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. BAUTC and ROBERT V. STORITI*

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

Communicated by Irving M. Klotz, August 18, 1983

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 (Mr, 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 cross-reactivity 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 NaDodSO4/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 (Mr, 34,000) and β-tropomyosin (Mr, 36,000) (3). Tropomyosin is also present in non-muscle cells (4–8). Most cytoplasmic tropomyosins are smaller than their muscle counterparts (Mr, 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.

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) BamHI restriction fragment of ADm85 into BamHI-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 HindIII/BamHI restriction fragment of ADm85.

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 125I to a speciﬁc activity of 107 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 ADm85 have been described (13). The 18 kbp of Drosophila DNA in ADm85 contain three transcribed regions (Fig. 1). Two of these regions (region I 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 [³⁵S]methionine-labeled hybrid-selected translation products were separated on one-dimensional NaDodSO₄/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 (Mr, 31,000) (Fig. 2, lanes A and B). This RNA is not selected by filters containing Escherichia coli DNA (lane C). The Mr 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 Mr 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 Mr 31,000 translation product is not very abundant (see Fig. 5E).

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/aceton Kc cell powder and equal volumes of the final sequentially obtained isoelectric precipitates were separated on a one-dimensional NaDodSO₄/polyacrylamide gel (Fig. 3). The precipitate collected at pH 5.0 was highly enriched with a Mr 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 pl 4.5 and pl 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 pl 4.5 precipitate from Kc cells were separated on one-dimensional NaDodSO₄/polyacrylamide gels and transferred to nitrocellulose filters. The pl 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 ³²P-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).

Conjugation 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 (Set 1, Pat 1, 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.
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. 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 [35S]methionine-labeled in vitro translation products were separated in a NaDodSO4/13% 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 (Mr, 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.](image2)

![Fig. 3. Coomassie blue-stained gel showing partial purification of Mr, 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 NaDodSO4/13% polyacrylamide gel and stained with Coomassie brilliant blue. Lane A, Kc cell acetone/ethanol pellet used as starting material. Lanes B-F, 5-

![Fig. 4. Two-step antitropomyosin antibody-binding assay to Drosophila proteins. Autoradiogram of separate nitrocellulose filters incubated with unlabeled chicken smooth muscle antitropomyosin anti-](image4)
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 32P-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 90% 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).

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 NaDodSO4/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 cross-reactivity with tropomyosins from rat REF-2A 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 (Mr, 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 kb 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.

We thank Dr. Jim Lin for providing the tropomyosin antibody, Dr. Paul Morris for providing Kc cells, and Dr. Steve Lobb for providing goat anti-rabbit IgG. We gratefully acknowledge the expert help of Drs. Steve Barclay and Alan Smith for the antibody-binding assay and Drs. Kay Knight and Ramesh Kumar for secondary-antibody labeling. This work was supported by National Institutes of Health Grants GM27611 and GM30181. R.V.S. is the recipient of a Research Career Development Award from the National Institutes of Health.