The Journal of Experimental Biology 203, 693–704 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2458

TRANSCRIPTIONAL CONTROL OF Ca^{2+}-ACTIVATED K^{+} CHANNEL EXPRESSION: IDENTIFICATION OF A SECOND, EVOLUTIONARILY CONSERVED, NEURONAL PROMOTER

RUDOLF A. BOHM, BIN WANG, ROBERT BRENNER AND NIGEL S. ATKINSON*

Section of Neurobiology and The Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712-1064, USA

*e-mail: NigelA@mail.utexas.edu

Accepted 9 December 1999; published on WWW 26 January 2000

Summary

Neuronal signaling properties are largely determined by the quantity and combination of ion channels expressed. The *Drosophila slowpoke* gene encodes a Ca^{2+}-activated K^{+} channel used throughout the nervous system. The *slowpoke* transcriptional control region is large and complex. To simplify the search for sequences responsible for tissue-specific expression, we relied on evolutionary conservation of functionally important sequences. A number of conserved segments were found between two *Drosophila* species. One led us to a new 5′ exon and a new transcriptional promoter: Promoter C0. In larvae and adults, Promoter C0 was demonstrated to be neural-specific using flies transformed with reporter genes that either contain or lack the promoter. The transcription start site of Promoter C0 was mapped, and the exon it appends to the 5′ end of the mRNA was sequenced. This is the second neural-specific *slowpoke* promoter to be identified, the first being Promoter C1. Promoter choice does not alter the encoded polypeptide sequence. RNAase protection assays indicate that Promoter C0 transcripts are approximately 12 times more abundant that Promoter C1 transcripts. Taken together, these facts suggest that promoter choice may be a means for cells to control channel density.

Key words: Ca^{2+}-activated K^{+} channel, ion channel regulation, neuronal promoter, transcriptional control, signalling, *slowpoke*, *Drosophila hydei*, *Drosphila melanogaster*.

Introduction

Nerve cells encode and transmit information in the form of electrical signals. The production of these impulses depends upon the cooperative effort of a number of distinct ion channels. These channels conduct the ionic currents responsible for the cell's electrical properties: its resting potential, its sensitivity to stimulation and the shape and duration of its action potential. In short, they shape the cell's input–output properties. Any given neuron will typically have one or more different inward currents and perhaps four or more outward currents. Together, these currents, each conducted by a different channel, give rise to the overall electrical properties of the cell. Not only do these channels shape their electrical environment, they also sense it and alter their activity in response to it. From this complex interplay between electrical environment and channel activity arises the cell's electrical character.

Eukaryotic genomes encode a staggeringly large number of distinct ion channels (Jan and Jan, 1990; Wei et al., 1996). The choice of which channels to express is an extremely significant cellular decision because it delimits the range of electrical properties that a cell can produce. It is therefore important to determine how this decision is made. Our model for studying the mechanics and consequences of channel gene regulation has been the *slowpoke* gene of *Drosophila melanogaster*. The *Drosophila slowpoke* gene was the first Ca^{2+}-activated K^{+} channel gene to be cloned (Atkinson et al., 1991) and it is the *Drosophila* homolog of the vertebrate BK-type Ca^{2+}-activated K^{+} channel gene. BK channels are well-known for their roles in determining the firing pattern of neurons and for modulating the contractile properties of muscles (Rudy, 1988; Latorre et al., 1989; Brayden and Nelson, 1992; Hille, 1992; Robitaille et al., 1993; Issa and Hudspeth, 1994; Nelson et al., 1995).

The complete *slowpoke* transcriptional control region was previously determined to be contained within 11 kb of genomic DNA (Brenner et al., 1996). Using a *lacZ* reporter gene and transgenic flies, we demonstrated that this genomic DNA could reproduce the entire *slowpoke* expression pattern. Within this region, tissue-specific promoters were mapped. Separate promoters for neural and midgut expression were identified, while muscle and tracheal cell expression were shown to arise from a shared promoter (Brenner and Atkinson, 1996; Brenner et al., 1996; Thomas et al., 1997). Presumably, tissue-specific promoters enable the gene to tailor the sequence or abundance of a channel to the specific needs of the cell.

To simplify the identification of sequences that drive *slowpoke* expression in specific tissues, we chose to rely on the fact that evolution favors the conservation of functionally important sequences. Here, we begin the hunt for sequences that direct expression in structures of the central and peripheral nervous systems. Using conservation as our metric, we have identified a new neural promoter and a large number of potential control elements.

Materials and methods

Isolation of the slowpoke *transcriptional control region from* Drosophila hydei

A 414 base pair (bp) *Bam*HI/*Apa*I fragment from the *slowpoke* cDNA Z54 (Becker et al., 1995), that contained exon C1 and C3, was used to probe a *Drosophila hydei* genomic library (O'Neil and Belote, 1992) carried in λEMBL4 (generously provided by Dr John Belote, Syracuse University) under reduced stringency. Hybridization and washing conditions were as follows. Hybridization: 20 % (v/v) formamide, 6× SSPE (prepared from a 20× stock solution containing 3.6 mol l^{-1} NaCl, 0.02 mol l^{-1} disodium EDTA and 0.2 mol l^{-1} $NaPO_4$, pH 7.7), 10× Denhardt's solution [prepared from a 50× stock solution containing 1 % Ficoll, 1 % bovine serum albumin, 1 % poly(vinylpyrrolidone)], 0.2 % SDS and 200 µg ml^{-1} salmon sperm DNA at 42 °C; wash: 2× SSPE, 0.1 % SDS at 65 °C. Of four purified clones, one also hybridized to an *Xho*I–*Bam*HI fragment containing the neural promoter C1 from *Drosophila melanogaster*. This one was chosen for further study.

Sequence analysis

DNA fragments from *Drosophila hydei* were subcloned into pBluescriptII using convenient restriction enzyme sites. Nested deletions were introduced using the enzyme *Bal*31 (Sambrook et al., 1989) and sequenced using the dideoxy chain termination method (Sanger et al., 1977). Accession numbers for the *D. melanogaster* and *D. hydei* sequences are U40221 and AF210728, respectively.

Reporter gene constructs

All the reporter genes described have a *lacZ* gene inserted into the 3′-most *Apa*I site of the *slowpoke* transcriptional control region (Fig. 1). Expression of all reporter genes were assayed by β-galactosidase staining as described in Brenner et al. (1996). The construction of reporter gene constructs P1 and P3 has been described (Becker et al., 1995; Brenner et al., 1996), and they are carried in the vector pCaSperβgal (Thummel et al., 1988). P1 contains the 11 kb full-length *slowpoke* transcriptional control region as presently defined. P3 was derived from P1 by deleting a region that contained all the *slowpoke* promoters except Promoters C0 and C1. P3 has been shown to reproduce the complete *slowpoke* neuronal expression pattern. P12 and P13 were derived from P3 using the ExoIII/S1 method (Sambrook et al., 1989). The construction of P12 has been described by Thomas et al. (1997). For both, the P3 plasmid was digested using *Sph*I and *Spe*I to generate a protected 3′ overhang at the *Sph*I site and a 5′ overhang at the *Spe*I site. Unidirectional deletions were then made using the ExoIII/S1 enzyme combination. Deletions were ligated, transformed into bacteria and then screened using restriction digests to identify those of proper length. The extent of the deletion was confirmed by sequence analysis. P12 contains a 950-nucleotide deletion that includes Promoter C0. P13 contains a 620-nucleotide deletion that does not remove this promoter.

Germline transformation

The transformation constructs (1 µg µl^{-1}) and the helper plasmid pπ25.7 (200 ng µl^{-1}) were co-injected into w^{1118} *Drosophila* embryos (Spradling, 1986). Transformants were identified on the basis of complementation of w^{1118} by the white gene of pCaSperβgal.

Reverse transcription/polymerase chain reaction (RT-PCR)

Total RNA was prepared from 0–24 h embryos, wandering third-instar larvae and whole adult animals. Reverse transcription (RT) was performed using the RABRT1 primer (see below) which specifically anneals to exon C3, an exon common to all known *slowpoke* transcripts.

Conditions for reverse transcription were as follows: 10 µg of total RNA and 40 pmol of RABRT1 primer were mixed, incubated at 70 °C for 10 min and then allowed to cool to 42 °C. The reaction was initiated by adding NEB M-MuLVRT buffer (50 mmol l^{-1} Tris-HCl, pH 8.3, 8 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} dithiothreitol, DTT), 2.5 mmol l^{-1} each of dGTP, dATP, dTTP and dCTP (dNTPs) and 12.5 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs). Following incubation at 42 °C for 45 min, the cDNA from each tissue type was subjected to the polymerase chain reaction (PCR) using primers RABP1 and GAMMA5 in the presence of 1.5 or 2.5 mmol l^{-1} $MgCl_2$ using standard conditions. Primer RABP1 anneals to exon C3 immediately upstream of the RABRT1 binding sites. GAMMA5 anneals to homology block 5 (see Fig. 2). Primer annealing temperature was determined using the OLIGO program (National Biosciences, Inc.). The primers used in this study were as follows: RABRT1, 5′-CGGCGTCGAATGGTGAATCTGTTGG-3′; RABP1, 5′-AATGATTCGACAGTGCTTTGAT-3′; GAMMA4, 5′-AAT-GTTATTTTGTTGCTTCCT-3′; GAMMA5, 5′-ATTGTA-TACGCTGCTGACGAGA-3′.

RNA-ligase-mediated rapid amplification of cDNA ends

The RNA-ligase-mediated rapid amplification of cDNA ends procedure (RLM-5′-RACE) was based on the procedure described by Schaefer (1995), taking into consideration the recommendations of Frohman (1995). Total DNAase-digested RNA (100 µg) was incubated with 30 units of calf intestinal alkaline phosphatase (New England Biolabs) in 100 µl of 1× digestion buffer (50 mmol l^{-1} Tris-HCl, pH 7.9, 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} DTT) at 42 °C for 1 h. The reaction was terminated with a single 300 µl

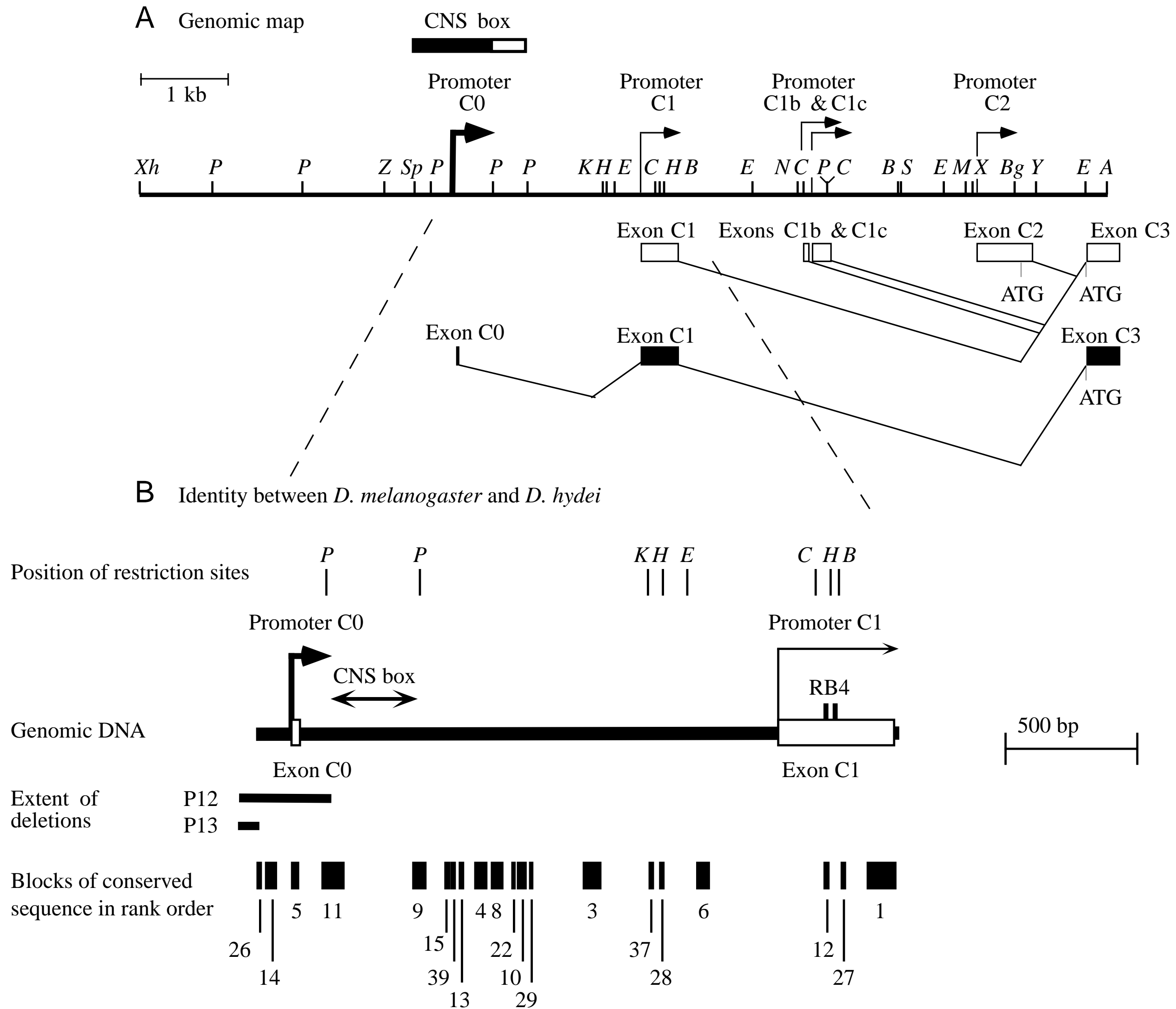


Fig. 1. Organization of the *slowpoke* transcriptional control region. (A) The long horizontal line represents 11 kb of genomic DNA that appears to contain the entire *slowpoke* transcriptional control region. This DNA includes only the first five *slowpoke* exons, which encode 86 amino acid residues of the amino terminus of the *slowpoke* channel protein. DNA that encodes the remainder of the protein (approximately 1200 amino acids residues) is not shown but would be found to the right of exon C3. The tick marks are restriction sites. The named arrows identify the positions of promoters C0, C1, C1b, C1c and C2. The 5′-terminal exons produced by each promoter are similarly named and are represented by open boxes below the line. Also shown is the identified splicing pattern of each 5′-terminal exon. Exon C3 is the first exon common to all *slowpoke* transcripts. In A, the position of the CNS box, as defined by Brenner et al. (1996), is marked by a box. This sequence was described as DNA that does not contain a promoter but that is required for central nervous system expression from Promoter C1. This mapping was refined to the smaller area covered by the open portion of the box. The dashed lines show the relationship between A and B. (B) Map of the position of DNA sequences conserved between *Drosophila melanogaster* and *D. hydei*. The open boxes represent exons C0 and C1. Also shown as black bars are the P12 and P13 deletions. The 3′ end is accurately depicted; however, the 5′ end is beyond the leftmost end of this map. In B, the CNS box is smaller than in A because the P12 deletion described in this paper maps its position more finely. Black filled rectangles at the bottom represent the position of blocks of homology between the *slowpoke* transcriptional control region of *D. melanogaster* and *D. hydei*. These are numbered according the goodness of the alignment. *A*, *Apa*I; *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hind*III; *K*, *Kpn*I; *M*, *Mun*I; *N*, *Nco*I; *P*, *Pst*I; *S*, *Sma*I; *Sp*, *Spe*I; *X*, *Xba*I; *Xh*, *Xho*I; *Y*, *Xmn*I; *Z*, *Sph*I. RB4, repetitive element found in *slowpoke* control region.

phenol:chloroform:isoamyl alcohol (50:49:1; PCIA) extraction. The nucleic acid was precipitated with 2.5 mol l^{-1} ammonium acetate and 200 µl of ethanol, washed once with 70 % ethanol, and resuspended in 100 µl of water. The 7-methyl guanosine triphosphate cap was removed with 10 units of tobacco acid pyrophosphatase (TAP; Epicentre) in 50 mmol l^{-1} sodium acetate (pH 6.0), 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} DTT, 0.01 % Triton X-100 and 0.1 % β-mercaptoethanol at 37 °C for 1 h. The mixture was extracted with PCIA, once with chloroform:isoamyl alcohol (49:1; CIA),

precipitated with 2.5 mol l^{-1} ammonium acetate and two new volumes of ethanol, washed once with 70 % ethanol and resuspended in 50 μl of water. A 135-nucleotide RNA anchor was generated by *in vitro* transcription (Krieg and Melton, 1987; Krieg, 1990). The RNA was digested with DNAase I (37 °C for 15 min) and purified by agarose gel electrophoresis, PCIA- and CIA-extracted and precipitated, dried and resuspended in 100 μl of RNAase-free water. A sample (10 μl) of the anchor was added to 10 μg of TAP-treated RNA and ligated using T4 RNA ligase (New England Biolabs) in the buffer recommended by the manufacturer at 18 °C for 16 h. The ligation product was PCIA-extracted, precipitated, washed and resuspended in 10 μl of water. RT-PCR was performed using the ThermoScript RT-PCR kit (Gibco-BRL). Reverse transcription was performed using 15 units of thermoscript reverse transcriptase (Gibco-BRL) in a volume of 30 μl using a primer RABRT1 (see above) that annealed to exon C3. A sample (2 μl) of the cDNA synthesis was used to seed the PCR reaction. Two rounds of LA-PCR (long and accurate PCR) were used to amplify the product (Barnes, 1994; Cheng et al., 1994). The first round used the gene-specific primer RABP1 (see above) and an anchor-specific primer GL1 (5′-CACCTCAGGTTCAGGCTCTT-3′). The second round of LA-PCR used the gene-specific primer 184 (5′-CCGTCTTGATCGATAGTTGTTCGTTC-3′) and the anchor primer GL2 (5′-ATTGCTGCCTTTGAAGTCTCCA-3′). The products were Southern-blotted, and the exon-C0-containing products were identified by hybridization. This band was gel-purified, cloned into the vector pBlunt (Invitrogen) and identified by colony hybridization.

RNAase protection assay

RNAase protection assays (RPA) were performed using the Ambion Maxiscript kit and Ambion RPAII kit (Ambion, Austin, TX, USA). A single RPA probe which contained exon C0 and C1 sequences was used to identify Promoter C0 and Promoter C1 products. Digestion products were electrophoresed on 8 mol l^{-1} urea, 5 % acrylamide gels. DNA sequencing ladders derived from the template were used to determine the size of the products. The relative abundance of the products was determined by densitometrically scanning the lanes and determining the areas under each peak. Areas were normalized for the number of radiolabeled nucleotides incorporated into each protection product.

Results

The previously mapped *slowpoke* promoters (C1, C1b, C1c and C2) are shown within the transcriptional control region in Fig. 1 (Brenner et al., 1996). RT-PCR, RPA (RNAase protection assay) and deletion analysis experiments indicate that Promoter C1 is active in the *Drosophila* nervous system (Brenner and Atkinson, 1996; Brenner et al., 1996). Thomas et al. (1997) provided evidence for a second neuronal promoter by deletion analysis. However, these expression studies were performed only in the embryo, and the position of this promoter was only crudely mapped to a 5 kb region 5′ of Promoter C1. This promoter was called Promoter Ce because it was responsible for *slowpoke* expression in the embryonic central nervous system. Here, we show that it is also expressed in the adult and larval brain in a pattern largely overlapping with Promoter C1. Since it is not specific to the embryo, we have renamed it Promoter C0 in keeping with the numerical numbering of *slowpoke* promoters.

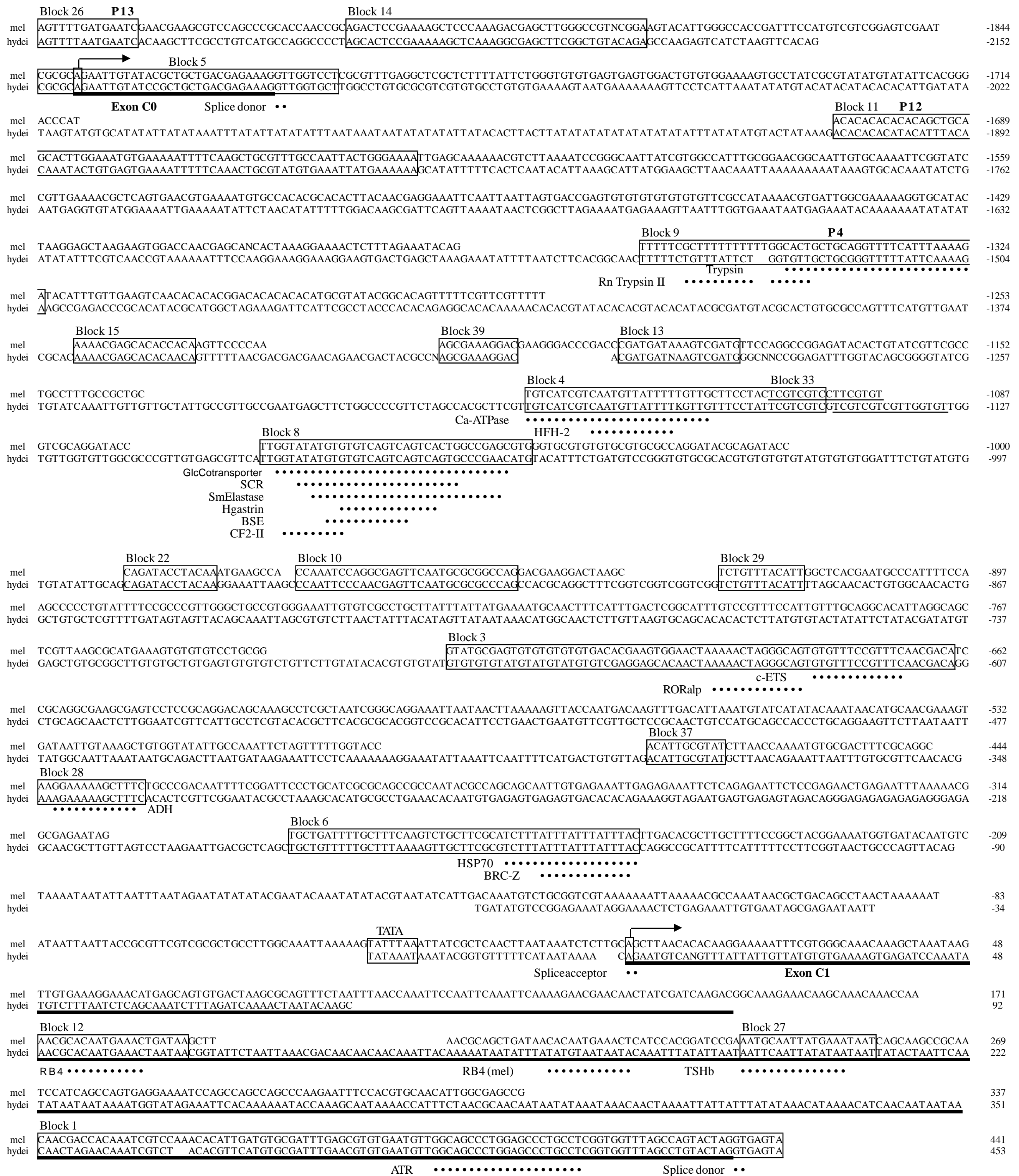
Evolutionary conservation to map putative cis*-acting elements*

To help identify sequences important for normal *slowpoke* expression, we have cloned parts of the *slowpoke* transcriptional control region from *Drosophila hydei*. The two

Fig. 2. Evolutionary conservation of genomic DNA adjacent to *slowpoke* Promoter C1 from *Drosophila melanogaster* (mel) and *D. hydei* (hyd). The sequence is numbered in relation to the transcription start site of Promoter C1. The bold underline identifies exons. C0 is the first exon and C1 is the second exon. The GT of the 5′ splice donor and the AG of the 3′ splice acceptor sites are identified below the sequence. Arrows identify transcription start sites. The *D. melanogaster* start sites have been physically mapped (Brenner et al., 1996). The proposed *D. hydei* start sites were identified on the basis of sequence similarity to the *D. melanogaster* sequence or by using programs designed to identify transcription start sites. A box is drawn around the Promoter C1 TATA box and the transcription start site. Conserved blocks of sequence are boxed and labeled above each line. All blocks were ranked and numbered from 1 to 39 according to the quality of the match. A lower number represents stronger conservation. Strong matches to transcription factor binding sites or to other transcriptional control regions are identified by name below each line of sequence. Black dots below a line identify the sequences involved in the match. Only matches observed in both *D. melanogaster* and *D. hydei* have been included. The labels P13 (−1959), P12 (−1698) and P4 (−1344, from Brenner et al., 1996) identify the 3′ endpoint of deletions in the transcriptional control region. The extent of these deletions is more clearly identified in Fig. 1. RB4, repetitive element found in the *slowpoke* control region (Brenner et al., 1996). Matches to transcription factor binding motifs are as follows: BSE, positively acting element first identified in rat embryonic myosin heavy-chain promoter (Yu and Nadal-Ginard, 1989); CF2-II, *Drosophila* chorion transcription factor CF2 (Gogos et al., 1992); RORalp, ROR alpha binding site (Giguere et al., 1994); c-ETS, proto-oncogene c-Ets binding site (Chen, 1988; Woods et al., 1992); BRC-Z, ecdysone-responsive *Drosophila* broad-complex transcription factor (von Kalm et al., 1994). The following abbreviations identify sequences that show strong similarity to other transcriptional control regions: ATR, primate insulin gene (Seino et al., 1987); SmElastase, *Schistosoma mansoni* elastase gene; Hgastrin, human gastrin gene; HSP70, *Drosophila* HSP70 gene; Rn G Protein, neuron-specific G-protein gamma 7 subunit 3′ UTR (Watson et al., 1994). Rn Trypsin II, rat trypsin II gene; Trypsin, mouse trypsin alpha gene; TSHb, mouse and rat thyrotropin β-subunit (Carr et al., 1987; Gordon et al., 1988); SCR, *Drosophila* sex combs reduced homeodomain protein (Gindhart and Kaufman, 1993). Ca-ATPase (Kessler et al., 1992; Shull and Greeb, 1988); HFH 2 (Overdier et al., 1994); GlcCotransporter (Veyhl et al., 1993); ADH (Kreitman, 1983).

species, *D. melanogaster* and *D. hydei*, diverged from a common ancestor approximately 60 million years ago (Patterson and Stone, 1952). We expect that control elements will be conserved between the species and that sequences not important for control of expression will have diverged.

In this paper, our analysis has been limited to a genomic sequence beginning approximately 1.5 kb upstream of neuronal Promoter C1 and terminating in the downstream intron that abuts exon C1. To identify conserved regions, we used the Macaw program (National Center for Biotechnology Information). Macaw ranks each conserved block of sequence on the basis of its overall length and similarity. As expected,



we observed blocks of similar or identical sequence separated by strikingly dissimilar regions (Figs 1B, 2). We have identified each block of conserved sequence by a number (1–39) that reflects its ranking with respect to the other blocks. Blocks with lower numbers received a higher similarity score than blocks with higher numbers.

These blocks are also conserved in another manner. For both *D. melanogaster* and *D. hydei*, the relative position of all the blocks with respect to one another and to Promoter C1 is conserved (Fig. 2). Blocks whose position was not conserved are not shown. Such blocks tended to be tiny and to be composed of simple sequence. Within the region compared, we observed no evidence that chromosome rearrangements have occurred since the evolutionary separation of the two species. Such rearrangements would reorganize the transcriptional control region.

These blocks are likely to have been conserved because they represent functionally important transcription factor binding sites. Conservation of position would be selected for if the groups of factors that bind to the blocks interact with one another and if productive interactions require a particular order and spacing. This added layer of conservation adds credence to the hypothesis that these elements are important for proper *slowpoke* expression.

Identification of the Drosophila hydei *Promoter C1*

One of the first features that we looked for was conservation of Promoter C1. The *D. melanogaster* Promoter C1 was previously mapped by 5′-RACE, by RPA and by cDNA cloning (Brenner et al., 1996). In *D. melanogaster*, Promoter C1 is located between conserved blocks 6 and 12 (Figs 1B, 2). We used the NNPP program (Reese, 1994) to search the *D. hydei* sequence in this area for potential TATA boxes followed by reasonable transcription start sites. TATA boxes direct transcription initiation to a unique nucleotide. The best match was found at the 2228th nucleotide in the *D. hydei* sequence. This aligns nicely with a TATA box in the *D. melanogaster* sequence that is 30 bp 5′ to the physically mapped *D. melanogaster* transcription start site (Fig. 2). Other criteria (see below) support this identification.

Identification of a new exon

Exon C1 was the most strongly conserved block of sequence even though it contributes only 5′ untranslated region (UTR) to the *slowpoke* transcript. Therefore, it occurred to us that some of the other conserved blocks might represent undiscovered *slowpoke* exons. Each conserved block was examined to determine whether it contained a consensus splice donor and therefore might represent an exon. Solely on the basis of sequence analysis, two blocks appeared to be good exon candidates. These are blocks 4 and 5 (Figs 1B, 2). To determine whether these blocks encoded exons, we used RT-PCR to determine whether different batches of mRNA contained transcribed versions of blocks 4 and 5. Since all known *Drosophila slowpoke* cDNAs include exon C3, we employed a reverse transcription primer and a 3′ PCR primer within exon C3. The 5′ PCR primer was specific for block 4 or block 5. Using this primer set, RT-PCR was performed on RNA samples purified from embryos, larvae, pupae and adults. The RT-PCR reaction using the block 4 primer was non-productive, indicating that block 4 does not serve as an exon in these developmental stages. However, the reaction using the block 5 primer amplified a band of approximately 500 bp from RNA purified from all developmental stages (Fig. 3). The only other PCR product was a small artifactual band that appeared when the PCR was carried out at very high Mg^{2+} concentration (not shown). Both fragments were cloned and sequenced. Sequence analysis of the 500 bp band indicated that the amplification product was actually 495 bp. The exon contained wholly or partially within conserved block 5 will from henceforth be referred to as exon C0. It should be noted that block 5 and exon C0 are not identical since block 5 also contains conserved intronic sequences that are probably required for splicing. DNA sequencing of the band amplified in a solution containing a high concentration of $MgCl_2$ showed that it was composed of primer concatomers.

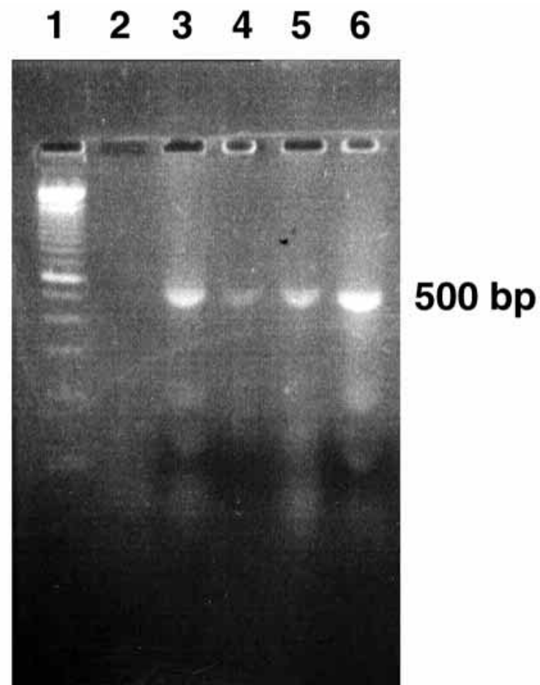


Fig. 3. RT-PCR using block 5 and exon-C3-specific primers demonstrates that block 5 is part of a *slowpoke* exon that is expressed in all developmental stages. The figure shows PCR products separated on a 2 % agarose gel. Lane 1 contains a size standard (100 bp ladder Gibco-BRL), and lane 2 represents a no-template control. Lanes 3–6 are RT-PCR amplifications from different RNA sources: lane 3, embryonic RNA; lane 4, larval RNA; lane 5, pupal RNA; lane 6, adult RNA. The approximately 500 bp amplification product corresponds to the C0:C1:C3 splice variant.

A new promoter

To map the transcription start site of Promoter C0, we used RLM-5′-RACE. This approach positively selects for full-

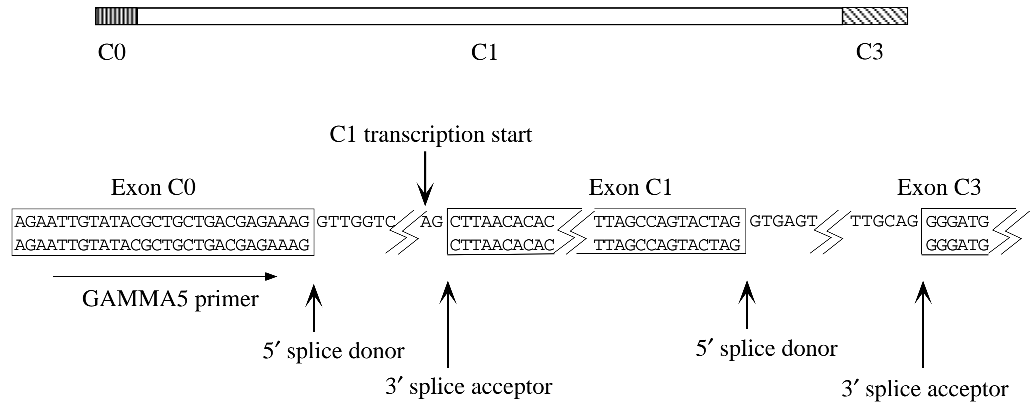
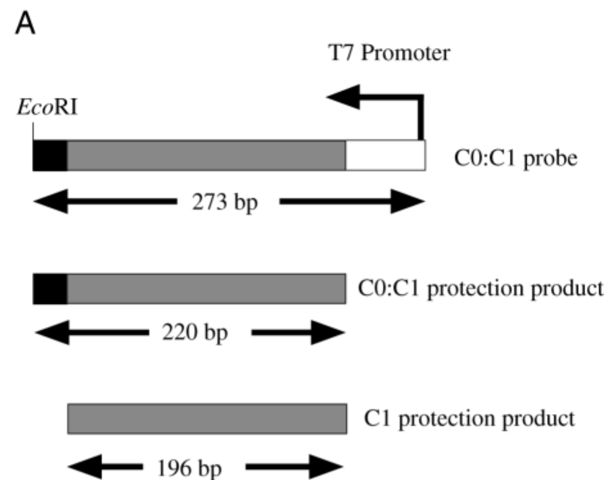


Fig. 4. Sequence of the C0:C1:C3 splice variant. (A) A schematic diagram of the 495 bp RT-PCR product from adult tissues. It splices together exon C0, exon C1 and exon C3. (B) The sequence surrounding the splice sites. The top sequence is genomic, the bottom sequence is from the RT-PCR product. Sequences matching the RT-PCR products are exons and are boxed. Splice donors and acceptors are labeled. Note that the splice acceptor site for the C1 exon is two base pairs from the transcription start site as mapped by Brenner et al. (1996). The GAMMA5 primer used in block 5 is shown with a horizontal arrow. The downstream primers are outside the range of the figure in exon C3 (see Materials and methods).

length messages by ligating an RNA linker of known sequence only to the 5′ end of mRNAs that retain their 5′ CAP. The 5′ CAP provides a unique identifier of the first nucleotide transcribed by RNA polymerase II. mRNA fragments that do not have a 5′ CAP are dephosphorylated with calf intestinal alkaline phosphatase so that they cannot participate in a subsequent ligation reaction. Treatment with tobacco acid pyrophosphatase converts the 5′ CAP, which is found only on the first nucleotide of the mRNA, into a 5′ phosphate group. Since, productive ligation of RNA linkers will only occur at these remaining phosphates, one can selectively attach the linker to mRNAs that represent full-length products. RT-PCR using linker- and gene-specific primers will selectively amplify products derived from 5′-CAP-containing mRNAs. Since, the linker is added to the mRNA, only full-length reverse transcription products are available for PCR amplification.

Using this approach, we were able to generate and clone 5′-RACE products that contained sequences derived from exon C0. Eight independently generated C0-containing clones were identified by colony hybridization using an oligo that anneals to exon C0. All eight had the same 5′ end and, by aligning the sequence of these cDNAs to the genomic sequence, we were able to map the transcription start site of Promoter C0 (identified in Figs 1, 2). Unlike Promoter C1, the transcription start site of Promoter C0 is not preceded by a recognizable TATA box. TATA-less transcriptional promoters are associated with both house-keeping genes and genes with tissue-specific expression patterns (Latchman, 1998).

The four previously mapped 5′ exons (C1, C1b, C1c and C2) directly splice to exon C3. Their splicing patterns can be summarized as C1:C3, C1b:C3, C1c:C3 and C2:C3 (Fig. 1A). One might anticipate that the new exon would also splice directly to exon C3. However, the RT-PCR-amplified cDNA fragments and the 5′-RACE products showed a novel splicing pattern: C0:C1:C3 (Fig. 4). Our surprise was compounded by the fact that this splicing pattern retains all but the first two



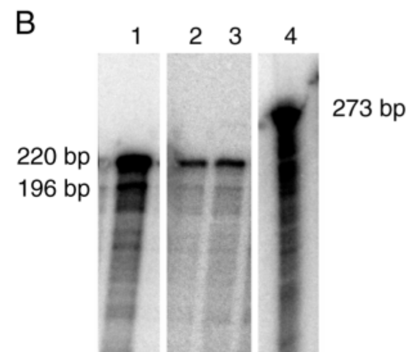


Fig. 5. Relative abundance of Promoter C0 and C1 transcripts. (A) RNAase protection probe and predicted digestion products. The C0:C1 probe is 273 nucleotides in length and was derived from a cDNA representing a Promoter C0 transcript. Transcripts from Promoter C0 produce a C0:C1 splice product generating a 220-nucleotide protection product. The black and gray boxes together represent this region. Promoter C1 transcripts do not contain any exon C0 sequence and produce a 196-nucleotide protection product. The gray box represents this product. (B) RNAase protection assay using the C0:C1 probe. Lanes 1–3 contain RNAase protection products produced using RNA purified from different developmental stages. Lane 1, poly(A+) embryonic RNA; lane 2, total RNA from larvae; lane 3, total RNA from adults; lane 4, the unprotected C0:C1 probe.

nucleotides of exon C1. The exon C0 5′ splice site was indeed the site identified by examination of the genomic sequence. Its 3′ splice acceptor site was the third nucleotide of exon C1.

Developmental specificity of Promoter C0

As previously noted, Fig. 3 presents the results of an RT-PCR experiment using RNA purified from embryos, larvae, pupae and adults. The amplification was performed using a primer set that would amplify only mRNAs that contain both exon C0 and C3. The 495 bp product representing the C0:C1:C3 splice variant was amplifiable from all developmental stages (Fig. 3).

Relative activity of the promoters

To quantify the relative expression levels of Promoter C0 and C1, we performed RNAase protection assays using a probe composed of portions of exon C0 and C1. This 273-nucleotide probe contains sequences from both exon C0 and C1 and is derived from an actual Promoter C0 transcript. When used, this probe detects a transcript starting either at Promoter C0 (producing a 220-nucleotide protection product) or at Promoter C1 (generating a 196-nucleotide protection product). Fig. 5 shows that the C0 product is expressed at higher levels than the C1 product in embryos, larvae and adults. After normalizing for the number of labeled nucleotides in each protected product, we determined that, in all developmental stages, the ratio of Promoter C0 to Promoter C1 transcripts is approximately 12:1. Therefore, at a gross level, Promoter C0 is responsible for most of the expression in the adult.

Tissue specificity of the promoters

We had previously shown that a reporter gene called P3 (Fig. 6) reproduces the *slowpoke* neuronal expression pattern but is not expressed in other tissues (Brenner et al., 1996). The P3 construct includes both Promoter C0 and C1, but it does not contain any of the other *slowpoke* tissue-specific promoters (Fig. 6). Therefore, the newly discovered Promoter C0 must be neuronal-specific. In the larval brain, P3 is expressed in the brain lobes, central brain, mushroom bodies and ventral nerve cord (Fig. 7A). In the adult, P3 is expressed in the optic lobes, central brain, mushroom bodies and eyes (Fig. 7B). Expression in the eye is believed to be in photoreceptor cells (Brenner et al., 1996).

To help determine the relative contributions of Promoter C0 and Promoter C1 to larval and adult neuronal expression, we employed two derivatives of P3: P12 and P13. The expression patterns of transformed flies were determined by β-galactosidase staining. The transformed animals being compared were stained for the same time in the same solution so that the relative expression level could be crudely compared. P12 contains a 950 bp deletion that removes Promoter C0 but is otherwise identical to P3 (Fig. 6). In the absence of Promoter C0, P12 should report the expression pattern of Promoter C1. The P13 construct is essentially identical to P12 except that

Reporter gene constructs

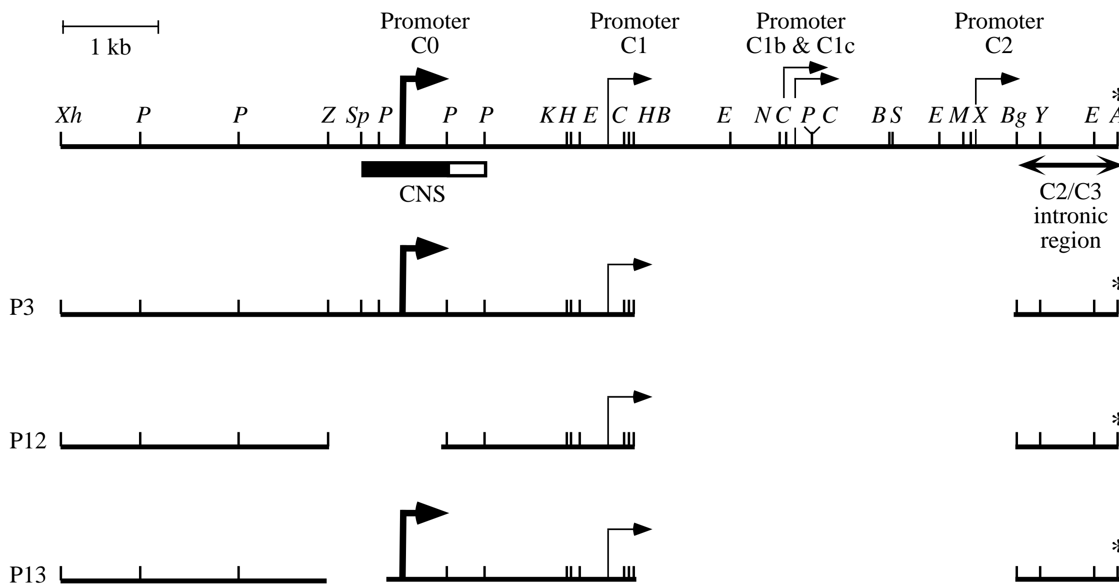


Fig. 6. Reporter gene constructs. The top line is a restriction map of the cloned *slowpoke* transcriptional control region. The positions of mapped promoters are identified by the labeled arrows. Promoter C0 was mapped in this study. The C2/C3 intronic region, which contains elements required for normal promoter activity, is identified by the line with double arrowheads. Arranged below this line are the P3, P12 and P13 deletion constructs. Gaps in the restriction map identify sequence that has been removed from each construct. In each construct, a *lacZ* gene has been inserted into the *Apa*I site. For clarity, an asterisk has been placed above this site. *Drosophila* stocks carrying each transgene were generated by P-element-mediated transformation. Abbreviations are as in Fig. 1.

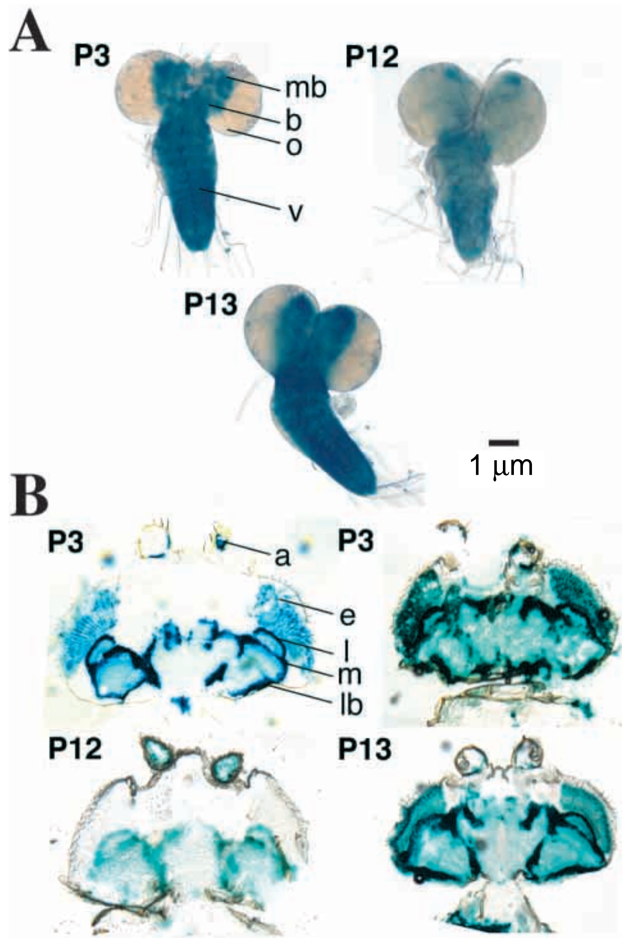


Fig. 7. Expression of reporter genes in larval and adult brain as determined by staining for β-galactosidase activity. Anterior is up. (A) Larval brains showing expression in the mushroom body, central brain, brain lobes and ventral nerve cord. P3 and P13 show similar levels of expression. P12 shows the lowest level of expression. All were stained together, in the same dish, to permit comparison of the relative expression levels. (B) Frozen adult head sections. Two P3 sections are shown; the left-hand section was lightly stained to preserve detail. The remaining three sections were all stained for the same time to permit comparison of the relative expression levels. Expression is seen in the lamina, medulla, lobula and central brain and the eye. P3 and P13 have similar or identical expression patterns and levels. P12 has a reduced expression level, and expression appears to have been lost in the eye. a, antenna; b, central brain; e, eye; l, cortical region of the lamina; m, cortical region of the medulla; lb, cortical region of the lobula plate; mb, a portion of the mushroom body; o, optic lobes of the brain; v, ventral nerve cord. Scale bar, 1 µm.

P13 has a slightly smaller deletion whose 3′ end is 121 nucleotides 5′ of the Promoter C0 transcription start site. The 5′ end of the deletion in both constructs is identical.

The P12 construct showed an expression level that was substantially reduced compared with P3, although all areas of the larval and adult brain seem to be represented (Fig. 7). Only in the adult eye does the P12 deletion cause a loss of expression. P13, which contains Promoter C0, shows essentially the same expression pattern and expression level as the P3 (wild-type) construct in both larval and adult brain (Fig. 7).

Use of evolutionary conservation to identify potential control elements

The evolutionary sequence conservation between *D. melanogaster* and *D. hydei* transcriptional control regions provides a detailed map that will speed the identification of important control elements. In this study, it helped to determine the position of a previously unmapped transcriptional promoter. This homology map will be used to guide future deletion analysis experiments aimed at identifying the control elements that regulate *slowpoke* expression.

Discussion

The transcriptional control of the *slowpoke* BK type Ca^{2+}-activated K^+ channel gene is remarkably complex. We have previously mapped a muscle/tracheal cell promoter, a midgut promoter and a central nervous system (CNS)-specific promoter (Brenner et al., 1996). These were all initially identified by isolating cDNAs representing transcripts from the gene. As an alternative approach, we mapped evolutionarily conserved portions of the transcriptional control region as a means of identifying promoters. This approached helped identify the new 5′ exon called exon C0 and the promoter that produced it: Promoter C0. The remaining blocks of conserved sequence are likely to represent control elements that regulate promoter activity. Functional testing will be required to determine the purpose of the conserved blocks. Our data indicate that Promoter C0 and Promoter C1 are active in all developmental stages and are responsible for almost all expression in the CNS. Of the two, transcripts arising from Promoter C0 are approximately 12 times more abundant than transcripts produced by Promoter C1.

Why does the fly require two neuronal promoters? The first translation start site in transcripts produced by either Promoter C0 or Promoter C1 is the second codon of exon C3. That is, all of exon C0 and exon C1 represent 5′ untranslated regions (5′ UTRs) of the mRNA. Therefore, promoter choice does not affect the sequence of the encoded polypeptide. Perhaps the two neuronal promoters provide a simple way for distinct cells to express the gene at different levels and thereby to produce cell membranes with different channel densities. The control of channel density can be just as important as channel type in determining a cell's electrical properties (Baro et al., 1997). Unfortunately, cell-to-cell differences in promoter use are not detectable in our gross histological assays. The facts that Promoter C0 is TATA-less and that Promoter C1 is preceded by a good match to a consensus TATA box support the idea that these two promoters are differentially regulated.

We were intrigued by the splicing pattern of Promoter C0 transcripts. The other four *slowpoke* transcripts that we have characterized all begin with an exon that is directly spliced to exon C3. However, transcripts that begin with exon C0 splice

Table 1. *Similarity of conserved blocks to known transcriptional control regions or 5′ untranslated regions*

Block 1

mel	ACACATTGATGTGCGATTTGAGCGTGTGAATGTTGGCAGCCCTGGAGCCCTGCCTCGGTGGTT
hyd	ACACGTTCATGTGCGATTTGAACGTGTGAATGTTGGCAGCCCTGGAGCCCTGCCTCGGTGGTT
Rn G Protein	AGGGTAGGATGTGCTGGTGTAGCCTGTGTACGTCCTAAGCACTGGAGCCCTGCCTCGGCCTAG
ATR	ACCTGGGGTCCAACTGCTCTGGAACCCCGAGTGGGGCAGCCCTGGGGCCCAGACTGGCTCGAA

Block 4

mel	TGTCATCGTCAATGTTATTTTTGTTGCTTCCTACTCGTCGTC
hyd	TGTCATCGTCAATGTTATTTTKGTTGTTTCCTATTCGTCGTC
rat Ca-ATPase	TGTCAACGGCATTGTTATTTTTGTT
human Ca-ATPase	TGTCAACAGCATTGTTATTTTTGTTG

Block 8

mel	TTGGTATATGTGTGTCAG TCAGTCACTGGCCGAGCGTG
hyd	TTGGTATATGTGTGTCAG TCAGTCAGTGCCCGAACATG
GlcCotransporter	GGTGTCTGTGGGGCAG GAAGGGACTGGCCGAGC
mel	TTGGTATATGTGTGTCAGT CAGTCACTGGCCGAGCGTG
hyd	TTGGTATATGTGTGTCAGT CAGTCAGTGCCCGAACATG
SCR	AAATATGTGTCAGT CAGTCAGTGAGCGAA
SmElastase	ATGTGTGTCAGT CAGTCAGTG
Hgastrin	TTAGTGTCAGT CAGTCA
BSE	GTGTCAGT CA

Block 9

mel	TGGCACTGCTGCAGGTTTTC
hyd	TGGTGTTGCTGCGGGTTTTT
trypsin ta	AAACATAGCTGCAGGTTTCT
trypsin tb	AAACACAGCTGTATTTTCCT
trypsin tc	AAACACAGCTGT GGTTTTC
trypsin td	AAGCACACCTGT GGTTTTC
trypsinII	AAGCACACCTGTGGGTTTCC
amy-2a	GTGCACAGCTGAAGGTTCTT

Block 10

mel	CCAAATCCAGGCGAGTTCAATGCGCGGCCAG
hyd	CCAATTCCCAACGAGTTCAATGCGCGCCCAG
mono-oxygenase	ATGGCCCCAGGCGAGGGCGATGCGCGGCCAG
plasminogen activator	CCAATTCCCAATGAAATCCATGCCCCCCCCC

Block 27

mel	AATGCAATTATGAAATAATCA
hyd	AATTCAATTATATAATAATTA
Mmthryotropin	AATGCAATTATATAAACAAGA
Rnthryotropin	AATGCAATTATATAAACAAGA

The *Drosophila melanogaster* and *D. hydei* blocks were used individually to query GenBank and EPD DNA databases using the NCBI Blast search engine. Only promoter regions or 5′ untranslated regions (UTRs) identified by both the *D. melanogaster* and *D. hydei* sequences were considered.

The abbreviations used, the name of the gene, the relative position of the match in the gene and the gene's accession number are as follows: ATR, *Arabidopsis trivirgatus* insulin, 5′ UTR, J02989; Rn G protein, *Rattus norvegicus* G-protein gamma subunit, 3′ UTR, L23219; rat Ca-ATPase, *R. norvegicus* brain Ca^{2+}-transporting ATPase, 3′ UTR, J03753; human Ca-ATPase, human Ca^{2+}-transporting ATPase, 3′ UTR, S49852; GlcCotransporter, *Sus scrofa* Na^{+}/D-glucose cotransporter, 3′ UTR, X64315; SCR, *Drosophila melanogaster* sex combs reduced, promoter region, U10507; SmElastase, *Schistosoma mansoni* elastase gene, promoter region, U31768; Hgastrin, human gastrin, promoter region, M15958; trypsin, *Mus musculus* trypsin gene family members (ta, tb, tc, td), promoter region, X04577; trypsinII, *R. norvegicus* pancreatic trypsin II promoter region, L00130; amy-2a, *M. musculus* α-amylase 2a, X02948; mono-oxygenase, *M. musculus* peptidylglycin alpha-amidating mono-oxygenase, 5′ UTR, U79523; plasminogen activator, *S. scrofa*, promoter region, X01648; Mmthyrotropin, *M. musculus* thyrotropin β subunit, promoter region, M22739; Rnthyrotropin, *R. norvegicus* thyrotropin β subunit, promoter region, M14499; BSE is an enhancer motif common to muscle-specific myosin heavy chain, actin and troponin genes (Yu and Nadal-Ginard, 1989).

mel, *D. melanogaster* sequence; hyd, *D. hydei* sequence.

first to exon C1 and then to exon C3. The Promoter C1 transcription start site and the splice acceptor site of exon C1 are only separated by two nucleotides. The cell seems to go to great lengths to ensure that almost all the untranslated exon C1 is included. It may be that untranslated sequences in exon C1 serve some important function. The fact that untranslated exon C1 contains the largest and most strongly conserved block of homology (block 1) lends support to this interpretation. These sequences might be important for mRNA stability or translatability or, alternatively, these sequences might be involved in targeting the mRNA to a specific portion of the endoplasmic reticulum. When Promoter C0 is used to drive expression, it appends 28 nucleotides of 5′ UTR to the message. The inclusion of these sequences might modulate one of these properties in a cell-specific manner.

It is known that deletion of the C2/C3 intronic region (see Fig. 1) causes a complete loss of adult neuronal expression from *slowpoke* reporter genes (Brenner and Atkinson, 1996). This had previously been interpreted to mean that Promoter C1 was dependent on these sequences for activity. With the discovery that a portion of CNS expression arises from Promoter C0, this interpretation can be expanded to mean that both Promoter C0 and Promoter C1 require the presence of the C2/C3 intronic region for activity. We interpret this to mean that transcription elements within the C2/C3 intronic region act on both these promoters.

In addition, Brenner et al. (1996) determined that a 1.3 kb region, named the CNS box (Fig. 1A), was required for neuronal expression in all developmental stages. At that time, no promoters had been mapped to this area and it was therefore postulated that this region contained regulatory elements required for neuronal activation of Promoter C1. We now

know that Promoter C0 maps to the 5′ half of the 1.3 kb CNS box (Fig. 1A). Brenner et al. (1996) observed that deletion of these sequences results in a complete loss of adult brain expression and an almost complete loss of larval brain expression. Does the removal of Promoter C0 alone account for this? The P12 deletion indicates that it does not. P12 removes approximately 1 kb of sequence from the 5′ side of the CNS box including Promoter C0. Even though Promoter C0 has been removed, P12 expression in the adult brain persists, albeit at a much reduced level. The simplest interpretation is that elements not removed by the P12 deletion, the 3′-most 300 bp of the CNS box (open box, Fig. 1A), contain sequences required for the normal activity of another neural promoter, presumably Promoter C1.

Which conserved sequences might represent these elements? The original 1.3 kb CNS box defined by Brenner et al. (1996) includes conserved blocks 26, 14, 5, 11 and approximately half of homology block 9. The loss of this 1.3 kb of DNA causes a loss of expression in the adult and larval brain (Brenner et al., 1996). The P12 deletion, however, removes only blocks 26, 14, 5 and nine base pairs of block 11 and does not eliminate expression. This suggests that blocks 11 and 9 are sequences required for Promoter C1 activity in the CNS.

We did not observe any reasonable similarity between the *Drosophila* sequence and the human *slowpoke* transcriptional control region recently characterized by Dhulipala and Kotlikoff (1999), who identified a single transcription start site and studied 1675 bp upstream of this site. The tissue-specificity of this promoter is not yet known. It may be that the human homolog to Promoters C0 and C1 awaits discovery or that vertebrates and invertebrates employ different mechanisms of regulation.

The remaining blocks of evolutionarily conserved sequence may also represent transcriptional control elements that modulate and direct the activity of the *slowpoke* promoters. While some showed similarities to known transcription factor binding sites, the most striking similarities were not to known transcription factor binding sites but to the transcriptional control regions of other genes (Table 1). These may represent undescribed transcriptional control elements. Some of these homologies were shown to exist between two different examples of the same gene. The position of these similarities is noted in Fig. 2. This type of analysis is of course speculative and should not be over-interpreted. However, it does provide an alternative method of ranking the conserved sequences for future deletion analysis studies and may be of use to others.

This research was supported by the National Science Foundation Grant IBN-9724088 to N.S.A. Accession numbers for DNA sequences are U40221 and AF210728. A portion of this work was reported at the 1998 meeting of the New York Academy of Sciences.

References

Atkinson, N. S., Robertson, G. A. and Ganetzky, B. (1991). A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science* **253**, 551–555.

Barnes, W. M. (1994). PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91**, 2216–2220.

Baro, D. J., Levini, R. M., Kim, M. T., Willms, A. R., Lanning, C. C., Rodriguez, H. E. and Harris-Warrick, R. M. (1997). Quantitative single-cell-reverse transcription-PCR demonstrates that A-current magnitude varies as a linear function of *shal* gene expression in identified stomatogastric neurons. *J. Neurosci.* **17**, 6597–6610.

Becker, M. N., Brenner, R. and Atkinson, N. S. (1995). Tissue-specific expression of a *Drosophila* calcium-activated potassium channel. *J. Neurosci.* **15**, 6250–6259.

Brayden, J. E. and Nelson, M. T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256**, 532–535.

Brenner, R. and Atkinson, N. (1996). Developmental and eye-specific transcriptional control elements in an intronic region of a Ca^{2+}-activated K^+ channel gene. *Dev. Biol.* **177**, 536–543.

Brenner, R., Thomas, T. O., Becker, M. N. and Atkinson, N. S. (1996). Tissue-specific expression of a Ca^{2+}-activated K^+ channel is controlled by multiple upstream regulatory elements. *J. Neurosci.* **16**, 1827–1835.

Carr, F. E., Need, L. R. and Chin, W. W. (1987). Isolation and characterization of the rat thyrotropin beta-subunit gene. Differential regulation of two transcriptional start sites by thyroid hormone. *J. Biol. Chem.* **262**, 981–987.

Chen, J. H. (1988). Complementary DNA clones of chicken proto-oncogene c-ets: sequence divergence from the viral oncogene v-ets. *Oncogene Res.* **2**, 371–384.

Cheng, S., Fockler, C., Barnes, W. M. and Higuchi, R. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA* **91**, 5695–5699.

Dhulipala, P. D. and Kotlikoff, M. I. (1999). Cloning and characterization of the promoters of the maxiK channel alpha and beta subunits. *Biochim. Biophys. Acta* **1444,** 254–562.

Frohman, M. A. (1995). Rapid amplifcation of cDNA ends. In *PCR Primer: A Laboratory Manual* (ed. C. W. Dieffenbach and G. S. Dveksler), pp. 381–409. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Giguere, V., Tini, M., Flock, G., Ong, E., Evans, R. M. and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. *Genes Dev.* **8**, 538–553.

Gindhart, J. G. and Kaufman, T. C. (1993). Developmental regulation of the *Drosophila* homeotic gene sex combs reduced. Dissertation, Howard Hughes Medical Institute at Indiana University.

Gogos, J. A., Hsu, T., Bolton, J. and Kafatos, F. C. (1992). Sequence discrimination by alternatively spliced isoforms of a DNA binding zinc finger domain. *Science* **257**, 1951–1955.

Gordon, D. F., Wood, W. M. and Ridgway, E. C. (1988). Organization and nucleotide sequence of the gene encoding the beta-subunit of murine thyrotropin. *DNA* **7**, 17–26.

Hille, B. (1992). *Ionic Channels of Excitable Membranes*, 2nd edn. Sunderland, MA: Sinauer Associates, Inc.

Issa, N. P. and Hudspeth, A. J. (1994). Clustering of Ca^{2+} channels and Ca^{2+}-activated K^+ channels at fluorescently labeled presynaptic active zones of hair cells. *Proc. Natl. Acad. Sci. USA* **91**, 7578–7582.

Jan, L. Y. and Jan, Y. N. (1990). How might the diversity of potassium channels be generated? *Trends Neurosci*. **13**, 415–419.

Kessler, F., Falchetto, R., Heim, R., Meili, R., Vorherr, T., Strehler, E. E. and Carafoli, E. (1992). Study of calmodulin binding to the alternatively spliced C-terminal domain of the plasma membrane Ca^{2+} pump. *Biochem.* **31**, 11785–11792.

Kreitman, M. (1983). Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster. Nature* **304**, 412–417.

Krieg, P. A. (1990). Improved synthesis of full-length RNA probe at reduced incubation temperatures. *Nucleic Acids Res.* **18**, 6463.

Krieg, P. A. and Melton, D. A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Meth. Enzymol.* **155**, 397–415.

Latchman, D. S. (1998). DNA sequences, transcription factors and chromatin structure. In *Eukaryotic Transcription Factors*, 3rd edn, pp. 1–20. San Diego: Academic Press.

Latorre, R., Oberhauser, A., Labarca, P. and Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol*. **51**, 385–389.

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J. and Lederer, W. J. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* **270**, 633–637.

O'Neil, M. T. and Belote, J. M. (1992). Interspecific comparison of the transformer gene of *Drosophila* reveals an unusually high degree of evolutionary divergence. *Genetics* **131**, 113–128.

Overdier, D. G., Porcella, A. and Costa, R. H. (1994). The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. *Mol. Cell. Biol.* **14**, 2755–2766.

Patterson, J. T. and Stone, W. S. (1952). *Evolution in the Genus* Drosophila. New York: The Macmillan Company.

Reese, M. G. (1994). Computational Genome Annotation in *Drosophila melanogaster* (http://www.fruitfly.org/seq_tools/promoter.html). Dissertation, German Cancer Research Center, Heidelberg.

Robitaille, R., Garcia, M. L., Kaczorowski, G. J. and Charlton, M. P. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* **11**, 645–655.

Rudy, B. (1988). Diversity and ubiquity of K channels. *Neuroscience* **25**, 729–749.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Schaefer, B. C. (1995). Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. *Analyt. Biochem*. **227**, 255–273.

Shull, G. E. and Greeb, J. (1988). Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. Structural and functional domains exhibit similarity to Na^+,K^+- and other cation transport ATPases. *J. Biol. Chem.* **263**, 8646–8657.

Seino, S., Steiner, D. F. and Bell, G. I. (1987). Sequence of a new world primate insulin having low biological potency and immunoreactivity. *Proc. Natl. Acad. Sci. USA* **84**, 7423–7427.

Spradling, A. C. (1986). P-element mediated transformation. In Drosophila*: A Practical Approach* (ed. D. B. Roberts), pp. 175–96. Oxford, Washington: IRL Press.

Thomas, T., Wang, B., Brenner, R. and Atkinson, N. S. (1997). Novel embryonic regulation of Ca^{2+}-activated K^+ channel expression in *Drosophila. Invert. Neurosci*. **2**, 283–291.

Thummel, C. S., Boulet, A. M. and Lipshitz, H. D. (1988). Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. *Gene* **74**, 445–456.

Veyhl, M., Spangenberg, J., Puschel, B., Poppe, R., Dekel, C., Fritzsch, G., Haase, W. and Koepsell, H. (1993). Cloning of a membrane-associated protein which modifies activity and properties of the $Na^{(+)}$-D-glucose cotransporter. *J. Biol. Chem.* **268**, 25041–25053.

von Kalm, L., Crossgrove, K., Seggern, D. V., Guild, G. M. and Beckendorf, S. K. (1994). The broad-complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J.* **13**, 3505–3516.

Watson, J. B., Coulter, P. M., Margulies, J. E., Lecea, L., de Danielson, P. E., Erlander, M. G. and Sutcliffe, J. G. (1994). G-protein gamma 7 subunit is selectively expressed in medium-sized neurons and dendrites of the rat neostriatum. *J. Neurosci. Res*. **39**, 108–116.

Wei, A., Jegla, T. and Salkoff, L. (1996). Eight potassium channel families revealed by the *C. elegans* genome project. *Neuropharmac*. **35**, 805–829.

Woods, D. B., Ghysdael, J. and Owen, M. J. (1992). Identification of nucleotide preferences in DNA sequences recognised specifially by c-Ets-1 protein. *Nucleic Acids Res*. **20**, 699–704.

Yu, Y.-T. and Nadal-Ginard, B. (1989). Interaction of nuclear proteins with a positive cis-acting element of rat embryonic myosin heavy-chain promoter: Identification of a new transcriptional factor. *Mol. Cell. Biol*. **9**, 1839–1849.