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Tissue-specific alternative splicing of BK channel transcripts in Drosophila

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BK-type calcium-activated potassium channels are large conductance channels that respond to changes in intracellular calcium and membrane potential. These channels are used in a wide variety of cell types and have recently been linked to drug sensitivity and tolerance. In both Drosophila and mammals, BK channels are encoded by the *slowpoke* gene. The Drosophila *slowpoke* gene includes 14 alternative exons distributed among five sites of alternative splicing. Presumably, the purpose of alternative processing is to provide transcripts tailored to the needs of the cell. The *slowpoke* gene is expressed in nervous, muscle and epithelial tissues. To determine whether splicing is controlled in a tissue- and/or developmental-specific manner, we built tissue- and developmental-specific cDNA libraries that preserved the relative frequency of various *slowpoke* splice variants. These libraries were screened by colony hybridization using alternative exon-specific DNA probes to document the frequency of individual alternative exons in different developmental stages and distinct tissue types. We demonstrate that *slowpoke* transcripts undergo tissue- and developmental-specific splicing in Drosophila and some exons are diagnostic for specific tissues.

Keywords: Alternative splicing, BK channel, Drosophila, potassium channel, *slowpoke*, tissue specific

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Genes encoding calcium-activated potassium channels are a small and specialized subset of the potassium channel superfamily. The BK-type calcium-activated potassium channels integrate calcium, electrical and metabolic signals. These channels are encoded by a single gene in both vertebrates and invertebrates. This gene, *slowpoke*, is highly conserved in both sequence and function (Butler *et al*. 1993; Piskorowski & Aldrich 2002; Schreiber & Salkoff 1997; Schreiber *et al*. 1999).

The *slowpoke*-encoded BK channels play important roles in determining the firing pattern and efficacy of synaptic transmission in the nervous system, in the control of blood pressure by smooth muscle and in the transport of ions in epithelial cells (Hanaoka *et al*. 1999; Nelson & Quayle 1995; Rudy 1988). Recently, BK channels have been shown to be important for the response of the nervous system to drugs such as ethanol and anesthetics. In *Caenorhabditis elegans*, a genetic screen for ethanol resistance identified mutations in the *slowpoke* gene (Davies *et al*. 2003), while Pietrzykowski *et al*. (2004) have linked a form of ethanol tolerance to the redistribution of BK channels in mammalian nervous system explants. In Drosophila, *slowpoke* mutants have been shown to have an increased sensitivity to volatile anesthetics (Leibovitch *et al*. 1995). Furthermore, changes in the expression level of the Drosophila *slowpoke* gene in the nervous system have been correlated with and are believed to underlie the rapid acquisition of tolerance to organic solvent sedation as measured in behavioral assays (Ghezzi *et al*. 2004).

The Drosophila *slowpoke* gene produces multiple gene products via alternative promoter activation and alternative mRNA splicing. The gene has five tissue-specific promoters that generate mRNAs with differing 5′ ends. Two of the promoters are responsible for the bulk of neural expression, two appear to be specialized for expression in the midgut and one is responsible for muscle and tracheal cell expression. The neural and midgut mRNAs all share the same translation start site while the muscle/tracheal cell message uses a novel translation start site (Bohm *et al*. 2000; Brenner & Atkinson 1997).

Alternative splicing of the *slowpoke* gene affects the amino acid sequence of the carboxy-terminal tail of the protein that has been shown to be involved in calcium sensing (Schreiber *et al*. 1999). Alternative splicing provides an economical way for cells to express channels fine-tuned to their specific needs and has been shown to affect the conductance, sensitivity to calcium and the distribution of phosphorylation sites (Lagrutta *et al*. 1994). Many of the resultant channel isoforms have been shown to have different properties and play specialized roles. In the turtle and chicken auditory system, it has been seen that alternative splice variants are tonotopically distributed and are involved in auditory tuning (Fettiplace & Fuchs 1999; Jones *et al*. 1998; Navaratnam *et al*. 1997). Furthermore, in mammals, *slowpoke* alternative splicing has been shown to be dynamically regulated in response to stress or hormones experienced by the animal

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(Erxleben *et al*. 2002; Lovell & McCobb 2001; Xie & McCobb 1998). In Drosophila, it has been demonstrated that the splice variants are not interchangeable. It has been demonstrated that a single splice variant complements only some of behavioral phenotypes caused by a null mutation in the gene (Brenner *et al*. 2000) and that in flight muscle the splice variants are not functionally interchangeable (Atkinson *et al*. 1998).

The Drosophila *slowpoke* gene has five sites of alternative mRNA processing. The first site has three alternative exons, the second has two, the third has two, the fourth has six and fifth has one. In theory, the gene could encode 1536 distinct polypeptides. However, it is possible that while a large number of alternative exons exist they only produce a limited number of distinct products and that the theoretical potential for diversity is never achieved. In any case, the significance of alternative splicing is dependent on the degree to which tissue-specific splicing occurs. We have conducted a tissue- and developmental-specific survey of the alternative splicing pattern of the Drosophila *slowpoke* gene.

Materials and methods

Collection of tissues for RNA isolation

The *slowpoke* gene is widely expressed in embryos, larvae and adults (Becker *et al*. 1995; Brenner & Atkinson 1997). For the study of *slowpoke* tissue-specific splicing, four body parts were isolated from Canton S flies by dissection. These were the larval brain, body wall and the middle midgut and the adult thorax. Larval body walls and adult thoraxes were used as a source enriched for muscle. The middle midgut that begins with the copper cell region and ends with the iron cell region was isolated from a second set of dissections. One hundred samples were collected for each body part. All dissections were conducted in phosphate-buffered saline (10 mM $NaPO_4$, 1.3 M NaCl pH 7.2), and the collected tissues were snap frozen in liquid nitrogen to preserve the RNA.

To investigate the RNA splicing pattern of *slowpoke* in different developmental stages, we collected approximately 500 μl of embryos, larvae or adult flies from a wild-type stock, Canton S, immediately before the RNA extraction. For the collection of embryos, 1-day-old female flies were collected and fed on yeast-seeded fresh food before transferring them to a small egg-collecting cage which contained 10% molasses 1% agarose supplemented with yeast paste. After 15–22 h, embryos were dechorionated with 50% bleach for 1.5 min and rinsed with deionized water. To isolate larvae, we dispersed the top layer of food from a culture bottle in water and adjusted to 3 M NaCl. Larvae float in this solution, and the medium sinks simplifying the collection of the larvae (Roberts 1986). Adults used for RNA preparation were all females that had been collected immediately after eclosion and fed with well-yeasted food for 24 h to deplete them of unlaid embyros or eggs. Total RNA was extracted and first-strand cDNA synthesized as described previously (Atkinson *et al*. 2000).

RT-PCR amplification of the alternatively spliced region

PCR primers were designed and their annealing temperatures calculated using the OLIGO 4.0 program (National Biosciences, Plymouth, MN). For all PCR amplifications, other than those from muscle cells, primers that annealed to the common exons immediately flanking the alternative splice region were chosen (Fig. 1a). These are called slo44 and slo45. The sequences of these primers are slo44 TGACGTTTAC TGTGAGACTGTCCT and slo45 CCGCCATTTTGATTCTGT GTG. Five microliters of the first-strand cDNA was used as template in a 100 μl of PCR reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton-X-100, 1.5 mM $MgCl_2$, and 50 μM each dNTP, 1 μM of each PCR primer and 2–5 U of Taq DNA polymerase). Prior to the addition of enzyme, the template was denatured for 2 min. Thirty-five cycles of amplification were carried out with the following temperature profile: 94 °C 30 seconds, 57 °C 45 seconds, 72 °C 2 min. In the last cycle, the extension time was 7 min.

For the amplification of *slowpoke* splice variants from the muscle-enriched preparations (larval body walls and adult thoraces), we wished to avoid contamination with neural-specific transcripts. To do so, we used an upstream PCR primer called slo43b in place of primer slo44. The slo43b primer (TGGCACTCGACTGCACTTGA) anneals specifically to the exon used to begin muscle transcripts and tracheal cell transcripts (Brenner *et al*. 1996; Chang *et al*. 2000). An annealing temperature of 53 °C was used with the slo43b/slo45 primers.

Library construction

Fifty to 100 ng of PCR product was ligated to 25 ng of PCR 2.1 vector or PCR-Blunt (Invitrogen, Carlsbad, CA). One microliter of the ligation reaction was used to transform TOP10 One Shot cells (Invitrogen). The whole transformation (250 μl) was plated on LB agar plates supplemented with appropriate antibiotics to generate colonies for cDNA clone screening. The PCR 2.1 vectors without inserts were eliminated using lacZ staining.

After an overnight 37 °C incubation, colonies presumably carrying insert were patched into microtiter dishes (96 wells) with 120 μl of LB/ampicillin (100 μg/ml) for PCR 2.1 or LB/Kanamycin (50 μg/ml) for PCR blunt in each well and incubated at 37 °C for 4 h. Then, the transformants were spotted on OMNI plates using a replicator (Boekel, Philadelphia, PA) and grown overnight at 37 °C and lifted with nitrocellulose membranes (MSI, Westborough, MA). To confirm that all replica spots contain a *slowpoke* cDNA insert, we performed hybridization with an oligomer probe that anneals to a common exon between alternative

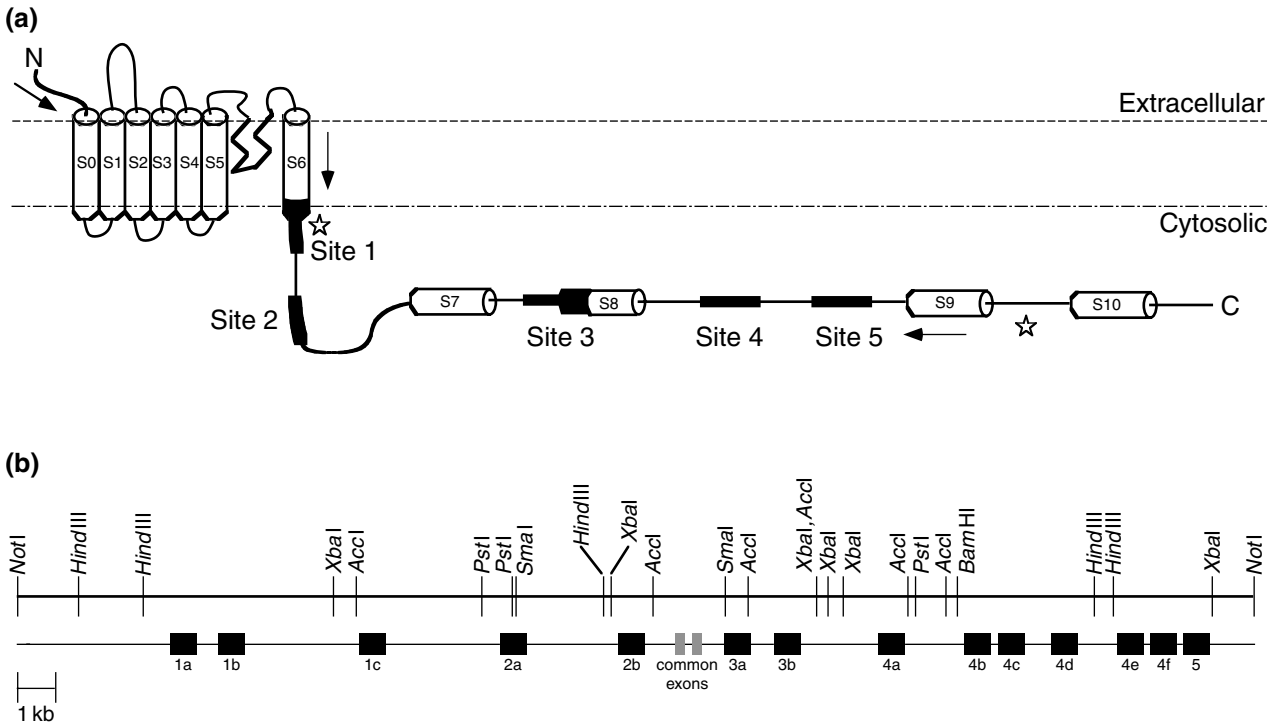


Figure 1: (a) Putative transmembrane organization of the Ca^{2+}-activated K^+ channel encoded by the *slowpoke* gene. S0–S6 represent transmembrane domains and S7 through S10 represent hydrophobic domains that may or may not interact with the membrane (Butler *et al.* 1993). Black thick lines designated as sites 1 through 5 show the relative positions of the alternative splice sites. The stars represent positions proposed to be involved in sensing Ca^{2+} (Schreiber & Salkoff 1997). The arrows identify the positions of primers used to amplify the alternative splice region. (b) Genomic DNA from *slowpoke* alternative splicing region. The black boxes are alternative exons. Oligonucleotides that specifically anneal to each alternative exon were prepared and used to probe tissue-specific cDNA libraries. Libraries were also screened with probes specific to two common exons. Only these two common exons are shown on the map (gray boxes). Unmarked common exons also exist between splice sites 1 and 2, 3 and 4 and 4 and 5. Restriction enzyme sites are shown above the map and a scale is shown below the map.

splice sites 2 and 3 (Fig. 1b). This probe has the sequence TCGATCAAGAACTGCAGCGACGACA.

Each titer dish filled with *slowpoke* cDNA clones was replicated onto 15 LB agar plates and incubated at 37 °C overnight. Colonies on the plates were lifted onto nitrocellulose membrane. One copy was transferred to an LB/25% glycerol plate as the master copy of the cDNA library. The membrane was allowed to equilibrate with the glycerol plate at 4 °C for 2 h before being stored at −80 °C.

Screening of libraries

Each nitrocellulose replica was sequentially submerged 5–10 min in 10% SDS, in 0.5 N NaOH, 1.5 M NaCl and then 1.5 M NaCl, 1 M Tris pH 8.0 (Sambrook *et al.* 1989). The membranes were rinsed in 2× SSC, 0.1% SDS briefly and placed on paper towel for 10–15 min to eliminate extra liquid. DNA was covalently bound to the membrane with a Stratalinker 1800 (Stratagene, La Jolla, CA).

To determine the alternative splice pattern of each cDNA, we performed hybridization of the libraries with oligonucleotide probes specific to each alternative exon. The sequence of each alternative exon probes are exon 1a, CTCGTC GGTAGTGGCAAT; exon 1b, TTGATTGGAACAAGAGCC; exon 1c, CTGGCTGCCCAAAGAAGC; exon 2a, CGCAGT CGACCTGGAGCGAGTC, exon 2b, TCCGATCGATCTTCAG AGGGTT; exon 3a, TGAAACATTGAGTCC; exon 3b, CGAAA CCCTATCACCTTCA; exon 4a, ACTGTTCGACCCAGGAGCAA ATTCGATGACTTA; exon 4b, GCCACCTTCCGGAAAGGCGTC CGAGCCGTACAA; exon 4c, TTGTATTCATCACCGACGTG ATGCCTTGGTTGACCATGCCCGTCTGCATCATAATCATGC; exon 4d, AAAGACGATGAATACTCGTTGTCAAATGAA; exon 4e, GAACACCATCCTGCACCCACATTTACTCCTCCA; exon 4f, GGTGATATCACTCGTGACAGAGAAGATACG and exon 5a, GAATGATGCTAACCCTTATGCGGGCTATCAACTTGCTTAC.

End labeling of probe

Fifty picomoles of each oligonucleotide probe was labeled with 25 μCi gamma-^{32}P ATP in 10 μl of 1× polynucleotide kinase (PNK) buffer with 4 U of PNK (New England BioLabs, Ipswich, MA) at 37 °C for 30 min. Sephadex G-50 filtration was used to remove unincorporated nucleotides.

Membrane hybridization

Non-specific binding to the membrane was blocked by incubation with the hybridization buffer (20% formamide, 5× Denhardts, 6× SSC, 0.2% SDS and 200 μg/ml salmon sperm DNA) at 42 °C with agitation for at least 4 h. Hybridization (16–18 h/42 °C) was initiated by adding the radioactive probe to this solution. Unbound probe was removed by washing with a 2× SSC, 0.2% SDS at the hybridization temperature (Sambrook *et al*. 1989). Membranes were then imaged in a phosphoimager.

Stoichiometry of the method

Each PCR product representing the alternative splice region is similar in size and is amplified by the same primer set. To confirm that the abundance of the PCR products represented the abundance of the starting material, we prepared mixtures of cDNA clones that represented the largest differences in size and nucleotide composition (cDNAs LB248, E16 and LM29). The template mixes were a 1 : 1 ratio of cDNA LM29 and of cDNA LB248 (0.25 ng of each), a 3 : 2 ratio of cDNA E16 (0.3 ng) and LB248 (0.2 ng) and a 2 : 3 ratio of cDNA E16 (0.2 ng) and LB248 (0.3 ng). For each, three PCR reactions were conducted for 31 cycles, 33 cycles and 35 cycles, respectively. All the PCR amplifications were conducted with the primers slo44 and slo45. PCR products were examined by vertical 1% agarose gel electrophoresis run under strong denaturation conditions (30 mM NaOH). The DNA molecule in the gel was visualized by staining with ethidium bromide and quantified with the computer program Imagequant (Molecular Dynamics Inc. Sunnyvale, CA).

To test the accuracy of the cloning method, we performed cloning of some tissue-specific cDNA libraries twice. Each library was plated and probed with exon-specific probes as described above. The mean frequency of exons was determined in both, and the difference in measurement in the two libraries did not exceed 3%. This confirms the reproducibility of the cloning, transformation and hybridization procedures.

Results

The *slowpoke* gene includes a large number of alternative exons (Fig. 1). Unfortunately, there has been no uniform method for naming these exons. Table 1 summarizes our nomenclature and relates it to the naming systems used in previous papers. In the mammalian systems, alternative *slowpoke* exons have been numbered according to their order in the gene. This is the approach that we have chosen. In this document, the five alternative splicing sites are named as site 1 through site 5 according to their 5′ to 3′ order in the gene. Alternative exons expressed at one site are named by the letters a, b, c, d, e and f in the order of the exon position in the genome. Thus, any alternative exon and its position can be specified by the number of the site followed by the letter name of the exon.

To determine the extent to which alternative mRNA processing of *slowpoke* is regulated in a tissue- and developmental-specific manner, we performed a quantitative RT-PCR survey of its alternative exon use. mRNA from different tissues or developmental stages was purified, and the alternative splice region of *slowpoke* mRNAs was reverse transcribed and PCR amplified. The PCR products were cloned and the resulting developmental or tissue-specific cDNA libraries screened by colony hybridization using exon-specific probes. In this manner, the frequency of exon use could be determined for each sample.

Because of the large number of alternative exons and because some alternative exons might remain unidentified, we chose to perform RT-PCR using primers that flanked the entire alternative splice region. The positions of these primers are shown in Fig. 1. No alternative exons have been found to lie outside of the region. This comparison was conducted between specific tissues and different developmental stages. In this manner, we could amplify a fragment representing any alternative splice product regardless of its composition. Using these primers, it would not be necessary to know the sequence of each alternative exon. All variants could be amplified and later characterized. The products amplified from this region were all of similar size (1272–1554 bp). The large number of different splice variants precluded testing the amplification efficiency of every PCR product representing a splice variant. However, we confirmed that splice variants with the greatest difference in size or extreme differences in alternative exon composition amplified with the same efficiency at least to 35 PCR cycles (Fig. 2). This was performed by preparing mixtures of cloned cDNA fragments that contained the alternative splice region. Templates LB248, LM29 and E16 (Fig. 2) represent cDNAs of the maximum, minimum and intermediate sizes, respectively. If all the templates amplify with the same efficiency, then the ratio of the abundance of PCR products should be the same as the ratio of the mass of cloned templates used to seed the PCR reaction. The amplifications produced almost overlapping amplification curves. The greatest observed difference is between cDNAs LM29 and LB248. At cycle 35, the abundance ratio between the PCR products from these two cDNAs showed a 2% difference from the mass ratio of the initial templates.

After amplification, the PCR products representing the alternative splice region were cloned into a vector to produce a representative library. As long as the libraries were plated at low density, the relative frequencies of each PCR product were preserved. Construction and screening of duplicate libraries with exon-specific probes showed that the library construction procedure caused a maximum of a 3% difference in exon frequency. Library replicas were screened by colony hybridization with a common exon probe to confirm that all carried *slowpoke* cDNA inserts. Fourteen replicas of each library were individually screened using oligonucleotide probes specific to each alternative exon.

Table 1: Nomenclature used to identify alternative exons in this report compared to other articles

This report and Atkinson *et al.* (1998)	1a	1b	1c	2a	2b	3a	3b	4cdef	4cf	4a	4aef	4af	4bef	5a
Atkinson *et al.* (1991)								A2			A1			B
Adelman *et al.* (1992)	A1	A2	A3	C1	C2	E2	E1	G1	G2	G3	G4	G5	G6	I
Number of amino acids	30	30	30	35	35	37	37	62	29	13	49	24	61	22

Multiple letters are used to indicate that some site 4 variants are combinations of six smaller exons.

One concern was that a library might contain cDNAs derived from unspliced or partially spliced mRNAs or that the library might be contaminated with amplified genomic DNA. Colonies carrying these materials would carry two or more mutually exclusive exons and would therefore hybridize to two or more mutually exclusive exon probes. Within the amplified region, there are five sites of alternative exon use. It has been previously reported that sites 1, 2 and 3 each house mutually exclusive exons (Lagrutta *et al.* 1994). None of the cDNA clones hybridized to more than one site 1 probe, more than one site 2 probe or more than one site 3 probe.

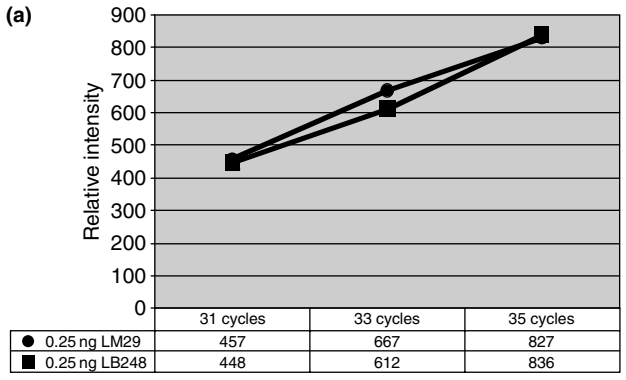
However, approximately 1% of all colonies failed to hybridize to any of the probes specific to site 1, 2 or 3 alternative exons. This might arise if the cDNAs contain unidentified alternative exons. Clones of this nature were sequenced and shown to be false negatives. Each one actually contained a previously identified exon at these sites.

We also identified many clones that failed to hybridize to any of the six probes specific to the site 4 exons. Sequencing indicated that these clones did not contain introns and that the splicing pattern preserves the *slowpoke* reading frame. This is the first description of splice variants that skip all of the site 4 exons. Finally, the alternative splice site 5 has been previously shown to have a single alternative exon that is either included or skipped. These were the only two site 5 splice variants that we observed. No additional alternative exons were identified in this procedure.

Both the hybridization patterns and sequence analysis of the cloned PCR products confirmed that alternative exons at sites 1, 2 and 3 are mutually exclusive. The six alternative exons at the splice site 4 are not mutually exclusive, and many different combinations of these exons exist. Previously, some of the exon combinations at site 4 have been described, but not the presence of six individual exons at this splice site. In the figures, the combinations of the letters a–f indicate the combination of site 4 exons that were found in the cDNAs. It is not yet clear whether any combinations are mutually exclusive. In the figures, the splicing pattern that skips all of the site 4 alternative exons is referred to as the 4X pattern. Site 5 can contain only a single alternative exon. When this exon is present, the site 5 splicing pattern is referred to as 5a. When the alternative exon is skipped, it is referred to as 5X. Both the 4X and 5X splice patterns maintain the *slowpoke* open reading frame.

Developmental-specific alternative exon use

Some exons are primarily associated with a specific developmental stage (Fig. 3). At splice site 1, alternative exon 1b is elevated from being rare in the larvae (1.7% of transcripts) to common in the adult (44%). To a large extent, this elevation has been at the expense of exon 1c which has dropped substantially in abundance between the larval and adult stage. Alternative exon 1c is found in about 60% of



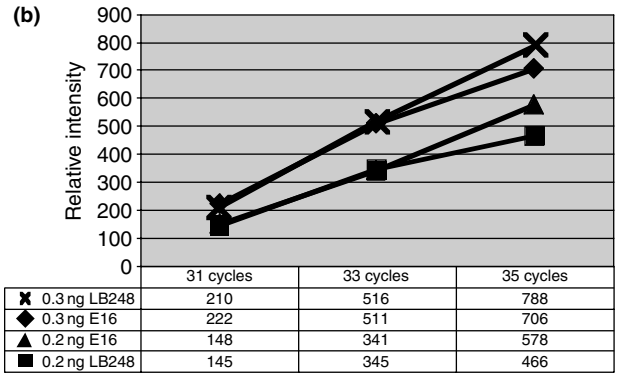


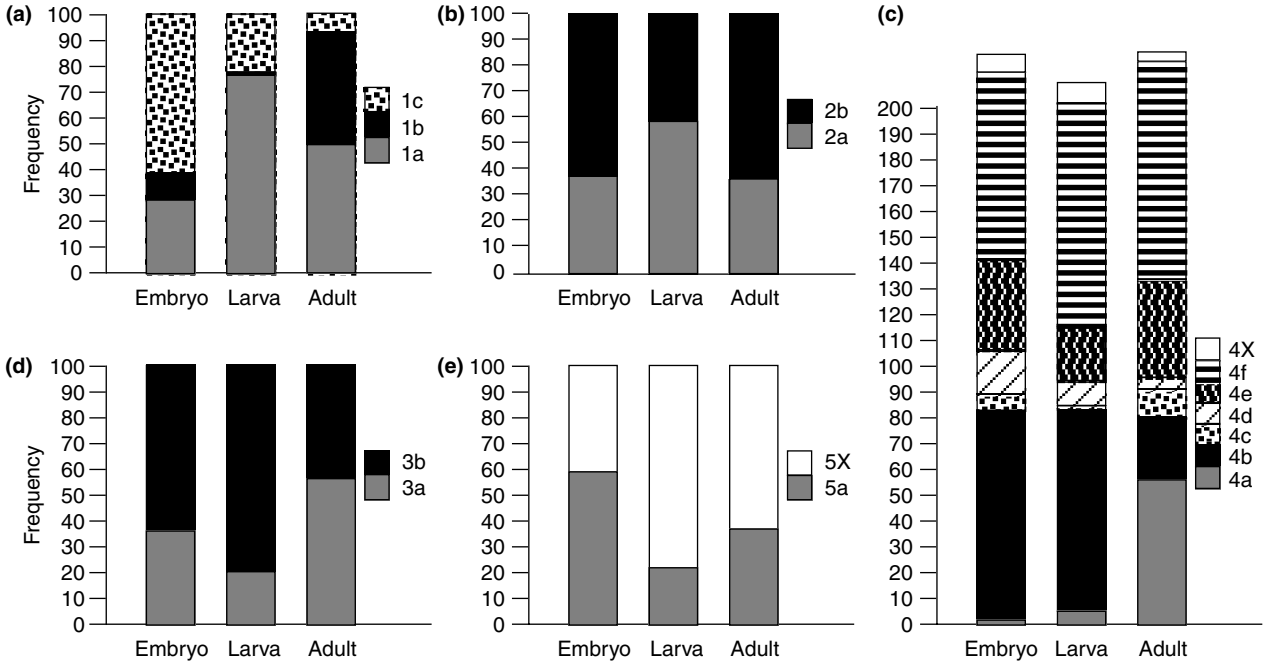
Figure 2: DNA spanning the largest and smallest cloned *slowpoke* regions have the same amplification efficiency. (a) Equal masses of two cloned DNAs were mixed and PCR amplified using the primers that flank the alternative exon region. Both amplify with the same efficiency. (b) PCR amplification of a mixture of splice variants of dissimilar size accurately reflects their initial concentration. Cloned splice variants E16 and LM 248 were mixed in either a 3 : 2 ratio or a 2 : 3 ratio and amplified with primers that flank the alternative splice region.

slowpoke transcripts in embryos, is moderately abundant in the larvae and is rare (7% of transcripts) in the adult. Another notable change is a 10-fold difference in the representation of alternative exon 4a. There is also a substantial reduction in the representation of exon 4b between larvae and adult (from 78 to 24%).

Tissue-specific splicing in larvae

Larvae can be easily dissected to isolate distinct tissue types. The ease of the larval dissection has made it a favorite preparation for electrophysiological studies. In larvae, *slowpoke* is expressed in the nervous system, musculature, tracheal cells and in a small region of the midgut (Becker *et al.* 1995; Brenner & Atkinson 1997). The midgut expression is in epithelial cells involved in translumen ion transport (Brenner & Atkinson 1997). These cells are not thought to be electrically excitable.

Third instar larvae were filleted (Jan & Jan 1976), and RNA was prepared from dissected brain, middle midgut and larval body walls. The body wall was used as a readily isolated source that is highly enriched for muscle. The larval body wall is a mixture of peripheral nervous tissue, muscle and a trace representation by tracheal cells. To avoid the amplification of neural transcripts, we relied on the fact that all muscle transcripts are expressed from a muscle/tracheal cell promoter that is distinct from the promoter used to produce neural transcripts (Chang *et al.* 2000). All transcripts from this promoter begin with an exon called C2 (not to be confused with exon C2 mentioned in Table 1). To limit the PCR amplification to cDNAs from muscle fibers, we designed the upstream PCR primer to specifically anneal to exon C2. The



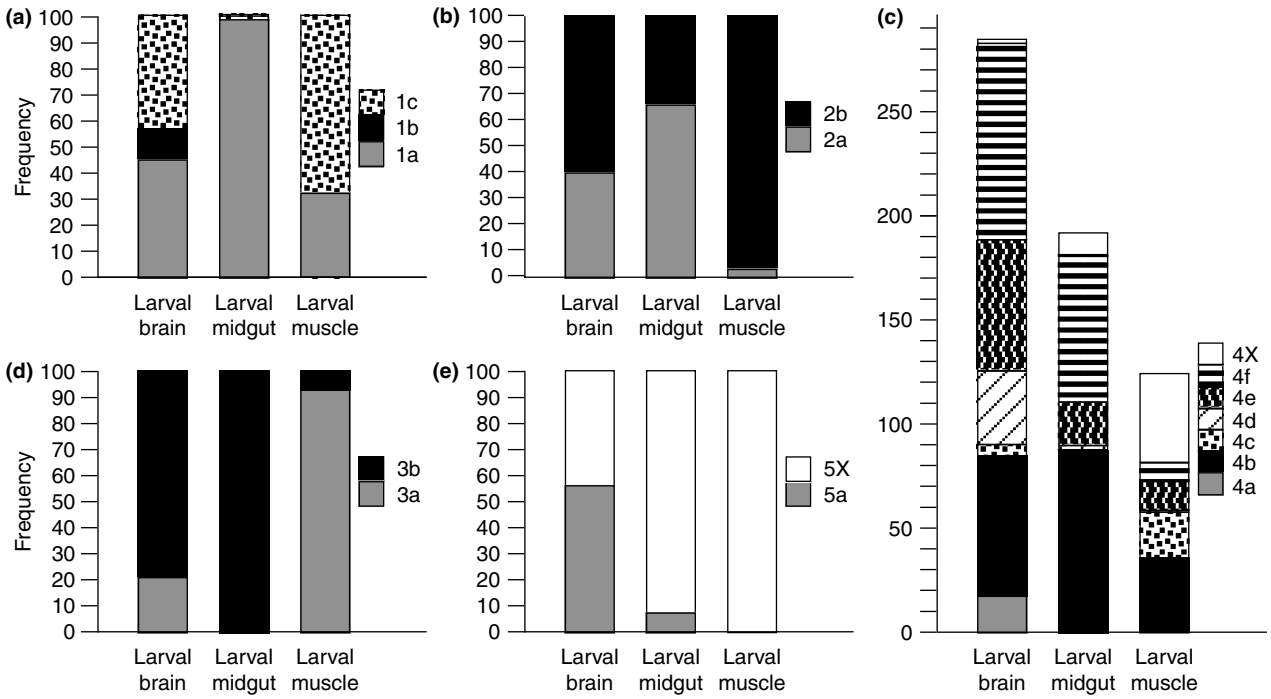
	1a	1b	1c	2a	2b	3a	3b	4a	4b	4c	4d	4e	4f	4X	5a	5X
Embryo	28	11	61	37	63	36	64	2	81	6	17	35	72	7	59	41
Larvae	76	2	22	58	42	20	80	5	78	2	10	21	86	8	22	78
Adult	49	44	7	36	64	56	44	56	24	11	5	38	84	4	37	63

Figure 3: Developmental profile of alternative exon in embryos, larvae and adults. The figure shows the relative frequency of the use of each alternative exon at (a) alternative splice site 1, (b) alternative splice site 2, (c) alternative splice site 4, (d) alternative splice site 3 and (e) alternative splice site 5. Each alternative exon is represented by a different shaded bar. The entry 4X refers to a splice variant that does not include any alternative exon cassettes at splice site 4. The table summarizes the same data in tabular form. All values are percent of total cDNA clones.

downstream primer anneals immediately 3′ to the region of the cDNA that houses all of the alternative exons. The PCR products produced in this manner will be free of neural contamination. However, because the same promoter is used in tracheal cells, there will be some contamination with tracheal cell transcripts. This is expected to be very small because of the huge difference in the density of muscle and tracheal cells (Chang *et al*. 2000). Furthermore, it has been previously shown, using reporter genes, that the *slowpoke* gene is expressed at relatively low levels in tracheal cells (Brenner & Atkinson 1997). Unfortunately, there is no practical way to remove this contamination.

Dissected larval midgut showed an extremely limited range of exon use, muscle body wall showed a somewhat more complex pattern, while neural tissue showed the most variety in the choice of alternative exons. The midgut completely avoids the use of four alternative exons. These are alternative exons 1b, 3a, 4a and 4c. Furthermore, alternative exon 1c is only rarely used (2% of splice variants). Midgut rarely uses exon 5a (Fig. 4).

The muscle samples also showed some striking biases. Larval muscle never uses alternative exon 1b and always uses the 5X splicing pattern in its messages (no alternative exon in site 5). Furthermore, alternative exons 4a and 4d are used in trace amounts (0.8%). In muscle, the most common site 4 splice pattern is to skip all alternative exons at site 4 (4X pattern). Forty-three percent of all larval body wall muscle transcripts use this pattern. Although common, this splicing pattern has never been previously described for *slowpoke*.



	1a	1b	1c	2a	2b	3a	3b	4a	4b	4c	4d	4e	4f	4X	5a	5X
Brain	45	12	43	40	60	21	79	17	67	6	35	63	94	2	46	64
Midgut	98	0	2	66	34	0	100	0	87	0	19	21	70	11	7	93
Muscle	32	0	68	3	97	93	7	1	35	22	1	14	9	43	0	100

Figure 4: Alternative exon frequency in dissected third instar larval tissues. Tissues were dissected from larvae and the mRNA assayed to determine relative frequency of each alternative exon at splice sites 1 through 5 (as described in the legend to Fig. 3). The table summarizes the same data in tabular form. Note that the cloned PCR products representing muscle transcripts are predicted to be contaminated with a low level of PCR products from tracheal cells (see *Results*).

Neural tissue shows the most widespread use of alternative exons. The larval brain also makes limited use of alternative exon 1b which was never selected for use in the larval muscle or midgut. In addition, exons 4a and 4d are common in the brain but rarely used in muscle. Exon 5a is also common in the brain and never used in muscle.

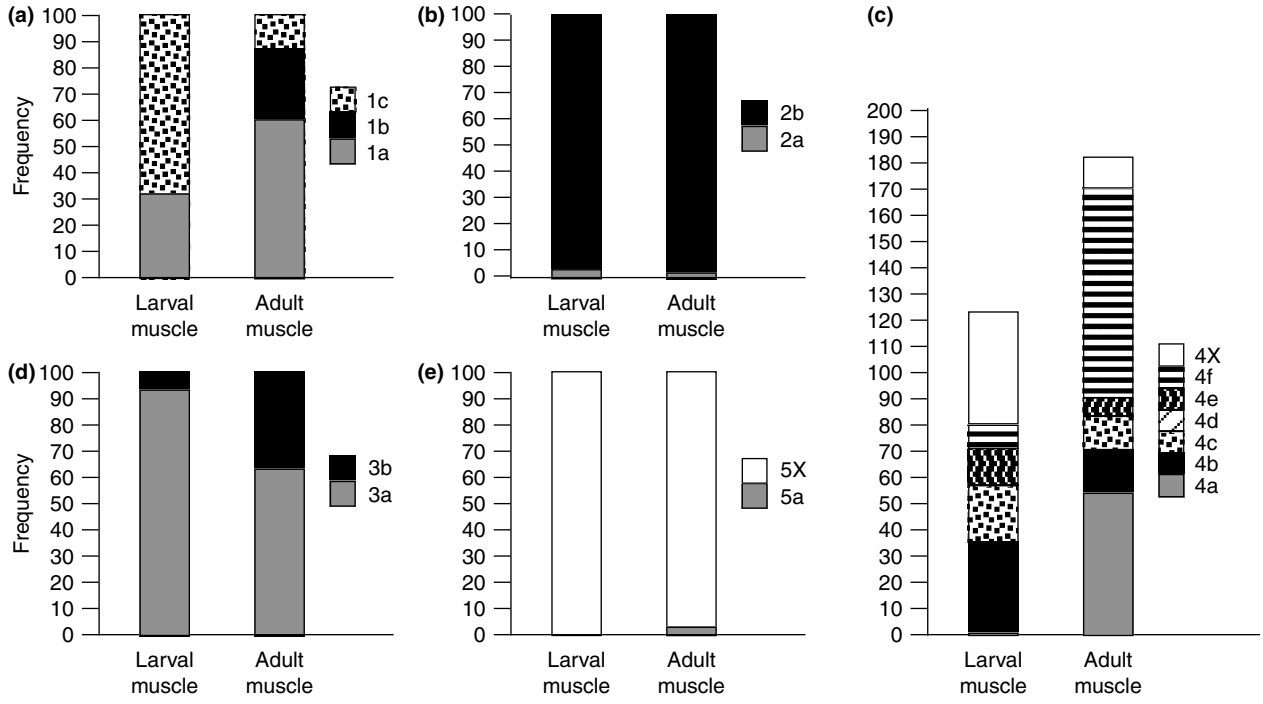
Distinctions in muscle subtypes

Previously, we observed differential regulation of the *slowpoke* muscle/tracheal cell promoter in larval body wall muscle and adult flight muscle (Chang *et al*. 2000). Larval muscles and thoracic muscles of the adult are fundamentally different muscle types. Larval muscles are isopotential and conduct impulses passively while muscles in the adult respond to stimulation with regenerative action potentials (Elkins *et al*. 1986; Jan & Jan 1976).

The muscles in the larval body wall preparation are somewhat homogenous; however, we recognize that the adult thorax does contain different types of muscles (indirect and direct muscles). We did not make any attempt to separate the different adult muscles. Nevertheless, we observed distinct differences in the splicing patterns of larval and adult muscles (Fig. 5).

The larvae completely avoided the use of exon 1b and showed a strong bias for the inclusion of exon 1c, whereas in the adult the use of exon 1b is quite prevalent and exon 1c is rare. Exon 1a is the predominant exon in the adult. Physiological studies in heterologous systems have shown that exon 1a produces channels of higher conductance and calcium sensitivity in comparison with channels expressed from messages containing exon 1c (Lagrutta *et al*. 1994).

Alternative exons 3a and 3b have been shown to produce channels with differing calcium sensitivities. Channels expressed using exon 3a require higher calcium activation



	1a	1b	1c	2a	2b	3a	3b	4a	4b	4c	4d	4e	4f	4X	5a	5X
Larval	32	0	68	3	97	93	7	<1	35	22	<1	14	9	43	0	100
Adult	60	27	13	2	98	60	40	54	17	13	0	7	79	12	3	97

Figure 5: Alternative exon frequency in larval and adult muscle. mRNA expressed in larval and adult muscle was assayed to determine the relative frequency of the use of each alternative exon as described in the legend to Fig. 3. The table summarizes the same data in tabular form. Note that the cloned PCR products representing muscle transcripts are predicted to be contaminated with a low level of PCR products from tracheal cells (see *Results*).

levels than do channels made using exon 3b. Larval muscle shows a strong preference for exon 3a whereas adult muscle is almost evenly split in its use of exons 3a and 3b. Another striking difference is the frequency with which 4a is used in larval vs. adult muscle transcripts. Larvae almost completely avoid the selection of this exon while in adults it is the very commonly chosen insert at this alternative splice site. Conversely, the 4X splice pattern (absence of any site 4 exons) is the most common larval pattern and is only moderately represented in the adult library. Unfortunately, there is no information on how these site 4 splice patterns affect channel properties.

Discussion

In animals, BK-type calcium-activated potassium channels are encoded by the *slowpoke* gene. These are high conductance channels whose open probability is governed by both membrane potential and calcium concentration. In the absence of calcium, they require extremely high membrane potentials to be activated (Schreiber *et al*. 1999). Therefore, at physiologically relevant membrane potentials, the channels act as calcium sensors.

These channels are expressed in most tissue types; however, among potassium channels, the BK-type potassium channels are unusual in that a single gene encodes the pore-forming subunits of all channels in the family in both mammals and Drosophila. This distinction means that evolutionary selection for functional diversity among BK and voltage-gated potassium pore-forming subunits has fundamentally distinct constraints. In the voltage-gated channels, each duplication of a mammalian gene provides an independent target for evolutionary change. For the BK-type channels, however, a modification in the gene sequence that enhances the performance of one cell type might detract from the performance of another. The fine-tuning of channel properties to the specific needs of the cell appears to be relegated to post-translation modifications, the addition of accessory subunits or alternative exon use.

Alternative exon use in *slowpoke* has two origins. In Drosophila, the gene is expressed from at least five distinct tissue-specific promoters. These promoters dictate the level of expression and select the exon with which the transcript begins. They thereby determine the translation start site of the message and the sequence of the amino terminus of the protein. In addition, *slowpoke* has a large number of alternative exons (14) that are selected or excluded by alternative mRNA processing. These alternative exons affect the sequence of the interior mouth of the ion pore (site 1 exons) or sequences adjacent to regions involved in controlling calcium sensitivity (Schreiber & Salkoff 1997). At least some of the Drosophila alternative exons have been shown to produce channels with distinct properties (Lagrutta *et al*. 1994).

Here we show that, in Drosophila, alternative mRNA splicing selects among alternative exons in a tissue-specific manner. We did not know whether all of the *slowpoke* alternative exons had been previously identified in cloned cDNAs. Genomic analysis may also have missed some exons because tiny alternative exons can be computationally difficult to identify. Therefore, to insure that we surveyed all splice variants, we chose to use a method that did not require prior identification of all exons used at each alternative splice site. We amplified the entire alternative splice region as a single fragment using PCR primers that recognized flanking exons which are common to all known *slowpoke* transcripts. We did not identify any new exons in this process which indicates that all of the alternative exons of the *slowpoke* gene have been correctly identified (Adelman *et al*. 1992; Atkinson *et al*. 1991).

Importantly, however, an unusual and previously unknown splicing pattern was identified. This is the 4X pattern in which all of the site 4 alternative exons are skipped. This is the most common variant used in larval muscle. It will be important to determine how the absence of all site 4 exons affects the channel properties. Analysis of this splice variant may provide the clearest understanding of the functional purpose of the very complex site 4 alternative exons.

The larval midgut is the most homogenous tissue that we examined. The expression of *slowpoke* is limited to the middle midgut that is subdivided into anterior, middle and posterior regions. The anterior midgut has a lumen pH of 6–8, whereas the middle midgut has an acidic lumen pH of about 3. Finally, the posterior midgut has an alkaline pH (Filshie *et al*. 1971; Hoppler & Bienz 1994; Poulson & Bowen 1952). It is believed that distinct types of digestion occur in these areas.

In the midgut, *slowpoke* is expressed in bands of epithelia at the beginning and at the end of the middle midgut. These bands flank the position of striking changes in pH within the midgut, and thus, the channel may play a role in maintaining the pH of the middle midgut (Brenner & Atkinson 1997; Dimitriadis 1991; Moffett & Koch 1992). At the anterior end, *slowpoke* expression is in the interstitial cells, and at the posterior end, *slowpoke* is expressed in the iron cells. Expression levels in these two bands of the middle midgut are vastly higher than in any other larval tissue (Brenner & Atkinson 1997).

One would expect that the most profound contrast in expression pattern of *slowpoke* would be between an electrically excitable cell, such as a neuron or muscle cells, and an epithelial cell, such as the interstitial and iron cells. However, this is not the case. While neural transcripts clearly show greater variety than either muscle or midgut transcripts, the larval muscle and larval midgut populations of transcripts have very similar degrees of complexity.

However, the selection of alternative exons employed in the middle midgut is dramatically distinct from those used in either muscle or nervous tissue. Our measurements indicate that almost all midgut messenger RNAs use exons 1a, 3b and 5X and that exons 1b, 3a, 4a and 4c are never used.

Based solely on the incidence of individual alternative exons, one would predict that all midgut splice variants have the 1a, 3b and 5X splicing pattern.

Exons 1a and 3b produce channels with high calcium sensitivity. Furthermore, exon 1a is associated with channels that have pores predicted to support higher levels of conductance than channels carrying exon 1c (Lagrutta *et al*. 1994). Barring modifications that would otherwise alter channel activity, it would seem that the midgut expression is geared toward the production of high levels of high conductance channels that are very sensitive to calcium activation. This suggests that potassium flux through the channel plays an important role in the midgut. These midgut channels probably recycle the potassium that accumulates during active transport and thereby help maintain the cellular resting potential necessary to drive transport and prevent the accumulation of intracellular potassium that might otherwise osmotically cause a change in cell volume (Brenner & Atkinson 1997; Dawson 1991; Welsh & McCann 1985).

A general property of larval and adult muscles is that they rely almost exclusively on alternative exon 2b and that they avoid using exons 4d and 5a. In addition, larval muscle avoids the use of alternative exons 1b and 4a and the 2b, 3a and 5X splice patterns appear to be most common. The newly described 4X pattern, in which all site 4 exons are skipped appears to be most frequently used in larval muscle. Adult muscle is further distinguished by the high frequency with which exons 2b and 4f are used. A specific channel property associated with the exon 2b has not been described. However, channels made using the exon 3a, as opposed to exon 3b, have dramatically reduced calcium sensitivity when expressed in Xenopus oocytes (Lagrutta *et al*. 1994).

In a sense, some tissues can be most simply defined by the exons that they avoid using. In this regard, the larval midgut shows the strongest selectivity and avoids using exons 1b, 3a, 4a and 4c, larval muscle avoids only the 1b and 5a splice patterns and adult muscle avoids only exon 4d. If we broaden the definition of disuse to include exons used in less than 1% of transcripts, then we observe that the midgut avoids exons 1b, 3a, 4a and 4c, while larval muscle transcripts do not use exons 1b, 4a, 4d and 5a, and finally adult muscle most simply avoids exon 4d.

The larval brain does not lend itself as well to this type of characterization. As a whole, the nervous system makes use of all of the alternative exons. This is expected because the brain represents the most complex tissue that we examined. However, some are more common than others. Ninety-four percent of all cDNAs carried exon 4f. Sixty-percent or more of the cDNAs carried exons 2b, 3b, 4b and 4e.

The *slowpoke*-encoded BK channels are used in a great variety of cell types. It has been previously demonstrated that the complex regulation of the five *slowpoke* transcriptional promoters leads to the production of tissue- and developmental-specific mRNA transcripts (Bohm *et al*. 2000; Brenner & Atkinson 1996; Brenner *et al*. 1996; Thomas *et al*. 1997). Transcriptional regulation of the *slowpoke* gene also appears to contribute to the rapid response of the nervous system to organic solvent intoxication. Here, we show that alternative splicing greatly augments the variety of gene products in a controlled manner. There is substantial evidence that transcriptional regulation can be functionally coupled to regulated alternative splicing of the mRNA transcripts (Kornblihtt *et al*. 2004). It is intriguing to speculate that the selection among the 14 different alternatively spliced exons might be made in a co-ordinate manner with the selection among the five transcriptional promoters. It will be interesting to see whether events such as drug sedation that enhances the initiation rate from the neural promoters also alters the splicing pattern of the mRNA.

Finally, here we only briefly glimpse the potential complexity of *slowpoke* products. The 14 alternative exons described herein and the two 'X' splice variants yield a possible 1536 unique combinations of exons. It is difficult to imagine that regulated alternative splicing can sensibly select among such a large number of possible variants. We hope that this initial examination of *slowpoke* mRNA splicing will lead to a complete description of expression from this gene.

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