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Invariance and heterogeneity in the major structural and regulatory proteins of chick muscle cells revealed by two-dimensional gel electrophoresis

(100-Å filaments/muscle contractile proteins/two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis)

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ABSTRACT **A two-dimensional gel electrophoresis system is used to investigate some of the properties of desmin, the major subunit of the 100-Å filaments from chick muscle cells, and to compare these properties to those of the other major contractile and regulatory proteins of muscle. Desmin from embryonic and adult smooth, skeletal, and cardiac muscle cells is resolved into two isoelectric variants, α and β, which possess slightly different electrophoretic mobilities in sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Both the α and the β variants from all six preparations appear to be identical in isoelectric point and apparent molecular weight. The α and β desmin are present in approximately equal amounts in all three types of muscle, suggesting that both isoelectric variants of desmin serve as the structural subunits of the 100-Å filaments in chick muscle cells.**

Tropomyosin also can be resolved into two subunits, α and β, in all three types of muscle. However, in each type of muscle both subunits differ from their counterparts in the other types of muscle, either by molecular weight or by isoelectric point. These results indicate that, with regard to apparent isoelectric point and molecular weight, desmin, a major muscle structural protein, is invariant, while tropomyosin, a major muscle regulatory protein, exhibits heterogeneity in the three types of muscle.

Intermediate-sized filaments, also known as 100-Å filaments, comprise a class of cytoplasmic structures distinct from actin filaments or microtubules. They are commonly found in epithelial cells, fibroblasts, nerve cells, glial cells, skeletal muscle, smooth muscle, and cardiac muscle cells (1–8). Although little is known about their function, the major subunit of these filaments has been isolated from neurons and glial cells. It has been characterized both biochemically and immunologically, and antibodies to this protein have been used to probe its localization in these cell types (9–13).

We have recently presented evidence that the major subunit of the 100-Å filaments from smooth muscle cells (chicken gizzard) is a protein with an apparent molecular weight of 50,000 that is immunologically distinct from any of the well known major contractile proteins of muscle (14). Antibodies elicited against this protein from smooth muscle crossreacted with skeletal and cardiac muscle cells, indicating that the subunit of the 100-Å filaments was at least antigenically related in the three types of muscle. On the basis of its immunofluorescent localization we have suggested that a cytoplasmic role of this molecule in differentiated muscle cells is structural, serving as a link between the plasma membrane and myofilaments at their Z lines. To indicate its putative linking role in muscle we have termed this protein desmin (14).

Actin heterogeneity in higher eukaryotic muscle and nonmuscle cells has been investigated by direct determination of the amino-acid sequences of selected tryptic peptides of muscle and nonmuscle actins (15), by tryptic peptide analysis (16), by sodium dodecyl sulfate ($NaDodSO_4$)/urea/polyacrylamide gel electrophoresis (PAGE) (17, 18), and by two-dimensional isoelectric-focusing (IEF) in conjunction with *in vitro* protein synthesis to detect isoelectric variants of actin in differentiating cultured muscle cells (19). The results suggest that distinct genes code for the muscle and nonmuscle forms of actin. In one of these studies (19) actin could be resolved into three isoelectric forms, with the same molecular weight. These forms have been referred to as α, β, and γ actin. α-Actin appears to be the predominant form in fully differentiated skeletal muscle cells, while the β and γ forms predominate in dividing prefusion myoblasts and in nonmuscle cells (19).

Several of the other major contractile and regulatory proteins of muscle tissues, such as tropomyosin and the heavy chain of myosin, also exhibit subtle chemical heterogeneity. These differences become apparent when comparing the isolated molecules from different types of muscle (20, 21), from different stages in muscle development (22–24), or from different types of muscle fibers (25–31). Such chemical heterogeneities have led to the idea that for each of these proteins a family of structural genes may exist that could share important characteristics with other multigene families such as the genes coding for the β chains of globin (for a review of multigene families see ref. 32). A member of any of these gene families may be expressed preferentially depending on the type of muscle, its function, and its developmental stage.

In this paper we use a two-dimensional gel electrophoresis system to examine heterogeneity in desmin, actin, and tropomyosin. This technique provides a sensitive measure of diversity for each of these proteins in skeletal, cardiac, and smooth muscle cells.

MATERIALS AND METHODS

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed as described (33). The IEF in the first dimension was run in cylindrical gels (2.5 × 120 mm) and was composed of 4% acrylamide (Eastman X5521), 0.21% *N,N'*-methylene bisacrylamide (Eastman P8383), 9.2 M urea (Schwartz-Mann, Ultrapure), 2% Nonidet P-40 (Shell Oil Co., No. 18164), 1.6% Carrier Ampholines pH 5–7 (LKB No. 11), 0.8% Carrier Ampholines pH 4–6 (LKB No. 26), and 0.2% Carrier Ampholines pH 3.5–10 (LKB No. 25). The gels were polymerized and prerun as described (33). Crude urea extracts of chick tissue were prepared by macerating fresh tissue in unbuffered 8 M urea. The tissue was extracted for 4 hr at 4° and then clarified by centrifugation at 500 × *g* for 10 min at 25°. Samples for IEF were made 9 M in urea, 2% in carrier ampholines pH 5–7, 1% in Nonidet P-40, and 5% in 2-mercaptoethanol. The extracts were warmed to 55° for 10 min, and 10-

Abbreviations: $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

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to 40-μl samples were subsequently loaded and run with the following voltage schedule: 450 V for 15 hr, 1.5 hr at 800 V. The IEF gels were eluted and equilibrated for 45 min in three changes of sample buffer. The gels from the first dimension were loaded on a 10% acrylamide, 0.23% *N,N'*-methylene bisacrylamide slab gel with a 2 cm 4.75% acrylamide stacking gel. After $NaDodSO_4$/PAGE in the second dimension, the gels were stained in 0.25% Coomassie brilliant blue in 50% methanol/10% acetic acid and destained in 25% isopropanol/10% acetic acid.

Each of the gel patterns shown in the figures represents material from a single animal unless noted otherwise. There was no apparent isoelectric variation in the proteins under investigation between muscle extracts from different members of a given population of chickens. The position of tropomyosin was determined by electrophoresis of highly purified rabbit skeletal muscle tropomyosin. As previously noted (31, 35), rabbit skeletal muscle tropomyosin is resolved into two subunits, α and β. In the gel system used here both subunits of rabbit skeletal tropomyosin have the same isoelectric focusing position with the two subunits of chicken gizzard or skeletal muscle tropomyosin.

The isoelectric positions of actins were assigned using rabbit skeletal muscle actin as a reference point for α-actin. The relative position of any of the three actins in a particular type of tissue was determined using as a reference point the isoelectric position of desmin, which is invariant in all three types of muscle. Actin and tropomyosin from rabbit skeletal muscle were purified as described (35).

RESULTS AND DISCUSSION

Desmin. In one-dimensional $NaDodSO_4$/PAGE analysis of chick skeletal, cardiac, and smooth muscle extracts, the protein previously designated as desmin (14) migrates as a closely spaced doublet (Fig. 1). Two-dimensional gel electrophoresis resolves desmin into two isoelectrically distinct forms in both embryonic and adult preparations of chick smooth (gizzard) (Figs. 5, 6, 10), cardiac (Figs. 2 and 4), and skeletal muscle (Fig. 9). Desmin that has been partially purified away from myosin and tropomyosin, by repeated extraction of smooth muscle with KCl and KI concentrations that solubilize actomyosin (14), also exhibits two isoelectric variants in two-dimensional gels (Fig. 10). Assuming that these variants represent closely related forms of the same protein, we have termed the more acidic component α desmin and the more basic component β desmin. The β subunit appears to have a slightly faster mobility in the $NaDodSO_4$/PAGE of the second dimension (Fig. 8).

Desmin is one of the major proteins in extracts of 1-day-old chick smooth and cardiac muscle. Desmin can also be identified in the extracts of 1-day-old chick skeletal muscle (Fig. 9). However, the quantity of this protein in skeletal muscle is less than that found in smooth and cardiac muscle at this age (compare Fig. 9 with Figs. 4 and 5; see also Fig. 1). Two-dimensional coelectrophoresis of extracts from gizzard and cardiac muscle (Fig. 8) indicates that neither α nor β desmin varies in either molecular weight or isoelectric point between these two tissues. Furthermore, in all three types of muscle the two isoelectric variants of desmin exist in approximately equal amounts, suggesting that both isoelectric variants of desmin are structural subunits of the 100-Å filaments. Thus, the immunological evidence presented previously (14) and the biochemical evidence presented here demonstrate that desmin is closely related in chick smooth, cardiac, and skeletal muscle. The results presented here have been consistently observed in muscle extracts from at least eight chickens in the same or different developmental stages. Therefore, the isoelectric

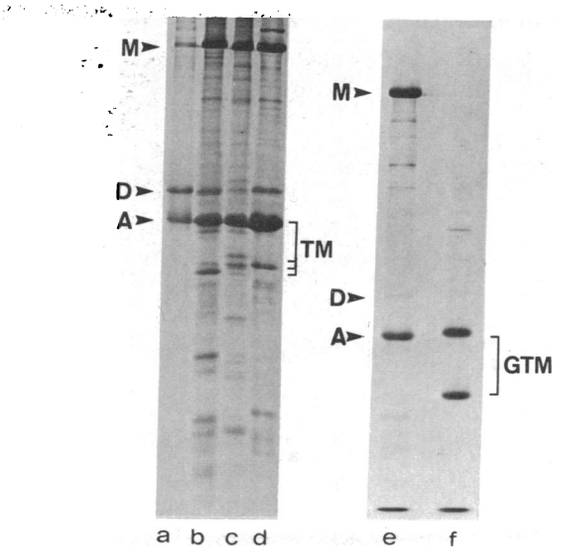
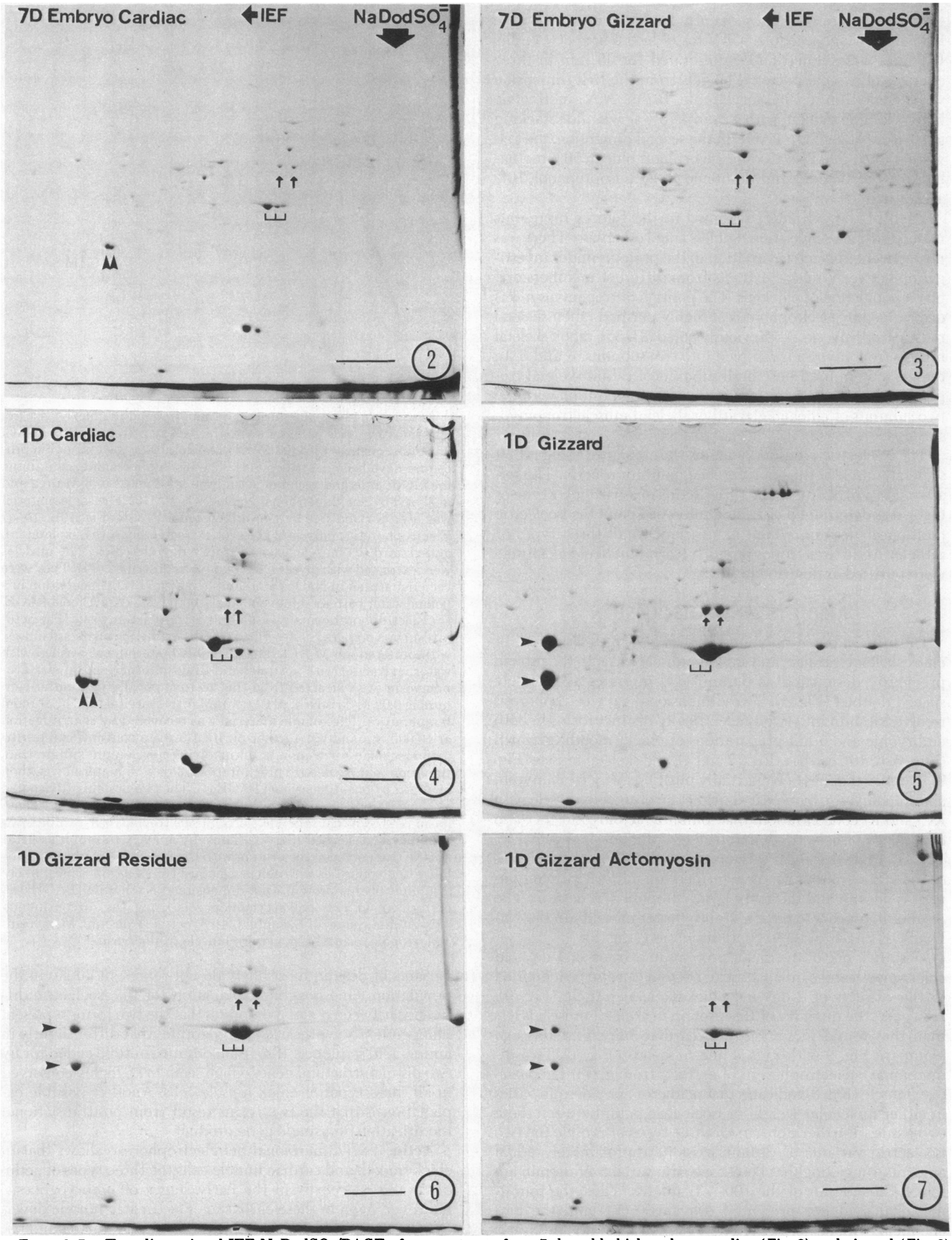


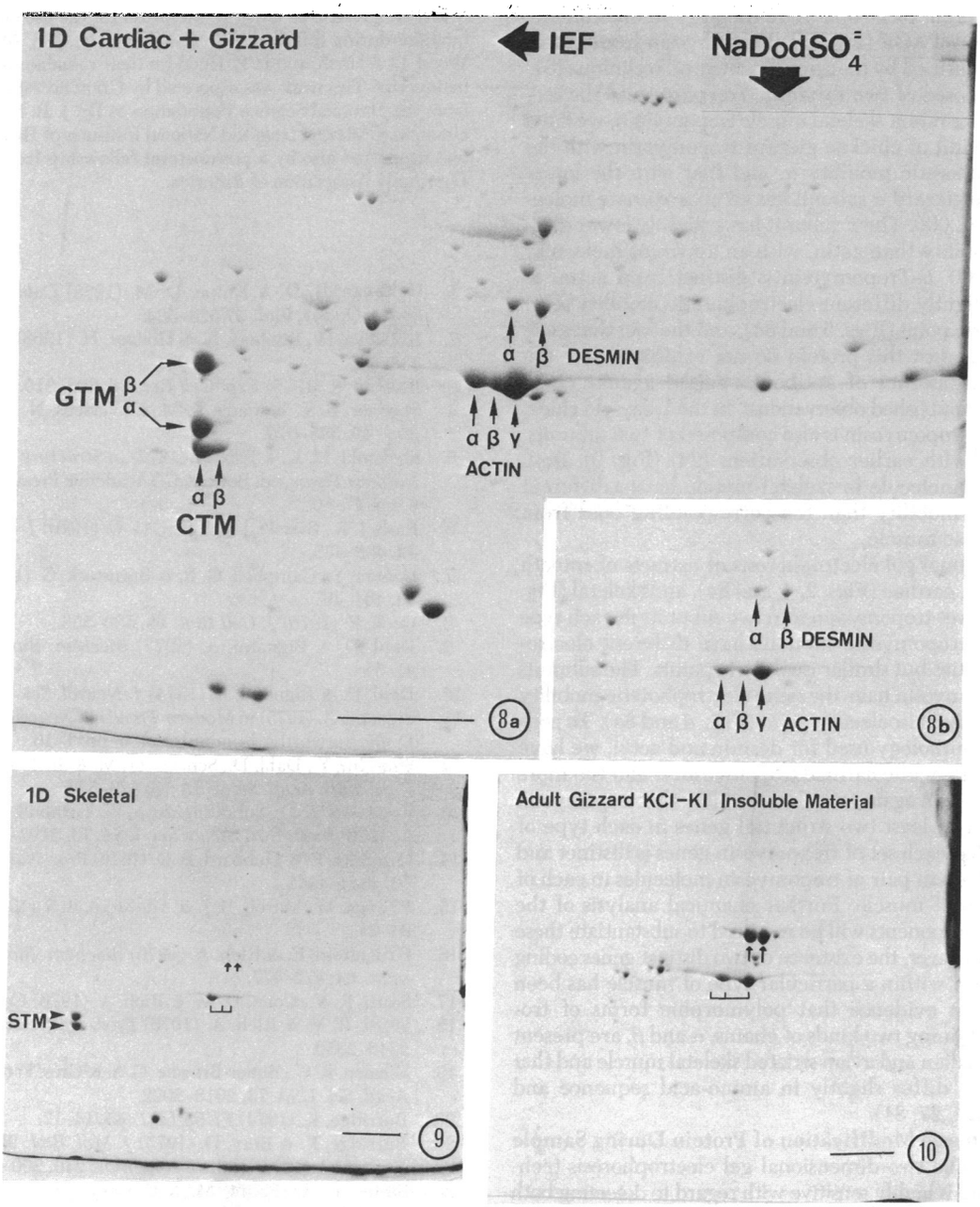
FIG. 1. (*a*) Partially purified desmin from adult chicken gizzard. The smooth muscle material was extracted extensively with 0.6 M KCl and subsequently with 0.6 M KI. The details of the extraction procedure have been described (14). The insoluble residue, containing mostly desmin and residual actin, was solubilized in 8 M urea and analyzed by one-dimensional $NaDodSO_4$/PAGE. The same material was used for analysis by two-dimensional PAGE (Fig. 10). (*b–d*) Electrophoretic analysis of glycerol-extracted heart (*b*), skeletal (*c*), and gizzard (*d*) muscle, excised from a day-old chick. The muscles were extracted with glycerol for 14 days as described (14). They were then denatured in $NaDodSO_4$ sample buffer (14) by incubation in a boiling water bath for 4 min and analyzed by one-dimensional PAGE. (*e*) Electrophoretic analysis of adult gizzard actomyosin. The actomyosin was prepared as described (14), by extraction of gizzard muscle with buffered 0.6 M KCl. The insoluble material was washed with water, extracted with acetone, and dried in ether and in air. Tropomyosin was extracted from this acetone powder in a buffer containing 0.02 M Tris·HCl, pH 7.6/1 mM dithiothreitol for 3 hr at room temperature. The soluble material was recovered by centrifugation at 10,000 × *g* and was further purified using a conventional purification scheme for tropomyosin involving ammonium sulfate fractionation and isoelectric precipitation (35). The material was then analyzed by one-dimensional PAGE (*f*). The two proteins, designated as α and β tropomyosin, copurify. Using the same purification scheme it can be shown that rabbit skeletal muscle tropomyosin exhibits two subunits as described (31, 35). Thus, by analogy to rabbit skeletal muscle tropomyosin, the protein with the lower electrophoretic mobility that copurifies with chicken gizzard tropomyosin is most likely a subunit of this protein. One-dimensional $NaDodSO_4$/PAGE was performed on 12% polyacrylamide gels using the discontinuous Tris-glycine system of Laemmli (34). A, actin; D, desmin; M, myosin; TM, tropomyosin; GTM, gizzard muscle tropomyosin.

variants of desmin most likely do not represent alleles in the population. One possible explanation for the isoelectric difference between α and β desmin is that the two forms represent independently synthesized polypeptides that differ slightly in amino-acid sequence. If so, then desmin must be coded for by two distinct structural genes which seem to be highly conserved in all three types of chick muscle cells. Another possible explanation is that the two forms result from posttranslational modification of a single gene product.

Actin. Two-dimensional gel electrophoresis shows that in chick smooth and cardiac muscle cells the three types of actin, α, β, and γ, coexist in the early stages of embryogenesis. However, even in these early stages of cardiac muscle development (7-day-old embryo) the α form predominates and becomes the main form of actin early after the embryos hatch (Figs. 2 and 4). In smooth (gizzard) muscle the γ form is the major isoelectric variant in both embryonic and adult muscle (compare Figs. 4, 5, and 8*b*). In gizzard actomyosin the β form



FIGS. 2–7. Two-dimensional IEF-NaDodSO$_4$/PAGE of urea extracts from 7-day-old chick embryo cardiac (*Fig. 2*) and gizzard (*Fig. 3*) muscle and 1-day-old chick cardiac (*Fig. 4*) and gizzard (*Fig. 5*) muscle. Arrows, isoelectric positions of α and β desmin; square brackets, isoelectric positions of α, β, and γ actin; arrowheads, tropomyosin spots. Actomyosin extraction of a day-old chicken gizzard with 0.6 M KCl leaves most of the desmin and a considerable amount of the actin in the insoluble residue (14). (*Fig. 6*) Desmin- and actin-containing residue insoluble in 0.6 M KCl. (*Fig. 7*) Actomyosin soluble in 0.6 M KCl. Bar in each gel is 2 cm.



FIGS. 8–10. (*Fig. 8*) (*a*) Coelectrophoresis of a mixture of urea extracts from cardiac and gizzard muscle from a day-old chicken. Note the difference in the sets of tropomyosin spots. (GTM and CTM, gizzard and cardiac muscle tropomyosin, respectively.) Also note that the desmin and actin isoelectric variants have the same mobilities on these gels. (*b*) The same sample as that of *a*, but underloaded to demonstrate the resolution of actin into α, β, and γ isoelectric forms. Under the conditions of tissue solubilization (8 M urea) and of two-dimensional gel electrophoresis (33) used here, myosin migrates poorly into the first isoelectric focusing dimension, and it is not identified in this work. (*Fig. 9*) Two-dimensional IEF-NaDodSO$_4$/PAGE of urea extract of 1-day-old chick skeletal muscle. STM, skeletal muscle tropomyosin. (*Fig. 10*) Material of adult chicken gizzard insoluble in 0.6 M KCl after prolonged extraction of the muscle with 0.6 KCl and subsequently with 0.6 M KI. The material represents the extract from approximately 20 gizzards. Details of the extraction procedure have been described (ref 14; see also Fig. 1*a*). The pH gradient was measured by cutting 5-mm pieces of an IEF gel and extracting each piece for 30 min with 0.25 ml of degassed 8 M urea. The gradient follows a slightly sigmoidal curve extending from pH 4.7 on the left side to pH 6.85 to the right of the gel. The bar in each gel equals 2 cm.

of actin appears to be the predominant one (Fig. 7), while a fraction of the β and the majority of the γ form appear to be less soluble under the conventional extraction procedures that solubilize actomyosin (Fig. 6). We have previously noted that a considerable amount of actin remains insoluble with desmin after prolonged extraction of gizzard muscle with KCl and KI concentrations that render the majority of the actomyosin soluble (ref. 14; see also Fig. 1*a*). The reasons for this dramatic difference in the solubility properties of the residual actin are unknown, but by its isoelectric point it appears to be predominantly the γ form of actin as well as some β actin (Figs. 6 and 10). Since the majority of the γ actin remains insoluble with desmin, it is possible that, like desmin, this form of actin may play a more structural cytoplamic role. The cytoplasmic roles of the α and β forms are unclear, but in accordance with previous observations (20), the α form of actin may be involved in the contractility of skeletal and cardiac muscle cells. Similarly, the β form of gizzard actin may be the contractile form of this protein in gizzard muscle, since it is extracted with actomyosin from these muscle cells.

Tropomyosin. Tropomyosin purified from chick gizzard by conventional biochemical techniques (35) is resolved into two

subunits of different electrophoretic mobility in one-dimensional NaDodSO$_4$/PAGE (Fig. 1*f*). Tropomyosin from rabbit skeletal muscle purified by the same biochemical technique (31, 35) also is composed of two subunits. To conform to the terminology used for rabbit skeletal muscle tropomyosin, we have termed the subunit of chicken gizzard tropomyosin with the higher electrophoretic mobility α, and that with the lower mobility β. The gizzard α subunit has an approximate molecular weight of 32,000. The β subunit has a slightly lower electrophoretic mobility than actin, with an apparent molecular weight of 43,000. β-Tropomyosin is distinct from actin, as judged by its slightly different electrophoretic mobility (Fig. 1*f*), its isoelectric point (Figs. 5 and 8*a*), and the fact that antibodies raised against this protein do not exhibit any of the characteristic properties of antibodies raised against chick gizzard actin (unpublished observations). In the 1-day-old chick, skeletal muscle tropomyosin is also composed of two subunits, in accordance with earlier observations (24) (Fig. 9). Both subunits of this molecule in skeletal muscle have a different electrophoretic mobility than the corresponding ones from smooth or cardiac muscle.

Two-dimensional gel electrophoresis of extracts of smooth (Figs. 5 and 8*a*), cardiac (Figs. 2, 4, and 8*a*), and skeletal (Fig. 9) muscles resolves tropomyosin into two subunits in each type of muscle. All tropomyosin subunits have different electrophoretic mobilities but similar isoelectric points. The subunits of cardiac tropomyosin have the same electrophoretic mobility but differ slightly in isoelectric point (Figs. 4 and 8*a*). To conform to the terminology used for desmin and actin, we have termed the more acidic cardiac tropomyosin α and the more basic β. The gel data again raise the possibility that tropomyosin is coded for by at least two structural genes in each type of muscle, and that each set of tropomyosin genes is distinct and codes for a different pair of tropomyosin molecules in each of the three types of muscle. Further chemical analysis of the tropomyosin components will be required to substantiate these possibilities. However, the existence of two distinct genes coding for tropomyosin within a particular type of muscle has been postulated from evidence that polymorphic forms of tropomyosin containing two kinds of chains, α and β, are present in both mammalian and avian striated skeletal muscle and that the two chains differ slightly in amino-acid sequence and composition (24, 27, 31).

Possible Charge Modification of Protein During Sample Preparation. The two-dimensional gel electrophoresis technique used here is highly sensitive with regard to detecting both charge differences between macromolecules and small differences in molecular weight (33). Therefore, artifactual charge modication or molecular weight alterations due to the presence of urea or due to proteolysis during sample preparation could give rise to artifactual isoelectric variants in a protein. We have noticed that prolonged exposure of either actin or desmin to urea causes the appearance of satellite isoelectric variants in two-dimensional IEF. Fig. 10 depicts the emergence of such artifactual variants. However, the constancy of charge and electrophoretic mobility seen in desmin, actin, and tropomyosin, as well as the constancy in the ratios observed in the isoelectric variants of these proteins, in conjunction with amino-acid sequence data for the latter two molecules leads us to believe that the isoelectric variants observed here do not result from artifactual charge modification. The tissue specificity of the relative quantities of each of the actin variants and the invariance of α and β desmin in all three tissues examined further suggest that the isoelectric variants observed for these proteins reflect real primary structure differences between the variants.

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