

# Muscle-specific Transcriptional Regulation of the *slowpoke* $Ca^{2+}$ -activated $K^{+}$ Channel Gene\*

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**Transcriptional regulation of the *Drosophila slowpoke* calcium-activated potassium channel gene is complex. To date, five transcriptional promoters have been identified, which are responsible for *slowpoke* expression in neurons, midgut cells, tracheal cells, and muscle fibers. The *slowpoke* promoter called Promoter C2 is active in muscles and tracheal cells. To identify sequences that activate Promoter C2 in specific cell types, we introduced small deletions into the *slowpoke* transcriptional control region. Using transformed flies, we asked how these deletions affected the *in situ* tissue-specific pattern of expression. Sequence comparisons between evolutionarily divergent species helped guide the placement of these deletions. A section of DNA important for expression in all cell types was subdivided and reintroduced into the mutated control region, a piece at a time, to identify which portion was required for promoter activity. We identified 55-, 214-, and 20-nucleotide sequences that control promoter activity. Different combinations of these elements activate the promoter in adult muscle, larval muscle, and tracheal cells.**

To acquire the appropriate electrical character, a neuron or muscle must express the correct subset of ion channels in the proper amounts (1). This is not a simple problem since even invertebrates have the capacity to produce more than a thousand different ion channel proteins (2). Obviously, one should expect the expression of channel genes to be heavily regulated.

Potassium channels belong to a large superfamily of genes. Particularly interesting are the calcium-activated potassium channels. These respond to changes in both calcium and membrane potential. The coupling of local calcium concentrations to a hyperpolarizing potassium current enables the cell to produce local circuits, which can rapidly and dynamically modulate both membrane potential and calcium influx (3, 4).

These channels participate in shaping the firing patterns of neurons and skeletal muscles, moderating synaptic efficacy, controlling smooth muscle tone, generating cyclical calcium waves during fertilization, active transport, controlling osmotic pressure, and demarcating the binding of ligands to receptors (5–13). The maxi-K-type calcium-activated potassium channels have conductances ranging upward of 200 picosiemens (14). Therefore, the activation of even a small number of such channels can effect the membrane potential of the cell and, as

a result, the activity of voltage-gated ion channels in the membrane.

The *slowpoke* gene encodes a maxi-K-type calcium-activated potassium channel that shows strong evolutionary sequence conservation and is expressed in a similar suite of tissues in vertebrates and invertebrates (15, 16). An independent metric of the similarity between invertebrate and vertebrate channels is the demonstration that the *Drosophila slowpoke* calcium sensor can activate the pore-forming domain of the mouse *slowpoke* protein (17).

We are using the *Drosophila* gene as a model to study how ion channel gene expression is regulated. The *slowpoke* transcriptional control region is extremely complex. To date, five tissue-specific promoters have been identified (2). These promoters are distributed over 7 kilobases of DNA and drive expression in the nervous system, larval midgut, muscle fibers, and tracheal cells (18, 19).

Here, we focus on a *slowpoke* promoter active in muscle and tracheal cells (Promoter C2). We use evolutionary sequence conservation coupled with deletion analysis to ask what cis-acting sequences activate the promoter in these cells and whether the promoter is regulated differently in distinct cell types. Promoter studies are usually performed *in vivo* in tissue culture lines; here, deletion analysis of the *slowpoke* transcriptional control region is performed *in situ*. We use animals stably and uniformly transformed with reporter genes. An advantage to this tack is that expression of a wild type or mutated transgene can be assayed in many tissues all situated in their native environment.

## MATERIALS AND METHODS

**Isolation of the *slowpoke* Transcriptional Control Region from *Drosophila hydei***—A 414-base pair (bp)<sup>1</sup> *Bam*HI/*Apa*I fragment from the *Drosophila melanogaster slowpoke* cDNA Z54 (15), which contained exon C1 and C3, was used to probe a *D. hydei* genomic library (20) generously provided by Dr. John Belote (Syracuse University) under reduced stringency (hybridization: 20% (v/v) formamide (Ambion), 6× SSPE, 10× Denhardt's solution, 0.2% SDS, and 200 μg/ml salmon sperm DNA at 42 °C; wash: 2× SSPE, 0.1% SDS at 65 °C). DNA fragments from *D. hydei* were subcloned into pBluescriptII. Exonuclease Bal31-generated nested deletions (21) were sequenced by the dideoxy chain termination method (22). Accession numbers for the *D. melanogaster* and *D. hydei* sequences are U40221 and AF208226, respectively.

**Reporter Gene Constructs**—The construction of P6 and P7 has been described (18). The construction of all other reporter genes has been described in detail by Chang (23) and is summarized below. Deletion constructs BR17 and EX remove sequences 5' of Promoter C2. To produce BR17, the P6 reporter gene was digested with *Bam*HI and

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<sup>1</sup> The abbreviations used are: bp, base pair(s); tss, transcription start site; DLM, dorsal longitudinal muscle; DVM, dorso-ventral muscle; dcm, direct control muscles, specifically the basalar and pterale I and II; TT, tergostrochanter muscle; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

*EcoRI* (partial) to release the 574-bp fragment between -975 and -401. Overhanging ends were converted to blunt ends and ligated to one another.

Deletion EX was built by deleting the material between the *EcoRI* and *XbaI* sites (-400 to -62). *EcoRI* is not unique in P6, so deletion EX is built by a three-step cloning process. The *XhoI-NotI* fragment from P614 was inserted into pBluescript to produce plasmid J10. The *EcoRI-XbaI* fragment was deleted from J10, the sticky ends converted to blunt ends using Klenow enzyme, and the plasmid ligated shut to produce the plasmid J10EX. This recreated an *EcoRI* site but destroyed the *XbaI* site. The J10EX insert was excised using a *XhoI* and *NotI* double digestion and used to replace the *XhoI* to *NotI* fragment from P614.

The GAL4BII reporter gene carries the entire transcriptional control region and includes Promoters C0, C1, C1b, C1c, and C2. This DNA fragment has been shown to reproduce the *slowpoke* expression pattern (15). In GAL4BII, the GAL4 gene has been inserted into a unique *BglII* site within exon C2, such that transcription from Promoter C2 expresses the GAL4 transcription factor. The translation start site is provided by the consensus start site within *slowpoke* exon C2. The Gal4 gene was derived from the promoter-less pGaTB plasmid kindly provided by Andrea Brand (24) and includes a hsp70 termination site. The GAL4B2.1 transgene is identical to Gal4BII, except that the C2/C3 intronic region (the *BglII-ApaI* fragment; Fig. 1A) has been deleted. Blast searches confirmed that the newly created junction fragments for BR17, EX, and Gal4BII did not themselves represent known transcription factor binding sites.

*Reinsertion of Conserved Elements into Deletion EX*—The EX deletion removed three evolutionarily conserved regions called the 55 box, the 4E region, and the 20 box. Each was added back into the EX deletion and then tested for activity. To make the construct 55/EX, the 55 box was produced by PCR. The primers 55 upper (5'-GTCTGATCACTCTGCCTTTTAATT-3') and 55 lower (5'-TATGGATCCGACCGCGAAAAGTGTCAG-3') were used to amplify the 55 box from P6. The PCR fragment was gel-purified and cloned into the vector PCRblunt (Invitrogen) to produce plasmid 55/pblunt. An *EcoRI* digestion was used to release the 55 box from 55/pblunt. The purified fragment was ligated into the unique *EcoRI* site of J10EX to produce 55/J10EX. Sequence analysis confirmed that the construct contained one copy of the 55 box and that it was in the positive orientation. This places the 55 box almost in its original position. The 55/J10EX insert was excised with *XhoI* and *NotI* and ligated into *XhoI-NotI*-digested P614 construct to produce plasmid 55/EX.

The 4E/EX reporter gene was built by adding the 214-bp 4E region back into the EX deletion construct. The 4E region was PCR-amplified from P6 DNA using the 4E upper (5'-TTCAGATCTTAGCCAAATGCCCGTATA-3') and 4E lower primers (5'-ACCGATCCACCGCACAACGGCG-3'). The product was blunt-end-cloned into the vector PCRblunt (Invitrogen) to produce 4E/pblunt. In this vector, *EcoRI* flanks the insert. The 4E insert from 4E/pblunt was excised with *EcoRI* and ligated into the recreated *EcoRI* site of J10EX produce 4E/J10EX. Sequence analysis confirmed that 4E/J10EX contained one copy of the 4E region in the positive orientation. The 4E/J10EX insert was then excised with *XhoI* and *NotI* and then ligated into a *XhoI-NotI*-digested P614 construct. The resulting transformation construct was called 4E/EX.

In the construct 20/EX, the 20 box has been inserted into plasmid EX at the site of the original deletion. A double-stranded oligomer representing the 20 box was prepared by annealing oligomer 20A upper (5'-AATTCGCGCCGCTTCGCTCGGTGCCTCCTTTTG-3') to oligomer 20A lower (5'-AATTCAAAAGGAGGCACCGAGCGAAGCGGCCGCG-3'). This produces a double-stranded oligomer that anneals to the 5' overhanging ends produced by *EcoRI*. An additional *NotI* site has been introduced to help identify the appropriate ligation product. The 20 oligomer was phosphorylated (polynucleotide kinase) and ligated directly into the *EcoRI* site of plasmid J10EX. This product is called 20/EX. The insertion was sequenced to confirm the number of copies of the 20 box and their orientation. Both one and two copies of the 20 box were obtained and are referred to as 1 × 20/J10EX and 2 × 20/J10EX, respectively. The 1 × 20/J10EX and 2 × 20/J10EX inserts were excised from the vector using *XhoI* and *NotI* and ligated into *XhoI-NotI*-digested P614 construct. These products are called 1 × 20/EX and the 2 × 20/EX, respectively. Both produced identical expression patterns in transformed flies; therefore, the transformants are collectively referred to as 20/EX.

*Drosophila Transformation*—P-element transformations were carried out largely as described by Spradling *et al.* (25). Potential transformants were crossed to *w*<sup>1118</sup>; *Sco/CyO*; *MKRS/TM6Tb*. The presence of the *w*<sup>+</sup> gene (orange to red eyes) was used to identify transformants.

*β-Galactosidase Staining*—Larvae and adults were stained for  $\beta$ -galactosidase activity as described by Brenner *et al.* (18). Relative expression levels were quantified by staining all transformants in the same dish at the same time and by monitoring the appearance of the blue reaction product throughout the staining period. The previously described P6 transgene (18, 19) was used as a positive control. Because the expression pattern of transgenes can be influenced by chromosomal position, the expression results were a consensus of no less than three independent P-element insertions. In each case all exhibited the same expression pattern. Homozygous transformants were used where possible, however, some transgene insertions were homozygous lethal and therefore were assayed as heterozygotes. In this case all animals in the comparison group were heterozygous. Animals carrying transgenes employing the Gal4 transcription factor as a reporter were first crossed to animals carrying the Gal4 responsive UAS-lacZ reporter (24). To determine the level and pattern of expression, control and experimental animals were stained together on the same slide or in the same dish.

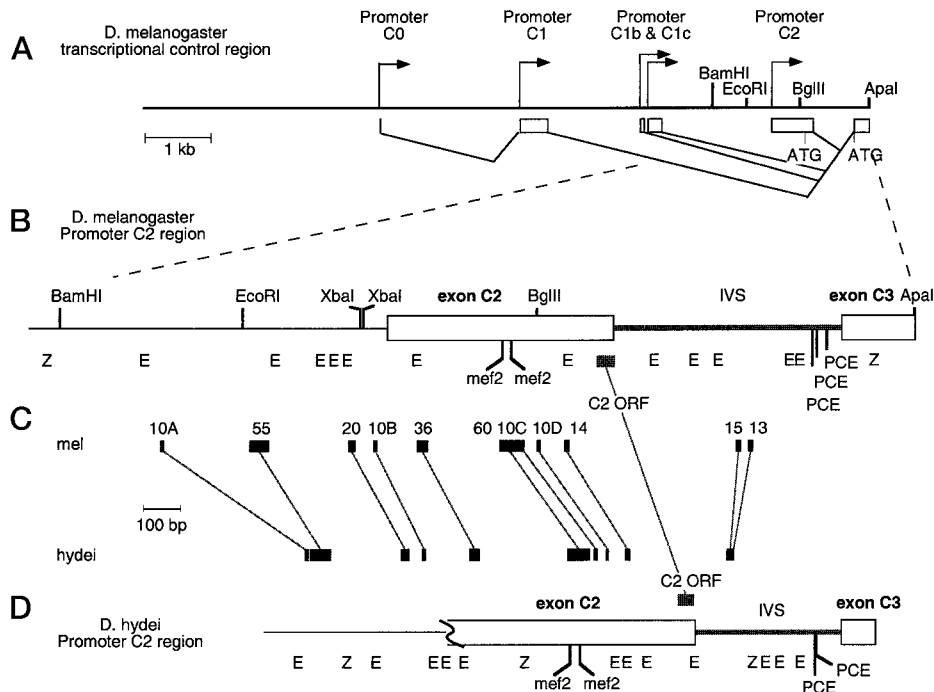
## RESULTS

Our understanding of the *slowpoke* transcriptional control region results from 1) the physical mapping of promoter transcription start sites by 5' rapid amplification of cDNA ends and 2) the assignment of promoter tissue specificity by deletion mapping (18, 19, 26). The *slowpoke* gene has been shown to have five transcriptional promoters (2). From 5' to 3', they are Promoters C0, C1, C1b, C1c, and C2 (Fig. 1A). Deletion analysis indicated that Promoters C0 and C1 are active in the nervous system, that the DNA fragment containing Promoters C1b and C1c is required for expression in two bands in the larval midgut, and finally that deletion of a fragment containing Promoter C2 causes a loss of expression in muscle fibers and tracheal cells (2, 18). Transcription from each of the *slowpoke* promoters begins with a unique 5' exon, which is subsequently spliced to exons common to all *slowpoke* transcripts. Each of these unique 5' exons is named after its promoter. Thus, exon C2 is a product specifically produced by transcription from Promoter C2. Following transcription, exon C2 is spliced to exon C3, which is an exon common to all *slowpoke* transcripts.

*Evolutionary Conservation*—We would like to identify sequences that regulate the activity of *slowpoke* Promoter C2. Promoter C2 is responsible for muscle and tracheal expression of *slowpoke*. While this promoter is also active in a small region of the larval brain (Ref. 18 and Fig. 5B), our focus here is on its regulation in muscle and tracheal cells. With respect to Promoter C2, transgenes that contain nucleotides -1902 to +1472, as in construct P6, reliably reproduce the *slowpoke* expression pattern in trachea and muscles (18, 19) and therefore are predicted to contain all elements required for normal activity of Promoter C2. Promoter C2 includes a single strong transcription start site followed by a number of minor start sites distributed within exon C2. In this document, nucleotides are numbered with respect to the Promoter C2 major transcription start site (18).

Random deletion analysis is an inefficient approach for identifying small control elements in such a large transcriptional control region. Therefore, we have chosen to use evolutionary conservation to guide our search for DNA elements important for controlling transcription from *slowpoke* Promoter C2. Toward this end, we have cloned and sequenced genomic DNA from *D. hydei* homologous to the Promoter C2 control region of *D. melanogaster*. These species diverged from a common ancestor approximately 60 million years ago (27). The program MACAW was used to identify and organize the sequences into blocks of homology (28). Eleven boxes of homology were identified (Figs. 1C and 2). All of these blocks were conserved in both sequence and relative position with respect to one another and to the *slowpoke* exons.

As expected, the most striking conservation was within the coding region of exons C2 and C3. However, three other rela-



**FIG. 1. Organization and evolutionary conservation of the *slowpoke* transcriptional control region.** A, map of the *D. melanogaster* *slowpoke* transcriptional control region. Arrowheads mark the major tss for five different promoters. Only relevant restriction sites have been identified. Boxes below the line represent exons and the connecting line the splicing pattern (2, 18). From left to right, the exons are named C0, C1, C1b, C1c, C2, and C3. Exon C3 is the first exon common to all *slowpoke* transcripts. ATG identifies consensus translation start sites. B, the line represents an expanded map of the *D. melanogaster* Promoter C2 transcriptional control region. Open boxes represent exon C2 and exon C3, which are separated by the C2/C3 intervening sequence (18). The 3' end of the exon C3 box represents the end of our sequence not the end of the exon. Immediately below this line, transcription factor binding site motifs are demarcated. The small gray box labeled C2 ORF represents the open reading frame provided by exon C2 (18). Abbreviations: E, E box; Z, zfh1 (zinc finger homeodomain-containing factor-1); mef2, myocyte enhancing factor 2; PCE, photoreceptor conserved element I (29, 36, 37). C, black boxes represent blocks of homology, in the vicinity of Promoter C2, between the *slowpoke* transcriptional control region of *D. melanogaster* and *D. hydei*. The top row represents the *D. melanogaster* sequence and is in register with the map in panel B. The boxes are named according to their length, and boxes with identical lengths are distinguished by the addition of a letter. The bottom row of boxes represent the *D. hydei* homologues, which are drawn to be in register with the map in panel D. Open reading frames have been intentionally excluded. D, map of the *D. hydei* transcriptional control region surrounding Promoter C2. The open boxes represent exon C2 and exon C3, whose end points have been determined based on similarity to the *D. melanogaster* sequence. Sequence similarity suggests that 5' end point of exon C2 is between homologous blocks 10B and 36. The 3' end of the exon is more clearly identifiable because of the strong conservation of the exon C2 open reading frame and the exon splice donor site. The 3' end of the exon C3 box represents the end of the sequence not the exon. Below the line are the positions of transcription factor binding site motifs. Abbreviations are as defined in B.

tively large homology blocks were identified. They are the 55 box, located upstream of the Promoter C2 transcription start site (tss) and the 36 and 60 boxes (Figs. 1 and 2) found within the 5'-untranslated region of exon C2. Smaller blocks of homology (10–20 nucleotides) were also considered significant if they were conserved in both sequence and position (Figs. 1 and 2).

Transcription start sites are difficult to identify by inspection of DNA sequence. The *D. melanogaster* Promoter C2 tss was previously mapped by 5' rapid amplification of cDNA ends and confirmed by RNase protection assays and deletion analysis (18). This site is flanked by the evolutionarily conserved 10B and 36 boxes. In the absence of physical mapping data, we assume that the *D. hydei* Promoter C2 tss is in the same relative position between these two conserved boxes.

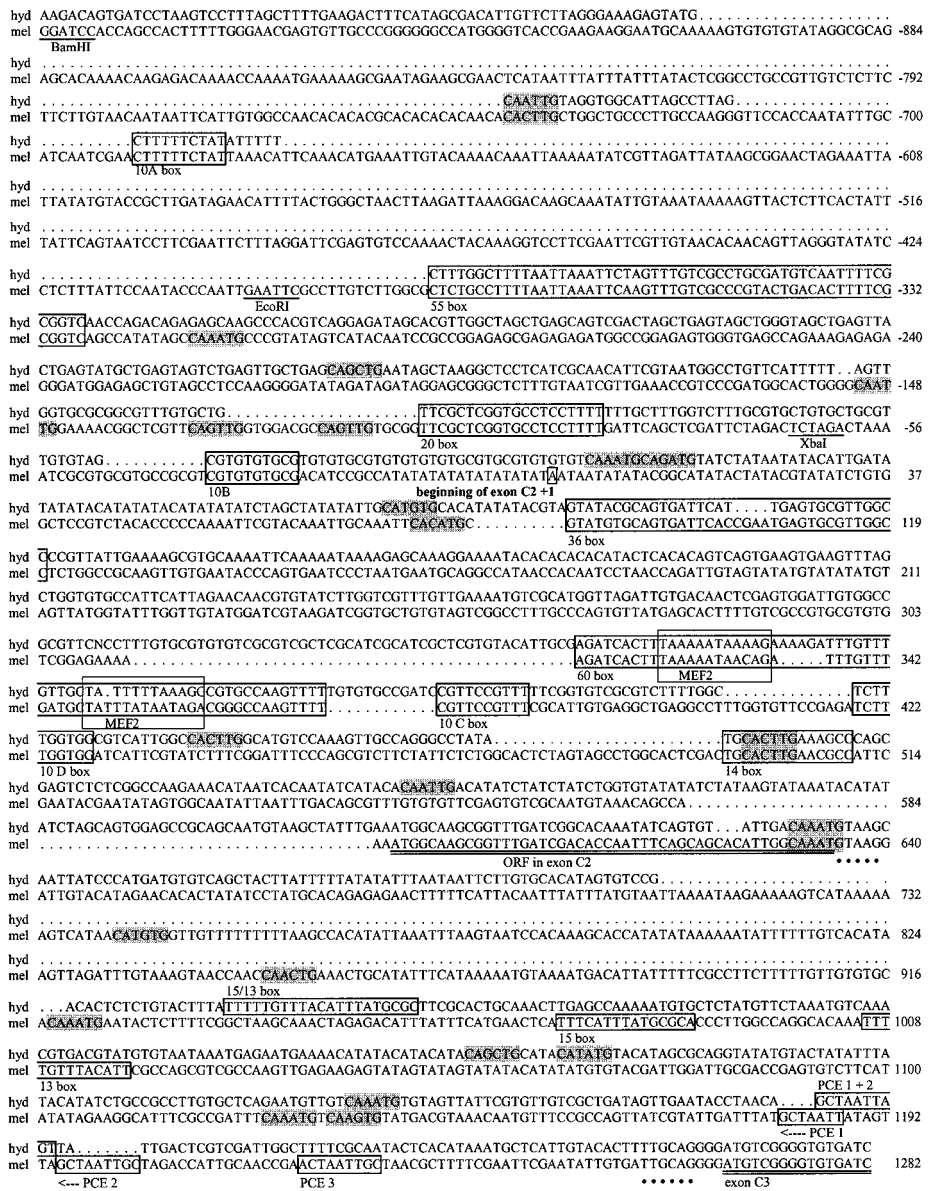
We also searched the sequence for known transcription factor binding motifs. Three mef2 and 20 E box motifs were identified. The mef2 and myoD family of transcription factors recognize these motifs and are key regulators of myogenesis (29). A single zfh1 motif was found 5' of exon C2 in the *D. melanogaster* and *D. hydei* sequences. Transcription factors that bind this site have been shown to be important in silencing muscle-specific genes in non-muscle tissue (30, 31). In a previous study (18), it was shown that the P7 deletion, which removed sequences 5' of Promoter C2 up to nucleotide -975 (the BamHI site in Fig. 1 and 2), was capable of reproducing the Promoter C2 expression pattern. This indicated that all essen-

tial, positively acting elements are 3' of this BamHI site. However, P7 was noted to be sensitive to chromosome position effects, which sometimes resulted in ectopic expression (23). This deletion removes a conserved zfh1 site. This suggests that this site may be a negative regulator of Promoter C2 expression, which serves to prevent expression in inappropriate tissues. Nevertheless, this study has focused on elements between nucleotides -975 and +1472, the fragment of DNA carried in the P7 construct.

**Deletion of Evolutionarily Conserved Elements**—The conserved blocks were tested for functional importance by deletion analysis. The P6 transgene was used as the starting material, since it reproduces the *slowpoke* muscle and tracheal cell expression pattern and is not sensitive to chromosome position effects (18, 19). Transgenic flies were used to compare the expression pattern of the deleted and intact versions of P6. Animals being compared were sectioned or dissected together and stained on the same slide or in the same dish. Table I provides a summary of the data discussed below.

The BR17 derivative of P6 has suffered a deletion that removes the nucleotides flanked by the BamHI and EcoRI sites of P6 (nucleotides -975 to -401) eliminating the 10A box (Fig. 3). In transformant lines, BR17 expressed  $\beta$ -galactosidase in the same pattern and with the same relative intensity as the P6 reporter gene in both larval and adult muscles and in tracheal cells (data not shown). It appears that the BR17





**FIG. 2. Alignment of the *D. melanogaster* and *D. hydei* Promoter C2 transcriptional control regions.** The *D. melanogaster* exon boundaries have been physically mapped (18). The corresponding *D. hydei* sites were determined based on similarity to the *D. melanogaster* sequence. In the *D. melanogaster* sequence, exon C2 begins at +1 and terminates at the end of the exon C2 open reading frame (*double underline*). This open reading frame is common to both *D. melanogaster* and *D. hydei* exon C2. Conserved blocks of sequence identified in Fig. 1C are boxed and labeled. Sequences representing E boxes are shaded gray but are otherwise unlabeled. Mef2 sites and PCE sites are labeled and boxed. The conserved splice donor (3' of exon C2) and splice acceptor (5' of exon C3) sites are identified by filled circles below the sequence. Trivial alignments of sequence were not included, and the relative position of unannotated sequence is not meant to imply a preferred alignment. Dots represent gaps introduced to maximize the alignment. Restriction enzyme sites used to construct reporter genes are underlined.

**TABLE I**  
*Summary of expression patterns*

Table gives a summary of the muscle expression pattern of *sloupoke* transgenes. In part A, the number of pluses represents a visual estimation of the relative expression level in stained animals. A minus indicates a lack of expression. Muscle subtypes are grouped as indirect asynchronous, indirect synchronous, and direct synchronous flight muscle. Abbreviations are as defined in Fig. 5. In part B, dependence of muscle subtypes and tracheal cells on different conserved regions is shown for expression from Promoter C2.

A.	Indirect, asynchronous		Indirect, synchronous		Direct, synchronous			
	DLM	DVM	TT	PS	Basalare	Pterale I	Pterale II	Leg
P6	++	++	++++	++++	++++	++++	++++	++++
EX	-	-	-	+	+	+	+	+
20/EX	-	-	-	+	+	+	+	+
4E/EX	+	+	+++	++++	++++	++++	++++	++++
55/EX	-	-	-	+	+	+	+	+
Gal4BII	++++	++++	-	++++	++++	++++	++++	++++
Gal4B2.1	-	-	-	+++	++	++	++	++

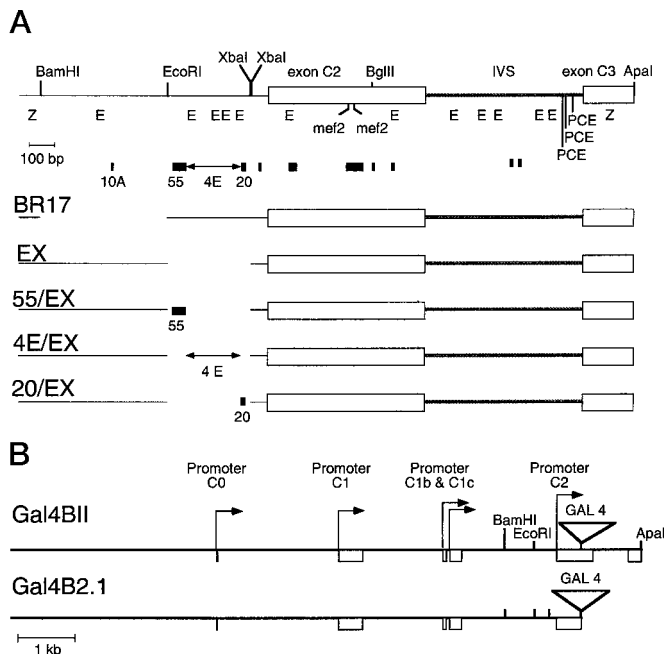
  

B.	55		4E		20		Intronic region
	Adult asynchronous muscle	Adult synchronous muscle	Larval body wall muscle	Larval tracheal cells	Adult asynchronous muscle	Adult synchronous muscle	
Adult asynchronous muscle	Not required	Not required	Sufficient	Not required	Not required	Not required	Required
Adult synchronous muscle	Not required	Not required	Not required	Not required	Not required	Not required	Not required
Larval body wall muscle	Sufficient	Sufficient	Sufficient	Not required	Not required	Not required	Not required
Larval tracheal cells	Sufficient	Sufficient	Not required	Not required	Sufficient	Sufficient	Not required

deletion causes no alteration in the pattern or the intensity of the muscle expression in larvae or adult. Clearly, the conserved 10A and E box removed by this deletion are not

essential for normal activity in muscles.

The EX deletion removes nucleotides -400 to -62 and eliminates the 55 box, the 4E region, and the 20 box. In *D. melo-*



**FIG. 3. Deletion constructs.** *A*, at the top is a map of the genomic DNA in the vicinity of *slowpoke* Promoter C2. Boxes immediately underneath the line identify the positions of evolutionarily conserved blocks. Please refer to Fig. 1 for the relationship of this fragment to the entire transcriptional control region. The subsequent lines represent the sequences remaining in the BR17 and EX deletion constructs. BR17 removes the conserved 10A box, while the EX deletion removes the 55 box, the 4E region, and the 20 box. In all of these constructs, the *lacZ* reporter gene has been inserted into the *ApaI* site shown at the 3' end of the sequence. Abbreviations are as in Fig. 1. *B*, maps of transgenes Gal4BII and Gal4B2.1. Gal4BII contains the entire *slowpoke* transcriptional control region modified by the insertion of a Gal4 reporter gene into the *BglII* site of exon C2. Gal4B2.1 is identical to Gal4BII, except that the latter is missing the C2/C3 intronic region. The arrowheads represent the position of transcription start sites for (from left to right) Promoters C0, C1, C1b, C1c, and C2. Exons are named after the corresponding promoters except for exon C3, which is the first exon common to all *slowpoke* messages (18).

*novaster*, the 214-nucleotide 4E region includes four E boxes. In larvae, this deletion caused the loss of all muscle expression (Fig. 4A).

The effect of the EX deletion in the adult can be summarized as a loss of expression in the fibrillar power muscles and a reduction in expression in other adult muscles (Fig. 4B). The muscle groups showing a loss of expression were the dorsal longitudinal muscles (DLM), the dorso-ventral muscles (DVM), and the tergotrochanter muscle (TT). The DLM and DVM provide the mechanical power for the wing beat, while the TT generates the jump used to initiate flight (32). Reduced expression was observed in the pterale direct control muscles (steering muscles), and in an indirect flight control muscle, the pleurosternal I muscle. The pterale muscles are involved in directly controlling wing kinematics while the pleurosternal muscles are thought to modulate wing beat frequency. A reduced but readily detectable level of expression was also observed in the prothoracic, mesothoracic, and metathoracic leg muscles (data not shown). The expression in these muscles indicates that the promoter is still functional and that the deletion has merely affected its tissue specificity.

**Reinsertion of Conserved Elements**—The 339-nucleotide EX deletion removes the conserved 55 and 20 boxes and the 214-bp 4E region. To determine the relative contribution of these sequences to Promoter C2 activity, each was individually added back to the EX deletion and the modified reporter gene assayed for expression in transformed flies. The EX deletion has an

*EcoRI* site at the site of the deletion. Oligonucleotides representing the 55 box, the 4E region, and the 20 box were individually prepared and inserted with their original polarity into this site. The products are called 55/EX, 4E/EX, and 20/EX to designate which oligonucleotide they contain. To be able to compare expression levels of the constructs, different genotypes were stained for the same length of time in the same dish or microscope slide.

In larvae, the insertion of the 55 box restored larval body wall muscle expression to a level indistinguishable from P6 (Fig. 4A). The 4E region, however, only partially restored larval muscle expression, and, finally, the insertion of one copy or two copies of the 20 box back into the EX deletion was unable to activate Promoter C2 in larval muscle (Fig. 4A).

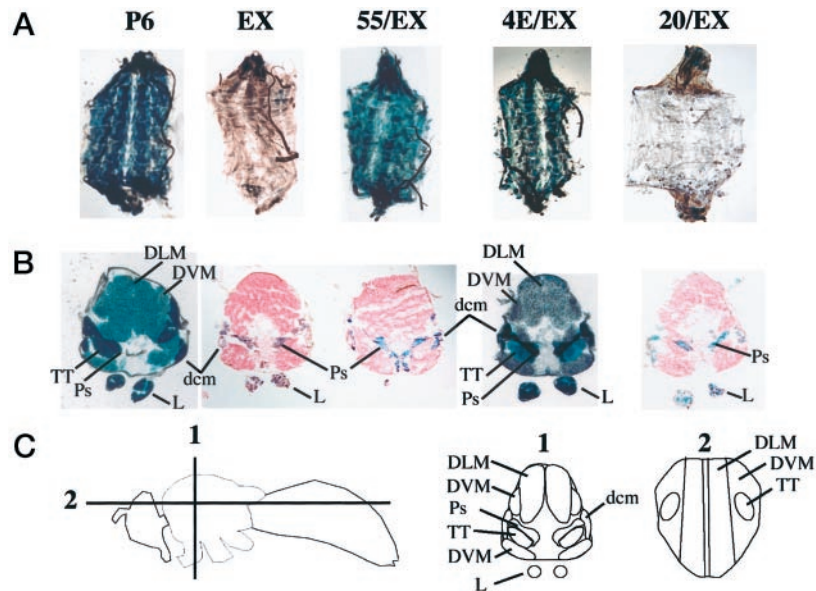
The expression levels of these reporter gene constructs in the adult are substantially different than that observed in the larvae. In adults, the 55/EX construct is expressed at extremely low levels (Fig. 4B). Thus, the 55 box is not sufficient in the absence of the 20 box and the 4E region to properly activate Promoter C2 in adults. However, the reinsertion of the 4E region alone restored expression in muscles of the thorax to near normal levels (Fig. 4B). Expression in muscles in the head and legs was also augmented (data not shown). The reinsertion of the 20 box had no effect on adult muscle expression and sections from these animals expressed the reporter in the same pattern and level as the original EX deletion (Fig. 4B). Taken together, the results suggest that it is the 4E region but not the 55 box or the 20 box that activates Promoter C2 in adult muscle.

**Direct Tagging of Exon C2**—We were quite surprised to observe that the intron located between exons C2 and C3 showed only slight evolutionary conservation since previous works indicated that a deletion from the *BglII* within exon C2 to the *ApaI* of exon C3 (intronic region; Ref. 19) had robust effects on the activity of the upstream promoters in both muscle and neurons. Removal of the intronic region eliminated expression in embryonic muscle and dramatically reduced adult muscle expression (19). Therefore, we anticipated strong conservation in this area. However, the data implicating the intronic region was collected using a transgene in which the reporter gene was inserted into exon C3 (Fig. 1B). Because of this, the deletion of the intronic region altered the structure of the reporter protein transcript and therefore might perturb, not the transcription initiation rate, but the translatability or stability of the resultant mRNA.

To confirm the importance of this sequence, we directly tagged exon C2 with a Gal4 reporter gene cassette, which includes its own termination site (33). In this transgene, the intronic region is 3' of this termination site. Therefore, the presence or absence of the intronic region should not affect the stability or translatability of the message. The reporter genes constructed in this manner are called Gal4BII and Gal4B2.1. These transgenes are identical, except that the latter has suffered the loss of the intronic region (Fig. 3B).

Previous work by Brenner *et al.* (18) indicated that Promoter C2 is responsible for expression of *slowpoke* in muscles. This work employed the P6 *slowpoke* transgene. In P6 all of the other *slowpoke* promoters have been deleted and exon C3 has been marked with a reporter gene. In transgenic flies, P6 and Gal4BII are expressed in essentially the same pattern in larvae and adults (Fig. 5, exceptions noted below).

In larvae, Gal4BII is expressed in body wall muscles and tracheal cells in the same pattern as P6. This confirms that Promoter C2 is responsible for muscle and tracheal cell expression. Neither Gal4BII nor P6 is expressed in larval visceral muscle, brain, or midgut. It should be noted that P6 is also



**FIG. 4. Larval and adult muscle expression show different dependence on sequence within the EX region.** *A*, larval fillets X-gal-stained to visualize expression of reporter genes in the body wall muscles. Each fillet is labeled according to the transgene it carries. The EX deletion removes the 55, 4E, and 20 boxes and appears to eliminate expression. The constructs 55/EX, 4E/EX, and 20/EX restore the 55 box, 4E region, and 20 box, respectively, to the EX deletion. *B*, cross-sections (orientation 1) of adult thoraces display the expression pattern in flight and leg muscles. P6 is expressed in all muscle groups. The EX deletion eliminates expression in all muscles except the leg, pleurosternal and direct control muscles (basalare and pterale I and II). The restoration of the 55 box (55/EX) or the 20 box (20/EX) into the EX deletion does not restore expression. The restoration of the 4E region to the EX deletion (4E/EX), however, results in nearly a complete restoration of the wild type expression pattern. To determine relative expression levels the samples were stained together. Examples shown are those with the best morphology. *C*, orientation of sections used in the paper. Schematic of an adult animal showing the relative position of the transverse section (1) used in Fig. 4 and the sagittal section (2) used in Fig. 5. Schematics of each section show the idealized position of muscles. Ps, pleurosternal.

expressed in a small number of cells in the larval brain (18, 19). We did not observe expression of Gal4BII in the larval brain. Either brain expression from P6 is an artifact of its construction or the insertion of Gal4 into exon C2 acts as a mutation that precludes activation of Promoter C2 in the brain.

In the adult, P6 is expressed in the direct and indirect flight muscles. In general Gal4BII recapitulates this expression pattern, with the exception that staining of the DLM and DVM is much more intense. This was not unexpected since the binary Gal4 system amplifies expression of the  $\beta$ -galactosidase reporter protein. However, in the TT indirect control muscle, the converse was observed. As assayed using the  $\beta$ -galactosidase reporter, Gal4BII expression is weak or absent in the TT while the P6 transgene produces its most robust staining in these muscles (Fig. 5C). The simplest interpretation is that only the TT muscle is sensitive to the interruption caused by the GAL4 gene. The Gal4 reporter may interrupt or displace a control element required for normal expression in the TT. In any case, it suggests the TT and the other classes of adult muscle regulate Promoter C2 activity in distinctly different ways.

**Role of the Intronic Region**—The GAL4B2.1 reporter is identical to GAL4BII except that it is missing the C2/C3 intronic region. The loss of these sequences did not affect the larval muscle expression pattern (Fig. 5A). Both Gal4BII and Gal4B2.1 are expressed in comparable levels in the larval body wall muscles (Fig. 5A). No ectopic expression was observed, indicating that the deletion does not remove a silencer element that suppresses promoter activity in inappropriate tissues.

However, in the adult thorax, loss of the intronic region causes a substantial change in expression. Gal4B2.1 shows a loss of expression in asynchronous flight muscles (DLM, DVM). With the exception of the TT, expression persists in the direct control muscles (Fig. 5C, Table I). The intronic region apparently contains elements required for expression in specific muscle subtypes.

**Tracheal Cell Expression**—Promoter C2 is also responsible

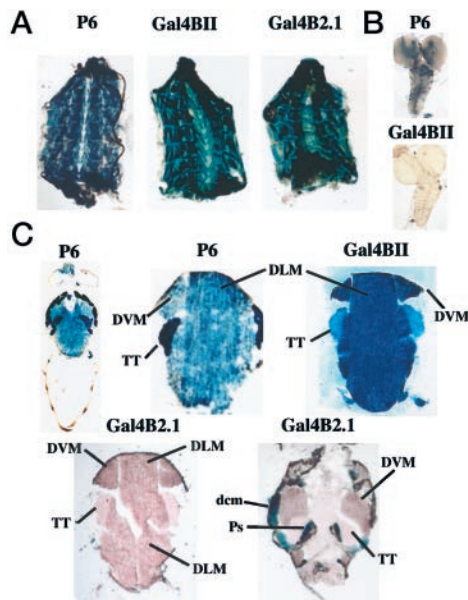
for expression in larval tracheal cells. Analysis of reporter gene expression in these cells produced very interesting results (Fig. 6). The P6 and Gal4BII lines showed strong reporter activity in the dorsal trunk, dorsal branch, visceral branch, lateral branch, and ganglionic branch of the tracheal system. The BR17 deletion did not affect expression in these cells (data not shown), indicating that the eliminated sequences are not required for tracheal cell activity. However, the EX deletion, which removed the 55, 4E, and 20 boxes, eliminated expression in tracheal cells. Restoration of the 55 and 20 boxes restored this expression while restoration of the 4E region did not. Both Gal4BII and Gal4B2.1 showed essentially identical patterns of expression in tracheal cells, indicating that tracheal cell activity of Promoter C2 is not dependent on the intronic region (summarized in Table I).

## DISCUSSION

Based on the expression pattern of mutated reporter constructs, we grouped muscles into four categories. Our data indicate that each group differentially regulates Promoter C2. These groups are 1) larval muscle, represented by the larval body wall muscles, 2) adult asynchronous muscle, represented by the DLM and DVM flight muscle; 3) adult synchronous muscle, represented by the pleurosternal, basalare, pterale, and leg muscles; and finally 4) jump muscle, represented by a single member, the TT muscle. Please refer to Table I (parts A and B) during the following discussion.

We chose to use evolutionary conservation as a rational approach for identifying important transcriptional control elements. Easily identifiable conserved blocks exist between the Promoter C2 control regions of *D. melanogaster* and *D. hydei*. Additional deletions were used to further cull unimportant from important sequences. The first, called BR17, removed nucleotides  $-975$  to  $-401$ , while the second, called EX, removed nucleotides  $-400$  to  $-62$ . In conjunction with the previously described P7 deletion, this provides an uninterrupted

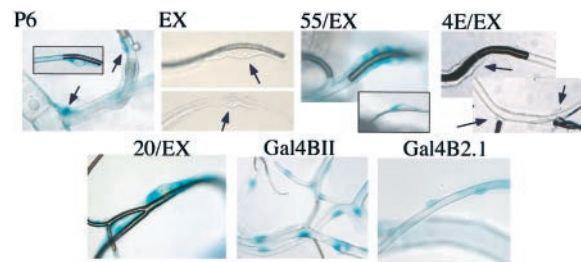




**FIG. 5. The intronic region affects adult but not larval muscle expression.** Each panel is labeled with the reporter gene being expressed. Expression patterns are visualized by X-gal staining. P6 and Gal4BII carry the reporter gene in exon C3 and exon C2, respectively. The P6 reporter reproduces the wild type muscle expression pattern. The Gal4B2.1 transgene is identical to Gal4BII except that it is missing the downstream intronic region. *A*, from left to right, the panels are: P6, Gal4BII, and Gal4B2.1 expression in a larvae filleted and eviscerated to display the body wall muscles. *B*, P6 and Gal4BII larval brains. Neither Gal4BII nor Gal4B2.1 showed expression in the larval brain. However, P6 shows limited, low level expression in the brain. *C*, expression of P6, Gal4BII, and Gal4B2.1 in adult thoracic muscle (sagittal sections, orientation 2; Fig. 4C). In the first panel, an adult P6 transformant was sectioned to display the head, thorax, and abdomen. Expression is obvious in the thorax. The remaining panels are magnified views of sectioned thoraces. P6 and Gal4BII appear to be expressed in the same muscles, with the exception that Gal4BII is not expressed in the TT. The last two panels are stained thoraces of Gal4B2.1 transformants. In these, the tissue not stained by X-gal has been visualized with a safranin counterstain. The last section is near the center of the animal. Gal4B2.1 shows expression only in the dcm and pleurosternal area (*Ps*) but not in the DLM, DVM, or TT. To determine relative expression levels, the samples were stained together. Examples shown are those with the best morphology. Abbreviations are as defined in Fig. 4.

set of deletions that approach Promoter C2 from the 5' end. BR17 removes weakly conserved sequence and therefore might be expected to have little effect on Promoter C2 activity. Indeed, the BR17 deletion did not alter the muscle or tracheal cell expression pattern. Deletion EX, however, which removed the strongly conserved 55 box, the 4E region, and the 20 box, had a larger effect. This loss silences Promoter C2 in both adult asynchronous muscle and larval muscle groups. Low level expression persisted in most members of the synchronous muscle group. This was the first indication that some muscles differentially regulate Promoter C2 activity.

Each conserved region was inserted back into the EX deletion construct and tested for the capacity to reactivate Promoter C2. The P6 construct represents the intact control region. The rank order of expression in larval muscle is P6  $\approx$  55/EX > 4E/EX >>>> 20/EX  $\approx$  EX. In adult asynchronous and synchronous muscle this order was quite different: P6 > 4E/EX >>>> 55/EX  $\approx$  20/EX  $\approx$  EX. This clearly illustrates the distinct differences in regulation between the larval muscle and adult muscle groups (asynchronous and synchronous). Whereas the 55 box and the 4E region strongly stimulate larval muscle expression, only the 4E region stimulated expression in adult muscle. Promoter C2 is clearly regulated differently in larval and adult muscle.



**FIG. 6. Expression of reporter genes in larval tracheal cells.** The label above each panel identifies the reporter gene. Some panels have multiple examples. Tracheal cells create and encompass a hollow tube used for gas exchange. In some photographs, the tube has partially filled with air, which causes it to be a dark brown. Arrowheads serve to identify tracheal cells. In P6, 55/EX, and 20/EX, the tracheal cells and their processes show expression of the reporter gene. In EX and 4E/EX, no expression is observed. This indicates that restoration of the 55 and 20 boxes but not the 4E region are sufficient to restore expression to the EX deletion. P6, Gal4BII, and Gal4B2.1 show the same tracheal cell expression pattern, indicating that tracheal expression is not dependent on the intronic region. When a reporter gene showed tracheal cell expression, it showed expression in all areas of the larval tracheal cell system (dorsal trunk and the dorsal, visceral, lateral, and ganglionic branches).

Previous studies showed that removal of the intronic region (+416 to +1473) reduces or eliminates expression in most adult flight muscle, but does not affect expression in larvae (19). This region includes the intron between exon C2 and C3 (downstream of the Promoter C2 tss) and portions of each exon. Unfortunately, this deletion altered the 5'-untranslated region and splicing of the mRNA encoding the reporter and consequently may alter the translatability or stability of the mRNA. Therefore, the loss of expression might not result from impaired transcription but from a change in mRNA stability.

The Gal4BII and Gal4B2.1 transgenes address this caveat. The former contains the intronic region in question, while the latter is lacking it. In both, exon C2 is directly tagged with a Gal4 reporter gene. Exon C2 is the first exon expressed by Promoter C2 and is not found in transcripts expressed by any of the other *slowpoke* promoters (2, 18). Because the intronic region is downstream of the Gal4 insertion and not part of the reporter gene mRNA, its removal cannot affect message stability. Interestingly, in the Gal4B constructs, removal of the intronic region eliminates expression in adult asynchronous muscles but does not reduce expression in larval muscle. This is a second illustration of the difference in the regulation of larval and adult muscle groups. Expression in larval muscle is independent of the intronic region, while adult DLM and DVM expression is absolutely dependent on this fragment of DNA.

Even within the adult, distinct muscle subtypes showed different sequence requirements. Adult thoracic muscles may be categorized as asynchronous or synchronous. Asynchronous flight muscles are optimized for generating force and rapid, repetitive, beating contractions. Neural stimulation makes this muscle competent for contraction but does not trigger a contraction. The synchronous muscles have fewer contractile fibers, a more developed SR, and serve to control flight and move the legs. In this subtype, excitation is tightly coupled to contraction (32).

Our data indicate that asynchronous and synchronous muscle regulate Promoter C2 differently (Table I). When the C2/C3 intronic region was deleted (Gal4B2.1 construct), a loss of expression in the asynchronous DLM and DVM was observed. The deletion did not, however, prevent expression in the synchronous pleurosternal, basalare, pterale, and leg muscles (the TT muscle is a special case discussed in detail below). A second, less robust, example of this dichotomy between asynchronous and synchronous muscle is provided by the EX deletion. EX eliminated expression in the asynchronous DLM and DVM but did not completely eliminate expression in the synchronous

pleurosternal, basale, pterale, and leg muscles.

The Gal4BII reporter gene provides a final example of muscle subtype regulation. The insertion of Gal4 into exon C2 caused a specific loss of expression in the TT muscle. This is a synchronous muscle that the animal uses to jump during flight initiation. Expression in other muscle types appeared unaffected. The conclusion that Promoter C2 is normally active in the TT is based on the expression pattern of seven different reporter gene constructs (18, 19) and is not in question. The insertion must be responsible.

In Gal4BII the structure of the message itself has been altered, which might affect the stability or translatability of the mRNA and result in the specific loss of expression in the TT. However, the most parsimonious explanation is that the insertion, which is adjacent to two evolutionarily conserved mef2 motifs, prevents the binding of factors required for expression in the TT but not in the other muscle types.

A consequence of our use of transformed animals for these transcription studies is that we could determine the effect of the same set of deletions upon Promoter C2 activity in tracheal cells. Only the EX deletion eliminated tracheal cell expression. Reinsertion of either the 55 or 20 box, but not the 4E region, reactivated the promoter.

The *slowpoke* transcriptional control region is complex, containing at least five tissue-specific promoters. We show that this complexity is mirrored in the regulation of a single *slowpoke* promoter; Promoter C2. The simplest model consistent with our results is as follows. 1) In general, promoter activation in muscle involves E boxes located in the flanking 4E and intronic regions. These may coordinate the binding of a muscle-activating transcription factor belonging to the myoD basic-helix-loop-helix superfamily. Adult tergoprochanter and asynchronous muscle regions have an absolute dependence for both regions. In larval body wall muscle, however, the intronic region is not required and the requirement for the 4E region can be supplanted by the 55 box. 2) Tracheal cell expression is not absolutely dependent on either of the E box regions that stimulate muscle expression. However, expression in these cells also employs a redundant system requiring the presence of either the 55 or the 20 boxes. The cis-acting 20 box is proposed to bind a transcription factor that stimulates tracheal cell but not muscle expression. It is therefore more specific than the 55 box. 3) It is possible that the capacity of the 55 box to stimulate expression in two very different larval cell types indicates that it participates in developmental stage rather than tissue-specific stimulation and that it will enhance expression in any larval cell that does not actively prevent activation. However, it is not uncommon for a single transcription factor binding site to be involved in tissue-specific stimulation of transcription in distinctly different cell types.

Thus, we identified sequences that specify expression in tracheal cells and two distinct muscle subtypes. This evidence for muscle subtype-specific regulation is of particular interest in light of the importance of calcium-activated potassium channels in controlling the tone and contractile properties of vertebrate muscle (10) and because little is known about how or

whether ion channel genes are differentially regulated in muscle subtypes.

Most of the important regulatory cascades affecting developmental gene expression were originally discovered in or shown to exist in *Drosophila*. At the molecular level, both *Drosophila* and vertebrate muscle development are strikingly similar (34, 35). Thus, our description of the control of *slowpoke* expression in *Drosophila* is relevant to the understanding of transcriptional regulation of ion channel genes in higher organisms.

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