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Tissue-Specific Expression of a Ca^{2+}-Activated K^+ Channel Is Controlled by Multiple Upstream Regulatory Elements

Robert Brenner, Tarita O. Thomas, Marie N. Becker, and Nigel S. Atkinson

Department of Zoology, The University of Texas at Austin, Austin, Texas 78712-1064

The electrical properties of a cell are produced by the complement of ion channels that it expresses. To understand how ion-channel gene expression is regulated, we are studying the tissue-specific regulation of the *slowpoke* (*slo*) Ca^{2+}-activated K^+ channel gene. This gene is expressed in the central and peripheral nervous system, in midgut and tracheal cells, and in the musculature of *Drosophila melanogaster*. The entire transcriptional control region has been cloned previously and shown to reproduce the tissue and developmental expression pattern of the endogenous gene.

Here we demonstrate that *slo* has at least four promoters distributed over ~4.5 kb of DNA. Promoter C1 and C1c display a TATA box-like sequence at the appropriate distance from the transcription start site. Promoters C1b and C2, however, are TATA-less promoters. C1, C1b, and C1c transcripts differ in their leader sequence but share a common translation start site. C2 transcripts incorporate a new translation start site that appends 17 amino acids to the N terminus of the encoded protein.

Deletion analysis was used to identify sequences important for tissue-specific expression. We used a transgenic *in vivo* expression system in which all tissues and developmental stages can be assayed easily. Six nested deletions were transformed into *Drosophila*, and the expression pattern was determined using a *lacZ* reporter in both dissected tissues and sectioned animals. We have identified different sequences required for expression in the CNS, midgut, tracheal cells, and muscle.

Key words: Ca^{2+}-activated K^+ channel; gene regulation; Drosophila; slowpoke; *reporter gene; tissue-specific expression*

A properly functioning nervous system requires great variety in the electrical properties of its cells. This variety must be controlled to ensure that any given cell has the precise attributes it needs to perform its duties. The electrical properties of a cell are produced by the coordinated activity of a suite of channels that conduct ions across the cell membrane. To manifest the appropriate electrical character, a cell must regulate gene expression so that the proper subset of ion channels is expressed at appropriate levels.

K^+ channels are a diverse family of channels, and this diversity is a major cause of the manifold differences in the electrical properties of cells. Although electrophysiological and molecular techniques have been used to catalog the K^+ channels expressed in nervous systems, relatively little is known about the regulation of K^+ channel gene expression. Certainly some K^+ channel genes have been shown to be transcriptionally regulated during development and by cell–cell interactions, cAMP, kinases, hormones, opiates, and depolarization (Roberds and Tamkun, 1991; Attardi et al., 1993; Bosma et al., 1993; Matsubara et al., 1993; Mori et al., 1993; Perney and Kaczmarek, 1993; Lindsdell and Moody, 1994; Mackler and Eberwine, 1994; Sontheimer, 1994). Information concerning how the tissue-specific expression is implemented, however, is largely absent. The need for controlled and coordinated expression makes it likely that the transcriptional control region of K^+ channels will be complex, exhibiting multiple tissue-specific promoters and enhancer elements which may result in the production of nonidentical proteins.

We are studying the transcription control region of the *Drosophila slowpoke* (*slo*) Ca^{2+}-activated K^+ (CAK) channel gene. This was the first CAK channel gene to be cloned (Atkinson et al., 1991) and has since been used to clone, by homology, CAK channel genes from other organisms (Butler et al., 1993; Dworetzky et al., 1994; Pallanck and Ganetzky, 1994; Tseng-Crank et al., 1994). The *slo* gene encodes a charybdotoxin-sensitive CAK channel that is functionally and structurally homologous to the vertebrate BK-type (large conductance) K^+ channel. BK-type channels typically participate in shaping action potentials, determining firing patterns, controlling smooth muscle tone, and modulating Ca^{2+} entry (Rudy, 1988; Latorre et al., 1989; Toro et al., 1990; Revest and Abbott, 1992).

In *Drosophila*, this channel is expressed in all or nearly all muscles, in neurons of the central and peripheral nervous systems, in a subset of the embryonic and larval midgut cells, and in tracheal cells (Becker et al., 1995). The entire *slo* transcriptional control region has been cloned in 11 kb of genomic DNA. This DNA has been attached to a reporter gene, transformed into *Drosophila*, and shown to drive expression in the same tissues as the endogenous gene (Becker et al., 1995). Here we describe the physical mapping of promoters and the functional mapping of sequences responsible for expression in the CNS, muscles, and tracheal and midgut cells.

MATERIALS AND METHODS

Isolation of the *slowpoke* promoter-containing DNA fragment, called P1, and cDNA clones has been described (Atkinson et al., 1991; Becker et al., 1995). Alternative first exons C1 and C2 were identified in adult head and embryonic cDNA libraries (Becker et al., 1995). Exons C1b and C1c were

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Correspondence should be addressed to Nigel Atkinson, Department of Zoology, The University of Texas at Austin, Austin, TX 78712-1064.

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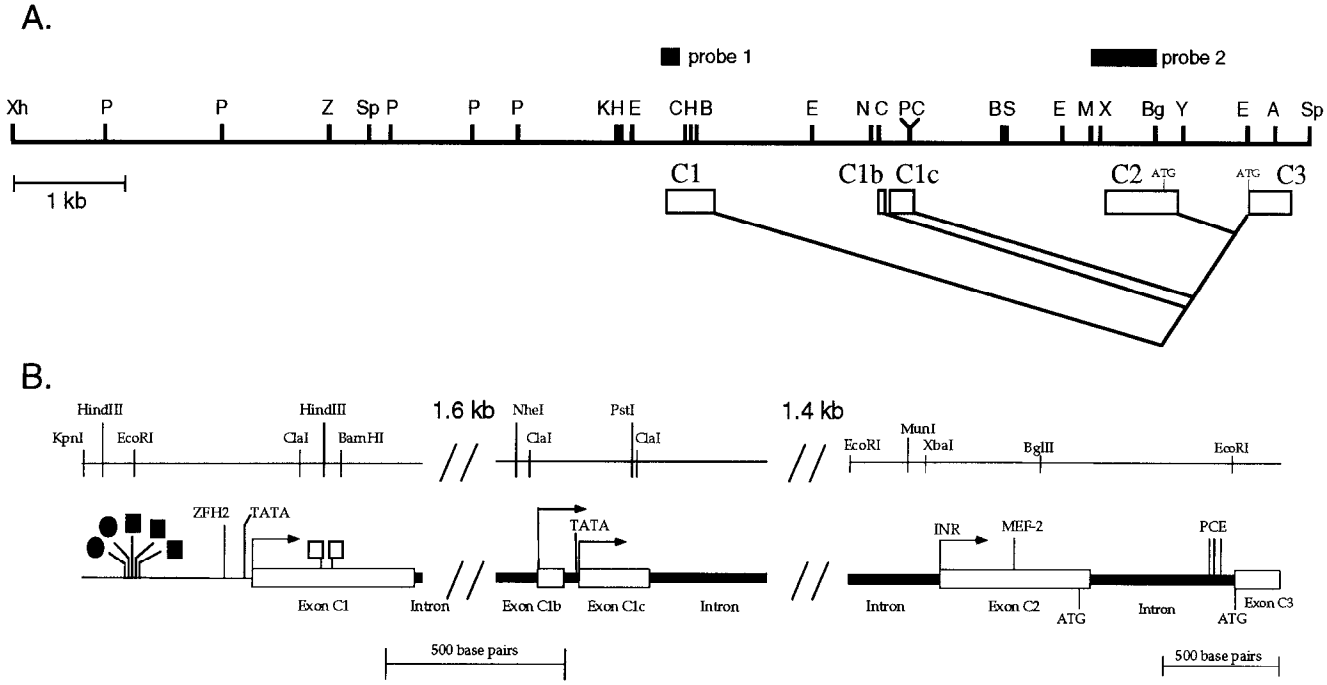

Figure 1. *A*, Map of genomic DNA in the vicinity of the *slo* promoters. The remainder of the gene extends 3′ at least 50 kb. *Tick marks* are restriction sites. *Open boxes below the line* identify exons. *Lines* connecting the exons indicate their splicing pattern. Exons *C1*, *C1b*, *C1c*, and *C2* are produced by different promoters. *ATG* represents translation start codons identified by sequence analysis. *Closed boxes* represent RPA probes. Probe 1 is a 175 nucleotide RPA probe used to fine-map promoter C1. Probe 2 is a 599 nucleotide RPA probe used to map promoter C2. Abbreviations: *A*, *Apa*I; *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *M*, *Mun*I; *N*, *Nhe*I; *P*, *Pst*I; *S*, *Sma*I; *Sp*, *Spe*I; *X*, *Xba*I; *Xh*, *Xho*I; *Y*, *Xmn*I *Z*, *Sph*I. *B*, Expanded view of each promoter represents sequenced DNA. *Open boxes* are alternative 5′ exons. *Arrows* identify transcription start sites. Proximal promoter and enhancer elements such as *TATA*, *ZFH2*, *MEF-2*, and *PCE* have been identified when found. Reiterated sequences of unknown function (RB1, 2, 3, and 4) are identified by *circles* and *boxes*; 8 nucleotide RB1 repeat (●); 7 nucleotide RB2 and RB3 repeats (■); and 12 nucleotide RB4 repeat (□).

identified in 5′-RACE products (see below). Mapping of exons onto genomic DNA was performed by DNA sequence alignment.

Deletion derivatives of P1, called P2 through P7 (see Fig. 4) were made by restriction enzyme digests and ligations at convenient restriction enzyme sites. Deletion constructs were carried in the vector pCaSperβgal (Thummel et al., 1988). Germline transformations were performed by co-injecting the helper plasmid pπ25.7wc (200 ng/μl) and transformation constructs (1 μg/μl) into w^{1118} embryos, as described (Spradling, 1986). Autosomes carrying insertions were maintained as homozygous stocks or using the balancer chromosomes *CyO* or *TM6*. To ensure that expression patterns did not reflect position effects, at least three different insertion positions were analyzed.

5′-RACE was performed using reagents and methods in the Clontech 5′-AmpliFINDER RACE KIT (Clontech Laboratories, Palo Alto, CA). The first-strand cDNA synthesis was performed using a primer inside exon C3 [5′-GCCAGCAAGGAATGTAAAGATGCTGGA-3′ (nucleotides 1378–1404)] (see Fig. 2*C*). Two rounds of PCR amplification were performed using nested primers in exon C3 and the Clontech anchor primer. The first-round primer was 5′-GCACCAGTACTTGCGCACCTTGAGACA-3′ (nucleotides 1342–1368) (see Fig. 2*C*), and the second-round primer was 5′-CATCGAATGGTGAATCTGTTGGATC-3′ (nucleotides 1309–1333) (see Fig. 2*C*). The PCR protocol was 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 34 cycles. RACE products were cloned into the Promega pGem-3T cloning vector (Promega, Madison, WI) and sequenced using universal reverse and −20 sequencing primers.

5′-RACE products that included exon C1 were identified by Southern blotting the RACE products with a 1.6 kb *Eco*RI genomic DNA fragment that overlaps exon C1 (Fig. 1*A*). All positively hybridizing clones contained a 600 nucleotide insert. Promoter C2 products were identified by hybridizing a blot with an exon C2-specific *Eco*RI/*Bgl*II fragment (nucleotides −401 to 416, Fig. 2*C*). To exclude those C2 products resulting from genomic DNA, a duplicate blot was hybridized with an *Xmn*I/*Eco*RI fragment (nucleotides 678-1241) (Fig. 2*C*) derived from the C2/C3 intron. To determine whether start sites existed between exons C1 and C2, the bacterial colonies containing the library of 5′-RACE clones were probed with the 2.2 kb of genomic DNA contained in the *Eco*RI fragment located between exons C1 and C2. All 5′-RACE products were sequenced in their entirety as was the corresponding genomic DNA. Sequence analysis confirmed that none of the clones were derived from contaminating genomic DNA. All of the unique 5′ ends (exons C1, C1b, C1c, and C2) are spliced to exon C3.

RNase protection assays (RPAs) were performed using antisense [α-^{32}P]cytidine 5′-triphosphate-labeled RNA that was synthesized using the Ambion Maxiscript kit (Ambion, Austin, TX). Antisense RNA (2×10^5 cpm) was hybridized to 25 μg of total adult RNA (exon C1 assays) or 10 μg of polyA$^+$ RNA (exon C2 assays). The protection assay was performed using the Ambion RPAII kit as described by the manufacturer. The protected probes were analyzed in an 8 M urea/6% acrylamide gel and exposed to x-ray film. The protected product size was determined by comparison to a DNA sequencing ladder and RNA markers.

For exon C1 containing transcripts, the initial probe was a genomic *Eco*RI/*Bam*HI fragment; nucleotides −344 to 230 (Fig. 2*A*) were cloned in pBluescript KS$^+$. A protected RNA product of ~240 n was observed (data not shown). To map the transcription start site more accurately, a smaller probe spanning the transcription start site was generated by PCR amplification (probe 1 in Fig. 1*A* and nucleotides −63 to 76 in Fig. 2*A*). The 3′ primer included the T7 RNA polymerase promoter sequence.

RPA mapping of exon C2-containing transcripts was performed using a probe derived from the genomic *Eco*RI/*Bgl*II DNA fragment that overlaps exon C2 (probe 2 in Fig. 1*A* and nucleotides −401 to 416 in Fig. 2*C*). This fragment was subcloned into plasmid pJKm (Kirschman and Cramer, 1988) and linearized with *Mun*I. Transcription from the SP6 promoter of the plasmid produced a 599 nucleotide RNA probe containing the *Mun*I to *Bgl*II portion of the fragment. This probe spans nucleotides −150 to 420 of the genomic DNA surrounding promoter C2 (Fig. 2*C*). The protection product was 421 nucleotides in length. RPAs were not used to confirm the position of promoter C1b and C1c start sites.

Sectioning and β-galactosidase detection in adults and larval tissues was performed as described (Becker et al., 1995). DNA sequencing was

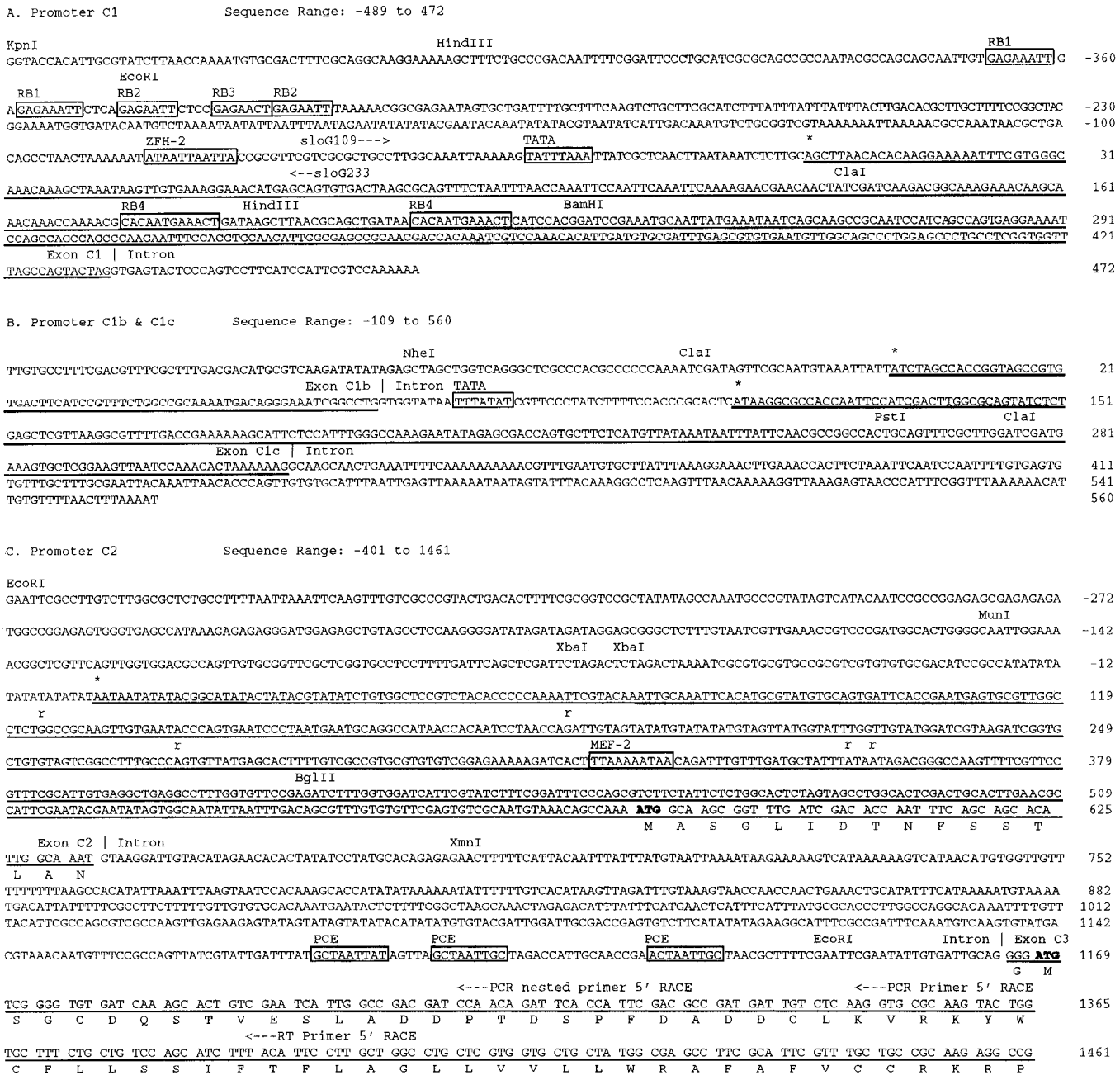

Figure 2. DNA sequence surrounding the *slo* promoters. Restriction sites, proximal promoter elements, repetitive sequences, exon/intron boundaries, and primers are annotated above the sequence. Transcription start sites are identified by an *asterisk*. Exons are underscored with a *double underline*. Translation of exons are presented below a line of sequence. Putative translation start codons are in *bold type*. *Boxed nucleotides* are sequence motifs referred to in Results and Discussion. *A*, Promoter C1 sequence −489 to 472. The transcription start site (+1) was identified by the coincidence of two 5′-RACE products (called C2–20 and C1–9) and also by RPA. sloG109 and sloG233 are PCR primers used to generate RPA probe 1 and define its ends. RB1 (GAGAAATT), RB2 (GAGAATT), RB3 (GAGAACT), and RB4 (CACAATGAAACT) identify distinctive repetitive elements (see Discussion). *B*, Promoters C1b and C1c sequence −109 to 560. Promoters C1b (+1) and C1c (+111) start sites as mapped by 5′-RACE. *C*, Promoter C2 sequence −401 to 1461. *5′-RACE* and *RPA* identified multiple starts. The nucleotide designated as +1 is marked by an *asterisk* and represents the 5′ end of the major 5′-RACE product. The most common transcription start site as identified by RPA also maps to −1. Quite probably these two represent the same transcription start site. Minor 5′ RACE products are identified by *r*. PCE identifies a match to the motif for a photoreceptor cell enhancer element (Kikuchi et al., 1993). The reverse transcription primer (*RT Primer*), *PCR primer*, and nested PCR primer were all used for the identification of 5′ ends by 5′-RACE.

carried out using the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase enzyme (USB, Cleveland, OH). Sequence motifs identified within the transcription control region are MEF-2 (5′ YTAAAAATAACYYY 3′), PCE (5′ YCAATTAGS 3′), and ZFH-2 (5′ MYAATTRAWTW 3′) (Gossett et al., 1989; Yu and Nadal-Ginard, 1989; Lundell and Hirsh, 1992).

RESULTS

Mapping of the *slo* promoters

The entire transcriptional control region is contained within an 11 kb *Xho*I/*Apa*I genomic DNA fragment (Becker et al., 1995) (Fig.

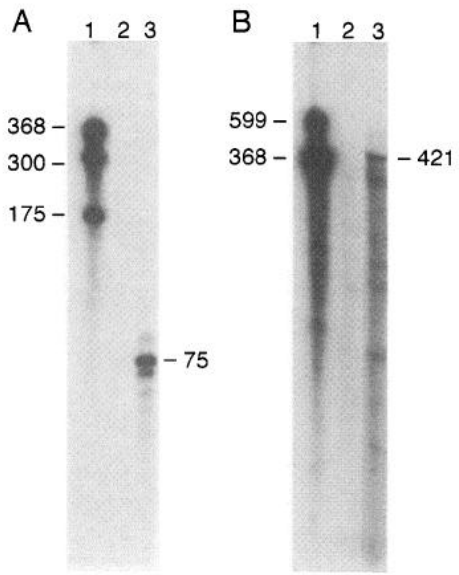

Figure 3. PAGE of RNase protection products. Sizes are in nucleotides and were determined on a DNA sequencing gel. *A*, Mapping of the promoter C1 transcription start site using probe 1. *Lane 1*, RNA standards. The 175 nucleotide standard is probe 1. *Lane 2*, Negative control in which the RNA probe was digested in the absence of *Drosophila* mRNA. *Lane 3*, 175 nucleotide probe 1 (Fig. 1*A*) transcript annealed to total RNA was digested with RNases to produce a major protection band of 75 nucleotides. *B*, Mapping of promoter C2 major transcription start sites using probe 2. *Lane 1*, RNA standards. The 599 nucleotide standard is probe 2. *Lane 2*, Negative control in which the RNA probe was digested in the absence of *Drosophila* mRNA. *Lane 3*, 599 nucleotide probe 2 (Fig. 1*A*) transcript annealed to polyA$^+$ RNA was digested with RNases to produce a major band of 421 nucleotides.

1). Three exons, C1, C2, and C3, have been mapped previously onto the genomic DNA by hybridization of cDNAs to genomic DNA. In our collection of cDNAs, C1 and C2 seemed to be alternative 5′ exons, both of which are spliced to the exon C3. Exons C1 and C2 hybridized to discrete sites within the genomic DNA, consistent with the hypothesis that *slo* had at least two major transcription start sites (Becker et al., 1995).

5′-RACE and an RPA were used to map directly the transcription start sites. RACE was performed on total fly RNA using 3′ primers specific for exon C3 (Fig. 2*C*). This exon is common to all known *slo* transcripts (Becker et al., 1995). After PCR amplification, the RACE products were cloned and Southern blotting and sequence analysis were used to map precisely the 5′ ends of the RACE clones onto genomic DNA. The positions of the C1 and C2 transcription start sites were also determined by RPA, using probes derived from genomic DNA.

The promoter C1 transcription start site was identified by aligning the 5′-most nucleotide of two independently isolated RACE products (Fig. 2*A*) and the 5′ end of cDNA H5 (Becker et al., 1995) with the sequence of the genomic DNA. Both RACE products align with the nucleotide designated as +1. The RPA also identified this position as the start site. The RPA probe, probe 1, was a genomic PCR amplification product that spans the putative start site. The position of probe 1 and the primers used to generate it are marked in Figures 1 and 2, respectively. This 175 nucleotide probe produced a 75 nucleotide RPA product when hybridized to *Drosophila* RNA (Fig. 3*A*). Seventy-five nucleotides from the 5′ end of our probe correspond exactly to the position designated as +1. Position +1 has been designated as the transcription start used by promoter C1 (Fig. 2*A*). Promoter C1 seems to be a stereotypical eukaryotic promoter with a TATA box at −36. TATA boxes direct transcription initiation to a unique nucleotide. Promoter C1 begins transcription with the exon C1.

Previous comparison of genomic DNA and cDNAs indicated that we should also expect to find a transcription start site at ~3.5 kb downstream in the vicinity of exon C2. In Figure 2*C*, the promoter C2 transcription start site has been identified by aligning the sequence of the 5′-RACE clone called C2–11 to genomic DNA. An RPA also indicates that this represents the major transcription start site for this promoter. In the RPA, a 599 nucleotide probe was transcribed from a genomic *Mun*I/*Bgl*II fragment that overlaps the putative start site (*probe 2* in Fig. 1). When hybridized against polyA$^+$ RNA, a 421 nucleotide protection product was produced. As shown in Figure 3, this is clearly the major protection product produced by this probe. Four hundred twenty-one nucleotides from the 5′ end of the probe correspond to genomic position −1. We believe that the 1-nucleotide discrepancy between the start sites mapped by 5′-RACE and RPA merely reflects the inherent difficulty in measuring the size of an RNA molecule.

Additional 5′-RACE products were isolated but mapped farther downstream within exon C2. These may represent actual transcription start sites or may have been produced by premature termination of the reverse transcription reaction. Additional evidence of multiple downstream transcription start sites was obtained by RPA. A large number of minor protection products smaller than 421 nucleotides were observed (Fig. 3*B*). All of these start sites map 5′ to the putative translation start codon at position 587. Therefore, all of the transcripts should encode the same protein. Sequence analysis showed that genomic DNA 5′ to exon C2 does not contain a recognizable TATA box (Conaway and Conaway, 1994). An INR motif, however, can be found centered at +1 and may substitute for a TATA box (Javahery et al., 1994). The 5′-RACE and RPA data are consistent with promoter C2 exhibiting multiple distributed transcription start sites.

This analysis confirmed and more finely mapped the promoter C1 and C2 start sites. Their position had been proposed on the basis of hybridization of cDNAs (isolated from a head-specific cDNA library) to genomic DNA (Becker et al., 1995). We were quite surprised when the analysis of RACE clones identified two additional transcription start sites. These are located between the C1 and C2 initiation sites and are called C1b and C1c (Fig. 1). Sequence analysis of the RACE products indicates that exons C1b and C1c are both spliced to exon C3, and therefore these products were copied from a processed mRNA transcript. Promoters C1b and C1c have been observed to be active in a limited way in the digestive system (see below). Because of the small number of cells that express these promoters, we did not determine the relative abundance of the C1b and C1c exons using RPA. Promoter C1b does not have a recognizable TATA or INR sequence; however, promoter C1c exhibits a TATA-like sequence at −34 (+77 in Fig. 2*B*). This expands the list of promoters and alternative 5′ exons to four.

Sequence analysis of cDNA clones indicated that all four promoters transcribe a unique 5′ exon that is then spliced to exon C3. We examined the exon/intron boundary for all of these exons. Canonical splice-donor sites were found adjacent to exons C1, C1b, and C2 (Fig. 2). Furthermore, the sequence 5′ to exon C3 matched the consensus for a splice-acceptor site. The sequence immediately 3′ to exon C1c, however, violates the so-called GT-AG rule for splice sites. Instead of GT as the 5′-most sequence of the intron, this intron displays GC (Fig. 2*B*). Although

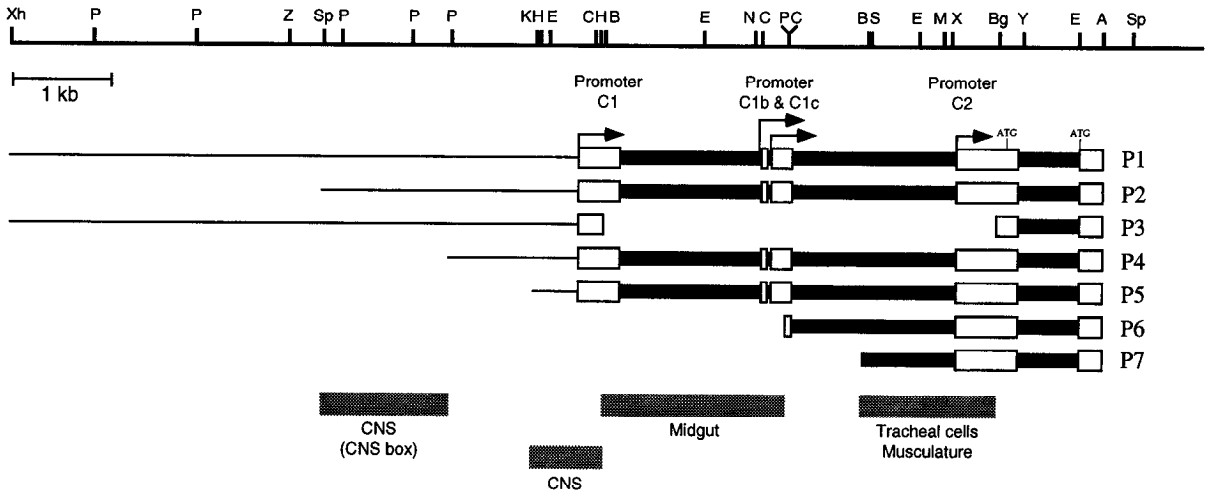

Figure 4. Transformation constructs, *P1–P7* are displayed below a genomic restriction map. A *thin line* represents DNA that is not part of a transcription unit, *open boxes* represent exons, and *thick lines* represent introns. In all constructs, a *lacZ* gene has been inserted into the *Apa*I site in exon C3. The *gray boxes* underscore sequences required for expression in particular tissue. Abbreviations are defined in Figure 1*A*.

unusual, this sequence is a common exception to the GT-AG rule (Jackson, 1991; Moore et al., 1993).

The 5′ exons C1, C1b, and C1c are 434, 67, and 206 nucleotides in length, respectively; however, these exons do not include an ATG in the same reading frame as the remainder of the coding sequence. Therefore, all are untranslated leaders, and translation of transcripts produced by promoters C1, C1b, and C1c must begin at the start codon of exon C3 (codon 2). The *Drosophila* consensus translational start site is a 10 nucleotide motif (RN-MAMNATGN) that promotes efficient translation initiation (Cavener, 1987). The C3 start codon matches the *Drosophila* consensus in only 6 of 10 sites. In contrast to promoters C1, C1b, and C1c, transcription from promoter C2 alters the coding capacity of the mRNA. Exon C2 is 634 nucleotides in length, as determined by RPA (Fig. 3*B*), and includes a start codon in the *slo* translational reading frame. This C2 translation start site perfectly matches the *Drosophila* consensus sequence. Furthermore, the use of C2 would add 17 amino acids onto the N terminus of the encoded protein.

Functional mapping of tissue-specific elements

We wanted to map the positions of elements involved in directing expression to specific tissue types. To do this, we constructed a series of nested deletions of the transcriptional control region and assessed their effect on the expression pattern of a reporter gene in transformed flies. The P1 transformation construct was used as the starting material. This plasmid contains all four promoters, all sequences required to reproduce the authentic *slo* expression pattern, and the first five exons of *slo*: C1, C1b, C1c, C2, and C3 (Fig. 4). Finally, a *lacZ* reporter gene has been inserted into C3 so that transcription from any of the promoters will cause the expression of β-galactosidase. Previous studies have shown that in transformed flies, P1 expresses β-galactosidase in the same tissue-specific pattern as *slo* (Becker et al., 1995). Deletions in P1 were made, and the modified plasmids were transformed into flies. The derivatives of P1 are presented in Figure 4, and a summary of their expression pattern can be found in Table 1.

Mapping of a CNS-specific transcriptional control element

In adults, expression of the P1 construct is clearly evident in the brain and thoracic muscles (Fig. 5*A*). P1 is also strongly expressed in the lobes and ventral nerve cord of the larval brain (Fig. 5*B*). In larvae, the body wall muscles uniformly express the reporter (Fig. 5*C*). P1 is also expressed in larval tracheal cells (Becker et al., 1995) (Fig. 6*A*).

Analysis of deletions P2, P3, P4, and P7 indicates that control of neuronal and muscle transcription is conveyed by distinct and independent DNA elements. These deletions define a 1.3 kb DNA fragment required for expression in the CNS and a non-overlapping 2.4 kb fragment that is sufficient to drive muscle but not neuronal expression. Deletions P2, P4, and P7 are progressive nested deletions from the 5′ end, and P3 is an internal deletion (Fig. 4).

Table 1. Summary of the expression pattern of transformants P1 through P7

	Larval brain	Larval muscle	Larval midgut	Larval tracheal cells	Adult brain	Adult muscles
P1	++++	++++	++++	++++	++++	++++
P2	++++	++++	++++	++++	++++	++++
P3	++++	−−−−	−−−−	−−−−	++++	−−−−
P4	+	++++	++++	++++	−−−−	++++
P5	+	++++	++++	++++	−−−−	++++
P6	−−−−	++++	−−−−	++++	−−−−	++++
P7	−−−−	++++	−−−−	++++	−−−−	++++

Four pluses indicates that expression is indistinguishable from the P1 transformant. A single plus indicates that the number of cells expressing β-galactosidase is substantially reduced. Four minuses indicate that reporter expression is nearly completely absent.

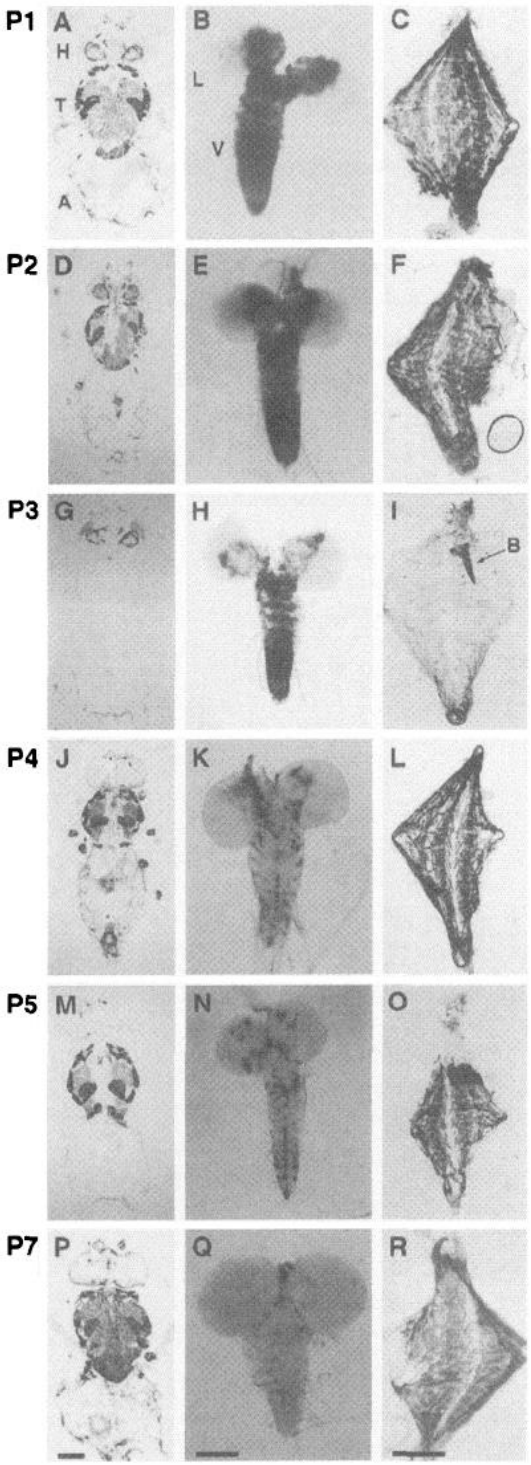

Figure 5. β-Galactosidase reporter gene expression. Adult and larval tissues have been stained with X-gal. Each row represents animals transformed with a different construct (*P1–P7*). Column 1 (*A, D, G, J, M, P*) shows horizontal sections of adults. Letters identify the head (*H*), thorax (*T*), and abdomen (*A*). In the head, only CNS expression is clearly visible. In the thorax, expression is in the direct and indirect flight muscles. Column 2 (*B, E, H, K, N, Q*) shows whole-mount larval brains. Letters identify a brain lobe (*L*) and ventral nerve cord (*V*). Column 3 (*C, F, I, L, O, R*) shows filleted larval body walls. Visible staining is in muscle fibers. In *I* (P3 transformant), the larval musculature does not stain; however, the larval brain (*B*) stains normally. Anterior is up. Scale bars: *P*, 0.25 mm; *Q*, 0.1 mm; *R*, 1.0 mm.

In deletion P2, the first 3.1 kb of P1 has been deleted. When transformed into flies, however, it expresses β-galactosidase in the CNS and musculature (Fig. 5*D–F*) with the exact same tissue-specific pattern as that of P1 transformants. This indicates that P2 has not lost any sequences required for generating the complete *slo* tissue-specific expression pattern. Deletion P4, however, does not express the complete pattern. P4 has lost 1.3 kb of 5′ terminal sequence that is present in P2. P4 is not expressed in the adult brain (Fig. 5*J*), and in the larval brain its expression has been reduced to a small number of cells in the lobes and ventral nerve cord (Fig. 5*K*). Expression in both adult and larval musculature, however, is normal (Fig. 5*J,L*). Notice that none of the promoters have been deleted in P4. Therefore, the missing 1.3 kb must remove one or more DNA elements that enable a *slo* promoter to be expressed in the CNS. We refer to this fragment as the CNS box (Fig. 4). The expression pattern of P5 (Fig. 5*M–O*) is indistinguishable from that of P4, in spite of the fact that P5 has suffered the loss of an additional 800 nucleotides from its 5′ end. These nucleotides seem to be devoid of *cis*-acting control elements.

P3 is identical to P1 except that all sequences between the *Bam*HI site in exon C1 and the *Bgl*II site in exon C2 have been removed. Therefore, P3 is missing portions of exon C1 and C2 and the intron between C1 and C2. Promoters C1b, C1c, and C2 are not present in this construct (Fig. 4). In the CNS, P3 expression is indistinguishable from P1 expression in the adult (compare Fig. 5, *A* and *G*) and larval brain (compare Fig. 5, *B* and *H*). No expression, however, is seen in either adult or larval muscle (Fig. 5*G,I*). Therefore, the P3 transformant shows that promoter C1, the CNS box, and the remaining sequences in P3 are sufficient, in the absence of promoters C1b, C1c, and C2, to direct expression to the CNS.

Mapping of muscle-specific and tracheal-specific elements

P7 represents a large 5′ deletion of the transcription control region. P7 was generated by removing all sequences upstream of the *Bam*HI site located midway between exons C1c and C2. This removes promoter C1, C1b, and C1c, leaving only 902 bp of sequence 5′ to the start of exon C2. P7 expresses β-galactosidase appropriately in adult and larval musculature (Fig. 5*P,R*) but not in the adult or larval CNS (Fig. 5*P,Q*).

Six independent transformants of P7 were examined for the expression pattern in tracheal cells. Four of the six show clear-cut expression in this cell type, whereas two show no expression (an example of a positive cell is shown in Fig. 6*C*). We believe that the lack of expression in two of the transformants was caused by a subtle chromosome position effect. A parallel effect on muscle expression was not observed, and all six transformants showed uniform muscle expression.

The P7 transformant demonstrates that promoter C2 and the flanking P7 sequence is capable of driving expression in muscle fibers and tracheal cells but is not transcriptionally active in other tissues and furthermore that sequences upstream of the 5′ end of P7 are not required for muscle or tracheal cell expression. This interpretation is supported by analysis of the P3 deletion (Figs. 5*G–I*, 6*B*). In P3, all of the C2/C3 intron and promoters C1b, C1c, and C2 have been deleted. P3 is not expressed in either muscle or tracheal cells.

Mapping of a midgut-specific element

The *slo* gene is also expressed in two discrete bands in the midgut of the larval digestion system (Becker et al., 1995). These two bands are

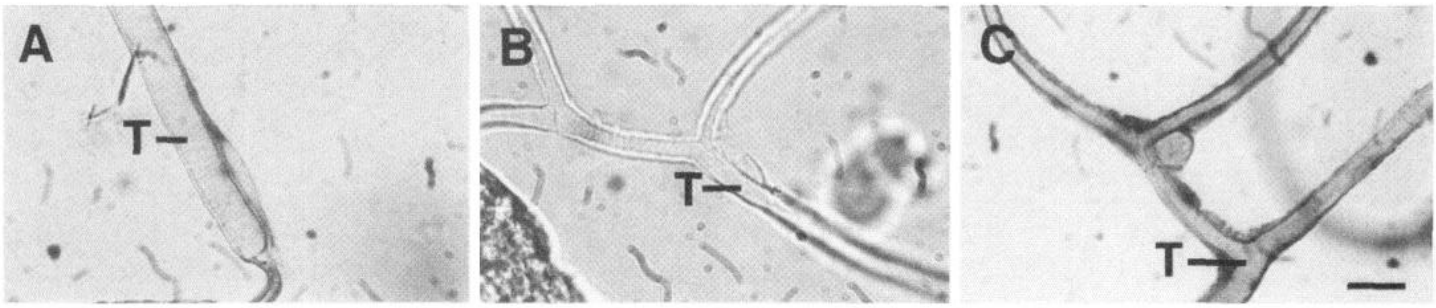

Figure 6. Reporter gene expression in X-gal-stained tracheal cells. The letter *T* identifies the trachea. Tracheal cell processes line the outside and form the trachea (Manning and Krasnow, 1993). *A*, P1 transformant shows *lacZ* expression throughout the tracheal cell. *B*, Transformants carrying the P3 deletion construct (missing promoters C1b, C1c, and C2) are not expressed in tracheal cells. *C*, Transformants carrying the P7 deletion construct (missing promoters C1, C1b, and C1c) are expressed in the tracheal cells. Scale bar, 0.1 mm.

situated between two bands of endogenous β-galactosidase activity of variable intensity (labeled as *E* in Fig. 7). Deletions P3, P5, and P6 (Fig. 4) enabled us to map sequences required for activation of *slo* promoters in the midgut. P3 does not express in the midgut (Fig. 7*A*), indicating that sequence between the *Bam*HI site of exon C1 and the *Bgl*II site of exon C2 are required for expression (Fig. 4). The deleted material includes promoters C1b, C1c, and C2.

The normal midgut expression of deletion P5 demonstrates that loss of sequences 5′ to promoter C1 does not interfere with midgut transcription (Fig. 7*B*). Deletion P6, however, which is missing all sequence 5′ to the *Pst*I site located in exon C1c, shows a complete loss of midgut expression (Fig. 7*C*). Deletion P6 contains only promoter C2. These three deletions indicate that an element, absolutely required for midgut expression, lies within the 1.8 kb of DNA flanked by the *Bam*HI site in exon C1 and the *Pst*I site in exon C1c (Fig. 4). Promoters C1b and C1c lie within this fragment. Therefore, one or both are likely to be responsible for midgut expression.

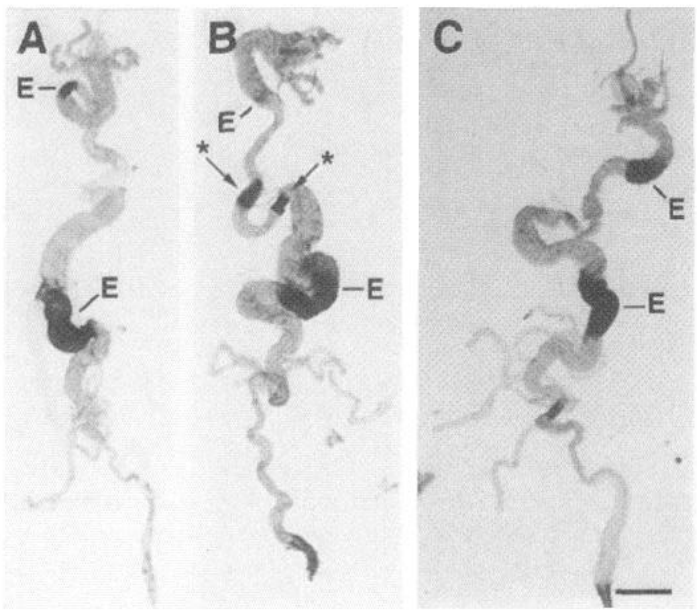

Figure 7. Expression of reporter genes in the larval digestive system. Two regions of the larval midgut show variable degrees of endogenous β-galactosidase activity (labeled as *E*) in both transformed and untransformed animals (Becker et al., 1995). *A*, Transformant P3 exhibits only endogenous staining. *B*, Transformant P5 shows endogenous staining (labeled as *E*) and expression characteristic of the *slo* gene (labeled with an *asterisk*). *C*, Transformant P6 shows only the endogenous staining pattern and no expression in the *slo*-specific pattern. Anterior is up. Scale bar, 0.5 mm.

Our analysis does not address how developmental-specific expression is regulated. It is likely that this will require an analysis of embryogenesis or pupariation and/or fine scale site-directed mutagenesis of suspected enhancer elements. Furthermore, we have yet to identify sequences responsible for expression in the peripheral nervous system.

DISCUSSION

The electrical properties of an excitable cell result from the combined activity of many different channels. It is safe to say that production of a cell membrane with specific electrical properties is a complex event, requiring the controlled expression of many different channel genes so that the appropriate amount of each is produced.

Tissue-specific expression of voltage-gated Na^+ channels results from positive regulatory mechanisms that indiscriminately stimulate transcription and negative regulatory mechanisms that prevent transcription in inappropriate tissues (Mandel and McKinnon, 1993; Sheng et al., 1994). Although transcription of a number of K^+ channel genes has been shown to be temporally and spatially regulated (Perney and Kaczmarek, 1993), how this occurs has not been described.

Here we show that the *slo* CAK channel gene has at least four promoters, each of which generates a transcript with a unique 5′ exon. We also mapped sequences that direct expression to the CNS, musculature, midgut, and trachea. Each promoter is named after the 5′ exon that it produces. After transcription, all four 5′ exons (C1, C1b, C1c, and C2) are spliced to exon C3. Exons C1, C1b, and C1c do not contain a translation initiation codon. Therefore, transcripts produced by promoters C1, C1b, and C1c must begin translation with the methionine codon found at nucleotide 4 of exon C3. Exon C2, however, appends another translation start site, 17 codons upstream of the C3 start codon. The promoter C2 messages, therefore, encode a protein with an altered N terminus. These additional 17 amino acids may alter the properties of the channel. How the presence or absence of these amino acids affects channel activity is not known, because only transcripts that encode these 17 amino acids have been expressed (Lagrutta et al., 1994). In voltage-gated K^+ channels, the N terminus is involved in fast inactivation and subunit assembly. Similar regions for CAK channels have not been identified. Neither of the polypeptides encoded by exons C2 or C3 are homologous to the inactivation balls or T-domains (subunit assembly domains) of voltage-gated channels (Hoshi et al., 1990; Shen and Pfaffinger, 1995). If these exons

perform a similar function, then they do so in a fundamentally different manner. Finally, neither are sufficiently hydrophobic to function as a signal sequence (von Heijne, 1994).

Transcriptional elements involved in tissue-specific expression were functionally mapped. Transformed stocks of six deletion constructs were established, and the expression pattern was assayed. As compared with the mapping of control elements using tissue culture lines, our approach has the advantage that all tissues and organs are simultaneously represented in their natural context.

Beginning from the 5′ end of the gene, we discovered a 1.3 kb region that we call the CNS box. Removal of it causes a nearly complete loss of expression in larval and adult brain. Because the CNS box is devoid of promoters, it must contain positively acting enhancers that modify the activity of a downstream promoter. Transformation construct P3 provides direct evidence that the CNS box can act on promoter C1 (1.2 kb downstream). In P3, only this promoter is present, and we observe normal CNS expression. We do not have proof, however, that the influence of the CNS box is limited to promoter C1. Similarly, deletion of a 2.5 kb fragment containing promoters C1b and C1c causes the loss of larval midgut expression (construct P6). Our hypothesis is that one or both promoters drive expression in the midgut and that all required enhancer elements are contained within this DNA fragment. Finally, a 902 nucleotide *Bam*HI/*Bgl*II DNA fragment containing promoter C2 is required for muscle and tracheal cell expression. When it was present (P1, P2, P4, P5, P6, and P7), we observed expression in muscles and tracheal cells, and when it was missing (P3), we saw a complete loss of expression in these tissues. The P7 construct, which carries this fragment absent any upstream sequences, expresses the complete muscle pattern in all transformants and the tracheal pattern in four of six transformants. From these results we propose a model in which promoter C1 is responsible for expression in the CNS, promoters C1b/C1c are responsible for expression in the midgut, and promoter C2 is responsible for expression in musculature and tracheal cells.

The tissue-specificity of many mammalian neural-specific genes, most notably the type II voltage-gated Na^+ channel, is enforced by the presence of silencers (Kraner et al., 1992). Silencers act negatively to repress transcription in inappropriate tissues. Silencers, unlike enhancers, are found in close association with promoters upon which they act. Therefore, should this mechanism be employed in the *slo* gene, we would expect the silencer to be located near the promoter, and small scale deletions would be required to detect it.

We have sequenced a total of 3492 nucleotides of the transcriptional control region. Comparison of the sequence with DNA databases produced three interesting matches (Fig. 2). A consensus ZFH-2 binding site exists 82 nucleotides 5′ to promoter C1. The ZFH-2 transcription factor is expressed throughout the CNS and regulates the expression of the *Drosophila* 3,4-dihydroxyphenylalanine decarboxylase gene in serotonergic and dopaminergic CNS neurons (Lundell and Hirsh, 1992). ZFH-2 may be involved in directing promoter C1 expression in the CNS.

Adjacent to promoter C2 is an MEF-2 binding site. This transcription factor activates transcription in striated and smooth muscle. It is associated with muscle-specific genes in both vertebrates and invertebrates (Gossett et al., 1989; Grayson et al., 1995). *Drosophila* MEF-2 is expressed ubiquitously in muscle (Lilly et al., 1994) and therefore might participate in promoter C2 expression in the musculature.

Finally, within the C2/C3 intron there exist three matches to the photoreceptor-conserved element I (PCE). This enhancer has been found associated with photoreceptor-specific genes in vertebrates and invertebrates (Kikuchi et al., 1993). We have shown previously that *slo* is expressed in the eye, although the specific cell type has not been determined (Becker et al., 1995). The PCEs might be responsible for eye expression.

Transcription factor binding sites often exist as reiterated elements and can sometimes be recognized on the basis of this property. We have found five clustered repeated elements centered on nucleotide −346 of promoter C1 (Figs. 1, 2). These repeats consist of two copies of the sequence GAGAAATT, called RB1, two copies of the related heptamer GAGAATT, called RB2, and one iteration of GAGAACT, called RB3 (Figs. 1*B*, 2*A*). Furthermore, a large repeat is found within the untranslated leader of exon C1. Two perfect copies of the 12 bp sequence CACAATGAAACT, called RB4, are found in this exon. The average frequency with which any given 12 nucleotide sequence should appear in a genome is once per 4^{12} nucleotides. Therefore, in the absence of functional selection, it is unlikely that two copies of a dodecamer would appear within 35 nucleotides. DNA database searches have not provided sensible matches to these repeats.

The close proximity of these elements to promoter C1 suggests that they may regulate its activity. Deletion analysis indicates that if they are positive-acting elements, then they are insufficient to generate the CNS expression pattern in the absence of the CNS box (Fig. 5; note expression of P4 and P5, which contain these elements); however, they could be silencer elements that prevent ectopic promoter C1 expression. Determination of their significance will require further testing.

The purpose of tissue-specific regulation might be to control channel density. Because these promoters are expressed in different numbers of cells, it may not be straightforward to quantitate their transcription rate in our *in situ* expression system. Promoters C1 and C2 are active in the CNS and musculature, respectively. In adult cDNA libraries, exon C1 is much more common than C2, notwithstanding the fact that muscle greatly exceeds CNS mass (data not shown). This suggests that promoter C1 is much more active than promoter C2. Transcripts from promoters C1b and C1c were not detected in cDNA libraries; however, this is probably because their activity is restricted to a subset of the digestive system that ensures under-representation in cDNA libraries.

The 5′ exon of each promoter might also affect channel abundance by altering translation efficiency. The presumed start site in C1, C1b, or C1c transcripts is the first ATG in C3 that matches the *Drosophila* consensus translation start site in only 6 of 10 sites. Use of exon C2, however, introduces a new start that perfectly matches the consensus and therefore might enhance translatability. This may play a role in determining protein abundance in different cells.

Like other CAK channels, the *Drosophila* channel is expressed in a wide variety of cells. Our work suggests that this results not from the activity of a promiscuous promoter but rather because of stringent regulatory control implemented in the form of distinct promoters, each producing a unique product in fundamentally different cell types. Either the biophysical character of the resulting ion channel or its abundance is likely to be tailor-made to suit the needs of the cell.

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