

Differentiation of Troponin in Cardiac and Skeletal Muscles in Chicken Embryos as Studied by Immunofluorescence Microscopy

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ABSTRACT The differentiation of troponin (TN) in cardiac and skeletal muscles of chicken embryos was studied by indirect immunofluorescence microscopy. Serial sections of embryos were stained with antibodies specific to TN components (TN-T, -I, and -C) from adult chicken cardiac and skeletal muscles. Cardiac muscle began to be stained with antibodies raised against cardiac TN components in embryos after stage 10 (Hamburger and Hamilton numbering, 1951, *J. Morphol.* 88:49-92). It reacted also with antiskeletal TN-I from stage 10 to hatching. Skeletal muscle was stained with antibodies raised against skeletal TN components after stage 14. It also reacted with anticardiac TN-T and C from stage 14 to hatching. It is concluded that, during embryonic development, cardiac muscle synthesizes TN-T and C that possess cardiac-type antigenicity and TN-I that has antigenic determinants similar to those present in cardiac as well as in skeletal muscles. Embryonic skeletal muscle synthesizes TN-I that possesses antigenicity for skeletal muscle and TN-T and C which share the antigenicities for both cardiac and skeletal muscles. Thus, in the development of cardiac and skeletal muscles, a process occurs in which the fiber changes its genomic programming: it ceases synthesis of the TN components that are immunologically indistinguishable from one another and synthesizes only tissue-type specific proteins after hatching.

It has been shown by a number of investigators that myofibrillar proteins of skeletal muscle of the higher animals exist in polymorphic forms that appear to be characteristic for the type of muscle from which they are prepared (2, 4, 5, 6, 9, 15, 16, 30). In the case of adult cardiac muscle, polymorphic forms of the myosin isozymes have also been reported (22, 29), as cardiac muscle consists of atrial, ventricular, and Purkinje fibers.

Evidence is increasing that myofibrillar proteins in the embryo are different from those in the adult and that, during development of skeletal muscle, they change to an adult form (7, 12, 15, 18-20). However, the developmental changes in myofibrillar proteins of the heart have thus far been examined only with respect to isozymes of myosin (14).

The present study was undertaken to investigate the developmental changes in cardiac muscle of the regulatory fibrillar proteins troponin T (TN-T), I (TN-I), and C (TN-C). The preparation of antibodies specific for cardiac (ventricular muscle) and skeletal (breast muscle) subunits of TN and their use with serial sections of whole embryos enabled us to study by

immunomicroscopy the chronological differentiation of TN components not only in cardiac but also in skeletal muscle. A part of the results described in this article has appeared in abstracts (23, 24).

MATERIALS AND METHODS

Preparation of Antigens

TN was extracted from adult chicken cardiac (ventricle) and skeletal (m. pectoralis major) muscles by the methods of Tsukui et al. (25) and Ebashi et al. (8), respectively. Cardiac and skeletal TN components (TN-T, I, and C), except cardiac TN-C, were separated by ion-exchange column chromatography in the presence of 6 M urea (8, 17). Cardiac TN-C was purified through SDS slab gel electrophoresis from cardiac TN (6, 13). The purity of each of the three TN components is shown in Fig. 1.

Production of Antisera

Antisera against cardiac TN components were raised in rabbits; those against skeletal TN components were raised in guinea pigs. The animals were immunized by subcutaneous injection of 1 ml of antigen solution containing 0.3-0.5 mg of a TN component in 0.4 M NaCl and 1 mM NaHCO₃ emulsified with an equal

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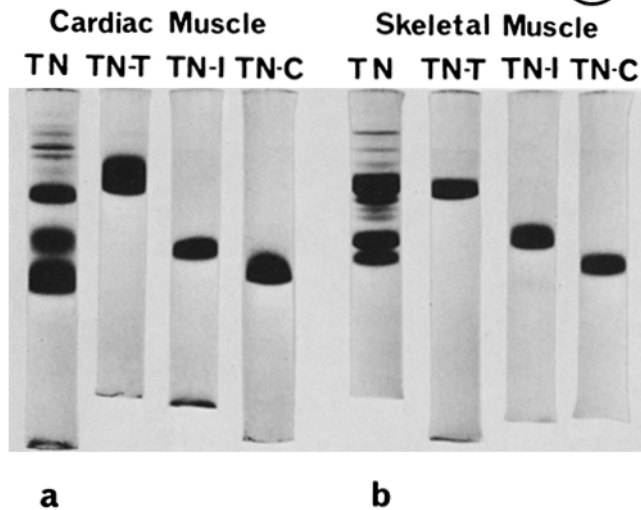


FIGURE 1 SDS-polyacrylamide gel electrophoresis of troponin (TN) from (a) adult chicken cardiac (ventricle) and (b) skeletal (breast) muscles. The three components (TN-T, I, and C) were separated from the original TN with ion-exchange chromatography or SDS slab gel electrophoresis (see text).

volume of complete Freund's adjuvant. A similar amount of antigen was injected after 2 wk, and the animals were tested for antibody production 1 wk after the second injection. Whenever necessary, additional boosts were given 1 wk later to obtain higher-titer antisera.

Specificities of Antibodies

The specificities of the antisera to all the proteins were examined by double immunodiffusion, immunoelectrophoresis, and the demonstration of specific staining of isolated myofibrils. The specificities were further confirmed by two rounds of absorption. First, each antiserum was absorbed against a small amount of homogenate from heterologous muscle (for example, anticardiac TN-T antiserum absorbed to skeletal muscle homogenate) and left at room temperature for 15 min and then at 4°C for 1 h. After the precipitate was removed by centrifugation, the same procedure was repeated with the supernatant fluid. The immunoglobulin (IgG) was fractionated from the absorbed serum by ammonium sulfate and purified by DEAE cellulose column chromatography (10). The reabsorption was further performed by passing the antibody through CNBr-activated Sepharose 4B conjugated with a heterologous TN component (for example, anticardiac TN-T absorbed against immobilized skeletal TN-T).

Electrophoresis, Double Immunodiffusion and Disc-Immunoelectrophoresis

SDS-polyacrylamide gel electrophoresis was performed in 10% acrylamide gel containing $\frac{1}{37}$ amount of methylene bisacrylamide according to Weber and Osborn (26). Electrophoresis was carried out in a buffer system containing 0.1 M Na-phosphate buffer (pH 7.0) and 0.1% SDS. The gels were fixed with 50% methanol containing 5% acetic acid and stained with 0.25% Coomassie Brilliant Blue R-250 in the presence of 7% acetic acid.

Double immunodiffusion was carried out with 1% agarose dissolved in phosphate-buffered saline (PBS). 45 μ l of antigen or antiserum solution was added to wells. Antigen concentrations were adjusted to 1–1.9 mg/ml in 0.4 M NaCl and 1 mM NaHCO₃. The agarose plates were developed for several days at 4°C until precipitin lines were complete. Then the gels were washed successively with PBS and distilled water, dried, and stained with 1% Amido black in 50% methanol, 7% acetic acid.

Immunodiffusion combined with SDS-acrylamide gel electrophoresis was performed by the method of Obinata et al. (16, 17). That is, SDS-acrylamide gel electrophoresis was first done in cylindrical gels of 10% acrylamide and, immediately after the completion of electrophoresis, the gels were embedded in a 1.0% agarose plate containing 0.05% SDS and PBS. Immunoreactions were elicited by applying the antisera against TN components in troughs parallel to the acrylamide gels.

Immunofluorescence Staining

Chicken embryos were staged according to Hamburger and Hamilton (11). Embryos younger than stage 20 were immersed in 50% glycerol solution containing KMP buffer (50 mM KCl, 2 mM MgCl₂, 2 mM EGTA, and 10 mM Na-phosphate buffer, pH 7.0) and stored at –20°C. They were then fixed in acetone at 4°C for 6–12 h, transferred to chloroform, and embedded in paraffin (Paraplast Plus Tissue Embedding Medium, Polysciences Inc., Warrington, Penn.). Sagittal sections were cut at 7 μ m, mounted on glass slides coated with an egg albumin-glycerine mixture, and the paraffin was removed with xylene. The sections were then soaked in a descending alcohol series. With embryos older than this stage and with adult chickens, the heart (ventricle) and skeletal (m. pectoralis major) muscles were excised and stored in 50% glycerol solution containing KMP buffer. The materials were embedded in cryoform, cut at 10 μ m in a cryostat, and mounted on albumin-coated slides. The sections were subsequently fixed in acetone at 4°C for 10 min. All materials were processed within 2 wk after glycerination.

Indirect immunofluorescence procedures were used with all the materials. After the sections were rinsed in PBS for 30 min, they were first treated with antibodies¹, washed with PBS, and reacted with fluorescein isothiocyanate (FITC)-labeled sheep antirabbit IgG or FITC-labeled sheep antiguinea pig IgG² (Miles Laboratories Inc., Elkhart, Ind.). Both incubations were carried out at 25°C for 30 min. The specificity of the reactions was tested with nonimmune serum in the first step.

Specimens were observed under a Zeiss microscope equipped with epifluorescence, and excitation (450–490 BP blue) and barrier (LP 520 and KP 560) filters were optimized for maximal FITC fluorescence. After fluorescence microscopy, the same specimens were further stained with hematoxylin and eosin.

RESULTS

Specificity of Antibody

To assess the specificity of antibodies, we applied an Ouchterlony double immunodiffusion test and a disc-immunoelectrophoretic method. Each antibody formed one precipitin line against its antigenic component (Figs. 2 and 3). Specificity of the antibodies was further assessed by immunofluorescence reactions with isolated myofibrils. Anticardiac TN-T, I, and C stained the thin filament region of cardiac myofibrils but never reacted with skeletal myofibrils, whereas antiskeletal TN components reacted only with skeletal myofibrils and never stained cardiac myofibrils (Fig. 4). These reactions were completely blocked by prior absorption of antibodies with corresponding immunogens and, further, no staining of myofibrils was observed by the treatment with preimmune serum. These results indicate that antibodies raised from TN components of cardiac and skeletal muscles are highly specific to those antigenic components and that there is no cross-reaction between antibodies raised from cardiac and skeletal muscles.

Immunofluorescence Microscopy

Because of the problems in getting good serial sections by cryomicrotomy of early chicken embryos, paraffin embedding was applied. Because identical results were obtained with paraffin and frozen sections, paraffin was used exclusively for small embryos in the present study.

Cardiac muscle began to be stained with antibodies in embryos at stage 9. The number of embryos that reacted with antibodies increased rapidly, and after stage 11 all the embryos

¹ Titer of antibodies was adjusted as follows: At first, antibodies were diluted to a concentration at which isolated myofibrils could not be stained by indirect immunofluorescence microscopy. The diluted antibodies were then concentrated threefold.

² Conjugates with different F/P ratios were separated by ion-exchange chromatography and only the fractions having a ratio of 1–2.5 were used in the immunofluorescence study. Conjugates were further absorbed with acetone powder of cardiac and skeletal muscles.

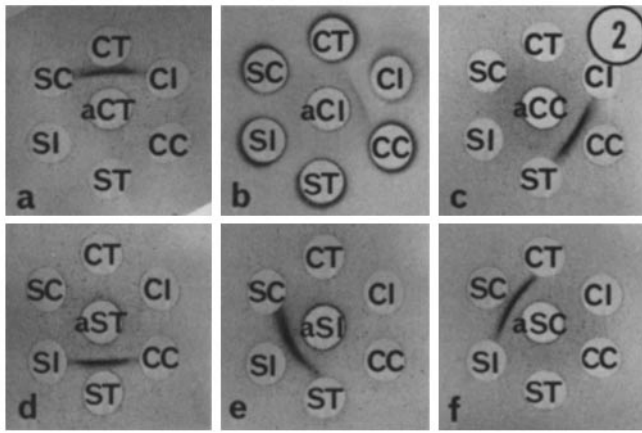


FIGURE 2 Ouchterlony double immunodiffusion assays. The central well contained: a, anti-cardiac TN-T (aCT); b, anti-cardiac TN-I (aCI); c, anti-cardiac TN-C (aCC); d, anti-skeletal TN-T (aS7); e, anti-skeletal TN-I (aSI); f, anti-skeletal TN-C (aSC). The outer wells beginning with the well at the top and proceeding clockwise contained: cardiac TN-T (CT), cardiac TN-I (CI), cardiac TN-C (CC), skeletal TN-T (ST), skeletal TN-I (SI), skeletal TN-C (SC). Each antibody formed one precipitin line against its antigenic component.

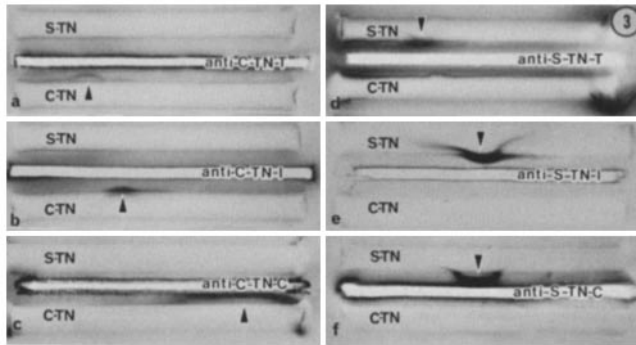


FIGURE 3 Immunoelectrophoresis showing the specificities of the antisera against TN components of heart and skeletal muscles. Electrophoresis of the original TN from cardiac and skeletal muscles was followed by immunodiffusion with antiserum against TN components of cardiac (a, b, c) and skeletal (d, e, f) muscles. Each antibody formed one precipitin line against its antigenic component at a specific gel portion corresponding to the electrophoretic mobility of each antigen (arrows).

examined were stained. Cardiac muscle reacted not only with antibodies against all of the three components of cardiac TN simultaneously (Fig. 5a-c), but also with antibody specific to skeletal TN-I (Fig. 5e). This result indicates that TN components of cardiac muscle were synthesized from about stage 10, and that embryonic cardiac TN-I has antigenicity that is different from that of adult cardiac TN-I and reacts with antibodies against TN-I from both cardiac and skeletal muscles.

At such early stages of development, skeletal muscle was not stained with any of the antibodies prepared (Fig. 5g-l). It began to be stained after stage 14 with antibodies directed against skeletal TN-T, I, and C simultaneously (Figs. 6 and 7d-f). It also reacted with antibodies raised against cardiac TN-T and C (Fig. 7a, c). This finding indicates that the onset of TN synthesis in skeletal muscle is at stage 14 and that TN-T and C present in embryonic skeletal muscle are different from adult skeletal TN-T and C; they react not only with

antiskeletal TN-T and C but also with anticardiac TN-T and C, respectively.

The reactivities of cardiac and skeletal muscles with antibodies against the TN components of the heterologous muscle disappeared before hatching. The stainability with heterologous antibodies in cardiac muscle disappeared at a somewhat

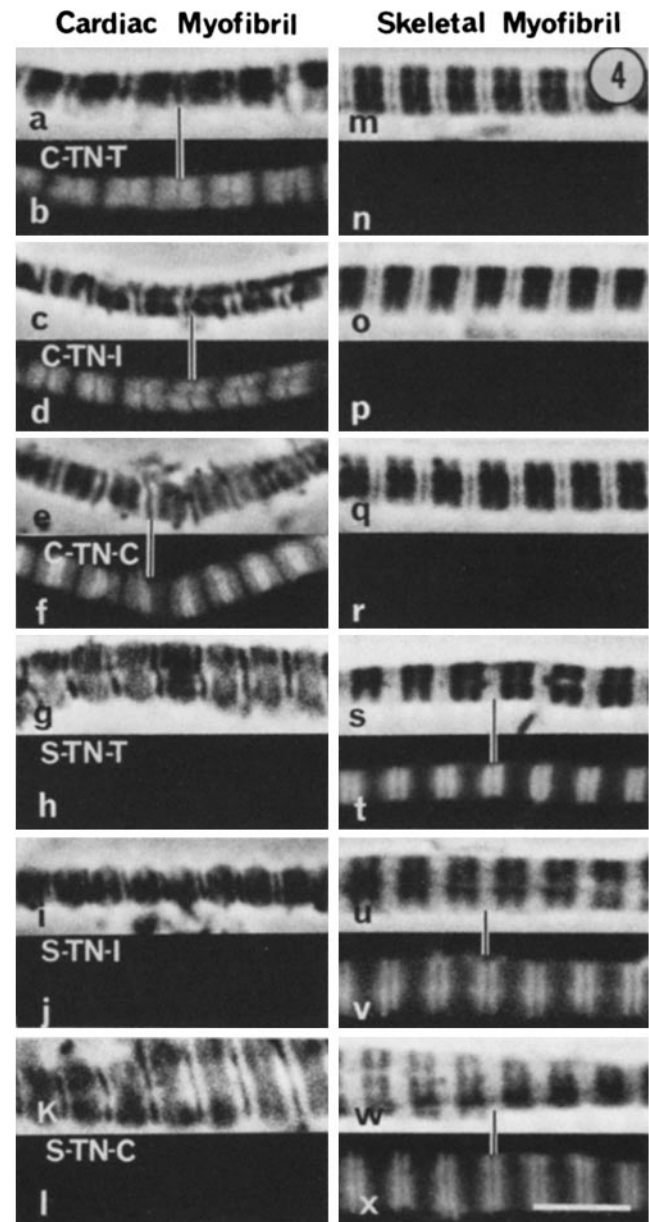


FIGURE 4 Staining of glycerinated myofibrils from adult cardiac (a-l) and skeletal (m-x) muscles with antibody against TN components. Affinity-purified IgG for each of the TN components was applied to myofibrils, that were then treated with FITC-conjugated sheep anti-guinea pig or anti-rabbit IgG. The phase-contrast micrographs, indicated a, c, e, g, i, k, m, o, q, s, u, and w, correspond to the fluorescence micrographs, b, d, f, h, j, l, n, p, r, t, v, and x, respectively. Stained with anti-cardiac TN-T (C-TN-T) (b and n), anti-cardiac TN-I (C-TN-I) (d and p), anti-cardiac TN-C (C-TN-C) (f and r), anti-skeletal TN-T (S-TN-T) (h and t), anti-skeletal TN-I (S-TN-I) (j and v), and anti-skeletal TN-C (S-TN-C) (l and x). The I bands of cardiac and skeletal myofibrils were stained with antibody against cardiac and skeletal TN components, respectively. No cross-reaction of myofibrils with heterologous antibodies was observed. Bar, 5 μ m. \times 2,600.

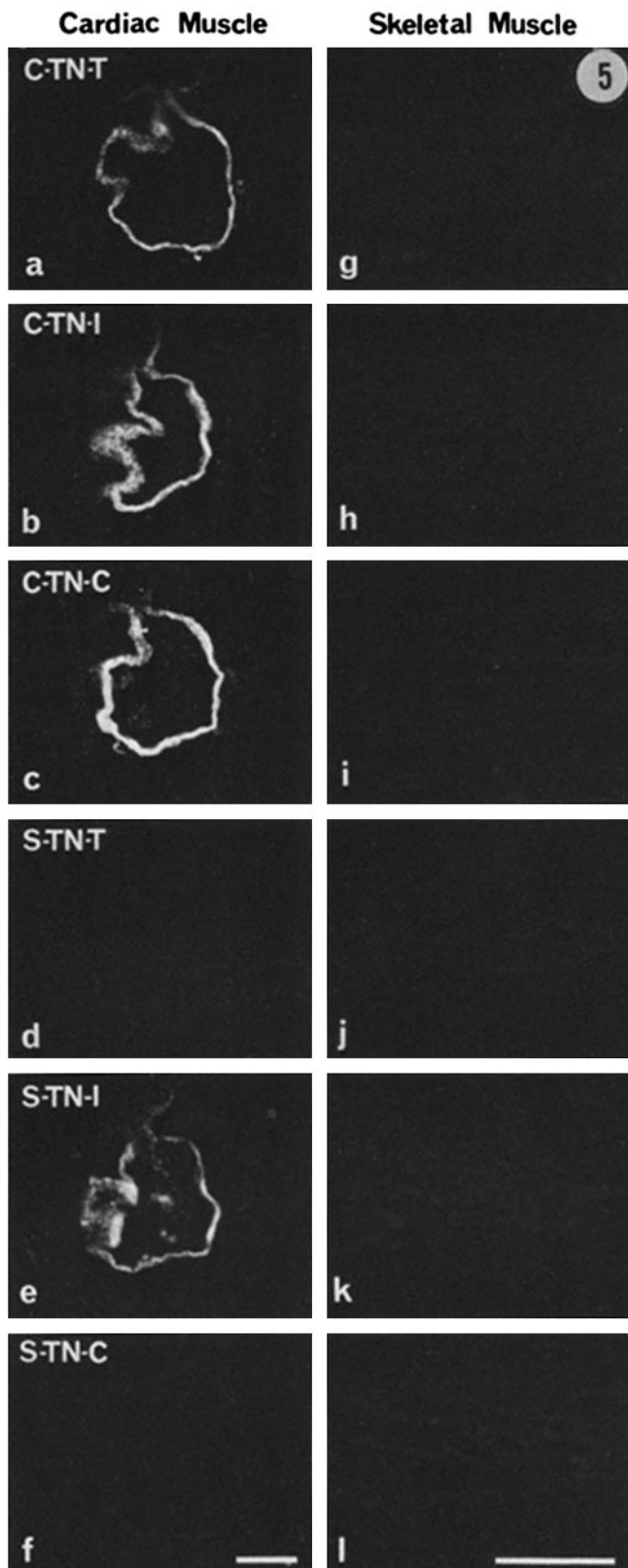
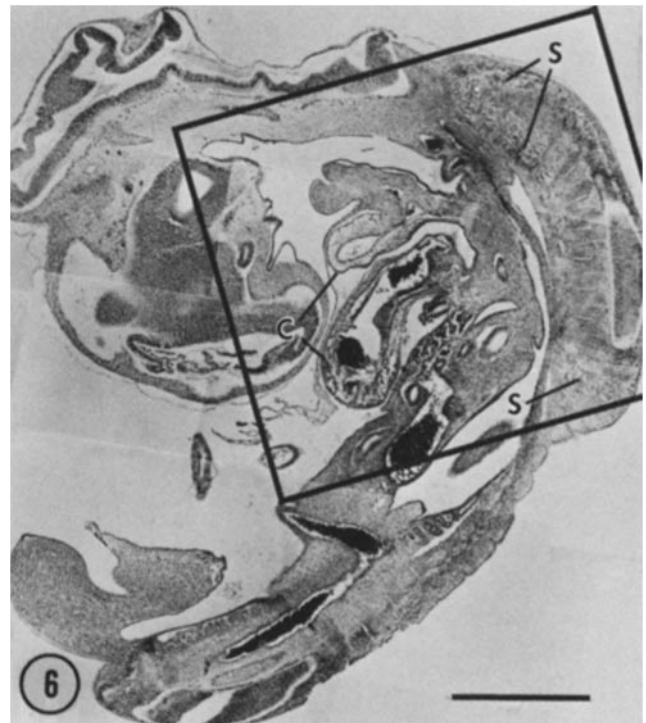


FIGURE 5 Chicken embryo at stage 10. Cardiac muscle was stained with antibodies against all of the cardiac TN components (a-c) and skeletal TN-I (e). Skeletal muscle was not stained at this stage (g-l). (a-f) Bar, 0.1 mm. $\times 80$. (g-l) Bar, 0.5 mm. $\times 32$. Figures 5, 7, 8, and 9 show immunofluorescence microscopy of embryonic and adult chickens stained with specific antibodies against cardiac TN-T (C-TN-T) (a and g), cardiac TN-I (C-TN-I) (b and h), cardiac TN-C (C-TN-C) (c and i), skeletal TN-T (S-TN-T) (d and j), skeletal TN-I (S-

earlier stage (stage 38) than that in skeletal muscle (stage 45) (Fig. 8 e). In developing skeletal muscles, the time of disappearance of stainability with antibodies against the heterologous TN components was not coordinated in different embryos, i.e., in some embryos stainability with antibody against cardiac TN-C disappeared slightly earlier than with anticardiac TN-T antibody, whereas in other embryos the reverse was observed. After hatching, muscle of each type was reacted only with antibodies specific for the TN components of the homotypic tissue (Fig. 9). The results are summarized in Table I.

DISCUSSION

The present immunoelectrophoretic and double immunodiffusion analysis showed that antibodies raised against TN-T, I, and C from cardiac and skeletal muscles are specific for the polymorphic forms of TN components against which they are raised. Dhoot et al. (5, 6) reported previously that the major form of TN-T, I, and C present in fast skeletal, slow skeletal, and cardiac muscles are immunochemically different proteins, except TN-C of slow and cardiac muscles. Thus, the results obtained from our work are in agreement with those reported previously concerning the existence of polymorphic forms that



FIGURES 6 and 7 Sagittal sections of a whole chicken embryo at stage 20 stained with hematoxylin and eosin (Fig. 6) and antibodies (Fig. 7). Immunofluorescence micrographs of the region corresponding to the rectangle in Fig. 6 are shown in Fig. 7. Cardiac muscle was stained with antibodies against cardiac TN components (Fig. 7 a-c) and skeletal TN-I (Fig. 7 e). Skeletal muscle was stained with antibodies against skeletal TN components (Fig. 7 d-f) and cardiac TN-T (Fig. 7 a) and C (Fig. 7 c). Cardiac muscle, C; skeletal muscle, S. Fig. 6. Bar, 1 mm. $\times 18$. Fig. 7. Bar, 1 mm. $\times 38$.

TN-I) (e and k) and skeletal TN-C (S-TN-C) (f and l). In Figs. 5, 8 and 9, serial sections through cardiac and skeletal muscles are shown in a-f and g-l, respectively. In Fig. 7, serial sections through entire embryos including regions of the heart and myotomes are shown.

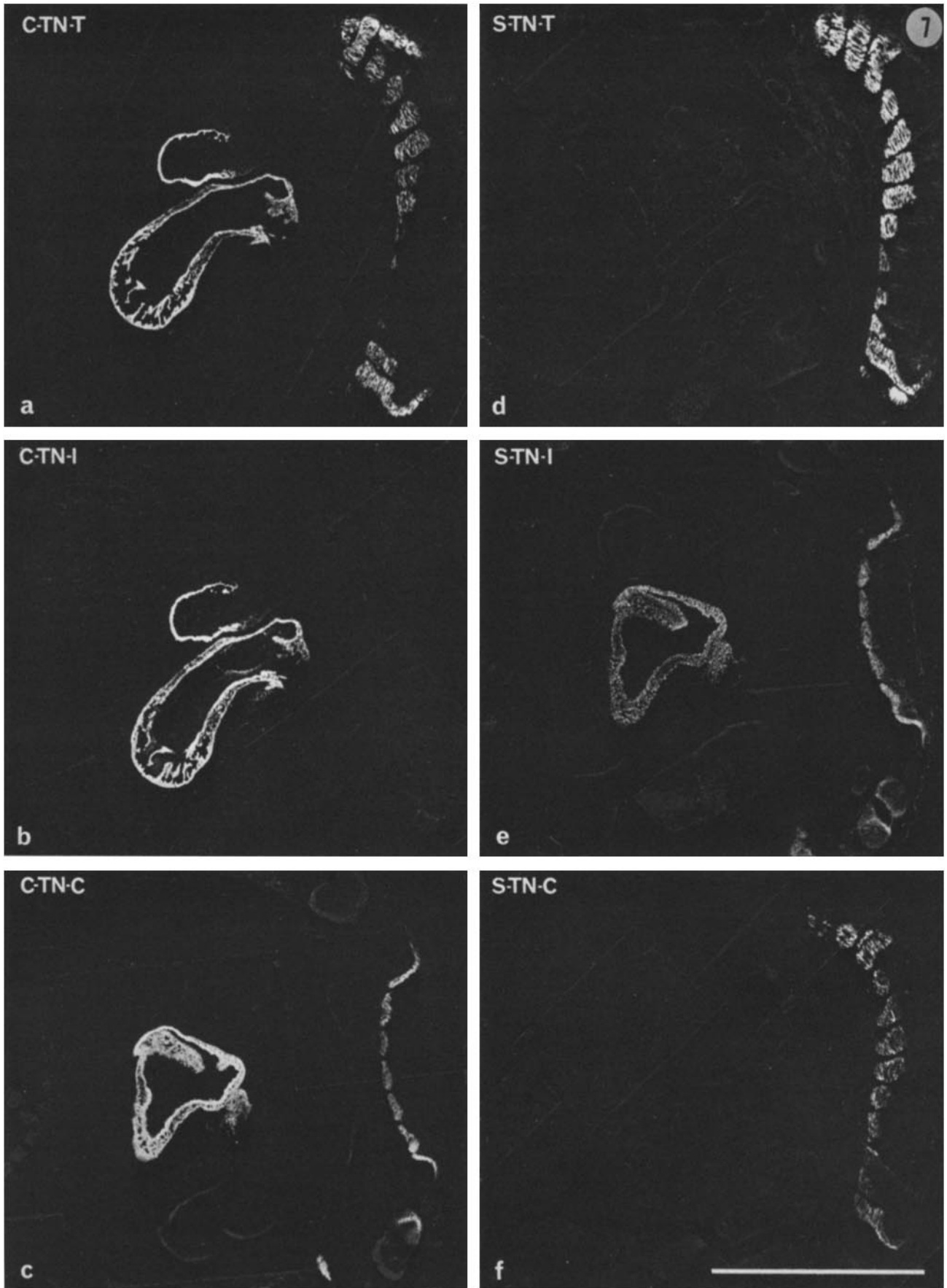


FIGURE 7

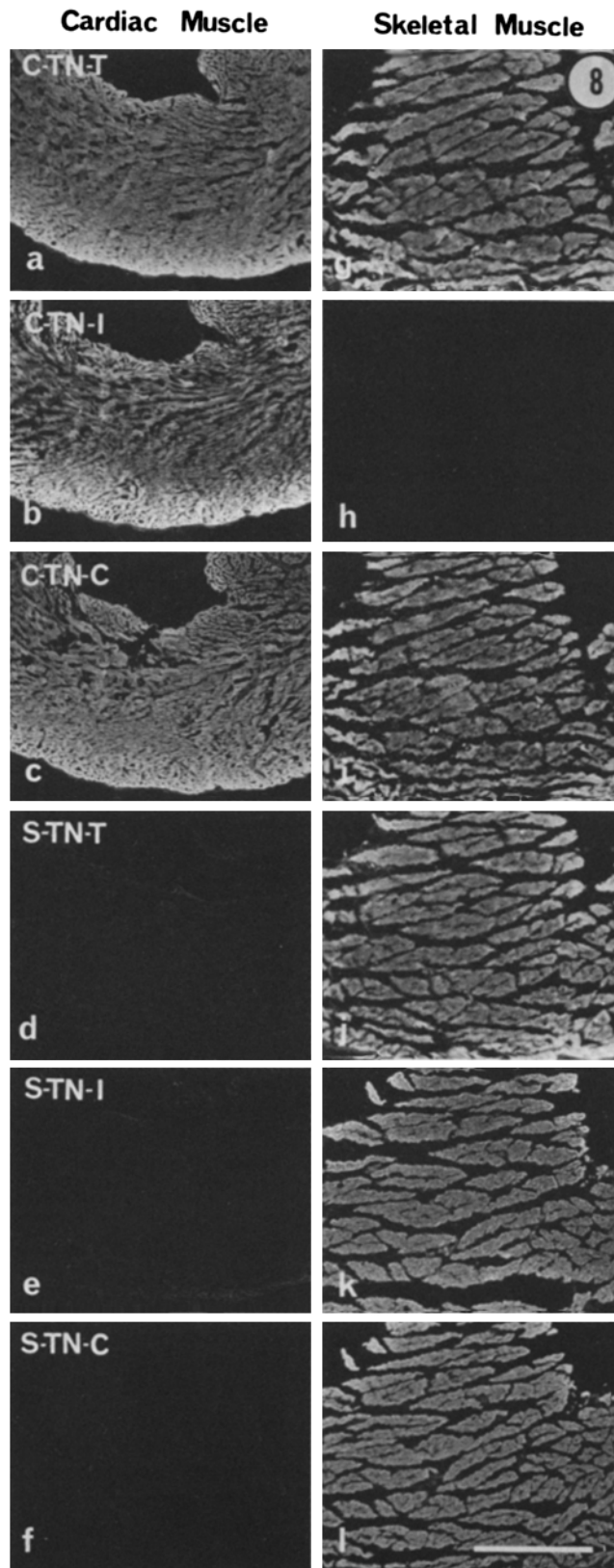


FIGURE 8 Chicken embryos at stage 45. Cardiac muscle reacted with antibodies against all of the cardiac TN components (a-c), but not with any antibody raised against skeletal TN components (d-f). Skeletal muscle was stained with antibodies against all of the skeletal TN components (j-i) and cardiac TN-T (g) and C (i). Bar, 0.5 mm. $\times 32$.

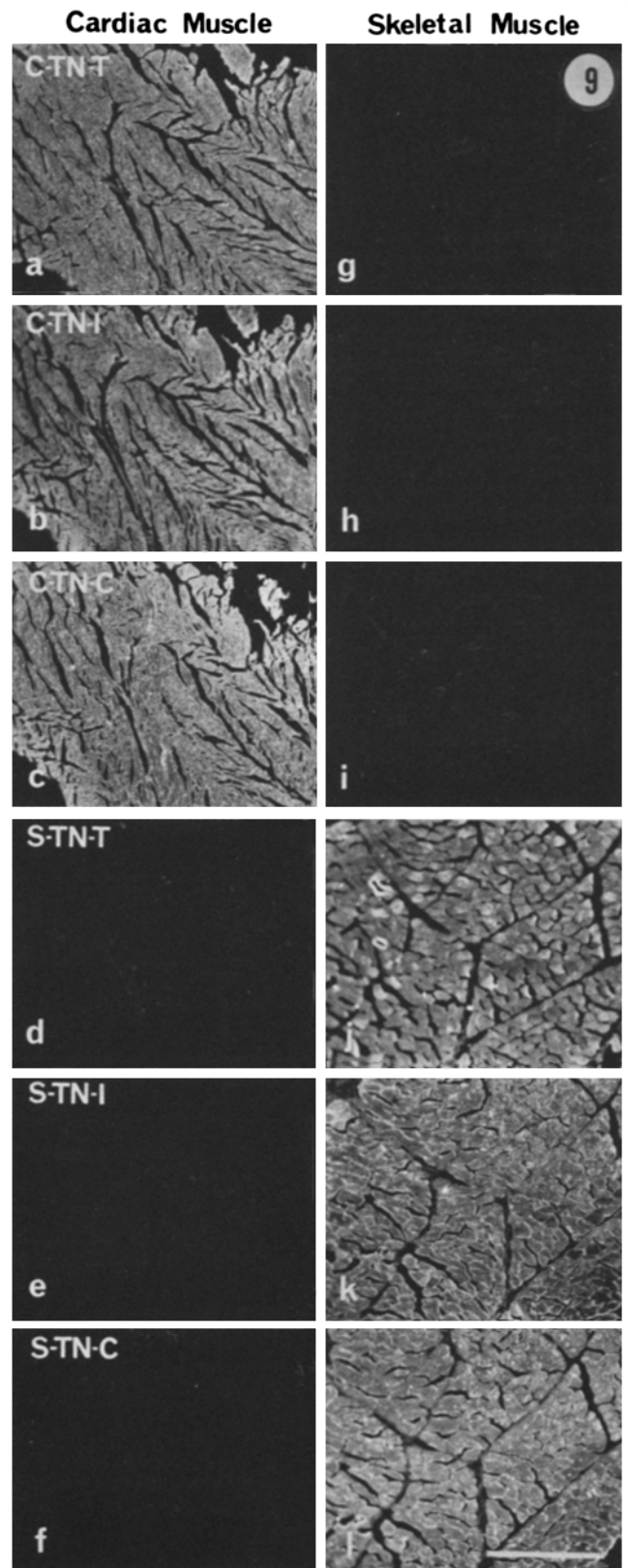


FIGURE 9 Adult chicken. Cardiac muscle was stained only with specific antibodies against TN components from heart (a-c). Skeletal muscle reacted only with antibodies raised against skeletal TN components (j-l). Bar, 0.5 mm. $\times 32$.

TABLE I
Differentiation of TN in Cardiac and Skeletal Muscles of Chicken Embryos

Tissue	Antibody against	Stages (Hamburger-Hamilton*) of Chicken Embryos					Adult
		8 (26-29 h)	10 (33-38 h)	14 (50-53 h)	20 (70-72 h)	45 (19-20 d)	
Cardiac muscle	Cardiac TN-T	-	+	+	+	+	+
	Cardiac TN-I	-	+	+	+	+	+
	Cardiac TN-C	-	+	+	+	+	+
	Skeletal TN-T	-	-	-	-	-	-
	Skeletal TN-I	-	+	+	+	-	-
	Skeletal TN-C	-	-	-	-	-	-
Skeletal muscle	Cardiac TN-T	-	-	+	+	±	-
	Cardiac TN-I	-	-	-	-	-	-
	Cardiac TN-C	-	-	+	+	±	-
	Skeletal TN-T	-	-	+	+	+	+
	Skeletal TN-I	-	-	+	+	+	+
	Skeletal TN-C	-	-	+	+	+	+

* V. HAMBURGER AND H. L. HAMILTON. 1951. *J. Morphol.* 88:49-92

are characteristic for the type of muscle from which they are prepared.

The present study further showed that some of the TN components synthesized by embryonic cardiac or skeletal muscles react with antibodies raised from both adult cardiac and skeletal muscles. That is, embryonic cardiac muscle displayed multiple reactivity to antibodies raised against two different TN-I's from adult heart and skeletal muscles. This suggests that embryonic cardiac muscle synthesizes TN-I's with antigenic determinants similar to those present in adult cardiac and skeletal muscles. Further, embryonic skeletal muscle reacted with antibodies against TN-T and C from the adult heart as well as those from skeletal muscles. This indicates that embryonic skeletal muscle synthesizes TN-T and C which share the antigenicities for both adult cardiac and skeletal muscles. However, the problem remaining to be solved is whether embryonic TN components are compatible with the presence of two types of distinct TN components corresponding to those formed in adult skeletal and cardiac muscles, or with the presence of one or more unique embryonic TN component(s) cross-reacting with antibodies to TN components of adult muscles.

Thus, it is clear from this study that some TN components of cardiac (TN-I) and skeletal (TN-T and C) muscles of the embryo are immunochemically different from those in the respective muscles of the adult. As development progresses, their forms change to the forms characteristic of their adult tissue type. There is an analogy between the findings reported here and those reported earlier concerning the patterns of myosin light chains, the myosin isozymes, tropomyosin, and TN-T in developing muscles (7, 12, 15, 18-20).

From our results it is apparent, in the development of cardiac and skeletal muscles, that a process occurs in which the fiber changes its genomic programming: it ceases synthesis of the TN components that are immunologically indistinguishable from each tissue-type, and synthesizes only tissue-type specific proteins after hatching. However, the reason is not clarified concerning why some TN components (cardiac TN-I, skeletal TN-T, and skeletal TN-C) change their forms whereas others (cardiac TN-T, cardiac TN-C, and skeletal TN-I) do not, and further, why the kind of TN components that change their forms during the development of cardiac muscle is different

from that of their skeletal muscle counterparts. In any case, it appears that a highly complex control mechanism is at work in the gene expression for the different muscle proteins. The problem of whether these developmental changes in the gene expression for TN components are programmed endogenously or are due to the influence of exogenous factor(s) requires further investigation. However, if exogenous factor(s) does affect the muscles, the role of innervating nerves cannot be ruled out, because it has been known in many instances that innervation leads to the differentiation of individual muscle fibers to specific types (1, 3, 21, 27, 28).

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