Characterization of muscle actin genes in *Drosophila virilis* reveals significant molecular complexity in skeletal muscle types


Department of Biology, University of New Mexico, Albuquerque, NM, USA; *Department of Biology, University of York, York, UK

Abstract

Actin is a ubiquitous and highly conserved eukaryotic protein required for cell motility and locomotion. In this manuscript, we characterize the four muscle actin genes of the insect *Drosophila virilis* and demonstrate strong similarities between the *D. virilis* genes and their homologues in *Drosophila melanogaster*; intron locations are conserved, and there are few amino acid differences between homologues. We also found strong conservation in temporal expression patterns of the muscle actin genes – the homologues of the *D. melanogaster* genes Act57B and Act87E are expressed throughout the life cycle, whereas the other two *D. virilis* genes, homologous to Act79B and Act88F are specific to pupal and adult stages. In situ hybridization revealed that each *D. virilis* gene is expressed in a unique pattern in the muscles of the thorax and abdomen. These muscle-specific patterns of actin isoforms suggest a greater physiological diversity for the adult muscles of insects than has been appreciated to date from their categorization into fibrillar, tubular (non-fibrillar) and supercontractile muscle types.

Keywords: *Drosophila melanogaster*, *Drosophila virilis*, actin, muscle, skeletal muscle.

Introduction

The diversity of muscle types within an animal is mirrored by a diversity in muscle protein isoforms. Actin is a ubiquitous and highly conserved protein, which in many organisms is encoded by multigene families in which individual isoforms probably perform cell-specific functions (see for example Rubenstein, 1990). Diversity in actin gene structure and expression has been studied extensively in *Drosophila melanogaster* (reviewed in Bernstein et al., 1993 and Mounier & Sparrow, 1993). The *D. melanogaster* genome contains six actin genes (Fyrberg et al., 1980; Tobin et al., 1980). Two genes, Act5C and Act42A are cytoplasmic, based upon their ubiquitous expression during embryogenesis (Burn et al., 1989; Tobin et al., 1990). The other four, Act57B, Act87E, Act79B and Act88F are predominantly muscle isoforms, but each is unique in its temporal and spatial expression patterns (Fyrberg et al., 1983; Tobin et al., 1990; Keller et al., 1997; Hiromi & Hotta, 1985; Ball et al., 1987; Courchesne-Smith & Tobin, 1989).

A basic premise when a multigene family is discovered, especially with complex expression patterns, is that different isoforms have evolved to function optimally in different physiological situations. Thus muscle and non-muscle actin isoforms emerged when muscle cells were first delineated, and other variants arose in response to requirements for muscles with different properties. However one can ask whether different actin isoforms have different biochemical properties? A number of studies have addressed the functional significance of different actin isoforms. Karlsson et al. (1991) partially rescued the lethality of a yeast act1 mutation by expression of chick β-actin. Inactivation of the mouse cardiac α-actin gene resulted in lethality that can be rescued, to some extent, by expression of a smooth muscle actin (Kumar et al., 1997). More recently, Fyrberg et al. (1998) used transgenic Act88F genes to assay genetically modified actins in Drosophila flight muscle function. They found that single amino acid substitutions corresponding to isoform-specific differences had relatively little effect upon actin function, however multiple substitutions (to a maximum of eighteen substitutions) rendered the modified ACT88F incapable of assembling into normal myofibrils. Similarly, Brault et al. (1999) showed that the human β-cytoplasmic actin, when expressed in Drosophila flight muscles in the absence of endogenous ACT88F did not

Received 18 December 2000; accepted after revision 13 March 2001.

Correspondence: Richard M. Cripps, Department of Biology, University of New Mexico, Albuquerque, NM 87131-1091, USA. Tel.: (505) 277 2822; fax: (505) 277 0304; e-mail: rcripps@unm.edu

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form fully normal nor functional myofibrils. These findings suggest that differences between muscle protein isoforms are significant either for muscle assembly, muscle function, or myofibril maintenance.

The evolutionary conservation of muscle protein gene expression patterns also provides evidence for functional requirements for different isoforms. However, in insects little is known concerning the structure and expression patterns of different actin genes between species. Loukas & Kafatos (1986) demonstrated that a number of Drosophila species including Drosophila viridis contain six dispersed actin loci, yet did not address gene structure nor expression pattern. Homologues of the 1995), however the expression patterns of these genes is

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nor expression pattern. Homologues of the six dispersed actin loci, yet did not address gene structure nor expression pattern. Homologues of Act88F have been identified in D. simulans (Belfuss & Durica, 1992) and the oriental fruitfly Bactrocera dorsalis (He & Haymer, 1994, 1995), however the expression patterns of these genes is not known. In the silkworm Bombyx mori three actin genes have been cloned, and there is probably a fourth (Mounier & Prudhomme, 1991). Despite the evolutionary distance that separates the Diptera and Lepidoptera the Bombyx genes show similar expression patterns to the D. melanogaster genes to which they show most sequence homology. These findings suggest considerable conservation in actin sequence and function, although not necessarily gene number, over a long evolutionary time.

This paper describes the isolation and characterization of the muscle actin genes of D. viridis, which diverged from D. melanogaster approximately 60 million years ago (Throckmorton, 1975; Beverley & Wilson, 1984). We found that the gene structures of each of the four muscle actins of D. melanogaster were strongly conserved in D. viridis. Furthermore, by Northern blot and in situ hybridization to mature pupae we have shown that the stage- and tissue-specific expression patterns of the homologues are very similar in both species. The adult expression patterns of the D. melanogaster Act87E and Act57B genes have not been previously studied in either D. viridis or D. melanogaster. The evolutionary conservation of muscle actin gene expression patterns provides support for functional requirements for different isoforms. Additionally these patterns reveal a greater diversity of skeletal muscles in insects than previously known, and suggest that multiple combinations of actin isoforms contribute to the physiological properties of individual insect muscles.

Results

Isolation of genomic DNA containing D. viridis actin genes

We performed PCR of D. viridis DNA using degenerate primers, to amplify a portion of the D. viridis actin gene sequence. These primers were designed to amplify a region within the D. melanogaster actin genes with variable amino acid sequences that differentiate the isoforms (amino acids 256–343). Amplification generated PCR products 400–600 bp in size, probably representing individual actin genes of which some contain introns in this region of the gene.

One of these fragments was used to probe a D. viridis bacteriophage genomic library (Hooper et al., 1992). Screening of 150,000 plaques resulted in the isolation of forty-five primary clones each of which was subjected to PCR using the degenerate primer set. Direct sequencing of PCR products revealed that fifteen clones represented six different actin genes. This independently confirms the results of Loukas & Kafatos (1986) that D. viridis has only six actin genes. We refer to these genes by the nomenclature of Loukas & Kafatos (1986).

The conceptual translation of the amplified region (amino acids 256–343) for all six genes is shown in Fig. 1, along with a comparison to the sequences of the D. melanogaster actins. All of the six D. viridis isoforms are distinguishable in this region of the protein. Furthermore, all are strongly conserved with homologous sequences from D. melanogaster. These findings demonstrate that significant evolutionary pressure has acted upon these genes to maintain a relatively constant coding sequence.

Actin gene structure and amino acid sequence

To characterize the structures of the D. viridis muscle actin genes, we purified clones for each of the muscle actins (ActC2, ActD1, ActE1 and ActE2), and sequenced the coding sequence and introns. Intron locations were determined based upon the presence of consensus splice sites, and by comparison with intron locations of D. melanogaster actin genes (Fyrberg et al., 1981; Sanchez et al., 1983; Manseau et al., 1988). We also identified the transcription start sites of each gene by comparison with D. melanogaster (K.K. Kelly, S.M.M. and R.M.C., manuscript in preparation, for Act57B; Manseau et al. (1988) for Act87E; Geyer & Fyrberg (1986) for Act88F; Sanchez et al. (1983) for Act79B).

Figure 1. Sequence alignment of the amplified coding regions of D. viridis and D. melanogaster actin genes. The most common residues at each position are shaded black; conserved changes are shaded grey. The top four sequences are from the adult-specific actins; the middle four sequences are from the larval and adult actins, and the lower four sequences are from the cytoplasmic actins. Note that each D. viridis actin gene is more closely related to its homologue in D. melanogaster than to any of the other actins.
Figure 2. Comparison of the genomic organizations of D. virilis and D. melanogaster muscle actin genes. (A) Gene structures. Exons are indicated by boxes: black boxes indicate coding sequence; open boxes indicate 5' untranslated sequence. Numbers indicate the sizes in nucleotides of the untranslated and translated portions of each exon, and the sizes of the introns (arrowed). Because we did not isolate full-length cDNAs for any of the genes, the extent of 3' introns (arrowed). Unique regions of 3' sequence used for Southern and Northern blotting and in situ hybridization are indicated by shaded boxes below the line. (B) 3' UTR probes recognize unique sequences in the genome. D. virilis genomic DNA digested with EcoR I (E) or HindIII (H) was probed with each of the indicated 3' probes. In each digest, single bands of hybridization were observed, in patterns unique to each probe. The sizes of hybridizing bands are indicated.

The structure of each D. virilis muscle actin gene is shown in Fig. 2(A) together with the structures of the D. melanogaster genes. There are considerable parallel features in organization between the actin genes of D. melanogaster and D. virilis. Introns locations are strongly conserved, and although there is some variation in intron size between the species, the characteristic intron-exon organization of each D. melanogaster gene is also recognizable in D. virilis. Because we used the D. melanogaster gene structures to aid us in characterizing the D. virilis genes, any features novel to the D. virilis genes, such as alternative exons, might not be detected. However, this is unlikely since in all actin genes characterized to date (more than ninety-five) from all species, no examples of alternative splicing occur; furthermore, intron locations are commonly conserved between closely related species (Shetlerline et al., 1998).

We compared the predicted amino acid sequences of each muscle actin isoform to its D. melanogaster homologue (Table 1). We found that the larval actins

<table>
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<th>Residue number</th>
<th>Dv residue</th>
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<td>ACTA1</td>
<td>ACT5C</td>
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<td>Q</td>
<td>H</td>
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<td>ACT42A</td>
<td>272</td>
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<td>I</td>
<td>L</td>
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<td>ACT77B</td>
<td>326</td>
<td>I</td>
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<td>ACTE1</td>
<td>ACT87E</td>
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<td>ACTE2</td>
<td>ACT87F</td>
<td>273</td>
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Table 1. Amino acid differences between D. virilis and D. melanogaster actin proteins

Listed are the amino acid residues where D. melanogaster (Dm) and D. virilis (Dv) actin homologues differ. *Note: the published sequence of ACT79B (Fyrberg et al., 1998) has an M at position 326, however the D. melanogaster genome project lists ACT79B as having an I at this position (Adams et al., 2000). This may simply reflect polymorphism at this residue.

ACT57B/ACTC2 and ACT87E/ACTE1 were identical between species. By contrast, the adult actins each showed a single amino acid difference between the two species: a cysteine residue at position 273 in ACT88F is replaced with a serine residue in ACTE2; and a methionine at residue 326 in ACT79B is replaced with an isoleucine in ACTD1. However, the latter difference may be a polymorphism because published ACT79B sequences differ at the same codon – Fyrberg et al. (1998) show a methionine at position 326, whereas Adams et al. (2000) show an isoleucine at that position.

Although we did not determine the complete coding sequence of the cytoplasmic actins, the portions amplified also show interspecific specific amino acid substitutions (see Table 1). These findings underline both the similarities in homologous genes between the two species, and the residue differences that may perform functions unique to each species.

Temporal expression of muscle actin genes

To determine whether actin gene expression patterns during development have been conserved, we generated 3' UTR probes for each gene. The location of each probe is indicated on Fig. 2(A). To ensure that the probes recognize unique genomic sequences we probed Southern blots of D. virilis genomic DNA with each of the four 3' UTR probes (Fig. 2B). In all cases, only a single hybridization band was detected, demonstrating that each probe recognizes a unique genomic sequence.

By Northern blot analysis (Fig. 3), each gene showed varied levels of expression during development, consistent with roles in muscle development. At 0–15 h after egg lay (AEL), muscles have not yet formed and none of the actin genes were expressed. At 16–24 h AEL, embryos showed expression of ActC2 and ActE1, but not expression of ActD1 and ActE2. ActC2 and ActE1 transcripts were also detected during larval development, demonstrating that these two genes are the only muscle actins expressed during the development and growth of the larval muscles. These findings

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correlate with the expression of Act57B and Act87E, as the only larval muscle actins in D. melanogaster.

By 24 h after puparium formation (APF), most of the larval muscles are being histolysed and little muscle differentiation is taking place. Consistent with this, expression of each actin gene at this stage was either low or undetectable. Subsequently, all four genes were active in the pupa at the pharate adult stage, demonstrating that each gene plays a role in adult muscle development. This indicates that ActD1 and ActE2 are uniquely adult actins, similar to their respective homologues in D. melanogaster, Act79B and Act88F (Fyrberg et al., 1983).

**Distribution of actin transcripts in the developing adult**

To study the distribution and complexity of actin gene expression in the adult muscles of D. virilis, we performed in situ hybridization with antisense riboprobes to transverse sections of pharate adults (Fig. 4).

**Figure 3. Temporal expression of muscle actin genes in D. virilis.** Total RNA (7 μg) isolated from the indicated developmental stages and 10 μg of RNA ladder (Gibco BRL, Life Technologies Inc., Rockville, MD) was separated by formaldehyde-agarose electrophoresis. The ethidium bromide stained gel was photographed (bottom panel) to ensure that equivalent amounts of RNA were present. Subsequently the gel was blotted and probed with genespecific probes for each of the muscle actin genes (top four panels). Note that each gene shows a dynamic pattern of expression during development, consistent with a role in muscle differentiation. The sizes of two RNA ladder fragments are indicated.

**ActE1** expression was predominantly detected in tubular muscles of the ventral thorax and in the internal muscles of the leg (Fig. 4A). Interestingly, ActE1 expression was also detected at low levels in the tergal depressor of the trochanter (TDT) muscle (data not shown), indicating that this muscle expresses two actin genes, ActD1 (see below) and ActE1. ActE1 was also expressed at low levels in the abdominal body wall muscles (data not shown). The ActE1 sense probe showed no hybridization in pharate adults (Fig. 4B).

Expression of ActC2 was most clearly detectable in the abdomen in the body wall muscles, in visceral muscles surrounding the gut (Fig. 4C), and in the heart (inset in Fig. 4C). This makes ActC2 the most broadly expressed actin gene in the adult abdomen. No significant signal was observed in any of these tissues with an ActC2 sense probe (Fig. 4D). Little reproducible expression was observed elsewhere in the pharate adult. Although the ActC2 probe hybridized significantly to the indirect flight muscles (IFMs), the control sense probe showed a comparable signal, suggesting that the IFM signal represented non-specific background staining. Despite using three different ActC2 probes from the 3'UTR and the 5'UTR, we were unable to distinguish sense and antisense probes in the thoracic musculature.

The predominant expression of ActD1 was in the TDT muscle (Fig. 4E). Interestingly, few of the ventral muscles of the thorax that showed ActE1 expression showed strong expression of ActD1, although it is not possible to tell if the domains of expression of these genes are exclusive. ActD1 is also expressed in some of the muscles of the leg. No hybridization of ActD1 probe was observed to the abdominal muscles, nor did a sense probe hybridize to any of the muscles of the pharate adult (Fig. 4F).

ActE2 expression was observed in the dorsoventral and the dorsal longitudinal IFMs (Fig. 4G), similar to the expression of Act88F in D. melanogaster. There was clearly no expression in the TDT, nor in the majority of tubular muscles of the thorax. However, we did observe some expression of ActE2 in a subset of the tubular muscles of the leg (inset in Fig. 4G), indicating that the expression of ActE2 is not exclusive to the fibrillar muscles of the thorax. No hybridization of ActE2 probe was observed to the abdominal muscles, nor did a sense probe hybridize to any of the muscles of the pharate adult (Fig. 4H).

In summary, our in situ hybridization analyses indicate that there is significant diversity among the thoracic muscles of D. virilis. Despite their similar ultrastructure, the tubular muscles are highly heterogeneous in that different muscles accumulate transcripts for different actin genes. These findings represent the first demonstration of such diversity within the tubular muscles of Drosophila.
Discussion

Significant evolutionary time separates *D. melanogaster* and *D. virilis*, however studies of these species have demonstrated a strong conservation in gene structure (see for example Hooper et al., 1992). This is also true for the four muscle actin genes. Each gene has strongly conserved intron-exon organization, exon size, and conceptual translation product. There is some variability in intron size, a feature observed previously in comparisons of *D. melanogaster* and *D. virilis* (Hooper et al., 1992). The strong conservation of gene structure, coupled with our finding of a maximum of six actin genes in *D. virilis*, confirms that we have indeed isolated the *D. virilis* homologues of all the *D. melanogaster* actin genes.

Four of the five amino acid substitutions found between the six actin genes of *D. virilis* and *D. melanogaster* (Table 1) cluster non-randomly within the actin structure.
Although this is not always the case (Miller et al., 1993), the expression of the homologous genes in D. melanogaster:

Distinct muscles of the D. melanogaster adult accumulate distinct actins. For example, the IFMs accumulate only ActE2, which is the only muscle actin gene expressed in the IFMs, but is co-expressed with ActE1. These analyses of muscle actin isoform distribution in D. virilis reveal a remarkable complexity to these adult insect muscles. This is the most detailed analysis to date of actin isoform distribution in an adult insect, and lays the groundwork for future studies defining the functional significance of muscle protein isoform usage.

Consistent with this model, many of the physiologically distinct muscles of the D. melanogaster adult accumulate distinct actins. For example, the IFMs accumulate only Act88F (Ball et al., 1987), and several of the tubular muscles accumulate Act79B (Ball et al., 1987; Courchesne-Smith & Tobin, 1989).

All of our findings using D. virilis are consistent with the expression of the homologous genes in D. melanogaster: ActE2 is the only muscle actin gene expressed in the IFMs, as with Act88F. Furthermore, we also find a small number of tubular muscle fibers in the leg that express the ActE2 isoform. Thus this actin is not unique to the stretch-activated fibrillar muscles. Recent findings by Roy & Vijayaraghavan (1997) using an Act88F-lacZ reporter and by Nongthomba et al. (2001) using an Act88F antisense riboprobe indicate that Act88F is expressed in specific tubular muscles of the adult. For ActD1, our results mirror the known expression pattern of Act79B, in which expression is restricted to the tubular muscles.

Little work has addressed the muscle-specific expression of two other actin genes in the D. melanogaster adult, Act87E and Act57B (Fyrberg et al., 1983; Tobin et al., 1990). Our detailed analysis of the expression patterns of the D. virilis homologues of these two actin genes revealed novel and interesting expression patterns. ActC2 is predominantly expressed in the body wall muscles of the adult abdomen, as well as in the heart and visceral muscles surrounding the gut. There was little specific hybridization of the ActC2 probe to mRNA in the thorax. For ActE1, a significant gene expression was detected in many of the tubular muscles of the thorax. Each of these expression patterns is the first described for these genes or their homologues in the Diptera.

The striking finding was that each actin gene has a unique expression pattern, yet several muscles accumulate different combinations of actin isoforms. ActE2 is the only muscle actin gene transcribed in the IFMs, but is co-expressed with ActE1 in specific leg muscles. ActE1 transcription is detected in the TDT muscle, where it is co-expressed with ActD1. ActE1 transcripts were detected at lower levels in the abdominal body wall muscles, along with ActC2. Finally, some muscles of the thorax and leg only accumulate ActE1. These analyses of muscle actin isoform distribution in D. virilis reveal a remarkable complexity to these adult insect muscles. This is the most detailed analysis to date of actin isoform distribution in an adult insect, and lays the groundwork for future studies defining the functional significance of muscle protein isoform usage.
Experimental procedures

Maintenance of Drosophila virilis

D. virilis were obtained from the National Drosophila Species Resource Center (Bowling Green State University, OH) and maintained at 25 °C in plastic half-pint bottles containing Drosophila Jazz Food Mix (Applied Scientific Inc., South San Francisco, CA).

Cloning of D. virilis actin genes

D. virilis genomic DNA was purified according to a method from SI Bernstein (San Diego State University, San Diego CA). Briefly, approximately twenty flies were homogenized in 1 ml of buffer containing 100 mM Tris-Cl pH 8.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA and 0.5% (w/v) SDS. RNase A (50 μl of a 10 mg/ml solution) was added, and the homogenate was incubated at 65 °C for 30–60 mins. Salt and proteins were precipitated by addition of 8 μl potassium acetate (150 μl). The mixture was incubated on ice for 1 h and then centrifuged at 14 000 × g to pellet debris. The supernatant was removed and subjected to two phenol/chloroform extractions and one chloroform extraction. DNA was precipitated by addition of 800 μl 100% cold ethanol, and centrifugation at 14 000 × g for 5 mins. All other molecular biology techniques were according to standard procedures (Sambrook et al., 1989).

PCR of genomic DNA used two degenerate primers, 5C-88F + (5’-CAGGT5ATCCATCCYGGMANYGARCG3’)- and 5C-88F– (5’-CCTCCTTGG5AR5GTC5TGG-3’). PCR products were cloned into pGEM-T Easy (Promega Corp.), and a clone containing ActE2 was used for probe generation. A D. virilis genomic library generated in EMBL3 (Hooper et al., 1992) was screened (150 000 plaques) with this probe and forty-five primary clones were purified. Primary bacteriophage eluates were used in a PCR using the 5C-88F primers. PCR products were sequenced using a BigDye sequencing kit, and an ABI Prizm 377 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA), to determine which actin gene was harboured in each bacteriophage clone. One genomic clone for each muscle actin gene was purified, the inserted genomic DNA was excised and cloned into pBluescript II KS (Stratagene). Plasmid clones were initially sequenced with the degenerate actin primers, and appropriate new primers were generated from these sequences to characterize the rest of the genes.

RNA extraction and Northern blotting

Total RNA was purified using TRI reagent according to the manufacturer’s instructions (Molecular Research Center Inc., Cincinnati, OH). For Northern blotting, 7 μg of total RNA was separated on a formaldehyde agarose gel, and the RNA was transferred to Hybond-XL (Amersham Pharmacia Biotech Inc., Piscataway, NJ) for hybridization.

To study the expression of individual actin genes, we generated by PCR gene-specific probes from the sequence 3’ to the translation stop. The following gene-specific primer pairs were used: ActC2–5’TACTGCTATCATCCTC-3’ and 5’-ATACTTTGGAAACCAAGG-3’; ActD1–5’-CAAGCATTAAATACAGACGC-3’ and 5’-TTTGCGATGCGCCGTG-3’; ActE1–5’-ACACAGCAACGCAGAGC3’- and 5’-GTCACTCGCAACACTTC-3’; ActE2– 5’-AATCTTGGCGCGTGCG-3’ and 5’-TCCACCGAAGTCTCGCG-3’. PCR products were ligated into pGEM-T Easy. For Northern blotting, UTR fragments were gel purified and labelled with 32P-dCTP using a random prime DNA labelling kit (Roche Molecular Biologicals, Indianapolis, IN). For in situ hybridization (below), clones were selected from which sense and antisense riboprobes could be made using the T7 RNA polymerase.

In situ hybridization to cryosections

Probe preparation, hybridization and signal detection were as described by O’Neill & Bier (1994). For sample preparation, pharate adult pupae were selected based upon the presence of red eye pigment. These were embedded in OCT medium (Tissue-Tek, Torrance, CA) and frozen on dry ice. After temperature equilibration, sections (15 μm) were generated using a Tissue-TekII cryomicrotome (Miles Corp.), and placed upon slides. Sections were fixed in 4% formaldehyde in 1X PBS for 10 min, washed with PBS and then subjected to proteinase K treatment (20 μg/ml for 8 min). The remaining steps were as described O’Neill & Bier, 1994. Signal was detected using the alkaline phosphatase BCIP/NBT substrate (Vector Laboratories, Burlingame, CA).

Acknowledgements

We are grateful to Dr John Tamkun for providing the D. virilis genomic library and to Dr Eric S. Loker for the use of his microscope. This work was supported by grants from the Muscular Dystrophy Association and the American Heart Association, Desert/Mountain Affiliate to R.M.C., and the BBSRC to J.C.S. The DNA sequences of the genes described in this manuscript have been deposited to EMBL/GenBank/DDBI nucleotide sequence databases with the following accession numbers: ActC2: AF358264; ActD1: AF358266; ActE1: AF358265; ActE2: AF358263.

References


