# Cloning and Characterization of a Calcium Channel $\alpha_1$ Subunit from *Drosophila melanogaster* with Similarity to the Rat Brain Type D Isoform

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We report the complete sequence of a calcium channel  $\alpha_1$ subunit cDNA cloned from a Drosophila head cDNA library. This cDNA encodes a deduced protein containing 2516 amino acids with a predicted molecular weight of 276,493. The deduced protein shares many features with vertebrate homologs, including four repeat structures, each containing six transmembrane domains, a conserved ion selectivity filter region between transmembrane domains 5 and 6, and an EF hand in the carboxy tail. The Drosophila subunit has unusually long initial amino and terminal carboxy tails. The region corresponding to the last transmembrane domain (IVS6) and the adjacent cytoplasmic domain has been postulated to form a phenylalkylamine-binding site in vertebrate calcium channels. This region is conserved in the Drosophila sequence, while domains thought to be involved in dihydropyridine binding show numerous changes. The Drosophila subunit exhibits 78.3% sequence similarity to the rat brain type D calcium channel  $\alpha_1$  subunit, and so has been designated as a Drosophila melanogaster calcium channel  $\alpha_1$ type D subunit (Dmca1D). In situ hybridization shows that Dmca1D is highly expressed in the embryonic nervous system. Northern analysis shows that Dmca1D cDNA hybridizes to three size classes of mRNA (9.5, 10.2, and 12.5 kb) in heads, but only two classes (9.5 and 12.5 kb) in bodies and legs. PCR analysis suggests that the Dmca1D message un-

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dergoes alternative splicing with more heterogeneity appearing in head and embryonic extracts than in bodies and legs.

[Key words: calcium channel, Drosophila melanogaster, cDNA sequence, polymerase chain reaction, ion channel evolution, channel structure, ion selectivity filter, phenylalkylamine-binding site, dihydropyridine-binding site]

Calcium channels are ubiquitous and are found in species ranging from *Paramecium* to humans. They are involved in many cell functions, including membrane excitability, synaptic transmission, and differentiation (Tsien et al., 1988). These channels are comprised of multiple subunits designated  $\alpha_1, \alpha_2, \beta, \gamma$ , and  $\delta$  (Catterall, 1991a,b). The  $\alpha_2$  and  $\delta$  subunits are encoded by the same gene and are cleaved during post-translational processing, whereas each of the other subunits arise from different genes. Gene cloning studies, which have focused exclusively on vertebrate species, have elucidated the molecular nature of calcium channel structure and have suggested a remarkable degree of channel heterogeneity beyond that predicted from physiological and pharmacological approaches. This molecular diversity of calcium channels arises from several mechanisms. One mechanism involves the presence of a family of genes, each encoding genetic variants of a given subunit (Snutch et al., 1990, 1991; Hui et al., 1991; Starr et al., 1991; Dubel et al., 1992; Williams et al., 1992a,b; Soong et al., 1993). For each member of a gene family further diversity is introduced by alternative splicing (Biel et al., 1990; Koch et al., 1990; Perez-Reyes et al., 1990; Snutch et al., 1991). If each subunit variant interacts with more than one form of each of the other subunits to form functional channels, then there is a potential for even further molecular diversity.

Early electrophysiological studies on invertebrate preparations suggested the presence of multiple types of voltage-dependent calcium channels (reviewed by Hille, 1992). Although studies of the molecular diversity of calcium channels in invertebrates are just beginning, there is evidence for structural and functional heterogeneity. Binding of phenylalkylamines (calcium channel blockers) to *Drosophila* head extracts showed curvilinear Scatchard plots indicative of multiple receptor classes (Greenberg et al., 1989). Pelzer et al. (1989) reported at least eight distinct voltage-sensitive calcium channels in *Drosophila* head membranes reconstituted into phospholipid bilayers. Patchclamp studies on cultured embryonic *Drosophila* myocytes and neurons also showed variability of channel properties, suggest-

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ing at least two types of neuronal calcium channels in *Drosophila* (Leung and Byerly, 1991). Further evidence for channel heterogeneity comes from differential sensitivity of *Drosophila* neuronal calcium channels to a purified toxin from the spider *Hololena curta* (Leung and Byerly, 1991). In another insect (*Periplaneta americana*), radiotracer flux studies have indicated heterogeneity through the presence of dihydropyridine-insensitive and -sensitive components of phenylalkylamine-sensitive calcium uptake in nervous system and skeletal muscle membranes, respectively (Skeer et al., 1992).

Drosophila provides an ideal system to define the significance of channel diversity by mutating individual subunit genes and determining the physiological and behavioral consequences. In this report we describe polymerase chain reaction (PCR)-initiated cloning, sequencing, genetic mapping, and expression pattern analysis of an  $\alpha_1$  subunit from Drosophila. This approach allows rapid cloning of related genes from evolutionarily distant organisms and should be applicable for the cloning of  $\alpha_1$  subunits from other invertebrate preparations of physiological or economic importance.

### **Materials and Methods**

#### Polymerase chain reaction (PCR)

*Primer design.* Primer sites were selected by aligning cDNA sequences for  $\alpha_1$  subunits of calcium channels from rabbit skeletal muscle (Tanabe et al., 1987), heart (Mikami, 1989), and brain (Mori et al., 1991), rat aorta (Koch et al., 1990), and fish skeletal muscle (Grabner et al., 1991) to identify the most highly conserved regions with the least amount of codon degeneracy. Inosine was used when A, T, G, and C were all a possibility at a given site (Martin et al., 1985; Knoth et al., 1988). Figure 1 shows the positions of a successful primer pair (P6 and P7) as shaded areas in the consensus (con) sequence in the carboxy portion of the channel. Primer P6 lies within IVS5 and has the sequence 5'AT[C/T/A]G[T/C]IATG[C/T]TIT[C/T]TT[C/T]ATITA[C/T]GC3'. Primer P7 lies between IVS6 and the putative EF hand and has the sequence 5'TC[G/A]TCIA[G/A][G/A]TG[G/A]TGIGGICCIA[G/A][G/A][G/A]

Reaction conditions for cross-species amplifications. The template for the polymerase chain reaction was 150 ng of Drosophila genomic DNA prepared from adult flies as described by Jowett (1986). The 50  $\mu$ l reaction mixture contained 0.2 mM of each of the dNTPs, 10 mM Tris buffer, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.1  $\mu$ M of each primer, and 1.25 units AmpliTaq DNA polymerase from Perkin Elmer Cetus (Norwalk, CT). Following an initial 2 min at 95°C, the following cycle was repeated 35 times: denaturation 2 min at 95°C, annealing 2 min at 40°C, extension 2 min at 72°C. The final extension was 10 min at 72°C. PCR products were analyzed by electrophoresis of 10  $\mu$ l of reaction mix on a 1% agarose gel.

#### DNA sequencing

The band containing the PCR product of interest was extracted from the gel by the phenol/freezing method of Benson (1984), resuspended in TE buffer, pH 8.0 (Sambrook et al., 1989), to a concentration of 10-20 ng/µl, and 25 ng template was used for reamplification in 100 µl reactions prior to sequencing. The PCR conditions were as described above, except that the annealing temperature was 65°C. Sequencing templates were purified and concentrated using Centricon-100 columns (Amicon, Danvers, MA). Double-stranded DNA sequencing was performed on an Applied Biosystems Sequencer Model 373A using the dideoxy chain termination method with fluorescent dye-tagged M13 or SP6 primers according to instructions supplied with a Taq Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, CA). Using this approach, 300-400 bases were generally read from each template. Each segment of DNA was sequenced at least twice in each direction. For sequencing PCR products without subcloning or for sequencing phage clones, new tailed primers were synthesized adding an 18 nucleotide M13 or SP6 sequence to the 5' end of the original PCR primer sequence.

#### Screening for cDNA clones

A total of 2  $\times$  10<sup>s</sup> plaque forming units (pfu) of a Drosophila head cDNA library in \gt11 (generously provided by Dr. Paul Salvaterra, Beckman Research Institute, Duarte, CA) (Itoh et al., 1986) were screened on Nylon membranes (ICN, Costa Mesa, CA) using the 499 base pair amplification product from primer pair P6/P7. The probe was randomprime labeled with <sup>32</sup>P-dCTP using the Multiprime Kit (Amersham Corp., Arlington Heights, IL). Standard conditions were used for prehybridization, hybridization, and washing (Sambrook et al., 1989). A 4 kb cDNA clone (SH22C; encodes the 3' end of Dmca1D) was isolated initially and further clones (including W8A, which encodes a portion of the 5' end of Dmca1D, and SH22D, which encodes a portion of Dmca1D that overlaps W8A and SH22C) were obtained using the 5' end of SH22C. Since W8A did not contain the 5' end of the open reading frame, rapid amplification of cDNA ends (RACE) was done with the 5' RACE kit from Clontech (Palo Alto, CA) and a primer from the 5' end of W8A, and extended the sequence 360 bases upstream. Since this extension was still incomplete, the 5' end of W8A was also used to isolate the N1 cDNA clone encoding the 5' end of Dmca1Dc.

#### In situ hybridization to salivary gland chromosome squashes

The map position of the cloned cDNA was determined as described previously (Engels et al., 1985; Murtagh et al., 1993) using biotinylated probes hybridized to salivary gland chromosomes.

#### Northern blots

Heads, bodies, and legs were isolated from frozen adult flies as described by Schmidt-Nielsen et al. (1977). Total RNA was prepared and polyA+ mRNA isolated by the guanidinium isothiocyanate-CsCl gradient mcthod, followed by one passage over oligo (dT)-cellulose columns (Sambrook et al., 1989). Ten micrograms of polyA+ RNA in TE was added to each lane of an 0.8% agarose gel containing 6.3% formaldehyde and electrophoresed for 3 hr at 100 V using 1× MOPS buffer according to Sambrook et al. (1989). The gel was capillary blotted onto a nylon membrane (Schleicher and Schuell, Keene, NH) and fixed by UV crosslinking. Prehybridization was 6 hr at 42°C in 50% deionized formamide,  $5 \times$  SSPE,  $5 \times$  Denhardt's, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA, and then 106 cpm/ml 32P-labeled cDNA probe was added and the incubation continued for 16 hr at 42°C. The blot was washed two times for 15 min each at room temperature in  $2 \times SSC$ , 0.1% SDS followed by two more washes for 30 min each at 65°C in 0.1× SSC, 0.1% SDS. The blots were exposed to x-ray film at  $-70^{\circ}$ C. Standard solutions (SSC, SSPE, Denhardt's) are as described by Sambrook et al. (1989).

#### Reverse transcriptase-coupled PCR (RT-PCR)

First-strand cDNA synthesis in 50  $\mu$ l was conducted at 42°C for 60 min using 1200 units/ml AMV (avian myeloblastosis virus) reverse transcriptase and 80  $\mu$ g/ml polyA<sup>+</sup> mRNA (see preceding section) as described by Gubler and Hoffman (1983) with the following changes: 40  $\mu$ g/ml oligo dT primer, 50 mM KCl, 0.5 mM spermidine, 1 mM each dNTP, 800 units/ml RNasin. The reaction was stopped with 1 mM EDTA, then 0.5  $\mu$ l of the reaction mix was used for a 50  $\mu$ l PCR as described for the cross-species amplifications above except that 0.005% gelatin was used and the amplification was 35 cycles of 95°C 1 min, 60° 1 min, 72° 1 min, followed by a final 5 min extension at 72°. Forty microliters of the amplification reaction was electrophoresed and extracted from an agarose gel by the freezing phenol method described above. DNA pellets were resuspended in 20  $\mu$ l distilled water and 6  $\mu$ l was used for each restriction enzyme digestion described in Table 1.

### In situ hybridization to embryo whole-mounts

Whole-mount *in situ* hybridization to *Drosophila* embryos was done as described by Tautz and Pfeifle (1989) using the formaldehyde fixation method. A single-stranded digoxigenin-labeled cDNA probe was prepared from a PCR product [corresponding to the region encoding amino acids 2163–2259 in *Drosophila melanogaster* calcium channel  $\alpha_1$  type D subunit (Dmca1D) in Fig. 1] which had been extracted from the gel using an Ultrafree-MC filter unit from Millipore Corp. (Bedford, MA), and concentrated using a Centricon-30 spin column. This purified PCR product (200 ng) was used as template to prepare single-stranded and

Dmca1	MGGGELVNCI	AYDDNTLVIE	RKPSPSSPS1	SRRYLKAETE	TRGSRKYNRK	SSAKSDLEVV	VVKPEHHHQF	A RSPTITLPVE	ANPLTTSASA	90
Dmca1	* GSSPTGAGLA	AGLGTASGTV	LQQSCSALDP	PEDSNQPSGT	RRRATSTELA	LSNVTSQIVN	NATYKLDFKQ	RRHKSNNGGS	ESGSLTGIAT	180
Dmca1		GPTSSSGKRR								
Dmca1	DITGDNSTLH	GLGVGDVCSF VGEVDDNADV	IADCDDNSED	DDGDPNNQDL	SSQTLRTAAI	VAAVAAAAKE	QAQEQSLADO	ESFSDRRQDA	DEDVRIIQDC	360
Dmca1	CGGINNDSLEL	VGEVDDNADV	VVKKNSKNKP	SIRRICRITE	, EDDDEDENAL	IGDFDREDQE	LUDEEPEGTI	. IDIDEQEQQE	DQGDSAEEEL	450
con										
Ratbd Dmca1	DDEDUDEVEE	EEEDDTQAFS	DEVOCONEL I	DNEGGONORE		HQRQQQEDHA				
Diicai	DDEDVDEIFE	ELEDDIQARS	FFISSSAELI	DNIGGGAGRE	*	SGEGGFSFNG	NGGPG5GDV5	RIARIDSGEG	*	540
con	4	tm.p	5 a 2	rk a	kka p	PRIECIAL	NDIP SCT	TUOWKDEO	IS1	
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Dmca1	IDSMGIANIP	ETMNGTTIGP	SGAGGQKGGA	AAGAAGQKRQ	QRRGKPQPDR	PQRALFCLSV	KNPLRALCIR	IVEWKPFEFL	ILLTIFANCI	630
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Ratbd	6766	FRVLRPLRLV								317
Rabsk		FRVLRPLRLV								
Dmca1	DAFDVKALRA	FRVLRPLRLV	SGVPSLQVVL	NSILKAMVPL	FHIALLVLFV	IIIYAIIGLE	LFSGKLHKAC	RDEITG	EYEENIRPC.	793
				SS1		SS2		IS6		
	G.GrqC						· · · · · · · · · · · · · · · · · · ·			100000
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Dmca1		.GYKCYGGWD								
	FsKEReKAK.									washing a
	FSKEREKAKA FTKEREKAKS									
Rabar	FIRERERARD									
	FSKERNKAKN			LDWITQAEDI			DSTENLGEEM			
Dmca1	FSKERNKAKN	RGDFQKLREK	QQIEEDLRGY	LDWITQAEDI	EPDAVGGLIS	DGKGKQPNEM	DSTENLGEEM	PEVQMTES	RW	
Dmca1	FSKERNKAKN .kr.wrrw SKLSRRWRRW	RGDFQKLREK NRRr.Cr. NRFNRRRCRA	QQIEEDLRGY aVKSFYWL AVKSVTFYWL	LDWITQAEDI IIS1 VIVIVFLNT1 VIVLVFLNTL	EPDAVGGLIS .iasEHynQp TISSEHYNQP	DGKGKQPNEM dWLtQdia DWLTQIQDIA	DSTENLGEEM IIS2 N.VII.LFTC NKVLLALFTC	PEVQMTES EML1KMYsLG EMLVKMYSLG	RW lq.YFvSIFN LQAYFVSLFN	960 587
Dmca1 con Ratbd Rabsk	FSKERNKAKN .kr.wrrw SKLSRRWRRW .QFIRHWRQW	RGDFQKLREK NRRr.Cr. NRFNRRCRA NRVFRWKCHD	QQIEEDLRGY aVKSFYWL AVKSVTFYWL LVKSRVFYWL	LDWITQAEDI IIS1 VIVIVFLNTI VIVLVFLNTL VILIVALNTL	EPDAVGGLIS .iasEHynQp TISSEHYNQP SIASEHHNQP	DGKGKQPNEM dWLtQdia DWLTQIQDIA LWLTHLQDIA	DSTENLGEEM IIS2 N.VII.LFTC NKVLLALFTC NRVLLSLFTI	PEVQMTES EML1KMYsLG EMLVKMYSLG EMLLKMYGLG	RW lq.yfvslfn LQAYFVSLFN LRQYFMSIFN	960 587 497
Dmcal con Ratbd	FSKERNKAKN .kr.wrrw SKLSRRWRRW .QFIRHWRQW	RGDFQKLREK NRRr.Cr. NRFNRRRCRA	QQIEEDLRGY aVKSFYWL AVKSVTFYWL LVKSRVFYWL	LDWITQAEDI IIS1 VIVIVFLNTI VIVLVFLNTL VILIVALNTL	EPDAVGGLIS .iasEHynQp TISSEHYNQP SIASEHHNQP	DGKGKQPNEM dWLtQdia DWLTQIQDIA LWLTHLQDIA	DSTENLGEEM IIS2 N.VII.LFTC NKVLLALFTC NRVLLSLFTI	PEVQMTES EML1KMYsLG EMLVKMYSLG EMLLKMYGLG	RW lq.yfvslfn LQAYFVSLFN LRQYFMSIFN	960 587 497
Dmca1 con Ratbd Rabsk	FSKERNKAKN .kr.wriw SKLSRRWRW .QFIRHWRQW RKMKKDFDRV IIS3	RGDFQKLREK NRRr.Cr. NRFNRRCRA NRVFRWKCHD NRRMRRACRK	QQIEEDLRGY aVKSFYWL AVKSVTFYWL LVKSRVFYWL AVKSQAFYWL	LDWITQAEDI IIS1 VIVIVFLNTI VIVLVFLNTL VILIVALNTL IIVLVFLNTG IIS4	EPDAVGGLIS .iasEHynQp TISSEHYNQP SIASEHHNQP VLATEHYGQL	DGKGKQPNEM dWLtQdia DWLTQIQDIA LWLTHLQDIA DWLDNFQEYT	DSTENLGEEM IIS2 N.V11.LFTC NKVLLALFTC NRVLLSLFTI NVFFIGLFTC	PEVQMTES EML1KMYsLG EMLVKMYSLG EMLLKMYSLG IIS5	RW lq.YFvSlFN LQAYFVSLFN LRQYFMSIFN FQGYFVSLFN	960 587 497
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Ratbd Rabsk	LTRDWSILGP LTRDWSILGP LTRDWSILGP	HHLDEFKRIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG	K1CPHRVACK KLCPHRVACK KFCPHRVACK KLCPHRMACK	RLVAMNMPLN RLVGMNMPLN RLVSMNMPLN	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT	LFALVRTALK LFALVRTALK LFAVVRTSLS	1476
Ratbd Rabsk	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN.eqA	HHLDEFKRIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NeELRA.IKk	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR IWKrTsmKLL	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA	Klcphrvack Klcphrvack KFCphrvack KlcphrMack TfLIQdyFRk	RLVAMNMPLN RLVGMNMPLN RLVSMNMPLN FkKRkEqg	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT y.pkTv	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRt1.	1476 2021
Ratbd Rabsk Dmcal con Ratbd	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NEELRA.IKK NEELRAVIKK	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR IWKrTSmKLL IWKKTSMKLL	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA	Klcphrvack Klcphrvack KFCphrvack KlcphrMack TfLiQdyFrk TfLiQdyFrk	RLVAMNMPLN RLVGMNMPLN RLVSMNMPLN FkkRkEqg FkkRkEQGLV	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT y.pkTv GKYPAKNTTI	LFALVRTALK LFALVRTALK .lQaglRt1. ALQMLERML	1476 2021 1646
Ratbd Rabsk Dmcal con Ratbd Rabsk	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA IKTEGNFEQA	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NEELRA.IKK NEELRAVIKK NEELRAIIKK	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR IWKrTSmKLL IWKKTSMKLL IWKRTSMKLL	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D	RTIQPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA	KlCPHRVACK KLCPHRVACK KFCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK	RLVAMNMPLN RLVSMNMPLN FkKRkEqg FKKRKEQGLV FMKRQEEYYG	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE	1476 2021 1646 1563
Ratbd Rabsk Dmcal con Ratbd	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA IKTEGNFEQA	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NEELRA.IKK NEELRAVIKK	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR IWKrTSmKLL IWKKTSMKLL IWKRTSMKLL	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D	RTIQPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA	KlCPHRVACK KLCPHRVACK KFCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK	RLVAMNMPLN RLVSMNMPLN FkKRkEqg FKKRKEQGLV FMKRQEEYYG	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE	1476 2021 1646 1563
Ratbd Rabsk Dmca1 con Ratbd Rabsk Dmca1	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA IKTEGNFEQA IKTEGN IDDA	HHLDEFKRIW HHLDEFKRIW HHLDEFKRIW NeELRA.IKK NEELRAVIKK NEELRAVIKK NSELRATIKQ	SEYDPEAKGR SEYDPEAKGR SEYDPEAKGR IWKrTSmKLL IWKKTSMKLL IWKRTSMKLL IWKRTNPKLL	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND	RTIQPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA	K1CPHRVACK KLCPHRVACK KLCPHRVACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR	RLVAMNMPLN RLVSMNMPLN FkKRkEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE	1476 2021 1646 1563
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA IKTEGNFEQA IKTEGNFEQA IKTDGN IDDA .epr.	HHLDEFKRIW HHLDEFKRIW HHLDEFKAIW NeELRA.IKK NEELRAVIKK NSELRATIKQ isg.1	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR SEYDPDAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m.	TKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVVPPGND	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA	K1CPHRVACK KLCPHRVACK KLCPHRWACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR	RLVAMNMPLN RLVGMNMPLN RLVSMNMPLN FKKRKEQg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G	SDGTVMFNAT SDGTVLFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE TLQAGLRTL. a	1476 2021 1646 1563 2109
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con Rabsk	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .e.pr. EEAAPEIRRT	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NeELRA.IKK NEELRAVIKK NSELRATIKQ isg.1 ISGDLTAEEE	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM	TKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVVPPGND  EERIFRRTGG	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA LEVTVGKFYA	KlCPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR TYLIQDYFRR	RLVAMNMPLN RLVGMNMPLN FkKRkEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE TLQAGLRTL. a LEDFPQDART	1476 2021 1646 1563 2109 1653
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con Rabsk	LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP LTRDWS ILGP IKTEGN. eqA IKTEGNLEQA IKTEGNFEQA IKTEGNFEQA IKTDGN IDDA .epr.	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFIRLW NeELRA.IKK NEELRAVIKK NSELRATIKQ isg.1 ISGDLTAEEE	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM	TKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVVPPGND  EERIFRRTGG	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA LEVTVGKFYA	KlCPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR TYLIQDYFRR	RLVAMNMPLN RLVGMNMPLN FkKRkEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF	LFALVRTALK LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE TLQAGLRTL. a LEDFPQDART	1476 2021 1646 1563 2109 1653
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con Rabsk Dmcal con	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .e.p.r. EEAAPEIRRT HEVSPALKRA .l.	HHLDEFKTIW HHLDEFKRIW HHLDEFKRIW HHLDEFIRLW NeELRA.IKK NEELRAVIKK NEELRAVIKK NSELRATIKQ isg.l ISGDLTAEEE ISGNLDELDQ V	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM EPEPMHRRHH n	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND EERIFRRTGG TLFGSVWSSI e.g.	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf LFGQVDTFLE RRHGNGTFRR	KlCPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR RTNSLPPVMA SAKATASQSN .s.s.r.l	RLVAMNMPLN RLVGMNMPLN FKKRKEq.g FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a.	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i.	1476 2021 1646 1563 2109 1653 2199
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con Rabsk Dmcal con Rabsk	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTEGNFEQA IKTDGNIDDA .epr. EEAAPEIRRT HEVSPALKRA 1 NPLARANTNN	HHLDEFKRIW HHLDEFKRIW HHLDEFKRIW HHLDEFIRLW NeELRA.IKK NEELRAVIKK NEELRAVIKK NSELRATIKQ ISG0LTAEEE ISGNLDELDQ V ANANVAYGNS	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL LERAMVEAAM EPEPMHRRHH n NHSNNQMFSS	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND EERIFRRTGG TLFGSVWSSI e.g. VHCEREFPGE	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf LFGQVDTFLE RRHGNGTFRR AETPAAGRGA	K1CPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL Q	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i. LVQPGMPINQ	1476 2021 1646 1563 2109 1653 2199 1735
Ratbd Rabsk Dmcal con Ratbd Rabsk Dmcal con Rabsk Dmcal con Rabsk	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .e.p.r. EEAAPEIRRT HEVSPALKRA .l.	HHLDEFKRIW HHLDEFKRIW HHLDEFKRIW HHLDEFIRLW NeELRA.IKK NEELRAVIKK NEELRAVIKK NSELRATIKQ ISG0LTAEEE ISGNLDELDQ V ANANVAYGNS	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL LERAMVEAAM EPEPMHRRHH n NHSNNQMFSS	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND EERIFRRTGG TLFGSVWSSI e.g. VHCEREFPGE	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf LFGQVDTFLE RRHGNGTFRR AETPAAGRGA	K1CPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL Q	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i. LVQPGMPINQ	1476 2021 1646 1563 2109 1653 2199 1735
Ratbd Rabsk Dmcal Con Ratbd Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .e.pr. EEAAPEIRRT HEVSPALKRA .l NPLARANTNN DYLYDTLNRS	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW NEELRA.IKK NEELRAVIKK NEELRAVIKK NSELRATIKQ ISGDLTAEEE ISGNLDELDQ V. ANANVAYGNS VADGVNNITR	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM EPEPMHRRHH NNHSNNQMFSS NIMQARLAAA	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND  EERIFRRTGG TLFGSVWSSI eg. VHCEREFPGE GKLQDELQGA	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf. LFGQVDTFLE RRHGNGTFRR AETPAAGRGA GSGGELRTFG	KlCPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR  RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP ESISMRPLAK	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN NGGGAATVAG	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL GQ TLPPEANAIN	LFALVRTALK LFAVVRTSLS .lQaglRtl. ALQMLERML QIQAGLRTIE TLQAGLRTIE LEDFPQDART VLGSSDPAGG i. LVQPGMPINQ YDNRNRGILL	1476 2021 1646 1563 2109 1653 2199 1735
Ratbd Rabsk Dmcal Con Ratbd Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal Con	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTEGNFEQA IKTDGNIDDA .epr. EEAAPEIRRT HEVSPALKRA 1 NPLARANTNN	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW NEELRA.IKK NEELRA.IKK NEELRAVIKK NSELRATIKQ isg.1 ISGDLTAEEE ISGNLDELDQ V ANANVAYGNS VADGVNNITR P	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM EPEPMHRRHH nNHSNNQMFSS NIMQARLAAA .ss.d.	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND  EERIFRRTGG TLFGSVWSSI eg. VHCEREFPGE GKLQDELQGA SS	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf LFGQVDTFLE RRHGNGTFRR AETPAAGRGA GSGGELRTFG	KlCPHRVACK KLCPHRVACK KLCPHRMACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR  RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP ESISMRPLAK .lil	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN NGGGAATVAG gld.	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL  GQ TLPPEANAIN fv	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i LVQPGMPINQ YDNRNRGILL .am.pee.	1476 2021 1646 1563 2109 1653 2199 1735 2289
Ratbd Rabsk Dmcal Con Ratbd Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal Con	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .epr. EEAAPEIRRT HEVSPALKRA 1 NPLARANTNN DYLYDTLNRS .pp. APPAPCQQPS	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW NEELRA.IKK NEELRA.IKK NEELRAVIKK NSELRATIKQ isg.1 ISGDLTAEEE ISGNLDELDQ V ANANVAYGNS VADGVNNITR P	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKRTSMKLL IWKRTSMKLL IWKRTNPKLL m LERAMVEAAM EPEPMHRRHH NNHSNNQMFSS NIMQARLAAA .ss.d. TSLTGSLQDE	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPP.G.D DQVVPPAG.D DQVIPPIG.D DQVVPPGND  EERIFRRTGG TLFGSVWSSI eg. VHCEREFPGE GKLQDELQGA SS APQRRSSEGS	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf. LFGQVDTFLE RRHGNGTFRR AETPAAGRGA GSGGELRTFG TPRRPAPATA	KlCPHRVACK KLCPHRVACK KLCPHRWACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR  RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP ESISMRPLAK .lil LLIQEALVRG	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGUV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN NGGGAATVAG gld GLDTLAADAG	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL  GQ TLPPEANAIN fv FVMATSQALV	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i LVQPGMPINQ YDNRNRGILL .am.pee. DACQMEPEEV	1476 2021 1646 1563 2109 1653 2199 1735 2289 1825
Ratbd Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal Con Rabsk Dmcal	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .epr. EEAAPEIRRT HEVSPALKRA 1 NPLARANTNN DYLYDTLNRS .pp. APPAPCQQPS HPYNNVYAPN	HHLDEFKTIW HHLDEFKRIW HHLDEFKAIW HHLDEFKAIW NEELRA.IKK NEELRAVIKK NSELRATIKQ isg.l ISGDLTAEEE ISGNLDELDQ V ANANVAYGNS VADGVNNITR p TDPPERGQRR GALPGHERMI	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKKTSMKLL IWKRTSMKLL IWKRTNPKLL m. LERAMVEAAM EPEPMHRRHH NNHSNNQMFSS NIMQARLAAA .ss.d. TSLTGSLQDE QSTPASPYDQ	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPPAG.D DQVVPPAG.D DQVVPPGND  EERIFRRTGG TLFGSVWSSI eg. VHCEREFPGE GKLQDELQGA SS APQRRSSEGS RRLPTSSDMN	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf. LFGQVDTFLE RRHGNGTFRR AETPAAGRGA GSGGELRTFG TPRRPAPATA GLAE	KlCPHRVACK KLCPHRVACK KLCPHRWACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR  RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP ESISMRPLAK .lil LLIQEALVRG	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGUV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN NGGGAATVAG gld GLDTLAADAG	SDGTVMFNAT SDGTVTFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL  GQ TLPPEANAIN fv FVMATSQALV	LFALVRTALK LFAVVRTSLS .lQag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i LVQPGMPINQ YDNRNRGILL .am.pee. DACQMEPEEV	1476 2021 1646 1563 2109 1653 2199 1735 2289 1825
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Ratbd Rabsk Dmcal con Ratbd Dmcal con Rabsk Dmcal con Rabsk Dmcal con Rabsk Dmcal con	LTRDWSILGP LTRDWSILGP LTRDWSILGP LTRDWSILGP IKTEGN.eqA IKTEGNLEQA IKTEGNFEQA IKTDGNIDDA .epr. EEAAPEIRRT HEVSPALKRA .l NPLARANTNN DYLYDTLNRS .pp. APPAPCQQPS HPYNNVYAPN aa.l. EVAATELLKE	HHLDEFKRIW HHLDEFKRIW HHLDEFKAIW NeELRA.IKK NEELRAVIKK NSELRAVIKK NSELRATIKQ isg.1 ISGDLTAEEE ISGNLDELDQ V ANANVAYGNS VADGVNNITR p TDPPERGQRR GALPGHERMI SS.	SEYDPEAKGR SEYDPEAKGR AEYDPEAKGR IWKrTSmKLL IWKKTSMKLL IWKRTSMKLL IWKRTNPKLL m LERAMVEAAM EPEPMHRRHH nNHSNNQMFSS NIMQARLAAA .ss.d. TSLTGSLQDE QSTPASPYDQ .gss.	IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL IKHLDVVTLL DQVVPPAG.D DQVIPPIG.D DQVVPPGND  EERIFRRTGG TLFGSVWSSI e.g. VHCEREFPGE GKLQDELQGA SS APQRRSSEGS RRLPTSSDMN gg. GSLDQVQGSQ	RrIqPPLGFG RRIQPPLGFG RRIQPPLGFG RKISPPLGFG DEVTVGKFYA DEVTVGKFYA DEVTVGKFYA tf LFGQVDTFLE RRHGNGTFRR  AETPAAGRGA GSGGELRTFG  TPRRPAPATA GLAE 1 ETLIPPRP	KlCPHRVACK KLCPHRVACK KLCPHRWACK TfLIQdyFRk TFLIQDYFRK TFLIQEHFRK TYLIQDYFRR  RTNSLPPVMA SAKATASQSN .s.s.r.l LSHSHRALGP ESISMRPLAK .lil LLIQEALVRG SLIGGVLAAE	RLVAMNMPLN RLVGMNMPLN FKKRKEqg FKKRKEQGLV FMKRQEEYYG FKKRKEQE.G  NQRPLQFAEI GALAIGGSAS a HSKPCAGKLN NGGGAATVAG gld. GLDTLAADAG GLGKY.CDSE	SDGTVMFNAT SDGTVTFNAT SDGTVLFNAT y.pkTv GKYPAKNTTI YRPKKDTV KEGHPDSNTV  EMEELESPVF AALGVGGSSL  GQ TLPPEANAIN fv FVMATSQALV FVGTAAREMR	LFALVRTALK LFAVVRTSLS .1Qag1Rt1. ALQMLERML QIQAGLRTIE TLQAGLRTIE TLQAGLRTL. a LEDFPQDART VLGSSDPAGG i. LVQPGMPINQ YDNRNRGILL .a.m.pee. DACQMEPEEW EALDMTPEEM	1476 2021 1646 1563 2109 1653 2199 1735 2289 1825 2372 1873
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Figure 1. Comparison of deduced amino acid sequence of the cDNA encoding the Drosophila  $\alpha_1$  subunit (Dmca1D) with rabbit skeletal muscle (Rabsk, Tanabe et al., 1987) and rat brain D (Ratbd, Hui et al., 1991) homologs. The proposed transmembrane domains and the position of a proposed calcium-binding domain (the EF hand, Babitch, 1989) are indicated as labeled dark lines above the consensus (con) amino acid sequence. The positions of possible start site methionine residues are indicated by \* beneath the residue in Dmca1D. (See text for discussion.) The positions of the primers used in the initial PCR amplification of genomic DNA are indicated by the shaded gray areas (with labels P6 and P7 directly above them) within the consensus sequence. Putative binding domains for two classes of calcium channel blockers are indicated by bars lying between Rabsk and Dmca1D in the carboxy half of the molecule. The hatched bar indicates the phenylalkylamine-binding fragment as proposed by Striessnig et al. (1990). The black bars indicate the dihydropyridine-binding fragments as proposed by Nakayama et al. (1991) and Striessnig et al. (1991). In the region of these bars only, nonconservative amino acid substitutions in Dmca1D are indicated by  $\blacktriangle$  directly beneath the residues. This sequence has been submitted to GenBank (accession #U00690).

Table 1.	RT-PCR followed by restriction enzyme digestion reveals
more Dm	ca1D message heterogeneity in heads than in bodies or legs

Region amplified by RT-PCR	Source of mRNA	mRNA isoforms present	Diagnostic restriction enzyme	
Cytoplasmic loop be-	Heads	f1, f2	Hinf I	
tween II and III	Bodies	f1	Hinf I	
(bases 3830-4033)	Legs	f1	Hinf I	
IIIS3 to loop between	Heads	f3, f4	Pst I or Rsa I	
IIIS5 and S6 (bases	Bodies	f3	Pst I or Rsa I	
4251-4635)	Legs	f3	Pst I or Rsa I	

f1, f2, f3, and f4 refer to splice forms found in different cDNA clones (f1 = W8A; f2, f4 = SH22D; f3 = SH22C) in the regions indicated. Although the alternative forms were similar in size, they could be distinguighed in the PCR amplification products following digestion with the indicated restriction enzymes.

tisense DNA in a total volume of 25  $\mu$ l using 5  $\mu$ l of the nucleotide solution from vial 6 in the Genius Kit from Boehringer Mannheim (Indianapolis, IN), 2  $\mu$ l primer stock for the antisense strand (10  $\mu$ M), and 0.3  $\mu$ l Taq-polymerase (5 U/ $\mu$ l). Amplification conditions for the synthesis of this single-stranded probe were 94°C for 45 sec, 55°C for 30 sec, and 72°C for 60 sec for a total of 25 cycles. Labeled probe was stored at -20°C.

## Results

# Strategy for cloning an $\alpha_i$ subunit of Drosophila calcium channels

When we began these studies, it was evident that Drosophila had multiple calcium channel subtypes, at least some of which had a different pharmacological specificity from that reported for the cloned dihydropyridine receptor from vertebrate skeletal muscle (Pauron et al., 1987; Greenberg et al., 1989; Pelzer et al., 1989; Glossmann et al., 1991). It was not clear, however, how much structural conservation would exist between Drosophila calcium channel subunits and those which had been cloned from vertebrates (Tanabe et al., 1987; Mikami et al., 1989; Koch et al., 1990; Grabner et al., 1991; Mori et al., 1991). Since both the Drosophila head binding activity and the cloned vertebrate subunits were known to be phenylalkylamine sensitive, we reasoned that at least some regions of the sequence were likely to be conserved. Using a PCR-based strategy allowed us to focus on short regions for primer design which were most likely to be conserved across species. We used Drosophila genomic DNA as a template to avoid assumptions concerning the tissue and stage in development when calcium channels would be expressed. Products approximately the same size as (or larger than) that predicted from vertebrate  $\alpha_1$  subunits were sequenced to identify those which encoded deduced amino acid sequences with structural similarity to the corresponding region of vertebrate calcium channel  $\alpha_1$  subunits. By including products larger than predicted from the vertebrate sequences, we allowed for the occurrence of introns in the genomic DNA used as template.

The product from primer pair P6/P7 (spanning the region from IVS5 to a cytoplasmic region following IVS6, Fig. 1) had a deduced amino acid sequence very similar to that of vertebrate  $\alpha_1$  subunits except that the 3' end of the IVS5 coding region and the middle of the IVS6 coding region were disrupted by 59 and 60 base pair introns, respectively. These introns were readily recognized using codon preference analysis from the University of Wisconsin Genetics Computer Group (GCG) software package.

Northern analysis showed that this Drosophila genomic frag-

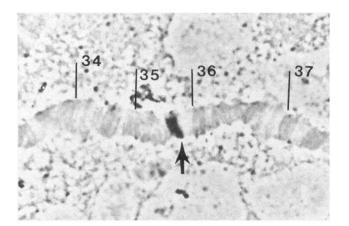


Figure 2. Chromosome mapping of the  $\alpha_1$  subunit Dmca1D. In situ hybridization to Drosophila salivary gland polytene chromosomes using a biotinylated probe (encoding amino acids 1417–1931, Fig. 1) mapped this gene to 35EF on the left arm of chromosome 2. Numbered divisions for this section of chromosome 2L are marked and the hybridization signal is indicated by the arrow. This same map position was seen using a variety of other probes from W8A and SH22C (data not shown), suggesting that these overlapping cDNAs are encoded by the same gene.

ment recognized a message that was expressed at a relatively high level in heads, as would be expected for a neuronal calcium channel component (Greenberg et al., 1989), so an adult head cDNA library was screened. The two longest cDNA clones, W8A and SH22C, with an overlap of 572 nucleotides were sequenced and combined. Although the sequence match between the two clones is excellent within the region of overlap (only three nucleotide discrepancies), there is a region of 149 nucleotides in W8A which shows no sequence similarity with SH22C. This nonmatch region begins in the intracellular loop between IIIS4 and S5 and extends into transmembrane domain IIIS5. In situ hybridization to salivary gland chromosomes (Fig. 2) showed that both W8A and SH22C mapped to the same position at 35EF on the left arm of the second chromosome, suggesting that the two cDNA clones are derived from the same gene. This was confirmed by sequencing a genomic clone and the SH22D cDNA clone in the regions flanking the nonoverlap section. Sequence analysis revealed two alternatively spliced exons in this region.

The 5' end sequence of the cDNA was derived from the N1 clone. In addition, 5' RACE was done starting with polyA<sup>+</sup> mRNA from Canton-S and a primer from the 5' end of W8A. The RACE product extended only 360 bases upstream from the end of W8A, whereas N1 clone provided 1116 bases upstream of the 5' end of W8A. In the 360 bases of overlap between the RACE product and clone N1, there was an exact match except for three bases within the proposed open reading frame. These differences did not affect the amino acid sequence and most likely represent sequence polymorphisms between DNAs from different wild-type sources.

# Structural features of the cDNA sequence

The complete deduced amino acid sequence for the *Drosophila*  $\alpha_1$  subunit is given in Figure 1, where it is compared with the two most closely related vertebrate  $\alpha_1$  subunit sequences. The carboxy terminus of the deduced protein is unambiguously determined by the TAG stop codon at nucleotide position 7549–7551, which is followed by 10 additional in-frame stop codons.

There is no polyadenylation consensus sequence (AAUAAA) in the 3' untranslated region, so there may be some additional 3' sequence which was not included in the SH22C clone. The total assembled cDNA sequence ( $\sim 8$  kb) is about 1.5 kb shorter than the smallest message observed in Northern blotting experiments (Fig. 3). This may be due to missing 5' and/or 3' untranslated regions in the cDNA clones sequenced and/or to extensive polyadenylation of the message.

Identification of the translation start site is somewhat problematical. The most likely start site is the methionine which was used in Figure 1, since it is preceded by three in-frame stop codons within the 156 bases upstream. However, there are four additional methionines encoded in the region between the first methionine and IS1. The area immediately upstream of each of these methionine codons was compared with the Drosophila translation start site consensus sequence (C/A AA A/C AUG) (Cavener, 1987). The first methionine shows 0/4 matches. Although it lacks an A at the crucial -3 position, it has the second most commonly used base (G) at this position. The second, third, and fifth methionines all have an A in the -3 position. In addition, the second (M494) and fifth (M553) methionines show three out of four nucleotide matches to the upstream consensus sequence for Drosophila. In Drosophila, the average fit to the four nucleotide consensus positions immediately upstream of a start codon is 3.1 matches. On the basis of nucleotide sequence, met494 and met553 might be start site candidates; however, there are no upstream in-frame stop codons preceding them. In this paper, we assume met1 is the start site.

# Tissue distribution and heterogeneity of Dmca1D message expression

The relative expression of Dmca1D transcripts in different body parts was determined by Northern blot analysis using rp49 (a uniformly expressed ribosomal protein mRNA) (O'Connell and Rosbash, 1984) as a control for the amount of RNA loaded into each lane. As shown in Figure 3A, polyA+ RNA from bodies (B), heads (H), and legs (L) was compared following hybridization with a probe from the 3' end of clone SH22C. This probe contains the coding sequence for the nonconserved carboxy terminus of the  $\alpha_1$  subunit. All three preparations show a major band at 9.5 kb and a minor band at 12.5 kb. The minor band is seen most clearly in the head preparation. In addition, the head preparation shows a second major band at 10.2 kb. A similar result (data not shown) was obtained using a probe derived from W8A. The relationship among the three mRNA size classes is not known. The largest size class (12.5 kb) is a very weak signal in all lanes, suggesting that it might be an unprocessed transcript or the product of another gene picked up by sequence similarity. Compared to messages expressed in heads, there is less heterogeneity in the message expressed in the bodies and legs since only one major band (9.5 kb) is visible.

To further investigate the difference in message heterogeneity among heads, bodies, and legs, we focused on two regions where sequence data from three different cDNA clones (W8A, SH22C, and SH22D) had shown differences. These differences could be most easily distinguished by RT-PCR (reverse transcriptasecoupled PCR) amplification followed by a diagnostic restriction enzyme digestion. It should be noted that the differences in the actual nucleic acid sequences were extensive as expected for alternative splice products and could not be explained by single base changes due to sequence polymorphisms (D. Ren and L. M. Hall, unpublished observations). As shown in Table 1, in

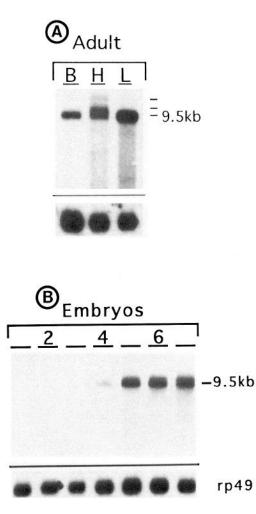


Figure 3. Tissue and temporal expression of the Drosophila  $\alpha_1$  subunit mRNA by Northern blotting. A, Message distribution in adult body parts. Northern blot of polyA+ mRNA (10 µg/lane) isolated from bodies (B), heads (H), or legs (L) was probed with a PCR fragment (region encoding amino acids 2047-2503, Fig. 1) from clone SH22C and washed at high stringency. The tics on the right indicate positions of bands at 12.5, 10.2, and 9.5 kb. The lower inset shows the results of reprobing with ribosomal protein 49 cDNA (rp49) to control for mRNA recovery and gel loading differences since rp49 is expressed uniformly throughout the organism and throughout the different developmental stages (O'Connell and Rosbash, 1984). B, Developmental profile of calcium channel  $\alpha_1$  subunit mRNA expression in embryos showing a peak of expression in the late embryonic stages. A Northern blot (as in A) consisting of mRNA isolated from embryos of different ages was hybridized with a 32P-labeled double-stranded probe from W8A (region encoding amino acids 2047-2503, Fig. 1). (Similar results were obtained with a probe from the region encoding amino acid 1888 in IVS6 to the end of cDNA clone SH22C. Data not shown.) Lanes 1-7 represent sequentially older embryos collected over 3 hr intervals and then aged appropriately at 25°C (i.e., 1 = 0-3 hr, 2 = 3-6 hr, 3 = 6-9 hr, 4 = 9-12 hr, 5 = 12-15hr, 6 = 15-18 hr, 7 = 18-24 hr).

each of the two regions tested for alternative splicing, two different major forms were found in heads, but only a single major form was found in bodies or legs. Embryos (which express this subunit only in the nervous system, as shown in Fig. 4 and discussed below) show the same pattern of heterogeneity seen in heads. Taken together, these results again suggest there may be more functional heterogeneity in Dmca1D-type calcium channels in neuronal tissue than elsewhere in the fly.

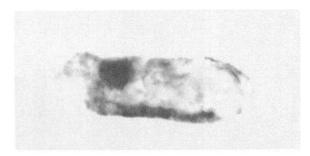


Figure 4. Localization of  $\alpha_1$  subunit mRNA in the embryonic nervous system using *in situ* hybridization to a whole-mount embryo. A single-stranded, antisense DNA probe labeled with digoxigenin was hybridized to embryo whole-mounts and the signal detected as described by Tautz and Pfeifle (1989). Dorsal is *up* and anterior is to the *left*.

#### Temporal pattern of expression of Dmca1D

To determine when the Dmca1D message is expressed in *Drosophila* embryos, a Northern blot (Fig. 3*B*) containing polyA<sup>+</sup> mRNA from a variety of embryonic stages was probed with two different Dmca1D specific probes: one from W8A (shown in Fig. 3*B*) and one from SH22C (from nucleotide 5665 in IVS6 to the end, data not shown). Regardless of which probe was used, expression of the 9.5 kb calcium channel message is detected faintly in embryos at 9–12 hr, corresponding to the time when condensation of the nervous system begins (Kankel et al., 1980). Expression increases rapidly as the nervous system matures within the embryo, peaking just prior to hatching. A second peak of expression of the 9.5 kb message is observed in late pupal stages around 73–108 hr postpuparium formation when the nervous system is completing a dramatic reorganization (F. Hannan, unpublished observations).

#### Embryonic whole-mount in situ hybridization

To determine where the message for this  $\alpha_1$  subunit is expressed, we used a digoxigenin-labeled antisense probe on embryonic whole-mounts. As shown in the 13–15 hr embryo in Figure 4, the Dmca1D subunit is preferentially expressed in the nervous system. The dark staining pattern highlights the round, dorsal cerebral hemisphere and the ventral ganglion which comes off the ventral side of the sphere and curves posteriorly on the ventral surface of the embryo.

## General structural features of the deduced amino acid sequence

Using the first in-frame AUG (met1) following a series of inframe stop codons as the translation start site, Figure 1 shows that the resulting open reading frame of the combined cDNA clones would encode a protein of 2516 amino acids with an expected molecular weight of 276,493 and a predicted pI of 5.04. If the second AUG is the actual translation start site, the Drosophila protein would consist of 2023 amino acids and have a predicted molecular weight of 224,369 and a pI of 6.49. If it begins with the fifth AUG, the protein would consist of 1964 amino acids with a predicted molecular weight of 218,580 and a predicted pI of 6.78. Just as in the vertebrate calcium channel  $\alpha_1$  subunits, the *Drosophila* subunit shows four repeat domains (designated I through IV), each with six hydrophobic domains (labeled 1-6) which would be long enough to span the membrane. The resemblance to the vertebrate  $\alpha_1$  homologs is striking. The regions of greatest difference are in the cytoplasmic amino and carboxy terminal tails. Both regions are much longer in

*Drosophila* than in the vertebrate homologs. Although there is striking similarity in the region of the carboxy tail closest to transmembrane region IVS6, the similarity falls off after about 160 amino acids from the end of the IVS6 region. On the amino terminal end the similarity to the vertebrate homologs falls off after about 26 amino acids upstream of the beginning of IS1.

The repeat structure and the pattern of the hydrophobic domains puts this newly cloned *Drosophila* protein in the same superfamily as the voltage-gated sodium and calcium channels. As shown in Table 2, when the deduced protein is compared with available sequences for sodium and calcium channels, in general there is more similarity in amino acid sequence between the *Drosophila* clone and vertebrate calcium channels (ranging from 63.4 to 78.3%) than between this sequence and sodium channels (57.9–58.9%), even if the sodium channel is from *Drosophila*. These differences are even more striking if amino acid identity is considered (42.7–64.2% identity for calcium channels vs 29.6–30.5% for sodium channels). Thus, based on overall sequence similarity, the newly cloned gene would be designated as a member of the calcium channel gene family.

Within the calcium channel group, the *Drosophila* sequence shows the closest relationship to rat brain type D. The next highest scoring channel from rabbit skeletal muscle shows ~8% less identity and ~6% less similarity than the rat brain type D channel. Based on this sequence similarity hierarchy and on its expression in the nervous system, we designate this *Drosophila* channel as *Drosophila melanogaster* calcium channel  $\alpha_1$  subunit type D (Dmca1D).

As for other members of the voltage-sensitive cation channel family, each of the S4 transmembrane domains of the newly cloned channel subunit shows positively charged amino acids (R = arginine or K = lysine) every third or fourth amino acid. In a commonly proposed model, this pattern would put all of the positively charged side chains on the same side of an  $\alpha$ -helix so that they sit in the membrane as the voltage sensor (Stühmer et al., 1989). The *Drosophila* protein shows the same general pattern as the majority of other calcium channels, with five positively charged side chains in the S4 helices in domains I, II, and IV, and six in domain III.

### Proposed calcium-binding EF hand region

Another feature commonly found in both sodium and calcium channel  $\alpha_1$  subunits is a protein motif known as the EF hand, which consists of two  $\alpha$ -helices flanking a calcium-binding loop (Babitch, 1990). As indicated by the bracketed area beginning 20 amino acids downstream from the IVS6 region in Figure 1, an EF hand is found in the *Drosophila* sequence. In the Tufty– Kretsinger test (Tufty and Kretsinger, 1975) the Dmca1D sequence has 11 matches (out of 16 possibilities) for residues important for calcium binding. The number of matches for Dmca1D can be increased to 14 by allowing conservative amino acid substitutions. Many vertebrate calcium channel  $\alpha_1$  subunits show a similar pattern of matching (Babitch, 1990). Again, the *Drosophila* sequence shows more similarity to calcium channels than to sodium channels in this critical area.

#### Ion selectivity filter

A portion of the sodium channel involved in the ion selectivity filter has been identified within short segment 2 (SS2) lying between S5 and S6 in all repeats (Heinemann et al., 1992). By changing a single amino acid residue (K1422 in repeat III or A1714 in repeat IV of rat sodium channel II) to a negatively

DHP sensitivity	Similarity	Identity	LoopII/III <sup>a</sup>	References
	(%)	(%)		, W 1
+ Rat brain-D	78.3	64.2	134	Hui et al., 1991
+ Rabbit skeletal muscle	72.4	56.1	138	Tanabe et al., 1987
+ Human brain	71.3	55.5	134	Williams et al., 1992b
+ Rabbit lung	70.2	54.1	125	Biel et al., 1990
+ Carp skeletal muscle	70.0	51.7	139	Grabner et al., 1991
+ Rat brain-C	69.9	54.1	150	Snutch et al., 1991
+ Rabbit heart	69.6	53.3	147	Mikami et al., 1989
+ Rat aorta	68.7	53.0	147	Koch et al., 1990
– Rat brain-A	65.2	45.1	479	Starr et al., 1991
- Rabbit brain-1	64.5	44.2	539	Mori et al., 1991
– Rat brain-B	63.4	43.7	438	Dubel et al., 1992
– Human N-type	63.4	42.7	451	Williams et al., 1992a
Na <sup>+</sup> channel (Drosophila)	58.9	30.5		Loughney et al., 1989
Na <sup>+</sup> channel (Rat skel. muscle)	57.9	29.6		Trimmer et al., 1989

Table 2. Comparison of a *Drosophila* calcium channel  $\alpha_1$  subunit with the vertebrate  $\alpha_1$  subunits at amino acid level

" This is the cytoplasmic loop between IIS6 and IIIS1. In Dmca1D the length of this loop is 129 amino acids.

charged glutamic acid (E) (as is found in calcium channels), the ion selectivity of the channel can be changed from that of a sodium channel to resemble that of a calcium channel. Recently, Tang et al. (1993) have done the reciprocal experiments on cardiac calcium channels and have shown that modification of conserved glutamate residues in the SS2 region of repeats I, II, or IV alters the ion selectivity and permeability of calcium channels. When the SS2 sequences of the newly cloned Dmca1D cDNA are compared with those of other sodium and calcium channels, the new Drosophila sequence resembles the calcium channel sequences more closely than it does the sodium channel sequences. In the crucial region of repeats I, II, III, and IV, all of the negatively charged glutamic acids (marked by a + within the SS2 regions) found in calcium channels have been conserved in the Drosophila sequence, providing further evidence that Dmca1D encodes a calcium channel subunit. The conservation of glutamate residues in all four SS2 regions is consistent with the suggestion of Tang et al. (1993) that these residues form a ring in the pore-lining SS1-SS2 region involved in ion selectivity and permeability.

### Possible sites for post-translational modification of the protein encoded by Dmca1D

There are two partially overlapping, possible N-linked glycosylation sites (NX[S/T]X) at N644 and N647 in the Drosophila  $\alpha_1$  subunit located in a region of the protein predicted to be external to the plasma membrane. (X generally is any amino acid, but in this site only X refers to any amino acid except P.) These asparagines (N) fall in the loop between IS1 and IS2 which is predicted to be extracellular. There are eight possible cAMPdependent protein kinase phosphorylation sites ([R/K]XX[S/ T]) lying in predicted cytoplasmic domains. Six are in the amino terminal region; one is in the region between IIS6 and IIIS1, which, in skeletal muscle L-type channels, has been implicated in excitation-contraction coupling processes (Tanabe et al., 1990); and one is in the carboxy terminus in the cytoplasmic region corresponding to the calcium-binding EF hand. In addition, there are 21 possible protein kinase C phosphorylation sites ([S/ T]X[R/K]). Twelve of these are in the amino terminus; two are in the region between IIS6 and IIIS1; and seven are in the carboxy terminal tail. There are also 27 possible casein kinase phosphorylation sites ([S/T]XX[D/E]): 12 in the amino terminus, one each in the loops IS6/IIS1 and IIIS6/IVS1, four in loop IIS6/IIIS1, one at the cytoplasmic end of IVS4, and eight in the carboxy terminal tail. The high concentration of potential phosphorylation sites within several regions (the amino terminus, the II/III cytoplasmic loop, and the C terminal tail) suggests that they may play roles in channel modulation by phosphorylation.

# Comparison of sequences in region of the proposed phenylalkylamine-binding domain

The phenylalkylamines constitute an important class of organic calcium channel blockers. A proposed binding site for phenylalkylamines has been localized to a 42 residue segment extending from E1349 to W1391 in the rabbit skeletal muscle subunit (Striessnig et al., 1990). This region (shown by hatched underline in Fig. 1) includes transmembrane domain IVS6 and adjacent intracellular and extracellular segments. Since phenylalkylamines exert their blocking effects from the inner surface of the membrane (Hescheler et al., 1982; Affolter and Coronado, 1986), the binding site for this class of blockers is thought to include the intracellular side of transmembrane segment IVS6 and the adjacent intracellular amino acids (Striessnig et al., 1990). In Figure 1, starting with the intracellular amino acids (right end of the hatched underline) and proceeding to the left into the transmembrane region IVS6, it is apparent that this segment is completely conserved between Drosophila and the two mammalian subunits shown until about half-way through the transmembrane region where there is a weakly conserved change from alanine (A) in the rabbit and rat to serine (S) in Drosophila and a highly conserved change from methionine (M) to valine (V). This high degree of conservation predicts that this Drosophila subunit should bind phenylalkylamines with high affinity.

# Sequence comparisons relevant to dihydropyridine sensitivity

It is interesting to note that among the calcium channel  $\alpha_1$  subunits listed in Table 2, the *Drosophila* subunit is most similar in sequence to those isoforms which have been shown to be dihydropyridine (DHP) sensitive (indicated by + in this table). The four isoforms which are known to be insensitive to dihydropyridines (rat brain-A and -B, rabbit brain-1, and human N-type) show the least similarity to the *Drosophila* sequence. Another interesting correlation is seen if the length of the cytoplasmic loop between repeats II and III is considered, since all the known dihydropyridine-sensitive subunits have a short loop (134–150 amino acids in length), whereas the insensitive subunits have a much longer loop, ranging in length from 479 to 539. By this criterion, the *Drosophila* sequence would also fall into the DHP-sensitive category, with a loop length of 129 amino acids.

A model for dihydropyridine-binding sites has been developed using photoaffinity labeling with dihydropyridines, and has implicated the extracellular sides of transmembrane segments IIIS6 and IVS6 and the extracellular amino acids immediately adjacent to these transmembrane regions (Nakayama et al., 1991; Striessnig et al., 1991; Catterall and Striessnig, 1992). The segments involved are shown by the black underline (lying between the Rabsk and Dmca1 lines) in Figure 1. In the portions of those two segments which include the left end (extracellular surface) of both S6 segments and the regions which extend to the left from the indicated transmembrane region, there are many amino acid differences (As point to the changes), including eight nonconserved amino acid substitutions in the region adjacent to IIIS6 and extending into the extracellular side of S6. In region IVS6 and the adjacent extracellular amino acids, there are three nonconserved substitutions and two deletions (involving one and two amino acids) in the Drosophila sequence compared with the rabbit and rat sequences. The functional significance of these changes can be addressed by expression of this new subunit. The large number of changes in this region is consistent with the cloned channel being the dihydropyridineinsensitive, phenylalkylamine-binding activity which predominates in Drosophila head membranes (Greenberg et al., 1989), even though the cloned channel falls into the same structural category as vertebrate dihydropyridine-sensitive subunits.

### Discussion

# Invertebrate voltage-dependent calcium channels belong to the same supergene family as those in mammals

When we began these studies, it was clear that Drosophila had calcium channels in both neurons and muscles, but the pharmacological specificity of these channels was apparently different from that described for the vertebrate L-type channel from skeletal muscle (Pauron et al., 1987; Greenberg et al., 1989; Pelzer et al., 1989; Glossmann et al., 1991) since the predominant channel in Drosophila heads was phenylalkylamine sensitive and dihydropyridine insensitive. In addition, other pharmacological differences were apparent in side-by-side comparisons of guinea pig skeletal muscle with Drosophila head extracts (Glossmann et al., 1991). Using PCR with degenerate primers, we were able to rapidly cross species to isolate this invertebrate calcium channel  $\alpha_1$  subunit using information from vertebrate homologs. Our studies show that despite pharmacological differences across species, insect calcium channel  $\alpha_1$ subunits belong to the same supergene family as mammalian  $\alpha_1$  subunits. The subunit described here shows the same four repeat structure, each containing six transmembrane segments, that is the characteristic pattern for voltage-dependent calcium channels. This *Drosophila* sequence highlights regions of  $\alpha_1$  subunits which have been conserved across large evolutionary distances and therefore will facilitate the design of primer pairs for cloning homologous subunits from other invertebrate preparations of physiological importance or for cloning this subunit from pest insects.

# Analysis of Dmca1D mRNA suggests heterogeneity of neuronal $\alpha_1$ subunits

In the tissues tested, the size of the mRNA on Northern blots is larger (9.5, 10.2, or 12.5 kb) than the cDNA sequence which we report here (8.0 kb). One possible explanation for this difference is that some untranslated regions are missing from the 5' and 3' ends. Indeed, we have not found a polyadenylation site on the 3' end. The finding of multiple, in-frame stop codons in both the 5' and 3' untranslated regions provides strong evidence that the sequence presented here contains the full-length open reading frame.

The predominant forms seen on the Northern blot (Fig. 3) may represent major differences due to alternative splicing. Preliminary comparisons between genomic and cDNA using PCR have demonstrated the presence of at least 22 introns ranging in size from 55 base pairs to ~3 kb (D. F. Eberl and D. Ren, unpublished observations). We demonstrate here that alternative splicing occurs in at least two of these intron regions, but there are still many additional regions to be characterized. Depending on how the alternative splicing is done, it is possible to generate a large variety of mRNAs which will encode subunit forms with potentially different properties. Our preliminary results suggest that this calcium channel subunit will show much heterogeneity due to alternative splicing. Indeed, the *Drosophila* sodium channel  $\alpha$  subunit shows 19 different combinations of alternative exons (Thackery and Ganetzky, 1994).

In view of the wide variety of potential alternative splice forms, it should be emphasized that the cDNA sequence shown in Figure 1 represents the synthetic fusion of sequence information from three cDNA clones. Because of the large size of the full-length message, it has not been possible to isolate a single cDNA clone that contains a complete open reading frame. One challenge of future work on this calcium channel subunit will be to identify physiologically relevant forms and define functional differences resulting from alternative splicing.

Using the Dmca1D cDNA as a probe in Northern blot analysis, there is more  $\alpha_1$  subunit heterogeneity in heads than in bodies and legs since a prominent band at 10.2 kb is seen in heads and is not detected in bodies and legs. Only the 9.5 kb band is seen in all preparations. The heads would be enriched for nervous system compared to bodies and legs so the heterogeneity which we see in size of mRNA from heads could, in part, be due to functional diversity of channels expressed in neurons. This is interesting because it mirrors the greater heterogeneity observed by Leung and Byerly (1991) in the physiological properties of neuronal compared to muscle calcium channels in primary cultures of neurons and muscle from *Drosophila* embryos.

Indeed, there could be much more heterogeneity than reflected by our Northern analysis with respect to the Dmca1D gene since alternatively spliced messages close in size would not be readily distinguished by Northern blot analysis of a message of this large size. PCR analysis of cDNA using strategically placed primers is a more sensitive approach. In the preliminary PCR experiment summarized in Table 1 we again see more heterogeneity in heads than in bodies and legs. Pelzer et al. (1989) found eight different conductance levels for calcium channels when *Drosophila* head membranes were reconstituted into lipid bilayers. These conductances were found in single channel activity records and did not interconvert, suggesting that each activity results from a different type of channel molecule. It is possible that these functionally distinct, nonconverting channel subtypes reflect, in part, the alternative splicing which we observe in Dmca1D expressed in *Drosophila* head mRNA. Functional expression of different splice variants of this cloned calcium channel subunit will allow us to define the molecular basis of these biophysically and pharmacologically distinct channel subtypes.

# Relationship of Dmca1D to previously studied calcium channel activities in Drosophila

The clones used to construct the full-length Dmca1D cDNA were all isolated from a head cDNA library. Thus, Dmca1D is a candidate for encoding the predominant phenylalkylamine-sensitive, dihydropyridine-insensitive binding activity found in *Drosophila* head membranes. The complete conservation of the phenylalkylamine-binding site in the Dmca1D-deduced protein coupled with the numerous changes in the proposed dihydropyridine-binding domains are consistent with this suggestion.

There is, however, one difficulty in equating Dmca1D with the previously characterized phenylalkylamine-binding activity in heads, and that is that there is a substantial size difference between the deduced amino acid sequence of Dmca1D (219-276 kDa) and the photoaffinity-labeled phenylalkylamine-binding components (136 and 27 kDa) (Pauron et al., 1987; Greenberg et al., 1989). Even if the two photoaffinity-labeled components are actually part of the same protein, they still add up to only 166 kDa. There are several possible explanations for this size discrepancy. It could be due to alternative splicing, and the predominantly expressed form of Dmca1D might be a different splice variant from the one presented here. Alternatively, it could be caused by a physiologically relevant proteolysis required for the maturation/activation of the subunit. The deduced Drosophila protein seems to be much larger in size than its vertebrate counterparts, and it will be interesting to determine whether the long amino and carboxy tails are required for physiological function. The size difference might also be an artefact reflecting anomalous electrophoretic mobility of a large membrane protein on SDS gels or an artefact reflecting proteolysis during and/or following photoaffinity labeling. Indeed, the carboxy tail of the deduced protein sequence of Dmca1D contains a motif resembling the active site of thiol (cysteine) proteases. Thus, this subunit might catalyze its own cleavage. A final possibility is that the cloned subunit might be the product of a different gene from the one encoding the product seen by phenylalkylamine-photoaffinity labeling in head extracts. Although this seems unlikely in light of the high degree of conservation of the phenylalkylamine-binding site in Dmca1D, there is preliminary evidence for a distinct gene encoding another calcium channel  $\alpha_1$  subunit in *Drosophila* (L. A. Smith and J. C. Hall, personal communication).

Determination of the pharmacological properties of Dmca1D will have to await functional expression. Regardless of its pharmacological specificity, this insect calcium channel  $\alpha_1$  subunit is, evolutionarily, the most distant of the sequences described to date. Sequence comparisons coupled with functional studies of chimeric molecules should provide useful information concerning the nature of drug-binding sites.

### Using genetics to define subunit properties in the organism

One of the primary motivating factors in extending calcium channel molecular biology studies to Drosophila is the ability to use genetics to inactivate subunit genes singly and in combination in order to define functional roles within the organism. The chromosome mapping studies described here show that the newly cloned Dmca1D gene falls within a well studied region of the Drosophila genome (see Ashburner et al., 1990). This region includes several lethal mutations. Recently, we have demonstrated that one of these embryonic lethal mutations causes a premature stop codon within the open reading frame of the Dmca1D gene (D. F. Eberl, D. Ren, G. Feng, and L. M. Hall, unpublished observations). Genetic analysis of double mutants from this and other calcium channel subunits will allow us to define which subunits actually interact in vivo. Transformation rescue experiments (Spradling, 1986) using this  $\alpha_1$  subunit will allow us to test whether there is functional overlap among the different genes encoding homologous subunits and to determine the role in vivo of the different splice variants of this gene.

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