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Biochemistry

Identification of a cytoplasmic tropomyosin gene linked to two muscle tropomyosin genes in *Drosophila*

(recombinant DNA/gene expression/hybrid-selection translation assay/polyclonal antibody)

VICTORIA L. BAUTCH AND ROBERT V. STORTI*

Department of Biological Chemistry, University of Illinois, Chicago, Health Sciences Center, Chicago, IL 60612

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ABSTRACT **A *Drosophila* cytoplasmic tropomyosin gene has been identified and is located on the same genomic DNA clone as two *Drosophila* muscle tropomyosin genes previously identified. A positive hybrid-selection translation assay using the subcloned gene and RNA from non-muscle cell sources yielded a protein with a size (M_r, 31,000) and isoelectric point (5.0) similar to vertebrate cytoplasmic tropomyosin. A modified protocol for the purification of vertebrate cytoplasmic tropomyosin was used to partially purify cytoplasmic tropomyosin from the *Drosophila* Kc cell line. The Kc cell protein was identified as a cytoplasmic form of tropomyosin on the basis of its size, isoelectric point, and crossreactivity with a polyclonal vertebrate antitropomyosin antibody in a two-step binding assay. The Kc cell cytoplasmic tropomyosin comigrates in two dimensions with the hybrid-selected *in vitro* translation product of the region 3 gene, and both proteins show a mobility shift in NaDodSO$_4$/urea/polyacrylamide gels that is characteristic of vertebrate tropomyosins. The cytoplasmic tropomyosin gene hybridizes to both *Drosophila* muscle tropomyosin genes under decreased stringency conditions. This cross-hybridization spans several internal restriction endonuclease sites in each muscle tropomyosin gene and indicates an overall partial homology among the three *Drosophila* tropomyosin genes. These results show that *Drosophila* tropomyosins are encoded by a closely linked family of differentially regulated genes.**

Tropomyosins are a highly conserved family of proteins. In muscle, tropomyosin is an abundant protein of the contractile apparatus that associates with troponin to regulate the calcium-sensitive interaction of actin and myosin (1). Vertebrate muscle tropomyosin consists of two major subunits that differ in primary amino acid sequence (2), α-tropomyosin (M_r, 34,000) and β-tropomyosin (M_r, 36,000) (3). Tropomyosin is also present in non-muscle cells (4–8). Most cytoplasmic tropomyosins are smaller than their muscle counterparts (M_r, 30,000) (9), although cytoplasmic tropomyosins with mobilities similar to muscle forms have recently been identified in fibroblasts (10, 11). The function of these non-muscle or cytoplasmic tropomyosins is unknown, although they are thought to participate in the contractile activities of non-muscle cells (for review, see ref. 12).

We have recently reported the isolation of two closely linked *Drosophila* muscle tropomyosin genes (13). These genes are single copy in the *Drosophila* genome and encode two electrophoretically distinct tropomyosins that are expressed only in muscle cells. We report here the identification of a third *Drosophila* gene that encodes a cytoplasmic tropomyosin. This protein is expressed in non-muscle cells and has biochemical properties similar to vertebrate cytoplasmic tropomyosins. The gene encoding the *Drosophila* cytoplasmic tropomyosin is also single copy and is found on the same genomic DNA clone as the two *Drosophila* muscle tropomyosin genes. Thus, the tropomyosins in *Drosophila* are encoded by a closely linked family of three genes.

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MATERIALS AND METHODS

Construction of Subclones. The construction of pVB85-1 has been described (13). pAS85-1 was constructed by ligating the agarose gel-purified 5.9-kilobase pair (kbp) *Bam*HI restriction fragment of λDm85 into *Bam*HI-digested pACYC184 and transformed according to the protocol of Kushner (14). pAS85-2 was constructed in the same way, using the gel-purified 2.0-kbp *Hin*dIII/*Bam*HI restriction fragment of λDm85.

Isolation of DNA and RNA. Purification of phage and plasmid DNA has been described (13). Staged embryos (Oregon-R) were collected at 25°C over a 4-hr period and kept at 25°C to give 1- to 5-hr-old embryos. Kc cells were maintained in spinner culture at 25°C. Poly(A)$^+$ RNA from embryos and Kc cells was isolated as described (13).

Hybrid Selection. Hybrid-selection translation assays were done according to the protocol of Ricciardi *et al.* (15). The translation products were separated in one or two dimensions, and the gels were prepared as described (13).

Protein Preparation. *Drosophila* cytoplasmic tropomyosin was prepared from 17 g of packed Kc cells by a modification of a protocol for preparation of chicken brain tropomyosin (7). The supernatant from the final heat-treated extract was acidified with 0.1 M HCl, and precipitates were collected by centrifugation (17,000 × *g*) at pH 6.0, pH 5.5, pH 5.0, pH 4.5, and pH 4.0.

Drosophila myofibrillar proteins were prepared according to Bullard *et al.* (16) as described (13).

Stringency Hybridizations. DNA was digested with appropriate restriction endonucleases, separated by agarose gel electrophoresis and transferred to nitrocellulose filter paper by the method of Southern (17). Hybridizations in various amounts of formamide at 35°C were performed according to the protocol of Howley *et al.* (18).

Antibody-Binding Assay. Electrophoretic transfer of proteins and antibody binding were carried out by a modification of the protocol of Towbin *et al.* (19). Proteins were transferred to nitrocellulose filter paper for 2.5 hr at 400 mA in 25 mM Tris·HCl, pH 8.1/192 mM glycine made 20% (vol/vol) in methanol. Filters were incubated in buffer as described (19). The secondary antibody was goat anti-rabbit light chain labeled with ^{125}I to a specific activity of 10^7 dpm/μg by the method of McFarlane (20).

Abbreviation: kbp, kilobase pair(s).

* To whom reprint requests should be addressed.

RESULTS

A Third Gene Is Closely Linked to Two *Drosophila* Muscle Tropomyosin Genes. The isolation and restriction endonuclease mapping of *Drosophila* genomic clone λDm85 have been described (13). The 18 kbp of *Drosophila* DNA in λDm85 contain three transcribed regions (Fig. 1). Two of these regions (region 1 and 2) encode the muscle tropomyosin I and tropomyosin II genes, respectively, and are separated by ≈3 kbp of DNA. A third, previously uncharacterized, transcribed region (region 3) is located adjacent to the tropomyosin II gene. The region 2 and 3 genes are separated by ≈1.3 kbp of DNA. The three genes have the same polarity of transcription (unpublished results). Subclones were constructed from each of the gene regions and used for subsequent analysis.

The Region 3 Gene Encodes a Protein Expressed in Nonmuscle Cells. We were unable to identify a protein product encoded by region 3 in a hybrid-selection translation assay performed with late embryo RNA (12–24 hr of development), a protocol successfully used for identification of the muscle tropomyosin genes. The results of RNA blot experiments indicated, however, that the region 3 transcript is more abundant in tissue culture cells and early embryos than in late embryos (13). Accordingly, region 3 plasmid pVB85-1 DNA was used in a hybrid-selection translation assay with poly(A)$^+$ RNAs isolated from 1- to 5-hr-old embryos and Kc tissue culture cells in an attempt to detect a translation product. The [^{35}S]methionine-labeled hybrid-selected translation products were separated on one-dimensional NaDodSO$_4$/polyacrylamide gels (Fig. 2). Several endogenous translation products are present in each of the lanes, which result from the long fluorographic exposure (2 weeks). Because this background is present in lanes containing the endogenous and nonspecific DNA controls, it can be attributed to translation of residual rabbit reticulocyte RNA. Nevertheless, it can be seen that RNA homologous to region 3 DNA and selected in the assay directed translation of a unique protein (M_r, 31,000) (Fig. 2, lanes A and B). This RNA is not selected by filters containing *Escherichia coli* DNA (lane C). The M_r 31,000 translation product was detected in assays using either early embryo or Kc cell RNA; however, it was not detected in assays using RNA from embryos at 12–24 hr of development (data not shown). Transcripts homologous to region 3 are not very abundant in the latter RNA population (13). The fact that long autoradiographic exposures were necessary to clearly detect the M_r 31,000 translation product suggests that this protein is a minor component of the total translation products. This was verified by inspection of the total translation products of early embryo RNA separated in two dimensions, which shows that the M_r 31,000 translation product is not very abundant (see Fig. 5*E*).

Partial Purification of a Tropomyosin-like Protein from *Drosophila* Kc Cells. Because the hybrid-selected product of the region 3 gene is similar in size to vertebrate cytoplasmic tropomyosin, we set out to determine if *Drosophila* cells contained a cytoplasmic form of tropomyosin. This was done by using a modified protocol for the partial purification of vertebrate cytoplasmic tropomyosin (7) with *Drosophila* Kc cells as starting material. An aliquot of the ethanol/acetone Kc cell powder and equal volumes of the final sequentially obtained isoelectric precipitates were separated on a one-dimensional NaDodSO$_4$/polyacrylamide gel (Fig. 3). The precipitate collected at pH 5.0 was highly enriched with a M_r 31,000 protein (lane D). This is within the same pH range used to purify vertebrate cytoplasmic tropomyosin. We, therefore, tentatively identified this protein as a *Drosophila* cytoplasmic tropomyosin. The pI 4.5 and pI 4.0 precipitates contain decreased amounts of the cytoplasmic tropomyosin protein band.

A Vertebrate Tropomyosin Antibody Crossreacts with the *Drosophila* Cytoplasmic Tropomyosin. To further characterize the putative *Drosophila* cytoplasmic tropomyosin protein, its crossreactivity with a chicken anti-smooth muscle tropomyosin antibody was tested in a two-step antibody-binding assay. A partially purified preparation of *Drosophila* myofibrillar proteins and the pI 4.5 precipitate from Kc cells were separated on one-dimensional NaDodSO$_4$/polyacrylamide gels and transferred to nitrocellulose filters. The pI 4.5 precipitate was used because it contains additional contaminating proteins of equivalent or greater abundance relative to the cytoplasmic tropomyosin, and these proteins provided an internal control for nonspecific sticking of antibody to unrelated proteins on the filter. The filters were incubated with primary antiserum and then with ^{125}I-labeled secondary antibody. The results show that the chicken antitropomyosin antibody binds specifically to both the *Drosophila* muscle tropomyosins (Fig. 4, lane C) and the *Drosophila* cytoplasmic tropomyosin (lane D). The autoradiographic signal is more intense from the band corresponding to *Drosophila* muscle tropomyosins than from the Kc cell protein, and inspection of duplicate stained filters shows that relatively less protein is present in the former band (data not shown). Therefore, the chicken anti-smooth muscle tropomyosin antibody shows more crossreactivity to the *Drosophila* muscle tropomyosins than to the cytoplasmic tropomyosin. There are also additional higher molecular weight bands on each filter that show decreased but reproducible binding to the antibody. The identity of these bands is unknown; however, they probably share antigenic determinants with tropomyosin because these bands are not seen in control lanes and because the specificity of this antibody has been established (21).

Comigration of the Region 3 Gene Product with the Kc Cell Tropomyosin. The hybrid-selected translation product of the

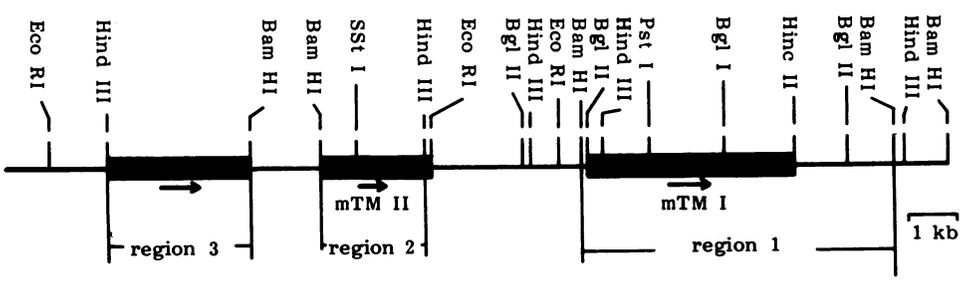

FIG. 1. Restriction endonuclease map of the tropomyosin gene region. The map was constructed as described (13). Only those sites referred to in the text are included for several enzymes (*Sst* I, *Pst* I, *Bgl* I, *Hinc* II). The darkened areas are those restriction fragments with homology to poly(A)$^+$ RNA from 12- to 24-hr *Drosophila* embryos. mTM I, muscle tropomyosin I gene; mTM II, muscle tropomyosin II gene. Regions 1, 2, and 3 indicate restriction fragments subcloned.

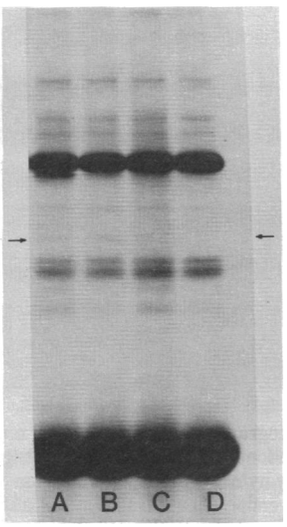

FIG. 2. Fluorogram of hybrid-selected translation product of region 3 DNA. Filter-bound DNA was used in a hybrid-selection translation assay with poly(A)$^+$ RNA. The [^{35}S]methionine-labeled *in vitro* translation products were separated in a NaDodSO$_4$/13% polyacrylamide gel and prepared for fluorography. The translation products of pVB85-1 DNA selection of poly(A)$^+$ Kc-cell RNA (lane A); pVB85-1 DNA selection of poly(A)$^+$ 1- to 5-hr embryo RNA (lane B); *E. coli* DNA selection of poly(A)$^+$ Kc-cell RNA (lane C); and products of rabbit reticulocyte cell-free translation without exogenous RNA (lane D) are shown. The arrow indicates the hybrid-selected translation product (M_r, 31,000) of pVB85-1 DNA. The polypeptide migrating just below the large endogenous band is actin and, because of its abundance, is a common contaminant.

region 3 gene and the cytoplasmic tropomyosin protein from Kc cells were further characterized. The *in vitro* translation products of early embryo RNA selected by the region 3 gene were mixed with the pI 4.5 precipitate fraction obtained from Kc cells. The pH 4.5 fraction was chosen because it contains a higher molecular weight protein band used as a relative mobility marker. This mixture was then subjected to two-dimensional gel elec-

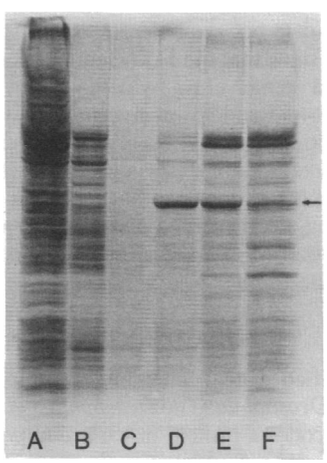

FIG. 3. Coomassie blue-stained gel showing partial purification of M_r 31,000 Kc cell protein. *Drosophila* Kc cells were subjected to a modified protocol for partial purification of vertebrate cytoplasmic tropomyosin (7). Protein preparations were separated in a NaDodSO$_4$/13% polyacrylamide gel and stained with Coomassie brilliant blue. Lane A, Kc cell acetone/ethanol pellet used as starting material. Lanes B–F, 5-μl aliquots of the final sequentially obtained precipitates: pI 6.0 (lane B), pI 5.5 (lane C), pI 5.0 (lane D), pI 4.5 (lane E), and pI 4.0 (lane F). The arrow indicates the M_r 31,000 Kc cell protein tentatively identified as tropomyosin.

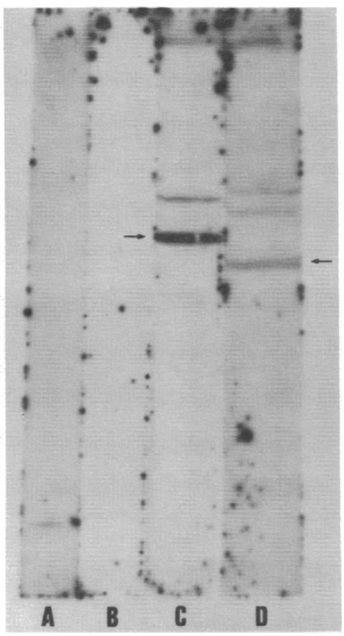

FIG. 4. Two-step antitropomyosin antibody-binding assay to *Drosophila* proteins. Autoradiogram of separate nitrocellulose filters incubated with unlabeled chicken smooth muscle antitropomyosin antiserum or control serum and ^{125}I-labeled goat anti-rabbit light-chain antibody. *Drosophila* proteins were separated in a NaDodSO$_4$/13% polyacrylamide gel before transfer. Protein preparations: lanes A, B, and D contain the Kc cell pH 4.5 isoelectric precipitate; lane C contains a partially purified myofibrillar protein preparation. Lane A, first incubation with normal rabbit serum; lane B, first incubation with no added serum; lanes C and D, first incubation with antitropomyosin antibody. Rightward pointing arrow indicates *Drosophila* muscle tropomyosins and leftward pointing arrow indicates Kc cell M_r 31,000 protein identified from duplicate stained filters.

trophoresis, and the gels were stained with Coomassie brilliant blue before fluorographic preparation. The results are shown in Fig. 5. Under these conditions, the cytoplasmic tropomyosin protein band from Kc cells consists of a single protein spot that comigrates with the region 3 hybrid-selected translation product (Fig. 5 *A* and *C*). These two proteins also comigrate in second-dimension gels containing 8 M urea, and they show the apparent increase in molecular weight when electrophoresed in urea that is characteristic of cytoplasmic and muscle tropomyosins (Fig. 5 *B* and *D*). The same results were obtained when the translation products of Kc cell RNA selected by the region 3 gene were used (data not shown). These results show that the hybrid-selected translation product of pVB85-1 DNA (region 3) is the same protein as the cytoplasmic tropomyosin obtained from Kc cells and that this *Drosophila* tropomyosin shows a characteristic mobility shift in the presence of 8 M urea.

The Kc cell pH 4.5 fraction was also mixed with the total *in vitro* translation products of unselected early embryo RNA and separated in two dimensions. A single [^{35}S]methionine-labeled translation product comigrated with the Kc cell tropomyosin (Fig. 5*E*). The fact that this translation product is not very abundant suggests that either the functional message is in low abundance in the RNA preparation or the message is not efficiently translated by the rabbit reticulocyte cell-free translation system.

pVB85-1 Cross-Hybridizes to Both Muscle Tropomyosin Genes Under Low Stringency Conditions. Because the cytoplasmic tropomyosin protein encoded by the region 3 gene is related to the muscle tropomyosins, we were interested in determining the relatedness of the genes at the DNA sequence

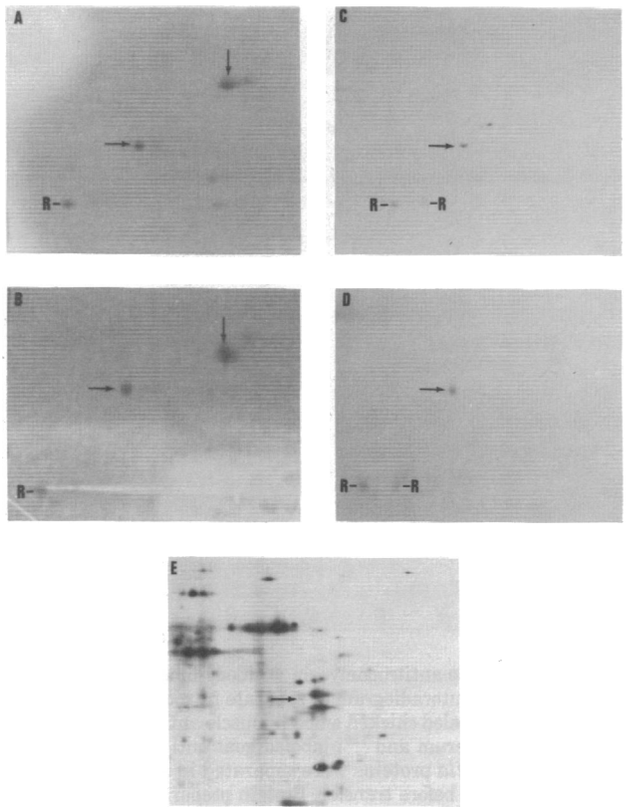

FIG. 5. Two-dimensional gel electrophoresis of Kc cell tropomyosin, hybrid-selected translation product of pVB85-1 DNA, and total *in vitro* translation products of 1- to 5-hr embryo poly(A)$^+$ RNA. Gels are pH 4–6 (basic to the left) in the isoelectric focusing dimension and $NaDodSO_4$/13% polyacrylamide with (*B* and *D*) or without (*A*, *C*, and *E*) 8 M urea in the molecular weight dimension. The *Drosophila* Kc cell pH 4.5 isoelectric precipitate was mixed with [^{35}S]methionine-labeled *in vitro* translation products of 1- to 5-hr embryo poly(A)$^+$ RNA selected by pVB85-1 DNA (*C* and *D*) or of total 1- to 5-hr embryo poly(A)$^+$ RNA (*E*) before separation. (*A* and *B*) Coomassie blue-stained gels of the mixed pI 4.5 precipitate and the hybrid-selected translation product of pVB85-1 DNA; (*C* and *D*) the corresponding fluorograms. (*E*) Fluorogram of gel containing total translation products of early embryo RNA. Horizontal arrows indicate Kc cell tropomyosin (*A* and *B*) and the *in vitro* translation product that comigrates under these conditions (*C*, *D*, and *E*). Vertical arrow in *A* and *B* is a Kc cell protein used as a relative mobility marker; R indicates polypeptides endogenous to the rabbit reticulocyte translation system.

level. Accordingly, plasmid DNAs pAS85-1 (muscle tropomyosin I gene) and pAS85-2 (muscle tropomyosin II gene) were digested with several different restriction endonucleases. The restriction fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters. Separate filters containing both plasmid DNAs were hybridized with ^{32}P-labeled pVB85-1 DNA (region 3 gene) under stringency conditions that were varied by changing the proportion of formamide in the hybridization buffer. Fig. 6 shows that, although vector DNA sequences cross-hybridize to homologous vector sequences in the probe under all conditions, the restriction endonuclease fragments of the *Drosophila* tropomyosin genes do not form hybrids in 60% formamide. Assuming an average G+C content of 40% for *Drosophila* DNA, it can be calculated that these stringency conditions allow for 15% base mismatch in the hybrids formed (lane F). As the stringency of hybridization is sequentially decreased to allow for up to 35% base mismatch (20% formamide), however, most of the tropomyosin I gene region restriction fragments form hybrids with the cytoplasmic tropomyosin gene probe, although the conditions under which the different fragments form hybrids vary (lanes B–E). The tropomyosin II gene also shows hybridization to the cytoplasmic tropomyosin gene probe, although the level of hybridization is significantly less than that observed for the tropomyosin I gene and is only observed under stringency conditions that tolerate 35% base mismatch (lane B). Although both restriction fragments derived from the tropomyosin II gene show hybridization to the probe under these conditions, the signal from the 0.88-kbp fragment is very weak and is only observed at long exposure times (data not shown).

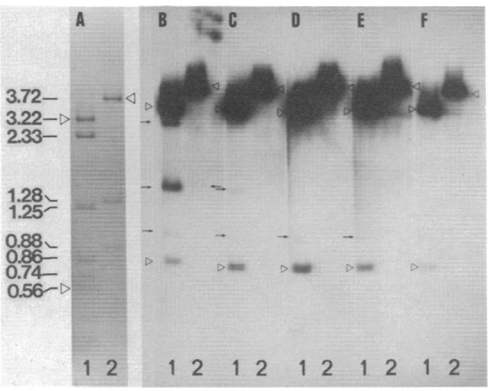

FIG. 6. Autoradiogram of filters containing muscle tropomyosin I and II restriction endonuclease-digested gene fragments subjected to decreased stringency hybridizations with a region 3 gene probe. Lanes 1 contain restriction endonuclease fragments of pAS85-1 DNA (muscle tropomyosin I gene) digested with *Bam*HI, *Bgl* I, *Pst* I, and *Hin*dIII; lanes 2 contain restriction endonuclease fragments of pAS85-2 DNA (muscle tropomyosin II gene) digested with *Hin*dIII, *Bam*HI, and *Sst* I. (*A*) Ethidium bromide-stained agarose gel before transfer. (*B–F*) Autoradiograms of separate nitrocellulose filters hybridized as described with 2.5×10^5 cpm of ^{32}P-labeled pVB85-1 DNA (region 3 gene) at 5×10^7 dpm/μg in 20% formamide (*B*), 30% formamide (*C*), 40% formamide (*D*), 50% formamide (*E*), or 60% formamide (*F*). Arrowheads indicate vector fragments, rightward pointing arrows indicate tropomyosin I gene fragments showing hybridization to the probe, leftward pointing arrow indicates tropomyosin II gene fragment showing hybridization to the probe. The restriction fragment sizes in kbp are in left margin and were determined using λ DNA digested with *Hin*dIII as size markers.

These results show that the DNA sequences in the two muscle tropomyosin genes cross-hybridize with the DNA sequences in the cytoplasmic tropomyosin gene under decreased stringency conditions. The differential hybridization of the filter-bound restriction endonuclease fragments to the cytoplasmic tropomyosin gene probe can be attributed to differing degrees of homology to the probe along the tropomyosin genes and to the fact that different fragments may contain different proportions of the tropomyosin genes.

DISCUSSION

We have identified a gene encoding a cytoplasmic form of tropomyosin in *Drosophila*. The *Drosophila* cytoplasmic tropomyosin gene encodes a protein in early embryos and Kc cells similar to vertebrate cytoplasmic tropomyosin. It has an electrophoretic mobility and isoelectric point similar to vertebrate cytoplasmic tropomyosin and was purified using conditions for purification of the vertebrate proteins. The *Drosophila* cytoplasmic tropomyosin also shows an increase in apparent molecular weight when electrophoresed in $NaDodSO_4$/urea/polyacrylamide gel, which is characteristic of vertebrate cytoplasmic tropomyosin.

The *Drosophila* cytoplasmic tropomyosin also crossreacts with a polyclonal chicken smooth muscle antitropomyosin antibody.

However, the *Drosophila* cytoplasmic tropomyosin shows less crossreactivity to the chicken antibody than do the muscle forms of tropomyosin. The chicken antibody also shows more crossreactivity with tropomyosins from rat REF-4A cells that are closer in size to muscle forms than with lower molecular weight forms of tropomyosin from the same cell line (21). Because the protein blotting technique used to assess binding probably destroys many of the conformational antigenic determinants, the relative degree of crossreactivity among the three *Drosophila* tropomyosins suggests that the two muscle forms are more homologous to each other at the amino acid sequence level than either is to the cytoplasmic form. This postulated relationship among the different *Drosophila* tropomyosins is analogous to the relationship of the different forms of vertebrate tropomyosins, which has been verified by amino acid sequence data (2, 9). The minor crossreactivity of the chicken antitropomyosin antibody with higher molecular weight proteins (M_r, 45,000–50,000) may represent crossreactivity with intermediate filament proteins. Intermediate filament proteins in *Drosophila* have electrophoretic mobilities in this range (22). The vertebrate intermediate-filament protein vimentin, which shares amino acid sequence homology with vertebrate tropomyosin, has also been shown to share antigenic determinants with tropomyosin (23, 24).

The *Drosophila* cytoplasmic tropomyosin gene is located close to the two previously identified muscle tropomyosin genes. The three genes are located within 18 kbp of genomic DNA, and hybridization of the subcloned genes to genomic DNA under decreased stringency conditions indicates that there are no other closely related tropomyosin sequences in the *Drosophila* genome (ref. 13; unpublished data). Therefore, in *Drosophila* the tropomyosins are encoded by three distinct genes that are closely linked. The tandem arrangement of the three tropomyosin genes suggests an evolutionary relationship among the members of this family of contractile protein genes. The fact that both muscle tropomyosin genes cross-hybridize with the cytoplasmic tropomyosin gene under decreased stringency conditions supports this hypothesis. The two muscle genes also cross-hybridize at decreased stringencies (unpublished results). Because sequences in the tropomyosin I gene region form hybrids with the cytoplasmic tropomyosin gene at higher stringencies than do the sequences in the tropomyosin II gene region, it is likely that the tropomyosin I gene is more homologous to the cytoplasmic tropomyosin gene at the DNA sequence level than is the tropomyosin II gene. The determination of the exact evolutionary relationship of the three *Drosophila* tropomyosin genes, however, must await further analysis.

The genomic organization of several multigene protein families in *Drosophila*, in which members are related structurally and functionally, is known. The chorion protein, cuticle protein, and yolk protein gene families each have a dispersed organization that includes subclusters of gene family members, whereas the actin, tubulin, and larval serum protein gene families have a totally dispersed organization (25–31). The organization of the tropomyosin multigene family is different from these gene families because all the *Drosophila* tropomyosin genes are clustered at a single locus. The clustered organization of the tropomyosin genes is more analogous to that of the small heat-shock protein genes in *Drosophila* (32). The evolutionary and regulatory implications of this clustered organization remain to be elucidated. However, the close proximity of three tropomyosin genes that exhibit both coordinate and differential expression suggests that the evolution of these genes is either a recent event or that there is a regulatory basis for this linkage.

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