MORI, T. TANABE, T. P. SNUTCH *et al.*, 1994 Calcium channel B-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α1-subunit. Nature **368:** 67–70.

REYES, A. A., S. J. SMALL and R. AKESON, 1991 At least 27 alternatively spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. Mol. Cell Biol. **11:** 1654–1661.

RUETER, S. M., C. M. BURNS, S. A. COODE, P. MOOKHERJEE and R. B. EMERSON, 1995 Glutamate receptor RNA editing *in vitro* by enzymatic conversion of adenosine to inosine. Science **267:** 1491–1494.

SAITOU, N., and M. NEI, 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **9:** 945–967.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANTONI, M. J., D. BARTHELS, G. VOPPER, A. BONED, C. GORIDIS *et al.*, 1989 Differential exon usage involving an unusual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. EMBO J. **8:** 385–392.

SCHILCHER, F. V., 1977 A mutant which changes courtship song in *Drosophila melanogaster.* Behav. Genet. **7:** 251–259.

SCHWARTZ, T. L., B. L. TEMPEL, D. M. PAPAZIAN, Y. N. JAN and L. Y. JAN, 1988 Multiple potassium-channel components are produced by alternative splicing at the *Shaker* locus in Drosophila. Nature **331:** 137–142.

SMITH, L. A., X. J. WANG, A. A. PEIXOTO, E. K. NEUMANN, L. M. HALL *et al.*, 1996 A Drosophila calcium channel α-1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J. Neurosci. **16:** 7868–7879.

SNUTCH, T. P., W. J. TOMLINSON, J. P. LEONARD and M. M. GILBERT, 1991 Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. Neuron **7:** 45–57.

SOLDATOV, N. M., 1992 Molecular diversity of L-type Ca^{2+} channel transcripts in human fibroblasts. Proc. Natl. Acad. Sci. USA **89:** 4628–4632.

SOLDATOV, N. M., 1994 Genomic structure of human L-type Ca^{2+} channel. Genomics **22:** 77–87.

STEA, A., T. W. SOONG and T. P. SNUTCH, 1995 Voltage-gated calcium channels, pp 114–151 in *Ligand- and Voltage-gated Ion Channels,* edited by R. A. NORTH. CRC Press, Inc., Boca Raton, FL.

STOLTZFUS, A., D. F. SPENCER, M. ZUKER, J. M. LOGSDON and W. F. DOOLITTLE, 1994 Testing the exon theory of genes: the evidence from protein structure. Science **265:** 202–207.

STRONG, M., K. G. CHANDY and G. A. GUTMAN, 1993 Molecular evolution of voltage-sensitive ion channel genes: on the origin of electrical excitability. Mol. Biol. Evol. **10:** 221–242.

SWIMMER, C., M. G. FENERJIAN, J. C. MARTINEZ-CRUZADO and F. C. KAFATOS, 1990 Evolution of the autosomal chorion cluster in Drosophila. III. Comparison of the *s18* gene in evolutionarily distant species and heterospecific control of chorion gene amplification. J. Mol. Biol. **215:** 225–235

TAMKUN, J. W., R. DEURING, M. P. SCOTT, M. KISSINGER, A. M. PATTATUCCI *et al.*, 1992 *brahma:* a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell **68:** 561–572.

TANABE, T., H. TAKESHIMA, A. MIKAMI, V. FLOCKERZI, H. TAKAHASHI *et al.*, 1987 Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature **328:** 313–318.

THACKERAY, J. R., and B. GANETZKY, 1994 Developmentally regulated alternative splicing generates a complex array of Drosophila *para* sodium channels isoforms. J. Neurosci. **14:** 2569–2578.

THACKERAY, J. R., and B. GANETZKY, 1995 Conserved alternative splicing patterns and splicing signals in the Drosophila sodium channel gene *para.* Genetics **141:** 203–214.

THOMAS, J. H., 1993 Thinking about genetic redundancy. Trends Genet. Sci. **9:** 395–399.

THOMPSON, J. D., D. G. HIGGINS and T. J. GIBSON, 1994 CLUSTAL W: improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

WILLIAMS, M. E., D. H. FELDMAN, A. F. MCCUE, R. BRENNER, G. VELICELEBI *et al.*, 1992 Structure and functional expression of α1, α2, and β subunits of a novel human neuronal calcium channel subtype. Neuron **8:** 71–84.

WU, C-F., and B. GANETZKY, 1992 Neurogenetic studies of ion channels in Drosophila. Ion Channels **3:** 261–314.

ZHENG, W., G. FENG, D. REN, D. F. EBERL, F. HANNAN *et al.*, 1995 Cloning and characterization of a calcium channel α1 subunit from *Drosophila melanogaster* with similarity to the rat brain type D isoform. J. Neurosci. **15:** 1132–1143.

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