Tropomyosin Isoforms in Chicken Embryo Fibroblasts: Purification, Characterization, and Changes in Rous Sarcoma Virus-transformed Cells

JIM JUNG-CHING LIN,* DAVID M. HELFMAN,‡ STEPHAN H. HUGHES,‡ and CHIN-SHENG CHOU‡

Department of Biology, University of Iowa, Iowa City, Iowa 52242; and ‡Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724. Dr. Chou's present address is Department of Microbiology and Immunology, National Defense Medical Center, Taiwan, Republic of China.

ABSTRACT Seven polypeptides (a, b, c, 1, 2, 3a, and 3b) have been previously identified as tropomyosin isoforms in chicken embryo fibroblasts (CEF) (Lin, J. J.-C., Matsumura, F., and Yamashiro-Matsumura, S., 1984, *J. Cell. Biol.*, 98:116–127). Spots a and c had identical mobility on two-dimensional gels with the slow-migrating and fast-migrating components, respectively, of chicken gizzard tropomyosin. However, the remaining isoforms of CEF tropomyosin were distinct from chicken skeletal and cardiac tropomyosins on two-dimensional gels. The mixture of CEF tropomyosin has been isolated by the combination of Triton/glycerol extraction of monolayer cells, heat treatment, and ammonium sulfate fractionation. The yield of tropomyosin was estimated to be 1.4% of total CEF proteins. The identical set of tropomyosin isoforms could be found in the antitropomyosin immunoprecipitates after the cell-free translation products of total poly(A)$^+$ RNAs isolated from CEF cells. This suggested that at least seven mRNAs coding for these tropomyosin isoforms existed in the cell. Purified tropomyosins (particularly 1, 2, and 3) showed different actin-binding abilities in the presence of 100 mM KCl and no divalent cation. Under this condition, the binding of tropomyosin 3 (3a + 3b) to actin filaments was significantly weaker than that of tropomyosin 1 or 2. CEF tropomyosin 1, and probably 3, could be cross-linked to form homodimers by treatment with 5,5′-dithiobis-(2-nitrobenzoate), whereas tropomyosin a and c formed a heterodimer. These dimer species may reflect the in vivo assembly of tropomyosin isoforms, since dimer formation occurred not only with purified tropomyosin but also with microfilament-associated tropomyosin. The expression of these tropomyosin isoforms in Rous sarcoma virus-transformed CEF cells has also been investigated. In agreement with the previous report by Hendricks and Weintraub (*Proc. Natl. Acad. Sci. USA.*, 78:5633–5637), we found that major tropomyosin 1 was greatly reduced in transformed cells. We have also found that the relative amounts of tropomyosin 3a and 3b were increased in both the total cell lysate and the microfilament fraction of transformed cells. Because of the different actin-binding properties observed for CEF tropomyosins, changes in the expression of these isoforms may, in part, be responsible for the reduction of actin cables and the alteration of cell shape found in transformed cells.

Tropomyosin is a ubiquitous protein associated with the thin filaments of muscle cells and with microfilaments of nonmuscle cells. In striated muscles, tropomyosin together with the troponin complex regulates the actin–myosin interaction in a calcium-dependent manner (50, 54). However, as no troponin-like protein has been detected so far in smooth muscle or nonmuscle cells, tropomyosin in these cells may not function in this way. Nonmuscle forms of tropomyosin have been isolated from human platelets (8), chicken brain (19), calf brain, pancreas, and platelets (18), Physarum (28),

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sea urchin eggs (26), cultured mammalian cells (49), porcine platelets (11), equine platelets (10), and rabbit lung macrophages (16). The reported molecular weights of these nonmuscle tropomyosins range from 29,000 to 35,000. Recently, multiple forms (or isoforms) of tropomyosin have been detected within a single culture of nonmuscle cells (21, 24, 35, 49, 53). By using our microfilament isolation method (42), we have previously detected at least five isoforms of tropomyosin from rat, mouse, and human fibroblasts (38, 42), and seven isoforms of tropomyosin from chicken embryo fibroblasts (CEF)[1] (37). The function and assembly of these multiple forms of tropomyosin are not yet understood. However, different forms of tropomyosin may play different roles in stabilizing and protecting actin-microfilaments in nonmuscle cells (3, 16, 24, 35, 38, 41).

In this report, we have extended our studies on the significance of tropomyosin isoforms by purifying seven forms of tropomyosin from CEF and by characterizing them in terms of their actin-binding properties and dimer formation. Different actin-binding properties for major and minor isoforms of CEF tropomyosin were observed.

Changes in the expression of tropomyosin isoforms have been previously reported to occur in many types of transformed cells, including cells transformed by DNA or RNA viruses, by chemical carcinogens, or ultraviolet irradiation, or by transfection with tumor DNA (38, 41). The general phenomena in these changes is that at least one of the major tropomyosin isoforms with a higher apparent molecular weight appears to be decreased or missing, and the level of at least one of the tropomyosin isoforms with a lower apparent molecular weight tends to be increased in all types of transformed cells. In this report, we also studied tropomyosin alterations in transformed CEF cells. In addition to the reduction of major tropomyosin in Rous sarcoma virus (RSV)-transformed CEF cells reported by Hendricks and Weintraub (24), we found that the levels of minor tropomyosin are increased. This is in agreement with the general pattern of tropomyosin isoform changes in transformed cells that we have previously reported (38, 41).

MATERIALS AND METHODS

Cell Culture: Primary cultures of CEF were prepared from 10-d-old embryos as described previously (37). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air. High titer stocks of RSV were used to infect CEF cells. Morphological transformation of CEF cells was evident at 3–4 d after infection.

Purification of Tropomyosin Isoforms: Muscle tropomyosins were purified from chicken leg, heart, and gizzard by a modification of Bailey's method (2). Briefly, muscle (20 grams) was homogenized in 200 ml of 10 mM Tris, pH 8.0, 0.1 M KCl, 2 mM EGTA, and 2 mM dithiothreitol (DTT) at 4°C. After centrifugation at 10,000 *g* for 10 min, the pellets were extracted twice with 200 ml each of 10 mM Tris, pH 8.0, 1 M KCl, 2 mM EGTA, and 2 mM DTT for 1 h at 4°C. The extracts were collected by centrifugation at 10,000 *g* for 10 min and boiled for 10 min. After being cooled on ice, the precipitates were removed by centrifugation at 12,000 *g* for 10 min. The supernatants containing tropomyosin were fractionated by ammonium sulfate with a concentration between 28 and 36 g/per 100 ml of the supernatant. The final tropomyosins were dialyzed extensively against 10 mM Tris, pH 8.0, 0.2 mM DTT, and 2 mM EDTA.

The CEF tropomyosin mixture was purified by a method similar to that for the isolation of microfilaments from CEF cells (37). Briefly, monolayer cells (20 100-mm dishes) at confluence were treated with Triton/glycerol solution (0.05% Triton X-100 in 0.1 M PIPES, 5 mM $MgCl_2$, 0.2 mM EGTA, 4 M glycerol) for 2 min at room temperature. After being washed three times with PBS containing 5 mM $MgCl_2$ and 0.2 mM EGTA, the Triton/glycerol-insoluble residues were collected in a minimal volume (2–3 ml). ATP and phenylmethylsulfonyl fluoride were added to give a final concentration of 5 mM each. The Triton/glycerol-insoluble residues were homogenized by 30 strokes in a motor-driven (glass/Teflon) homogenizer (Wheaton Scientific, Millville, NJ) at a 3.2 setting. After centrifugation of the homogenates at 12,800 *g* for 15 min to remove most of the intermediate filaments and nuclei (Pellet-1), the supernatants (Sup-1) were heated at 100°C for 10 min. After being cooled on ice, the precipitates were removed by centrifugation at 12,800 *g* for 10 min. The final supernatants (Sup-2) containing tropomyosin mixture were kept frozen at −70°C and accumulated to a large volume (~30 ml). The pooled Sup-2 was fractionated and dialyzed by the method given above for muscle tropomyosin purification.

Purification of Rabbit Skeletal Muscle F-Actin and Actin-binding Assay: Acetone powder of rabbit skeletal muscle was prepared as described by Ebashi and Ebashi (13). Skeletal muscle actin was extracted from this acetone powder for 1 h at 0°C with 30 vol of cold water. After addition of 3 M KCl to a final concentration of 30 mM, actin was allowed to polymerize overnight at 4°C. F-actin was collected by centrifugation (100,000 *g*, 2 h) and treated with 0.6 M KCl according to the method of Spudich and Watt (51). Again, the actin pellet was collected by centrifugation (100,000 *g*, 2 h) and washed once with 0.1 M KCl. The final F-actin was suspended in a 0.1 M KCl solution at a concentration of ~5 mg/ml and used for the actin-binding assay.

The actin-binding assay was based on the co-sedimentation method (12, 25, 31) and performed on a Beckman airfuge rotor A-100/18 (Beckman Instruments, Inc., Palo Alto, CA). Actin (100 μg) and tropomyosin (17 μg) were mixed in 175 μl of 10 mM Tris, pH 8.0, 0.2 mM DTT with various amounts of Mg^{++} and KCl and incubated at room temperature for 30 min. The mixtures were centrifuged for 30 min in a Beckman airfuge at 26 psi to separate the bound and unbound tropomyosin. Aliquots of the supernatants and pellets were analyzed on 12.5% SDS polyacrylamide gels.

Preparation of Antibodies and Isolation of Microfilaments: Methods for preparing and characterizing rabbit antiserum against chicken gizzard tropomyosin were described in a previous report (42). This antiserum recognized all isoforms of tropomyosin from CEF cells. Antitropomyosin monoclonal antibody LCK16 was prepared and characterized as described previously (36).

As reported previously, ascites fluid of LCK16 antibody was suitable for the isolation of tropomyosin-enriched microfilaments from various cultured cells (37, 38, 41, 42). The tropomyosin-enriched microfilaments were isolated from normal and RSV-transformed CEF cells as described (37). The yield of isolated microfilaments was estimated from [^{35}S]methionine-labeled cells and expressed as the actin content. Cells on 100-mm dish were labeled in vivo for 15 h with 250 μCi of [^{35}S]methionine (1,100 Ci/mmol) in 3 ml of methionine-free Dulbecco's modified Eagle's medium containing 2.5% fetal calf serum and subjected to the isolation of the tropomyosin-enriched microfilaments. Aliquots of samples from each step of the isolation procedure were analyzed on 12.5% SDS polyacrylamide gels. After staining and destaining, gels were then dried on filter papers. The actin bands of each fraction were cut out and sliced to a 1-mm thickness. Each slice was immersed in 0.2 ml of 2% SDS for 24 h to elute the radiolabeled protein. After addition of 4 ml of Aquasol (New England Nuclear, Boston, MA), the radioactivity was measured in a liquid scintillation counter. The radioactivities of tropomyosin isoforms associated with the isolated microfilaments were also obtained by a similar method and used for calculating the ratios of tropomyosin isoforms to 100 parts of actin.

Dimer Formation of Tropomyosin Isoforms: The tropomyosin dimer was formed as a result of intramolecular cross-linking by oxidation of opposing cysteines in tropomyosin with 5,5′-dithiobis (2-nitrobenzoate) (DTNB) (6, 32). The 0.1 M stock solution of DTNB was prepared as described previously by Lehrer (32). Purified tropomyosins (0.2–0.5 mg/ml) or the isolated microfilaments in PBS that contained 5 mM Mg^{++} and 0.2 mM EGTA were reacted with 10 mM DTNB for 30 min at room temperature. After the reaction was complete, samples were solubilized in SDS gel sample buffer without reducing agents and analyzed on 12.5% SDS polyacrylamide gel. In order to identify which isoforms were capable of forming dimers, we cut out the gel track from a one-dimensional gel and placed it on the top of a new SDS polyacrylamide slab gel. This second dimension gel was run under reducing conditions by overlaying the gel track with SDS gel sample buffer containing 100 mM DTT and 1% agarose.

Other Biochemical Procedures: One-dimensional SDS PAGE was carried out according to Laemmli (30) with a low concentration of bisacrylamide (12.5% acrylamide and 0.104% bisacrylamide) (4). Two-dimen-

[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblasts; DTNB, 5,5′-dithiobis (2-nitrobenzoate); DTT, dithiothreitol; RSV, Rous sarcoma virus.

sional gel electrophoresis was performed as described by a modified procedure of O'Farrell (46). The first-dimensional gels contained 4% polyacrylamide and pH 4–6 ampholytes. The second-dimensional gels were 12.5% SDS polyacrylamide slab gels with a low concentration of bisacrylamide. For autoradiography and fluorography of the gel, a modified method of Bonner and Laskey (5) was performed as described previously (37). Protein concentration was determined by the method of Lowry et al. (39) with BSA as standard.

Total cellular RNA was prepared from CEF cells by a modification (17) of the quanidine/hot phenol method (7). Oligo (dT)-cellulose columns were used to prepare poly $(A)^+$ RNA from the total cellular RNA (1). Poly$(A)^+$ RNA was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (47). The in vitro translational products were solubilized in SDS gel sample buffer and analyzed by immunoprecipitation and by SDS PAGE. Immunoprecipitation with rabbit antiserum against tropomyosin was carried out as described previously (42) in the buffer containing 50 mM Tris, pH 8.0, 0.05% SDS, 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 mM NaCl.

RESULTS

Tropomyosin Isoforms from CEF

The procedure used here for the isolation of CEF tropomyosin is similar to the microfilament isolation method we previously described (37, 42). It should be applicable to the isolation of tropomyosin from a variety of different cultured cells from which the initial materials for purification are relatively limited.

As Table I shows, ~20% of the total protein was removed by Triton/glycerol extraction of monolayer cells. However, as judged by SDS PAGE, no significant amounts of tropomyosin were extracted from CEF cells by this Triton/glycerol solution (containing 0.05% Triton X-100). The Triton/glycerol-insoluble residues were homogenized in 5 mM Mg^{++}-ATP to disperse the microfilaments. After low-speed centrifugation (12,800 *g* for 15 min), ~30% of the total protein remained in the supernatant (Sup-1). The pellet (pellet-1) contained major proteins for the intermediate filaments and nuclei (Fig. 1, lane *C*). Very little tropomyosin was lost in this pellet-1 fraction, as judged by gel analysis. Heat treatment (100°C, 10 min) of Sup-1 precipitated many contaminant proteins and left tropomyosin in the soluble fraction (Sup-2). Tropomyosin was greatly enriched in this Sup-2 fraction (Fig. 1, Lane *D*), which contained 9% of the total protein. Tropomyosin in the Sup-2 was further fractionated into the fraction between 28 and 36 grams of ammonium sulfate/100 ml of the Sup-2. A substantial amount of tropomyosin (120 μg) with a purity >90% was obtained from twenty 100-mm culture dishes (Table I and Fig. 1, lanes *G* and *H*). Therefore, the amount of tropomyosin obtained was estimated as 1.4% of total proteins after correcting for impurities.

Tropomyosin purified from CEF cells migrated as five components on one-dimensional SDS polyacrylamide gels (Fig. 1, lanes *G* and *H* and Fig. 2*A*, lane *CEF*). These tropomyosin isoforms, which we have called a, b, 1, 2, and 3 (37), had relative molecular weights of 45,000, 43,000, 38,000, 36,500, and 32,800, respectively. Tropomyosin 1 and 2 were considered the major forms. These differed from tropomyosin isoforms purified from chicken leg muscle, gizzard, and heart in their mobilities on SDS polyacrylamide gel (Fig. 2*A*). Tropomyosin a, b, and 3 were relatively minor forms. Tropomyosin 3 was further separated into 2 components (3a and 3b) on two-dimensional gels (Fig. 2*B*). In addition, another component, tropomyosin c, was distinguished from tropomyosin 1 on two-dimensional gels. In the previous report (37), we provided evidence demonstrating that these components (a, b, c, 1, 2, 3a, and 3b) were tropomyosin isoforms:

TABLE I

Total Protein Distribution during Purification of Tropomyosin from CEF Cells

Fraction	Total protein	Percent of total
	mg	
Triton/glycerol extract	1.5	19.0
Triton/glycerol-insoluble residues	6.4	81.0
Sup-1	2.4	30.4
Pellet-1	3.0	38.0
Sup-2	0.7	8.9
28–36% ammonium sulfate	0.12	1.5

The total protein recovered in each fraction during purification was calculated for the initial contents of twenty 100-mm dishes of confluent CEF culture.

(*a*) they were immunologically cross-reacting with both polyclonal and monoclonal antibodies against gizzard tropomyosin; (*b*) they contained neither tryptophan nor proline; (*c*) they were associated with the actin-containing microfilaments; and (*d*) they were resistant to heat denaturation (100°C, 10 min). By performing two-dimensional gel analyses with mixed tropomyosin samples, we were able to compare the mobilities of tropomyosin isoforms from chicken skeletal muscle, smooth muscle (gizzard), cardiac muscle, and CEF cells. Fig. 3 shows a schematic drawing of tropomyosin isoforms from chicken on two-dimensional gels. The mobilities of skeletal muscle tropomyosin (*α-CL* and *β-CL*) and cardiac tropomyosin (*CH*) were different from those of CEF tropomyosins, whereas the slow- and fast-migrating components of gizzard tropomyosin appear to have identical mobilities to tropomyosin a and c, respectively. Therefore, at least 10 tropomyosin isoforms could be detected from chicken material by our gel system. It should be mentioned that the phosphorylated variants of skeletal muscle tropomyosin and cardiac muscle tropomyosin, as reported by Montarras et al. (44, 45), were not included here.

To examine whether any of the CEF tropomyosin isoforms have resulted from the posttranslational modification of any other, we carried out experiments including [^{32}P]phosphate labeling, cell-free translation, and pulse-chase labeling. Analyses of the immunoprecipitates of ^{32}P-labeled CEF cell extract with antitropomyosin antiserum and the microfilaments isolated from ^{32}P-labeled cells have led us to suggest that CEF tropomyosin isoforms most likely contain no phosphate (data not shown). As Fig. 4 shows, rabbit antigizzard tropomyosin antiserum is able to precipitate five protein bands from the in vitro translational products of total poly$(A)^+$ RNA isolated from CEF cells. These five protein bands produced a pattern on a two-dimensional gel identical to that obtained either from the purified CEF tropomyosin or from the tropomyosin associated with microfilaments (data not shown). Thus, at least seven mRNAs are present for these tropomyosin isoforms in CEF cells. Furthermore, the result from pulse-chase experiments showed that the rates of synthesis of the tropomyosin isoforms were very similar (37), suggesting no precursor–product relationships.

Characterization of CEF Tropomyosin Isoforms

ACTIN-BINDING ABILITY: All binding experiments were carried out at a molar ratio of CEF tropomyosin to rabbit skeletal F-actin of 1:9 in order to obtain complete binding. This ratio was chosen based on the results (*a*) from in vitro binding assays, in which the molar ratios of muscle and

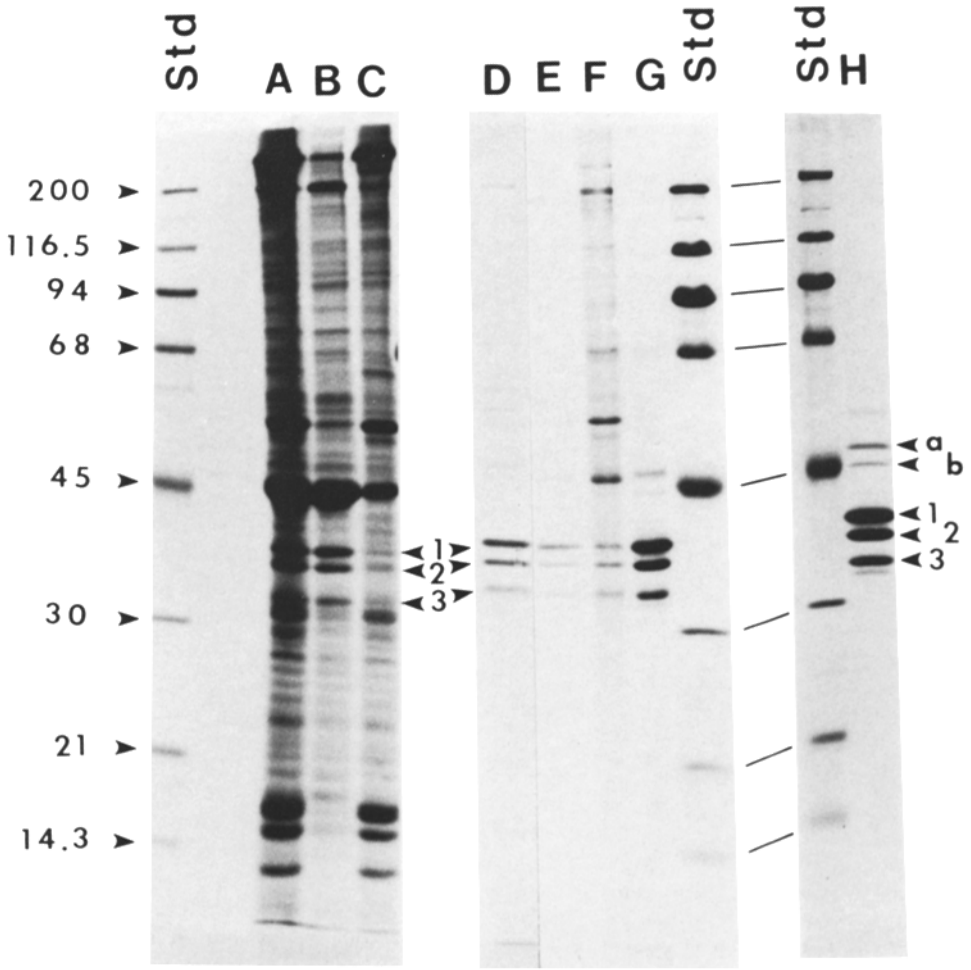

FIGURE 1 SDS polyacrylamide gels that show the protein profiles of various fractions during the purification of CEF tropomyosin. (*A*) Homogenate of Triton/glycerol-insoluble residues, 34 μg; (*B*) Sup-1, the supernatant fraction after the removal of nuclei and intermediate filaments from the homogenate by low speed centrifugation (12,800 *g*, 15 min), 12 μg; (*C*) pellet-1, the precipitate of the homogenate by low speed centrifugation, 15 μg; (*D*) Sup-2, the supernatant fraction after the removal of heat-denatured (100°C, 10 min) proteins from Sup-1 by low-speed centrifugation (12,800 *g*, 10 min), 2 μg; (*E*) the supernatant after fractionation of Sup-2 with 36 grams of ammonium sulfate/100 ml, 1.2 μg; (*F*) the pellet after fractionation of Sup-2 with 28 grams of ammonium sulfate/100 ml, 3.2 μg; (*G* and *H*) 28–36% ammonium sulfate, the fraction between 28 and 36 grams of ammonium sulfate/100 ml of Sup-2 solution, 3.5 μg in *G*, 7.5 μg in *H*; they were obtained from different preparations. (*Std*) Molecular weight marker proteins ($M_r \times 10^{-3}$). CEF tropomyosin isoforms are indicated by *a*, *b*, *1*, *2*, and *3*.

nonmuscle tropomyosin to actin are 1:7 and 1:6, respectively, at the maximal binding condition; and (*b*) from the previous analysis of the tropomyosin-enriched microfilaments isolated from CEF cells, in which the molar ratio is 1:7–8 (37). Thus, the 1:9 ratio should give complete binding. After centrifugation, the bound tropomyosin should co-sediment with F-actin filaments in the pellet fraction (Fig. 5, lanes *B*, *D*, *F*, *H*, *J*, and *L*) whereas the unbound tropomyosin should remain in the supernatants (Fig. 5, lanes *A*, *C*, *E*, *G*, *I*, and *K*). When purified tropomyosin was mixed with actin at a low salt concentration (30 mM KCl) in the absence of divalent cations, none of tropomyosin isoforms was able to bind to F-actin (Fig. 5, lanes *A* and *B*), as was expected for skeletal muscle tropomyosin (9, 55–57). However, in the presence of 5 mM Mg^{++}, ~95 and 100% binding of tropomyosins 1 and 2 (estimated from the staining intensity of tropomyosin in supernatant and pellet on the gel), respectively, were observed, whereas the binding of tropomyosin 3 to F-actin reached only ~75% (Fig. 5, lanes *C* and *D*). When the concentration of Mg^{++} in the binding solution was increased to 10 mM, the binding of tropomyosins 1, 2, and 3 was nearly complete (Fig. 5, lanes *E* and *F*). However, the increased amounts of tropomyosin in the pellet were not due to a simple precipitation of tropomyosin by Mg^{++}, because there was no precipitation of tropomyosin at 10 mM Mg^{++} (Fig. 5, lanes *G* and *H*). Moreover, when the binding experiment was performed at 100 mM KCl in the absence of Mg^{++}, the extent of binding for tropomyosins 1 and 2 appeared to be greater than that for tropomyosin 3 (Fig. 5, lanes *I* and *J*). At 100 mM KCl and 5 mM Mg^{++}, the binding of tropomyosins 1 and 2 reached maximal, while only ~50% of tropomyosin 3 was bound to F-actin (in Fig. 5, lanes *K* and *L*). Therefore, it appears that the actin-binding property of tropomyosin 3 is quite different from that of tropomyosins 1 and 2. As reported for platelet tropomyosin (9), troponin I also stimulated the binding of tropomyosins 1, 2, and 3 to F-actin (data not shown).

DIMER FORMATION BY TROPOMYOSIN ISOFORMS: It has been shown that rabbit skeletal tropomyosin in the native state can be intramolecularly cross-linked by DTNB or by air oxidation in the presence of Cu^{++}. The results suggest that

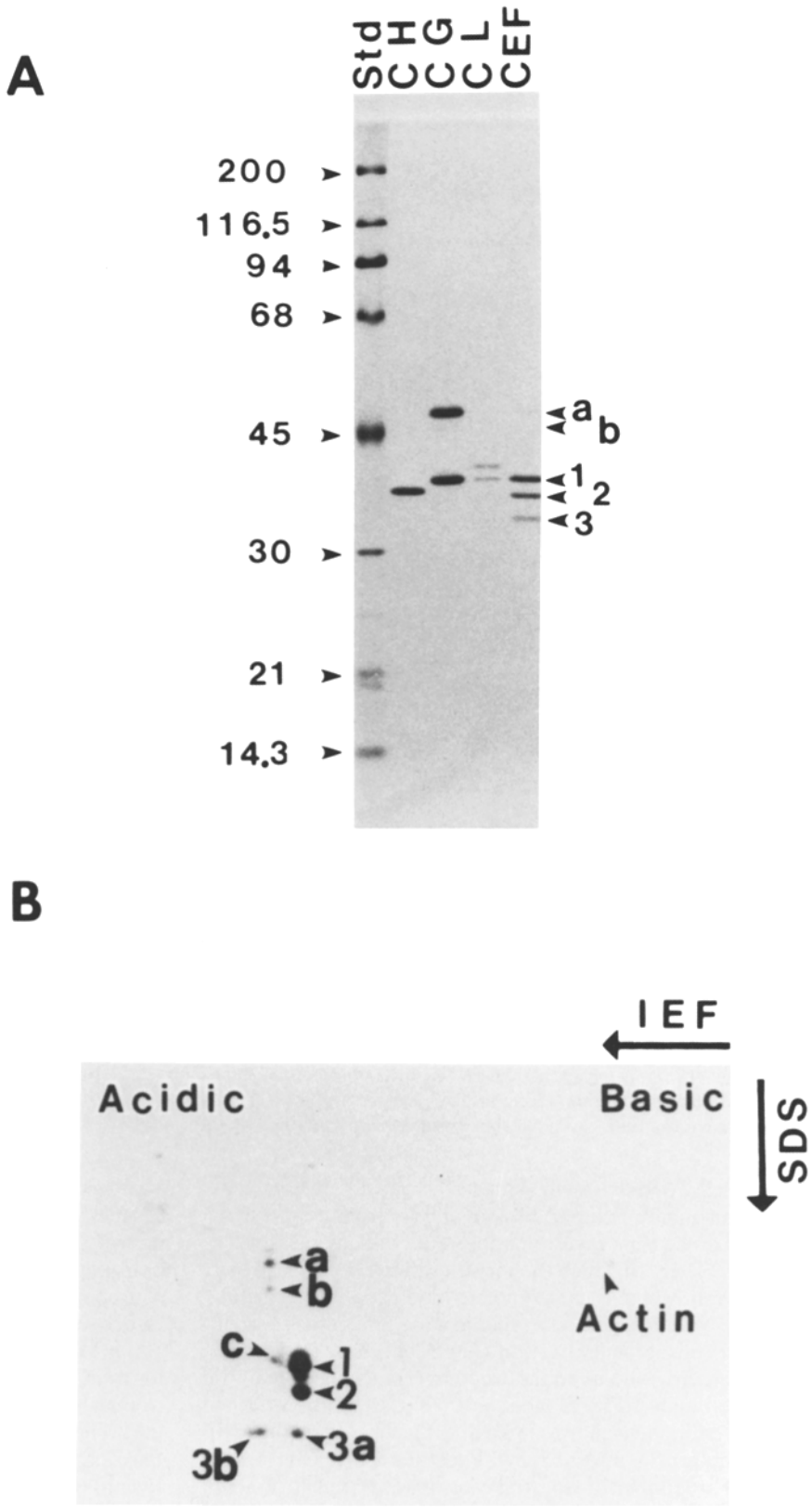

FIGURE 2 One- and two-dimensional gel analyses of tropomyosin isoforms purified from chicken. (*A*) Comparison of CEF tropomyosin isoforms (*CEF*) with muscle isoforms purified from chicken heart (*CH*), gizzard (*CG*), and leg (*CL*). (*B*) CEF tropomyosin isoforms are resolved into seven polypeptides, i.e., a, b, c, 1, 2, 3a, and 3b on the two-dimensional gel with pH 4–6 ampholytes for the first dimension (*IEF*, isoelectric focusing) and 12.5% SDS polyacrylamide for the second dimension. For unknown reasons, tropomyosins a, b, and 1 are usually found to split into two molecular weight variants on two-dimensional gels.

the tropomyosin dimer is arranged in register (27, 32, 52). Further analysis of the distribution of tropomyosin dimers in several muscle fibers revealed that tropomyosin dimers did not assemble randomly from α and β isoforms, but rather that $\alpha\beta$ was assembled preferentially in vivo (6). It was, therefore, of interest to find out what types of tropomyosin dimers, i.e., homodimers or heterodimers, could be formed in CEF cells. The information obtained could be useful in understanding the assembly of CEF tropomyosin. Therefore, we performed the cross-linking reaction both on purified CEF

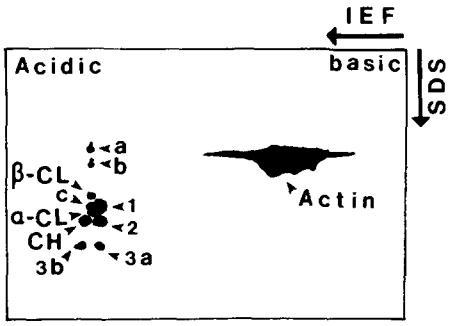

FIGURE 3 A schematic representation of the tropomyosin isoforms from chicken on two-dimensional gels. *α-CL* and *β-CL*, skeletal tropomyosin isoforms purified from chicken leg muscle; *CH*, cardiac tropomyosin isoform purified from chicken heart muscle; *a* and *c*, smooth muscle tropomyosin isoforms purified from gizzard; *a*, *b*, *c*, *1*, *2*, *3a*, and *3b*, tropomyosin isoforms purified from CEF cells.

tropomyosin and on microfilaments by the DTNB method. In contrast to air oxidation, the DTNB method has been shown to be an efficient, reproducible, and convenient method to cross-link tropomyosin into dimers (6, 32). The cross-linked samples were solubilized in the gel sample buffer without reducing agents and then analyzed on 12.5% SDS polyacrylamide gels. As shown in Fig. 6, CEF tropomyosins formed at least three species of dimers, although some of the isoforms still remained in the monomer state (lanes *4* and *5*). Under the same conditions for cross-linking, both chicken cardiac and leg tropomyosin were completely dimerized (Fig. 6, lanes *1* and *2*). On the other hand, only part of the gizzard tropomyosin was in dimer form (Fig. 6, lane *3* and Fig. 7*C*). The failure of the gizzard tropomyosin to cross-link well by the DTNB method has also been reported recently (34). The amino acid sequence derived from the nucleotide sequence of a cDNA clone containing coding region for the fast component of gizzard tropomyosin (Helfman, D. M., J. R. Feramisco, W. M. Ricci, and S. H. Hughes, manuscript in preparation) indicated that this gizzard tropomyosin did not contain cysteine residue at the position 190, which is important for the dimer formation. In striated tropomyosin, the localized chain separation occurred in a region containing cysteine residue at position 190. This is presumably the basis for the dimer formation (34). Furthermore, the apparent molecular weights of the cross-linked tropomyosins do not correspond to those expected. This may be due to the abnormal mobility of tropomyosins on SDS polyacrylamide gels.

To identify which tropomyosin isoforms formed which dimers, we subjected the cross-linked samples to two-dimensional SDS-PAGE with the first dimension run under oxidized conditions and the second dimension run under the reduced conditions. Any proteins that did not form a dimer should migrate along the diagonal of the two-dimensional gel. The dimers present in the first dimension of the gel should be reduced to their monomer components off the diagonal line of the two-dimensional gel. Figs. 7 and 8 show the results of this type of analysis. In Fig. 7*A*, dimer species of β_2, α_2, and $\alpha\beta$ can be readily identified from the cross-linked sample of chicken leg tropomyosin. Because only one isoform of tropomyosin was found in chicken cardiac muscle, the dimer species formed should be α_2 (Fig. 7*B*). In the case of gizzard tropomyosin, the fast-migrating (*f*) and slow-migrating (*s*) components formed the dimer species of f_2, sf, and s_2 (Fig. 7*C*). As Fig. 8 shows, CEF tropomyosin 1 formed a homo-

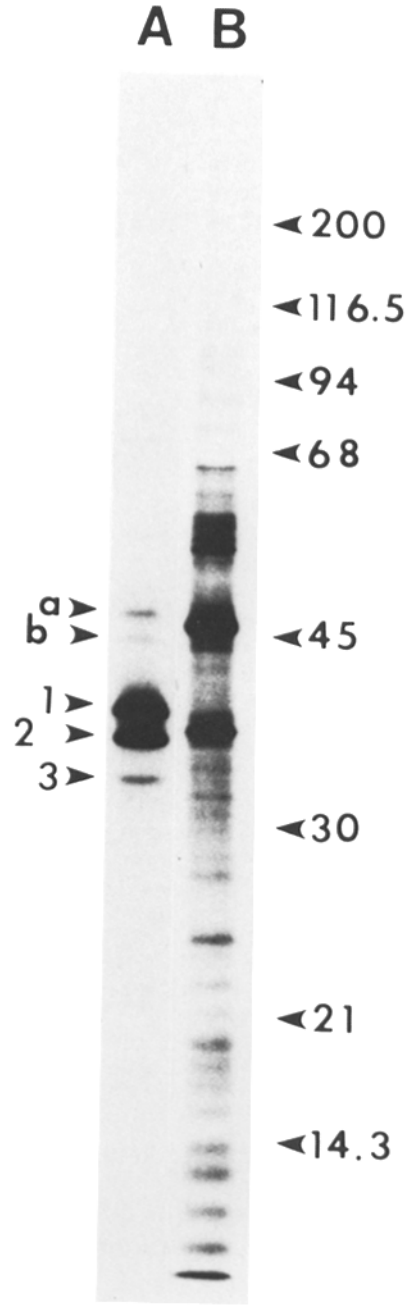

FIGURE 4 SDS polyacrylamide gel analysis of the immunoprecipitate of cell-free translation products of CEF total poly(A)$^+$ RNA with rabbit antiserum against gizzard tropomyosin. Translational products were labeled with [^{35}S]methionine and analyzed on 12.5% SDS polyacrylamide gels. After electrophoresis, the gels were processed for fluorography. (*A*), The immunoprecipitate; (*B*) total cell-free translational products. CEF tropomyosin isoforms are indicated.

dimer, although some was left in the monomer state. Tropomyosin 3 also formed a dimer. At the present time, we do not know whether tropomyosin 3a or 3b formed individual homodimers or both of them formed a heterodimer. Another dimer species was also formed by tropomyosin a and c. The evidence that indicates that tropomyosin c rather than 1 is involved in the formation of this dimer is indirect. Namely, the molecular weight of this dimer species was identical to that of the sf dimer formed from gizzard tropomyosin and tropomyosin a and c appear to be identical to the s and f of gizzard tropomyosin. Tropomyosin 2, either from the purified

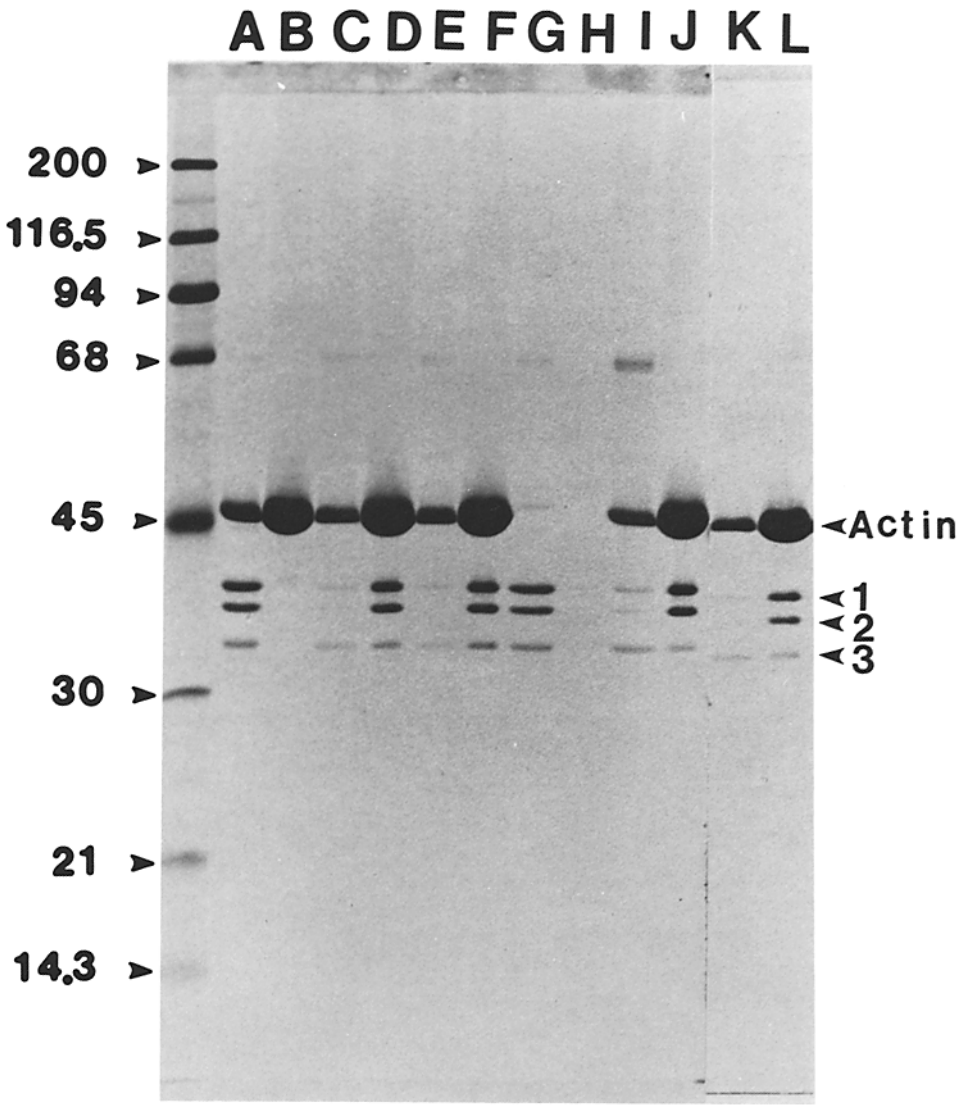

FIGURE 5 Tests for the actin-binding ability of CEF tropomyosin isoforms. Actin and tropomyosin were mixed in a buffer solution of 10 mM Tris, pH 8.0, and 0.2 mM DTT, containing either (*A* and *B*) 30 mM KCl, no Mg^{++}, (*C* and *D*) 30 mM KCl, 5 mM Mg^{++}, (*E* and *F*) 30 mM KCl, 10 mM Mg^{++}, (*I* and *J*) 100 mM KCl, no Mg^{++}, or (*K* and *L*) 100 mM KCl, 5 mM Mg^{++}. The sample for lanes *G* and *H* was tropomyosin alone at 30 mM KCl and 10 mM Mg^{++}. After incubation for 30 min at room temperature, the protein samples were centrifuged for 30 min in a Beckman airfuge rotor A-100/18 at 26 psi. Aliquots of the resulting supernatants (lanes *A*, *C*, *E*, *G*, *I*, and *K*) and pellets (lanes *B*, *D*, *F*, *H*, *J*, and *L*) were analyzed on 12.5% SDS polyacrylamide gels. CEF tropomyosins are indicated by *1*, *2*, and *3*. Tropomyosins a and b cannot be distinguished from the actin band in this experiment.

tropomyosin fraction or from the isolated microfilament fraction, did not form any dimer.

Changes in Tropomyosin Isoform Expression in RSV-transformed CEF Cells

We have isolated the tropomyosin-enriched microfilaments from normal and RSV-transformed CEF cells and analyzed their protein components on one- and two-dimensional gels. Fig. 9 shows the protein profiles of the isolated microfilaments from normal and RSV-transformed CEF cells. In agreement with the report by Hendricks and Weintraub (24), tropomyosins 1 and 2 were found to be decreased in the microfilaments isolated from RSV-transformed cells (Fig. 9, lane *1*), when compared with that from normal CEF cells (Fig. 9, lane *2*). The relative decrease in tropomyosins 1 and 2 was more obvious in the Coomassie Blue-stained gel (Fig. 9*B*), suggesting that the steady-state levels of tropomyosin 1 and 2 were reduced. However, in the autoradiogram (Fig. 9*A*), the level of tropomyosin 1 was also decreased, although there was no apparent change in the amount of tropomyosin 2. In contrast to these decreases, tropomyosin 3 was found to be greatly increased in the microfilaments of RSV-transformed cells. To quantitatively examine these changes in tropomyosin isoform expression upon transformation of CEF cells, we estimated ratios of tropomyosins 1, 2, and 3 to 100 parts of actin by measuring radioactivities of [^{35}S]methionine incorporated in each protein in isolated microfilaments as described in Materials and Methods. As can be seen in Table II, normal CEF cell microfilaments have 8.9 parts of tropomyosin 1, 6.7 parts of tropomyosin 2, and 2.0 parts of tropomyosin 3 to 100 parts of actin. Upon transformation by RSV, the level of tropomyosin 1 was decreased more than 2.5 times, but the level of tropomyosin 3 was increased threefold. It should be noted that the recovery of microfilaments from RSV-transformed cells was less than that from normal CEF cells. Approximately 30 and 11% of the total cell actin was recovered in the microfilaments isolated from normal and transformed cells, respectively. As estimated by two-dimensional gel analysis, the microfilaments isolated from both normal and transformed cells contained >90% of the total cell tropomyosins. Therefore, the total amount of tropomyosin in transformed cell was reduced significantly.

To examine more completely the changes in tropomyosin isoforms upon transformation, we analyzed the isolated microfilaments on two-dimensional gels to resolve all forms of tropomyosin. As shown in Fig. 10*B*, in addition to the decrease in the amounts of tropomyosin 1 and 2, tropomyosins

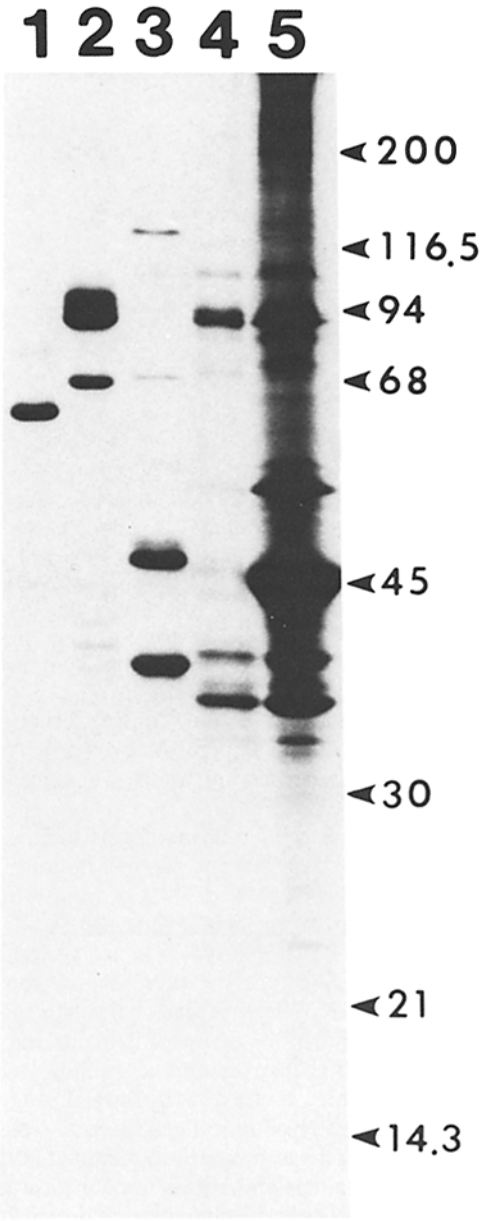

FIGURE 6 SDS polyacrylamide gel analysis of cross-linked samples. Tropomyosins purified from chicken heart (lane *1*), leg (lane *2*), gizzard (lane *3*), and CEF cells (lane *4*), and the microfilaments isolated from CEF cells (lane *5*) were intramolecularly cross-linked by oxidation of opposing cysteines with DTNB. The cross-linked samples were solubilized in gel sample buffer without the reducing agents and analyzed on 12.5% SDS polyacrylamide gels.

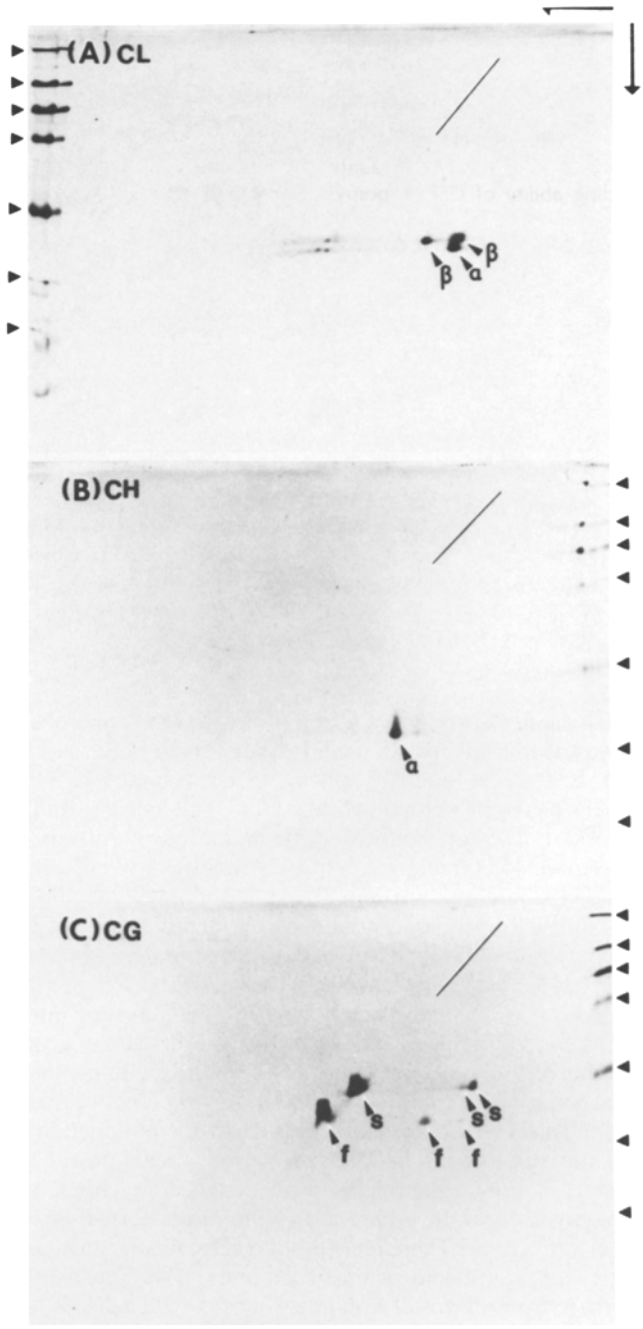

FIGURE 7 Identification of dimer species formed from chicken muscle tropomyosin by modified two-dimensional gel electrophoresis. Tropomyosin purified from chicken leg (*A*), heart (*B*), and gizzard (C) was intramolecularly cross-linked by oxidation of opposing cysteines with DTNB. The cross-linked samples were then subjected to 12.5% SDS polyacrylamide gel electrophoresis under the oxidized condition as the first dimension (from right to left). 12.5% SDS PAGE in the second dimension (from top to bottom) was performed under the reduced condition. Proteins having formed dimers would be expected to run off the diagonal line (line in each gel) after the reduction in the second dimension. Arrowheads indicate the positions for the molecular weight markers. The molecular weight marker proteins are identical to those used in Fig. 1. α and β in *A* indicate α- and β-tropomyosin of chicken skeletal muscle. α in *B* indicates α-tropomyosin of cardiac muscle. *s* and *f* in C indicate the slow- and fast-migrating components of gizzard tropomyosin.

a, b, and c appear to be undetectable in the transformed cell microfilaments. However, both tropomyosin 3a and 3b are greatly increased. The identical pattern of changes in the tropomyosin isoforms were also found in the total lysates of transformed cells (data not shown).

DISCUSSION

CEF tropomyosin has been identified and isolated to a high degree of purity. The purification procedure described here is suitable for purifying tropomyosin from various types of cultured cells. Taking into account the impurities after the final step of purification, we estimated that the amounts of

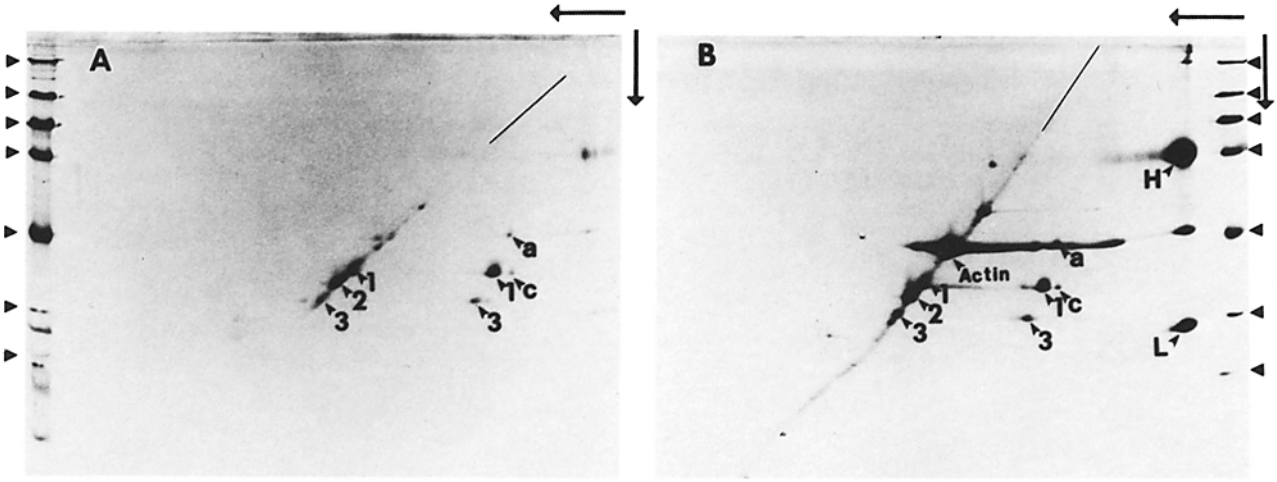

FIGURE 8 Identification of dimer species formed from CEF tropomyosin by modified two-dimensional gel electrophoresis. CEF tropomyosin (*A*) and the isolated microfilaments (*B*) were intramolecularly cross-linked by oxidation of opposing cysteines with DTNB. The cross-linked samples were then subjected to a modified two-dimensional gel electrophoresis, as described in the legend of Fig. 7. Proteins that have formed dimers would be expected to run off the diagonal line (line in each gel). CEF tropomyosin isoforms are indicated. *H* and *L* indicate the heavy and light chains of the LCK16 IgM monoclonal antibody. Arrowheads indicate the positions of the molecular weight markers ($M_r \times 10^{-3}$). The molecular weight marker proteins are identical to those used in Fig. 1.

CEF tropomyosin obtained were 1.4% of the total cell protein. The purified tropomyosin is composed of seven isoforms, i.e., a, b, c, 1, 2, 3a, and 3b, which can be separated by two-dimensional gels. Tropomyosins 1 (38,000 mol wt) and 2 (36,500 mol wt) are considered the major forms, and tropomyosins a (45,000 mol wt), b (43,000 mol wt), c (38,000 mol wt), 3a (32,800 mol wt), and 3b (32,800 mol wt) are considered minor forms. Earlier studies on the identification of CEF tropomyosin with rabbit antiserum against muscle tropomyosin have detected two proteins with apparent molecular weights of 35,000 and 33,000 (24, 40). These are equivalent to tropomyosins 1 and 2 described here. The lack of cross-reaction of antibodies used by these groups might hinder them from detecting the minor forms of tropomyosin. In the present and previous studies, we have carried out the purification of tropomyosin and the isolation of microfilaments from CEF cells (37). Both experiments were designed to enrich the tropomyosin isoforms, particularly the minor forms, in the isolated fractions. Therefore, with the help of polyclonal and monoclonal antibodies against tropomyosin, we were able to detect seven isoforms of tropomyosin from CEF cells.

The results from cell-free translation of total CEF poly(A)$^+$ RNAs, pulse-chase labeling experiments, and [^{32}P]phosphate labeling experiments strongly argue against the possibility that CEF tropomyosin isoforms resulted simply from posttranslational modifications. Furthermore, the cell-free translation results suggest that at least seven mRNAs are present for these multiple forms of tropomyosin. Whether these tropomyosin isoforms represent the products of different genes remains to be determined.

Tropomyosins a and c exhibit mobilities on two-dimensional gels that are identical to the slow- and fast-migrating components of gizzard tropomyosin. This may indicate contamination by a small population of smooth muscle cells in our CEF preparation. Alternatively, CEF cells prepared here may indeed express trace, but significant, amounts of gizzard forms of tropomyosin. At the present time, we are unable to distinguish between these two possibilities. However, because neither skeletal muscle nor cardiac muscle forms of tropomyosin can be detected in CEF tropomyosin, it is unlikely that there was contamination of striated muscle cells in our CEF preparation.

In addition to CEF cells, multiple forms of tropomyosin have also been found in many cultured nonmuscle cells, including human fibroblasts (21, 38, 53), mouse fibroblasts (38, 49), and rat embryo cells (41, 49). The significance of these multiple forms of tropomyosin is not yet understood. However, it is possible that the presence of tropomyosin isoforms in a cell may reflect regulatory mechanisms for the numerous discrete functions of microfilaments, such as the maintenance of cell shape, membrane ruffling, cell movement, and formation of cleavage furrows during mitosis. Knowledge concerning the intracellular assembly (or localization) and the biochemical properties of individual isoforms may allow us to evaluate this regulatory function of tropomyosin. To this end, two experiments, i.e., dimer formation and the actin-binding abilities of tropomyosin isoforms, have been carried out in the present report.

Analysis of tropomyosin dimer species reveals that tropomyosin 1, and probably 3, can form individual homodimers by the oxidation of opposing cysteine residues on the molecule, whereas under the same conditions, tropomyosins a and c form a heterodimer. The identical dimer species were obtained from the oxidation of both the purified tropomyosin fraction and the isolated microfilament fraction. In the latter fraction, tropomyosin isoforms are associated with native actin filaments (38). Therefore, it is likely that homodimers formed by tropomyosin 1 or 3 and heterodimers formed by tropomyosins a and c do indeed reflect the in vivo assembly of these isoforms. However, although both cardiac and skeletal muscle tropomyosins form dimers completely under the condition used for oxidation, there are some populations of tropomyosins 1 and 3 and all of tropomyosin 2 that remain in their monomer state. One possible explanation is that either there are no cysteine residues on these tropomyosins or cysteine residues are already oxidized during isolation. Alterna-

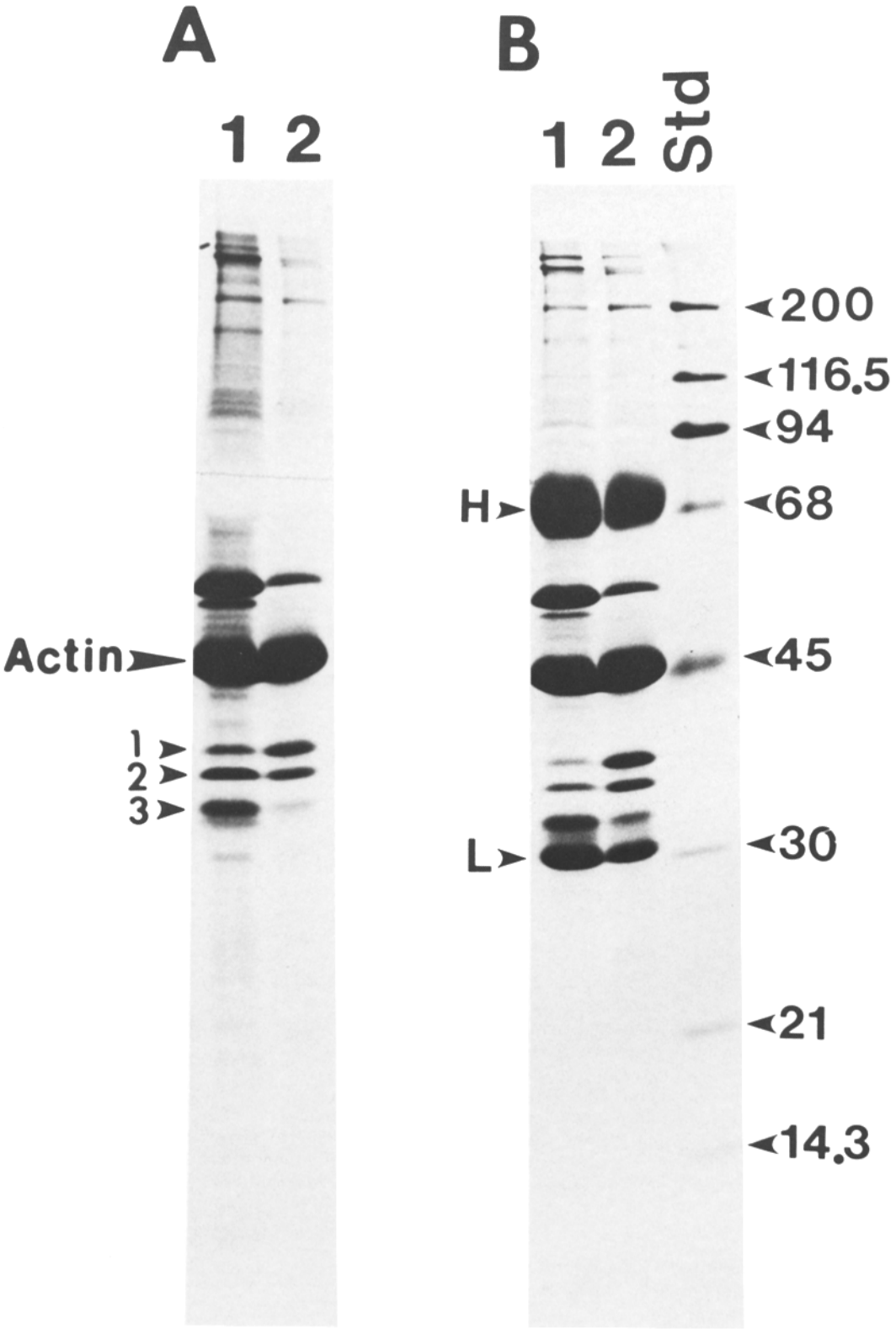

FIGURE 9 SDS polyacrylamide gel analysis of the microfilaments isolated from normal and RSV-transformed CEF cells. Normal and RSV-transformed CEF cells were labeled with [^{35}S]methionine and subjected to microfilament isolation. An equivalent amount of each microfilament were analyzed on 12.5% SDS polyacrylamide gels. (*A*) Autoradiogram; (*B*) Coomassie Blue-stained gel profile. (Lanes *1*) The microfilaments from RSV-transformed CEF cells; (*lanes 2*) the microfilaments from normal CEF cells. CEF tropomyosin isoforms are indicated by *1*, *2*, and *3*. *H* and *L* indicate the heavy and light chains of LCK16 IgM antibody.

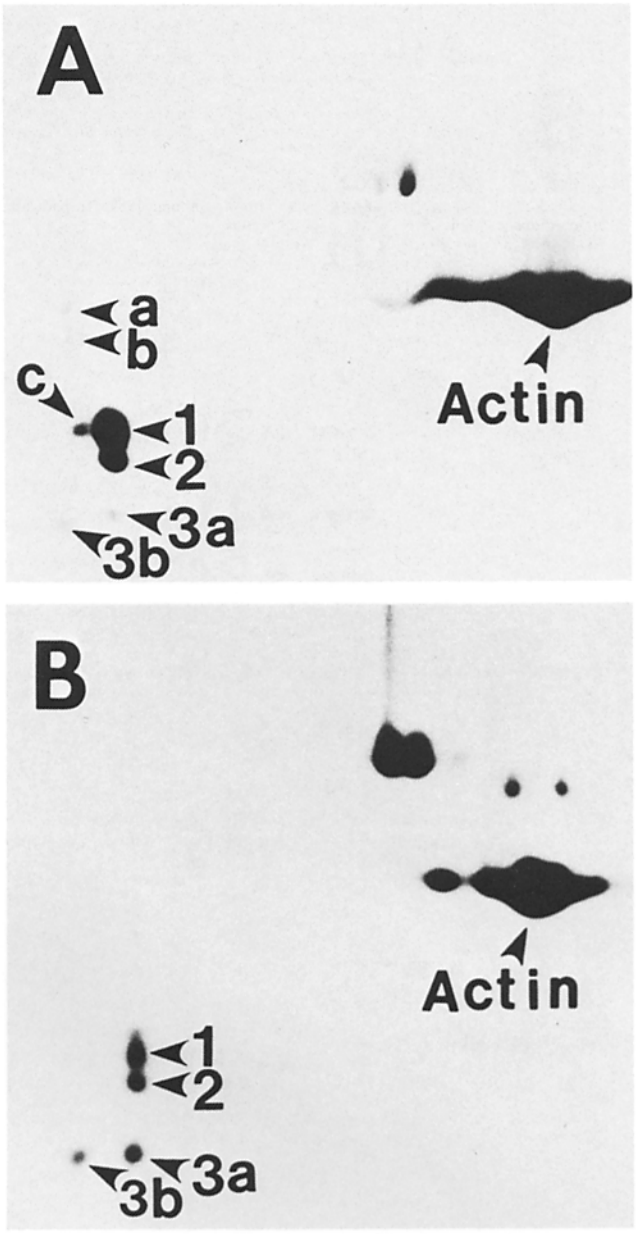

FIGURE 10 Two-dimensional gel analysis of the microfilaments isolated from normal and RSV-transformed CEF cells. Microfilaments were isolated from [^{35}S]methionine-labeled normal (*A*) and RSV-transformed (*B*) CEF cells and analyzed by two-dimensional gel electrophoresis. Fluorographs of a portion of the gels are shown with the acidic ends to the left. CEF tropomyosin isoforms are *a*, *b*, *c*, *1*, *2*, *3a*, and *3b*. Tropomyosin isoforms *1* and *2* were greatly reduced and tropomyosins *3a* and *3b* were increased in the transformed cell microfilaments as compared to those in normal cell microfilaments.

tively, it is possible that tropomyosin 2 have formed heterodimer in vivo with either tropomyosin 1 or 3. Because the positions of cysteine residues on these isoforms are out of register, no dimers can be detected by the present cross-linking method. Isoform-specific monoclonal antibodies may provide useful reagents for studying the intracellular assembly and localization of individual isoforms.

It has been shown that the binding of nonmuscle tropomyosin to rabbit F-actin is weaker than that of muscle tropomyosin (9, 19). In this paper, a direct comparison of the

TABLE II

Radioactive Ratios of Tropomyosin Isoforms to Actin in Tropomyosin-enriched Microfilaments Isolated from Normal and RSV-transformed CEF*

	Normal CEF			RSV-transformed CEF		
	I	II	Average	I	II	Average
Actin	100	100	100	100	100	100
Tropomyosins						
1 (38K)	8.8	9.0	8.9	3.2	3.8	3.5
2 (36.5K)	6.3	7.1	6.7	6.4	7.9	7.1
3 (32.8K)	1.4	2.6	2.0	6.2	5.4	5.8

* Values were calculated from [^{35}S]methionine incorporation into each protein band of the isolated microfilaments separated on SDS polyacrylamide gels. (*K*, $\times 10^3$.)

actin-binding abilities of CEF tropomyosin isoforms has been made. With 100 mM KCl and no Mg^{++}, tropomyosin 3 binds poorly to F-actin, while >85% of tropomyosins 1 and 2 are bound to actin under these conditions. This difference in actin binding may be of importance with respect to the in vivo functions of individual isoforms. Results from numerous studies have suggested that tropomyosin may play a role in stabilizing and protecting actin filaments in the cell (3, 16, 20, 29, 33, 43). Therefore, it is possible that different isoforms of tropomyosin with different actin-bind abilities would have different extents of actin filament stabilization and protection. That is, tropomyosins 1 and 2 are more effective in stabilizing and protecting actin microfilaments than tropomyosin 3. Because transformed cells had more disrupted microfilaments (14, 15, 22, 23, 48), one would expect to find a reduction in levels of tropomyosins 1 and 2, concomitant with an increased synthesis of tropomyosin 3, in RSV-transformed CEF cells. Indeed, we have observed this type of change in tropomyosin isoforms upon cell transformation (Figs. 9 and 10).

We would like to thank Dr. J. D. Watson for his enthusiastic support of this work. We are grateful to Drs. M. Solursh, N. Williams, and D. Soll for their invaluable suggestions and critical reading of the manuscript. The able assistance of N. Haffner and J. L.-C. Lin is gratefully acknowledged.

This work was supported by a National Institute of Health grant GM34105 and by a grant from the Muscular Dystrophy Association.

Received for publication 29 August 1984, and in revised form 29 October 1984.

Note Added in Proof: After this manuscript was accepted, Hendricks and Weintraub (Hendricks, M., and H. Weintraub, 1984, *Mol. Cell. Biol.*, 4:1823–1833) reported that CEF cells contained seven distinct tropomyosin isoforms and that the differential expression of these tropomyosin isoforms was found in RSV-transformed CEF cells.

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