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# Foot Protein Isoforms Are Expressed at Different Times during Embryonic Chick Skeletal Muscle Development

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*Abstract.* We have investigated the time course of expression of the alpha and beta triad junctional foot proteins in embryonic chick pectoral muscle. The level of [³H]ryanodine binding in muscle homogenates is low until day E20 of embryonic development, then increases dramatically at the time of hatching reaching adult levels by day N7 posthatch. The alpha and beta foot protein isoforms increase in abundance concomitantly with [³H]ryanodine binding. Using foot protein isoform-specific antibodies, the alpha foot protein is detected in a majority of fibers in day E10 muscle, while the beta isoform is first observed at low levels in a few fibers in day E15 muscle. A high molecular weight polypeptide, distinct from the alpha and beta proteins, is recognized by antifoot protein antibodies. This polypeptide is observed in day E8 muscle and declines in abundance with continued development. It appears to exist as a monomer and does not bind [³H]ryanodine. In contrast, the alpha isoform present in day E10 muscle and the beta isoform in day E20 muscle are oligomeric and bind [³H]ryanodine suggesting that they may exist as functional calcium channels in differentiating muscle. Comparison of the intracellular distributions of the alpha foot protein, f-actin, the heavy chain of myosin and titin in day E10 muscle indicates that the alpha foot protein is expressed during myofibril assembly and Z line formation. The differential expression of the foot protein isoforms in developing muscle, and their continued expression in mature muscle, is consistent with these proteins making different functional contributions. In addition, the expression of the alpha isoform during the time of organization of a differentiated muscle morphology suggests that foot proteins may participate in events involved in muscle differentiation.

T HE foot protein, or ryanodine receptor, is a key component of the triad junction in striated muscle (4, 6). It embodies a high conductance channel permeable to calcium ions (7, 11, 24), and with the junction, participates in coupling electrical depolarization of the surface membrane to the release of calcium from the sarcoplasmic reticulum. The in situ contributions made by the foot protein to muscle function are unclear and may include roles as (a) a structural element involved in maintaining muscle cell morphology; (b) a transduction element responding to signals, such as depolarization, calcium, and inositol triphosphate; and (c) an ion channel, providing the pathway for calcium release from the sarcoplasmic reticulum. We have recently obtained evidence for two foot protein isoforms in mature chicken skeletal muscle fibers (2), suggesting that they may be specialized for different functions.

To investigate the contributions made by the foot proteins to skeletal muscle structure and function, we are characterizing when these proteins are expressed, become incorporated into the triad junction, and begin to participate in the release of intracellular calcium in embryonic chick skeletal muscle. Such studies in intact embryonic chick pectoral muscle from early developmental stages are complicated by a lack of synchronization of the development of individual muscle fibers. In contrast, muscle cells maintained in primary culture develop relatively synchronously and are generally used instead of intact embryonic muscle to investigate muscle development. A potential disadvantage of the cultured cells, particularly for investigations of the assembly of the triad junction, is that although junctions are observed, there appears to be an overproduction of transverse tubular elements that become arranged in extensive arrays (5, 8). This tubule arrangement may be a consequence of the cells being aneural, since a similar situation has been observed in

denervated muscle (19). In view of this condition, we felt it important to investigate the time course of the expression of the foot proteins in embryonic muscle in ovo before using cultured muscle cells as a model for more detailed studies of these events.

In the studies described in this report, we have found that the foot protein isoforms are expressed at different times during chick pectoral muscle development in ovo. These results suggest that the foot protein isoforms have different roles in muscle cell structure and function and that they may contribute to events involved in muscle differentiation.

## Materials and Methods

### Materials

Fertilized white Leghorn chicken eggs were purchased from the Weber Egg Co. (Rio Linda, CA). Leupeptin, PMSF, CHAPS, L-alpha-phosphatidylcholine, polyethylenimine, agarose-linked goat anti-mouse IgG (whole molecule) antibodies, n-propyl gallate, and antiactin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO); Zwittergent 3-14 from Calbiochem Behring Corp. (La Jolla, CA); SDS-PAGE molecular weight standards from Bio-Rad Laboratories (Richmond, CA); Centriprep concentrators were from Amicon Corp. (Danvers, MA); alkaline phosphatase conjugated goat anti-mouse IgG from Tago Inc. (Burlingame, CA); paraformaldehyde from Polysciences, Inc. (Warrington, PA); OCT compound from Miles Laboratories Inc. (Elkhart, IN); and rhodamine-conjugated phallacidin from Molecular Probes Inc. (Eugene, OR), FITC-conjugated goat anti-mouse antibodies, normal goat and normal mouse sera were obtained from Organon Technika (Durham, NC).

### Preparation and Solubilization of Muscle Homogenates

Breast muscle was dissected from white Leghorn embryos and homogenized in 5 ml/gram of muscle of a solution containing 0.3 M sucrose, 10 mM imidazole, pH 7.4, 1.1 $\mu$M leupeptin, 230 $\mu$M PMSF either for three 1-min intervals at high speed in a Waring blender or for 3 × 30 s using a Polytron at a setting of seven. Homogenates were rapidly frozen in liquid nitrogen and stored at −90°C. Protein was assayed using the method of Lowry et al. (13). For immunoprecipitation experiments, homogenate protein (3 mg protein/ml) was solubilized with 4% CHAPS in the presence of 2% phosphatidylcholine. Solubilized protein was obtained after centrifugation at 100,000 g for 30 min. In some experiments the foot protein isoforms were further purified by sucrose gradient sedimentation. 3–5 ml of CHAPS-solubilized homogenate protein were layered on top of 30 ml 3–30% continuous sucrose gradients and centrifuged at 133,000 g for 14–16 h (2, 11). The gradients were divided into 3-ml fractions and the protein profile of each fraction visualized in SDS-PAGE gels stained with Coomassie brilliant blue. In the experiments illustrated in Fig. 3, muscle homogenate protein was solubilized with 1% CHAPS in the presence of 0.5% phospholipid as described above and concentrated fivefold using Centriprep 30 concentrators before sucrose gradient sedimentation.

### Antibodies, Immunoprecipitation, SDS-PAGE, and Western Blotting

The antichicken skeletal muscle foot protein mAbs used in these studies were partially purified (21) before use, and included 110F (antialpha), 110E (antibeta), and 34C (antialpha/beta) (2). Antibodies against the heavy chain of myosin and titin were the generous gifts from Dr. E. Bandman (University of California, Davis) and Dr. P.A. Maher (University of California, San Diego), respectively. The antimyosin antibody recognizes all isoforms of sarcomeric myosin (E. Bandman, personal communication). The characteristics of the antititin antibody have been described previously (26).

Solubilized foot protein isoforms were immunoprecipitated using antichicken skeletal muscle foot protein monoclonal and polyclonal antibodies as described previously (2). Proteins were separated in SDS-PAGE gradient (4–20%) gels and electrophoretically transferred to nitrocellulose (2). Blots were probed with antichicken skeletal muscle foot protein antibod-

ies and visualized using alkaline phosphatase-conjugated secondary antibodies.

### [³H]ryanodine and [³H]epiryanodine Binding

[³H]ryanodine binding by nonsolubilized muscle homogenates was measured by incubating 100–250 $\mu$g/ml of homogenate protein in 0.5 ml of 0.5 M KCl, 20 mM Tris, pH 7.4, 100 $\mu$M CaCl$_2$, 1.1 $\mu$M leupeptin, 230 $\mu$M PMSF, and 20 nM [³H]ryanodine (reference 25; 56.4 Ci/mmol) for 2 h at 37°C. Specific binding was defined as the [³H]ryanodine displaced by a 100-fold excess of unlabeled ryanodine. Binding was terminated by filtration through GF/B filters (Whatman Inc., Clifton, NJ) and washing four times with 4 ml of an ice-cold wash solution containing 0.5 M KCl, 20 mM Tris, pH 7.4, and 100 $\mu$M CaCl$_2$. The binding of [³H]ryanodine or [³H]epiryanodine (2) by solubilized homogenate protein was measured in a 100-$\mu$l vol of 0.5 M KCl, 20 mM Tris, pH 7.4, 200 $\mu$M CaCl$_2$, 1.1 $\mu$M leupeptin, 230 $\mu$M PMSF, 1% CHAPS, and 0.5% phosphatidylcholine, and either 100 nM [³H]ryanodine, or 540 nM [³H]epiryanodine (reference 25; 45.8 Ci/mmol) for 1 h at room temperature. Binding was terminated by filtration through Whatman GF/B filters treated with 1.0% PEI (3) and washing seven times with 4-ml aliquots of the wash solution described above.

### Immunofluorescent Labeling

3-d-old chicks were fixed by vascular perfusion and day E10, E15, and E18 embryos by immersion fixation with 4% formaldehyde (from paraformaldehyde) in 0.1 M PBS. Breast muscle was removed and further fixed in the same solution for 2 h at 4°C. Tissue was cryoprotected in 0.5 M sucrose in PBS for 30 min and then in 1.0 M sucrose in PBS for 1 h. Tissue was frozen in OCT compound in either dry ice or liquid N$_2$.

Immunofluorescent labeling was conducted as described previously (1). Briefly, 10-$\mu$m-thick cryostat sections were mounted on gelatin-coated slides and rinsed with 0.1 M PBS containing 0.05 M glycine. Nonspecific binding was blocked by incubating sections for 20 min in 1% normal goat serum, 0.5% BSA, and 0.5% gelatin in PBS. Sections were incubated with the monoclonal antifoot protein antibodies, either alone or with antibodies against actin, myosin, or titin, or with normal mouse serum for 1 h at 22°C. The sections were washed 10 times for 2 min in PBS and incubated in goat anti-mouse IgG-FITC conjugate for 1 h at 22°C. For double-labeling experiments with rhodamine-phallacidin, this conjugate was added to the secondary antibodies halfway through the incubation. The sections were again washed 10 times for 2 min in PBS and covered with an antifade media consisting of 4% n-propyl gallate, 90% glycerol in PBS.
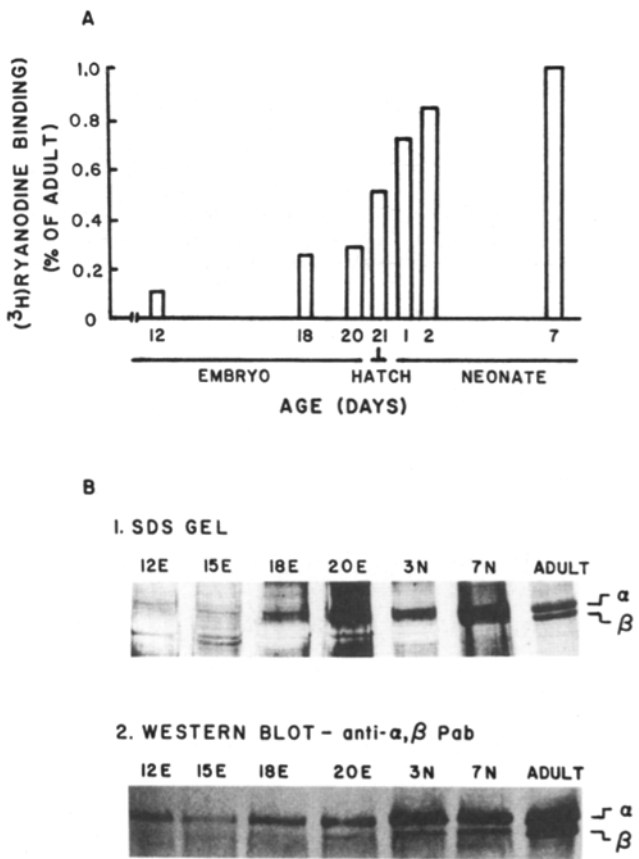
### Confocal Immunofluorescent Microscopy

Immunofluorescent images were obtained using a BioRad MRC-600 confocal laser scanning microscope fitted to a Zeiss Axiovert 35M inverted microscope in either single or dual channel modes. For double-labeled specimens, dual fluorescent images were simultaneously recorded from the same optical sections. For single-labeled specimens, Nomarski images were simultaneously obtained using a transmission detector. Images were stored on an AGA optical disk recorder and photographed with a Lasergraphics LFR camera using Kodak Ektachrome 100 film.

## Results

### Time Course of the Appearance of [³H]ryanodine Binding in Embryonic Chick Breast Muscle

[³H]ryanodine binding is a characteristic of a functional tetrameric triad junctional foot protein (11, 12), therefore, to determine the approximate time course of the expression of this protein we investigated specific [³H]ryanodine binding in embryonic chick pectoral muscle. Whole homogenates were used in these studies, since it is not known which subcellular fractions of immature muscle contain the foot proteins. As illustrated in Fig. 1 A, specific [³H]ryanodine binding increased slowly to ~30% of that observed in adult muscle between days E12 and E20 of embryonic development, and then more dramatically at the time of hatching

First, foot protein polypeptide subunits similar to those occurring in mature avian fast twitch skeletal muscle cells are present in embryonic pectoral muscle (Fig. 1 *B*). These proteins increase in abundance in parallel with the increase in specific [³H]ryanodine binding. This is evident from SDS polyacrylamide gels stained for protein with Coomassie brilliant blue (Fig. 1 *B1*), but can be visualized more clearly in Western blots probed with affinity-purified (18) antifoot protein polyclonal sera (Fig. 1 *B2*). A second observation is that the alpha and beta foot proteins are detected at different times during development. The alpha foot protein is present in day E12 embryonic muscle, while the beta isoform is not evident until about day E18 (Fig. 1 *B*).

We next sought to ensure that the difference in the temporal appearance of these proteins was not due to either a technical aspect of the methods used, or to the relatively low abundance of the foot proteins in muscle homogenates. For example, the alpha and beta isoforms may not transfer electrophoretically in an equivalent manner, or the beta isoform may be expressed at very low levels before day E15. We used both biochemical and immunocytochemical methods to assess these possibilities.

*Figure 1.* Time course of the expression of [³H]ryanodine binding activity and of the alpha and beta foot proteins during embryonic chick muscle development. (*A*) Specific [³H]ryanodine binding by pectoral muscle homogenates prepared from chicks of different developmental ages was normalized relative to that of muscle homogenates obtained from adult subjects. E and N refer to embryonic and neonatal ages, respectively. (*B*) The high molecular weight proteins present in the homogenates were visualized in SDS-PAGE gels stained with Coomassie brilliant blue and by probing Western blots with a rabbit antichicken foot protein polyclonal sera affinity purified against both alpha and beta polypeptides (18). Equivalent amounts of muscle homogenate protein were loaded in the lanes labeled 12E through 7N. The large quantities of myosin present in adult muscle homogenates precluded their use in SDS gels, therefore, more purified microsomal membranes were used as starting material for the lane labeled Adult. Consequently, a comparison of the quantities of the high molecular weight proteins present can be made between lanes 12E through 7N, but not between these lanes and that labeled Adult.
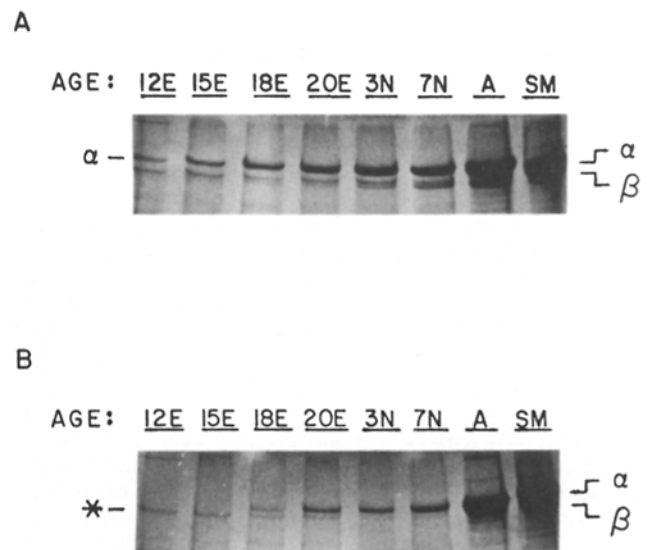
(day 21), reaching 85% of the adult levels by day N2 posthatch. Adult levels of [³H]ryanodine binding were reached by day N7 posthatch.

## Time Course of Foot Protein Biosynthesis in Embryonic Chick Breast Muscle

We investigated whether the increase in [³H]ryanodine binding observed at the time of hatching was due to the synthesis of new foot protein or to changes in the binding characteristics of existing protein. Analysis of homogenate protein in SDS polyacrylamide gels, or by Western blotting with foot protein–specific mAbs (2) yielded two observations.



*Figure 2.* Immunoprecipitation of alpha and beta foot proteins by foot protein-specific MAbs from homogenates of embryonic chick pectoral muscle obtained at different stages of development. E and N denote embryonic and neonatal ages, respectively, and A indicates adult muscle. SM indicates the solubilized material used for the immunoprecipitation experiment, therefore, this sample was the only one that was not immunoprecipitated. Before precipitation, proteins were solubilized with CHAPS in the presence of phospholipid to insure the maximal availability of the foot protein epitopes to the antibodies. (*A*) Alpha polypeptides were precipitated from homogenates obtained from muscles from all of the developmental stages tested by the antialpha polypeptide mAb, 110F. The lower molecular weight polypeptides evident in this panel have greater mobilities than the beta polypeptide and represent breakdown products of alpha and the gamma polypeptide. (*B*) The beta polypeptide was precipitated by the antibeta mAb 110E, from homogenates obtained from stages 18E and later. A lower molecular weight gamma polypeptide denoted by * is also recognized by this antibody in homogenates from stages 12E through 20E.
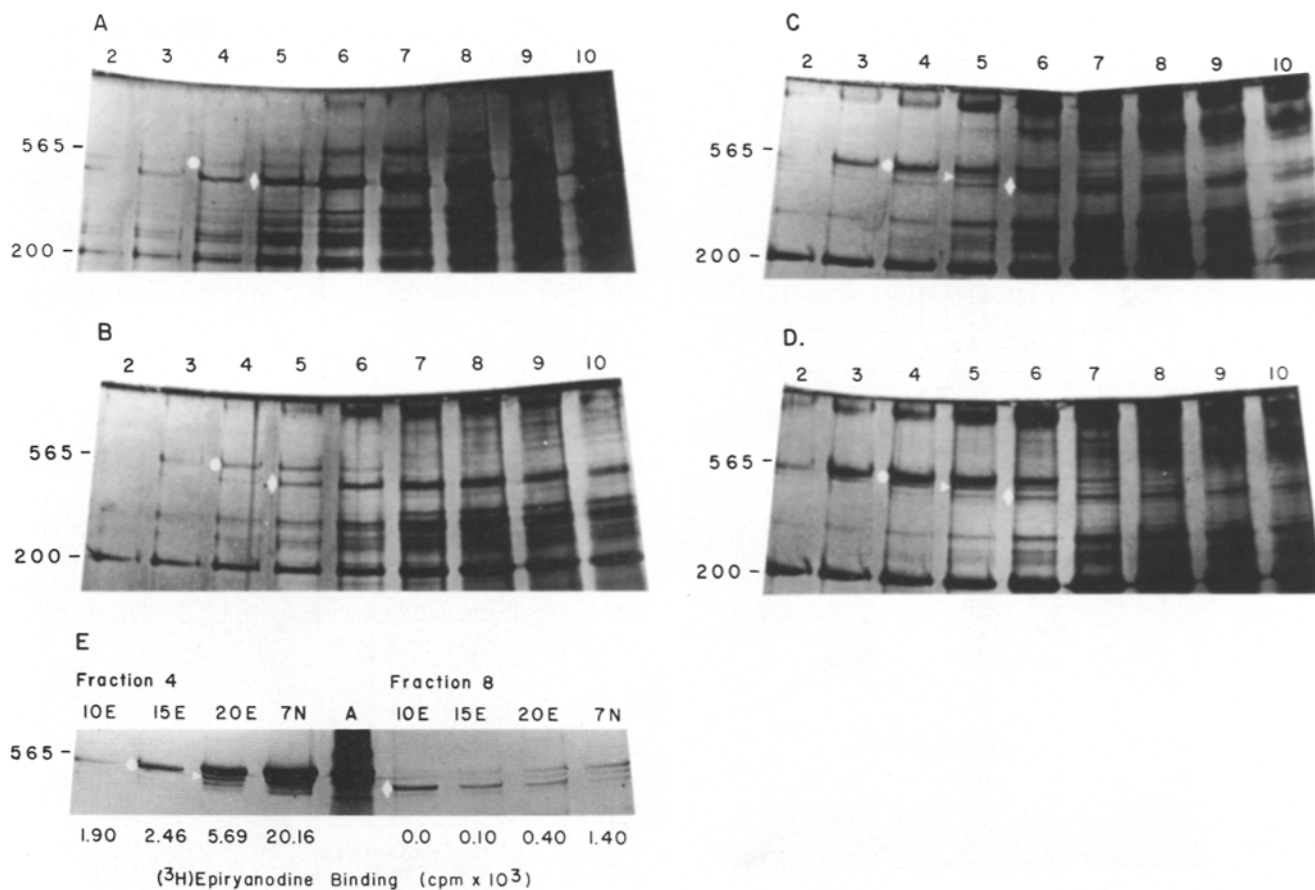
*Figure 3.* Comparison of the abundance, the native size and [³H]epiryanodine binding ability of the foot protein isoforms present in 10, 15, and 20 d embryonic and 7-d old neonatal chick pectoral muscle. (*A–D*) Proteins present in pectoral muscle homogenates from each developmental stage were solubilized with CHAPS in the presence of phospholipid, concentrated fivefold, and sedimented through individual continuous 3–30% sucrose gradients. The proteins present in gradient fractions 2–10 (numbered from the bottom of the gradient) were resolved in 4–20% continuous polyacrylamide gradient SDS gels stained with Coomassie brilliant blue. The alpha, beta, and putative gamma foot protein isoforms are indicated by circles, arrowheads, and diamonds, respectively. The rabbit skeletal muscle foot protein (565 kD) and the heavy chain of myosin (200 kD) were used as molecular mass standards. (*E*) Foot protein isoforms present in gradient fractions 4 and 8 from each of the preparations were immunoprecipitated using the mAb 34C. The proteins precipitated were resolved on 4–20% continuous gradient polyacrylamide SDS gels stained with Coomassie brilliant blue. 10E, 15E, 20E, 7N and A denote, 10, 15, and 20 d embryonic, 7 d neonatal and adult chicken muscle samples, respectively. Note that the adult sample represents intact microsomal membrane protein that was neither solubilized with CHAPS, nor immunoprecipitated. The specifically bound [³H]epiryanodine that coprecipitated with these samples is given below lane.

An immunoprecipitation protocol that both amplifies the level of antigen by 10–20-fold and does not involve electrophoretic transfer of protein was used as one approach. Membrane proteins were solubilized with CHAPS in the presence of phospholipid from homogenates prepared from embryonic muscle obtained at different developmental stages. Foot proteins were immunoprecipitated from the solubilized material using foot protein isoform-specific antibodies. The immunoprecipitated material was concentrated by resuspension in a reduced volume to amplify the signal at this step. As illustrated in Fig. 2, the results of these experiments confirmed the preceding observations. The alpha foot protein subunit polypeptide is present in day E12 embryonic muscle, and detectable levels of the beta foot protein polypeptide appeared at approximately day E18.

In a second study, we attempted to achieve a further selective amplification of the foot proteins by concentrating the homogenate proteins from days E10, E15, E20, and N7 chick muscles solubilized with CHAPS fivefold, and then purifying the foot proteins by sedimentation through continuous sucrose gradients. As shown in Fig. 3, *A–D*, the alpha foot protein isoform (indicated by the circles) is present in day E10 muscle, while the beta isoform (indicated by the triangles) is not detected until between days E15 and E20. The purified foot proteins were amplified further by precipitation with the antifoot protein antibody, 34C, as described in Materials and Methods. The results obtained were consistent with those just described, in that the alpha isoform was precipitated from day E10 muscle homogenates, while the beta isoform was only observed in muscle homogenates from days E20 and N7 (Fig. 3 *E*).

In using the preceding methods, it was necessary to sample homogenates of whole muscles; consequently, it is possible that low levels of the beta isoform present in a few fibers were not detected. Therefore, in a second approach, we utilized isoform-specific antibodies and immunocytochemical techniques to examine the foot protein content of individual fibers in muscles from days E10, E15, and E18 embryos (Figs.
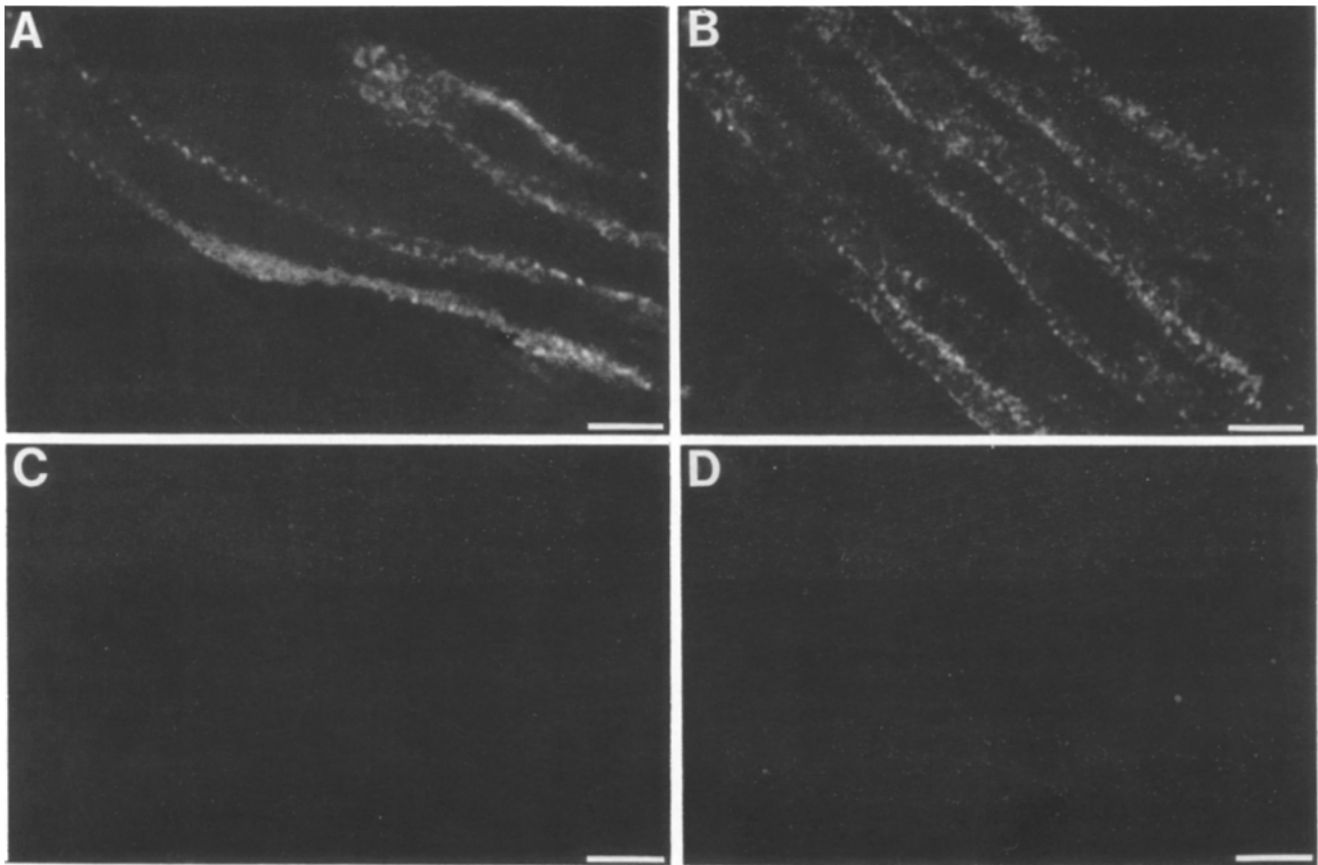
*Figure 4.* Immunofluorescent localization of alpha and beta, (*A*), alpha (*B*), or beta (*C*) foot proteins in serial sections of 10 d embryonic chick breast muscle obtained using the antialpha, beta antibody, 34C, the antialpha antibody, 110F, and the antibeta antibody, 110E, respectively. In *D*, normal mouse serum was substituted for the primary antibody as a negative control. Bars, 10 μM.

4–5). The alpha isoform was observed in all of the fibers examined in muscles from each stage. The beta isoform was not detected in any fibers in day E10 muscle (Fig. 4), observed in just a few fibers in day E15 muscle, and was relatively more abundant by day E18 (Fig. 5). Consistent with the results shown in Figs. 1–3, the intensity of the fluorescence associated with the staining of the beta isoform in day E15 muscle fibers was less than that observed for the alpha isoform in day E10 muscle. In conclusion, these results indicate that the foot protein isoforms are expressed at different times during chick skeletal muscle development. The alpha isoform is present at least as early as day E10, while the beta isoform first appears at low levels in a few fibers at day E15.

### Antifoot Protein Antibodies Recognize a Third High Molecular Weight Polypeptide in Embryonic Chick Breast Muscle

Antibodies specific for either the alpha or beta foot protein isoform recognize polypeptides having lower apparent molecular weights than either the alpha or beta isoform. One of these polypeptides, recognized by the antialpha antibody, parallels the alpha polypeptide in the time course of its appearance and in its relative abundance and, therefore, may represent the breakdown product of alpha described previously (2). Another polypeptide recognized by both antialpha and antibeta antibodies and denoted by an asterisk in Fig. 2 *B* was termed the gamma polypeptide. This polypeptide is

present in homogenates from day E10 embryonic muscle and became less abundant with continued development (Figs. 2 and 3). In other experiments, the gamma polypeptide was found to be in relatively greater abundance in breast muscle from day E8 embryos (data not shown). The recognition of this protein by several foot protein–specific antibodies and its transient expression suggests that it could be a developmental stage-specific foot protein isoform.

### Molecular Complexity and Ligand Binding Ability of Foot Proteins in Embryonic Chick Breast Muscle

The detection of the alpha foot protein isoform in developing embryonic muscle, and the existence of the transiently expressed putative gamma isoform indicate a possible role for the foot proteins in early myofibril formation. A tetrameric foot protein appears to be required for ion channel and ligand binding functions (12), whereas the gamma isoform, as a putative foot protein monomer, may have sufficient size to participate in the initial assembly of the triad junction prior to development of the calcium release function. To learn more about these early events, we investigated the oligomeric nature and [³H]epiryanodine binding ability of the foot proteins in breast muscle from day E10, E15, and E20 embryonic and day N7 neonatal chicks, as indices of the functional state of these proteins.

In these experiments, CHAPS-solubilized chick muscle homogenate protein was concentrated fivefold before the su-
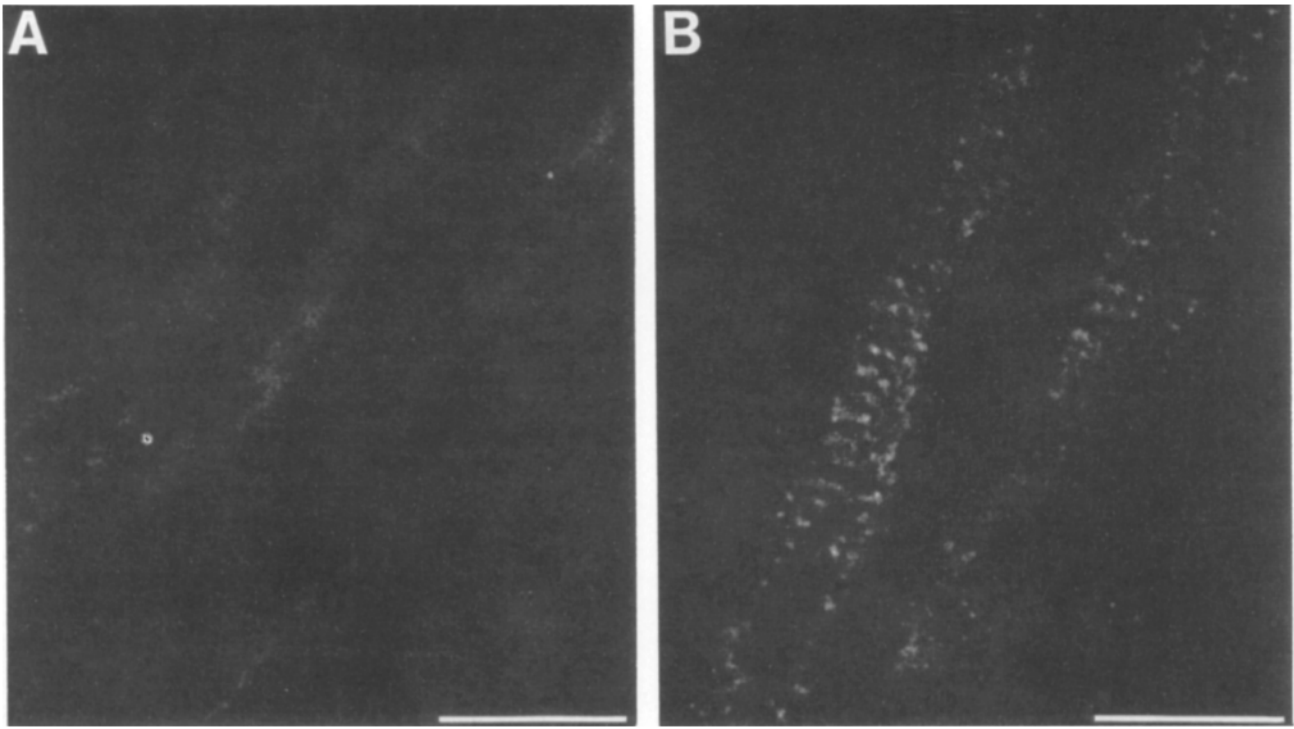
*Figure 5.* Immunofluorescent localization of the beta foot protein isoform in breast muscle from day E15 (*A*) and E18 (*B*) chick embryos using the antibeta antibody, 110E. Bars, 10 μM.
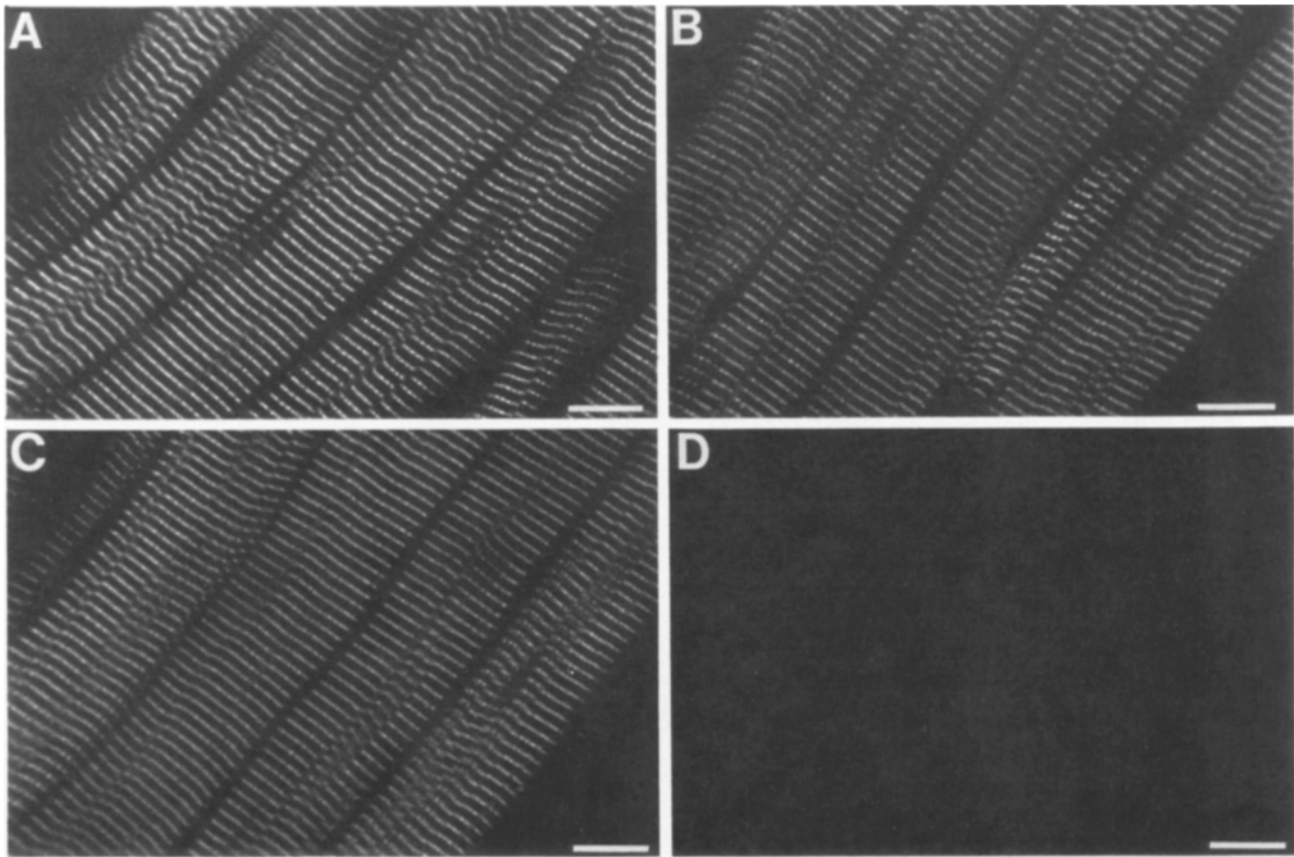


*Figure 6.* Immunofluorescent localization of alpha and beta foot proteins in serial sections of day N3 neonatal chick breast muscle fibers. As in Fig. 4, the alpha and beta isoforms were stained with 34C (*A*); alpha with 110F (*B*); beta with 110E (*C*); and the normal mouse serum control (*D*). Bars, 10 μM.
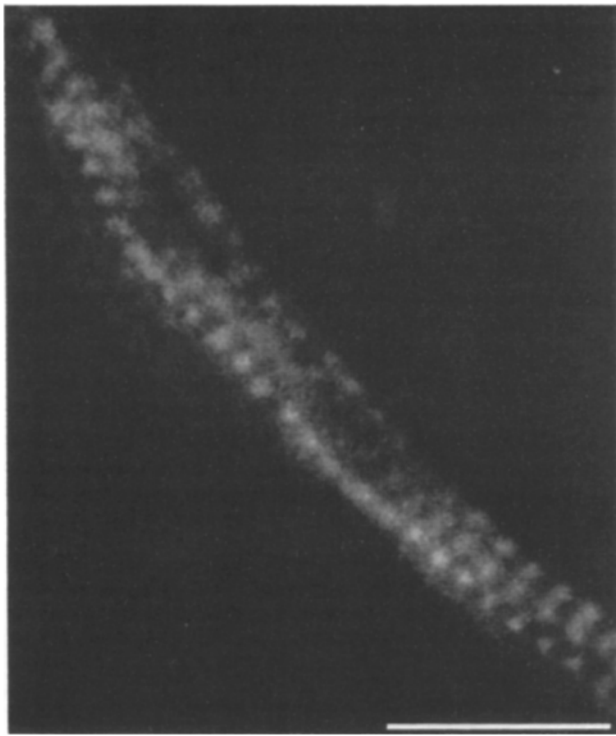
*Figure 7.* Localization of the heavy chain of myosin using an antimyosin antibody in day E10 embryonic chick breast muscle. Bars, 10 $\mu$M.

crose gradient purification step to enhance detection of foot proteins expressed at low levels in muscles from early developmental stages. The oligomeric state of the protein was assessed by sedimentation through continuous sucrose gradients (12). In control experiments, foot protein tetramers and monomers were produced by solubilization of adult chicken muscle microsomes with CHAPS and Zwittergent 3-14, respectively (12). The tetramers were found to sediment to sucrose gradient fractions 3–6, while foot protein monomers were found primarily in fractions 7–10 (fractions numbered from the bottom of the gradient, data not shown). The sucrose gradient fraction distributions of foot proteins found in days E10, E15, and E20 embryonic muscle and day N7 neonate muscle are shown in Fig. 3, *A–D*. Foot proteins were precipitated with the antibody, 34C, to amplify low levels of [$^3$H]epiryanodine binding, and the ability of proteins sedimenting in gradient fractions 4 and 8 to bind [$^3$H]epiryanodine was determined (Fig. 3 *E*).

The putative gamma isoform (indicated by the diamonds) is present predominately in sucrose gradient fractions 7–10 indicating a monomeric state. In agreement with the results shown in Fig. 2, the level of this polypeptide is greatest in day E10 embryonic muscle becoming less abundant as development progresses. The alpha isoform from each of the developmental stages (indicated by the circles) sediments primarily as a tetrameric protein in gradient fractions 3–6 and becomes more abundant with muscle development. The beta isoform (indicated by the arrowheads) is similar to alpha in its gradient fraction distribution and therefore appears to be predominately tetrameric. Similar sucrose gradient and developmental stage distribution patterns were observed after precipitation with the antibody, 34C (*E*). Additionally, with

the greater signal amplification obtained with the immunoprecipitation protocol, low levels of both alpha and beta isoforms are also detected in gradient fraction 8 obtained with each of the developmental stages. The levels of specific [$^3$H]epiryanodine binding present in these two fractions are given at the bottom of this panel.

The data in Fig. 3 suggest that the alpha and beta isoforms exist primarily as tetrameric proteins at the embryonic stages tested. There does not appear to be an accumulation of monomeric alpha and beta subunits, at these stages, as has been observed for the alpha subunit of the sodium channel (23) and suggested for the dihydropyridine receptor (16). Relatively low levels of the alpha and beta isoforms were observed together with measurable [$^3$H]epiryanodine binding after immunoprecipitation of fraction 8 (Fig. 3 *E*). It has been demonstrated that a tetrameric foot protein is required for ligand binding (11, 12). Consequently, this ligand binding activity could be due to the presence of tetrameric proteins associated with the trailing edge of sedimentation distribution, rather than to individual monomers, since the initial fivefold concentration of the solubilized proteins resulted in an overloading of the sucrose gradients. Alternatively, individual foot protein monomers resulting from a low level of denaturation of oligomeric proteins could reform oligomers during the ligand binding assay. The putative gamma isoform appears to be predominately monomeric and does not bind [$^3$H]epiryanodine.

### Intracellular Distributions of Foot Proteins in Embryonic Chick Breast Muscle

In other studies we utilized antichicken skeletal muscle foot protein mAbs, designated 110F, 110E, and 34C, which recognize the alpha, the beta and both the alpha and beta isoforms, respectively (2), to compare the intracellular localization of the foot proteins in muscle fibers obtained from day E10 embryos and day N3 neonates. Using these antibodies, we have demonstrated previously that both the alpha and beta foot protein isoforms are localized to the triad junction in mature chicken breast muscle (2). It should be noted that unlike mammalian skeletal muscle and other avian skeletal muscles, the triad junctions are located at the Z lines in chick breast muscle (17). Consistent with these findings, serial sections of muscle from 3-d-old neonatal chick muscle stained with the three antifoot protein antibodies exhibited a similar, strongly banded, punctate fluorescence (Fig. 6) expected for the peri-Z line localization of the foot proteins and the triad junctions found in mature pectoral muscle fibers (2, 17).

The cellular distribution of the alpha foot protein in day E10 muscle fibers suggests that this isoform is expressed during myofibril assembly and perhaps Z line formation. The relationships existing between these events were investigated by comparing the cellular localization of the foot protein with those of the sarcomeric proteins, f-actin, and myosin (heavy chain), and titin in double-labeled sections of muscle from day E10 embryos and day N3 neonates. Variability in the extent of development of individual fibers was observed in day E10 muscles. Some fibers exhibited well-developed sarcomeric myofibrillar arrays, whereas in other fibers, such organization was just beginning. For example, compare the fiber stained for the heavy chain of myosin shown in Fig. 7, with those stained for either actin or titin (Figs. 8 and 9). In
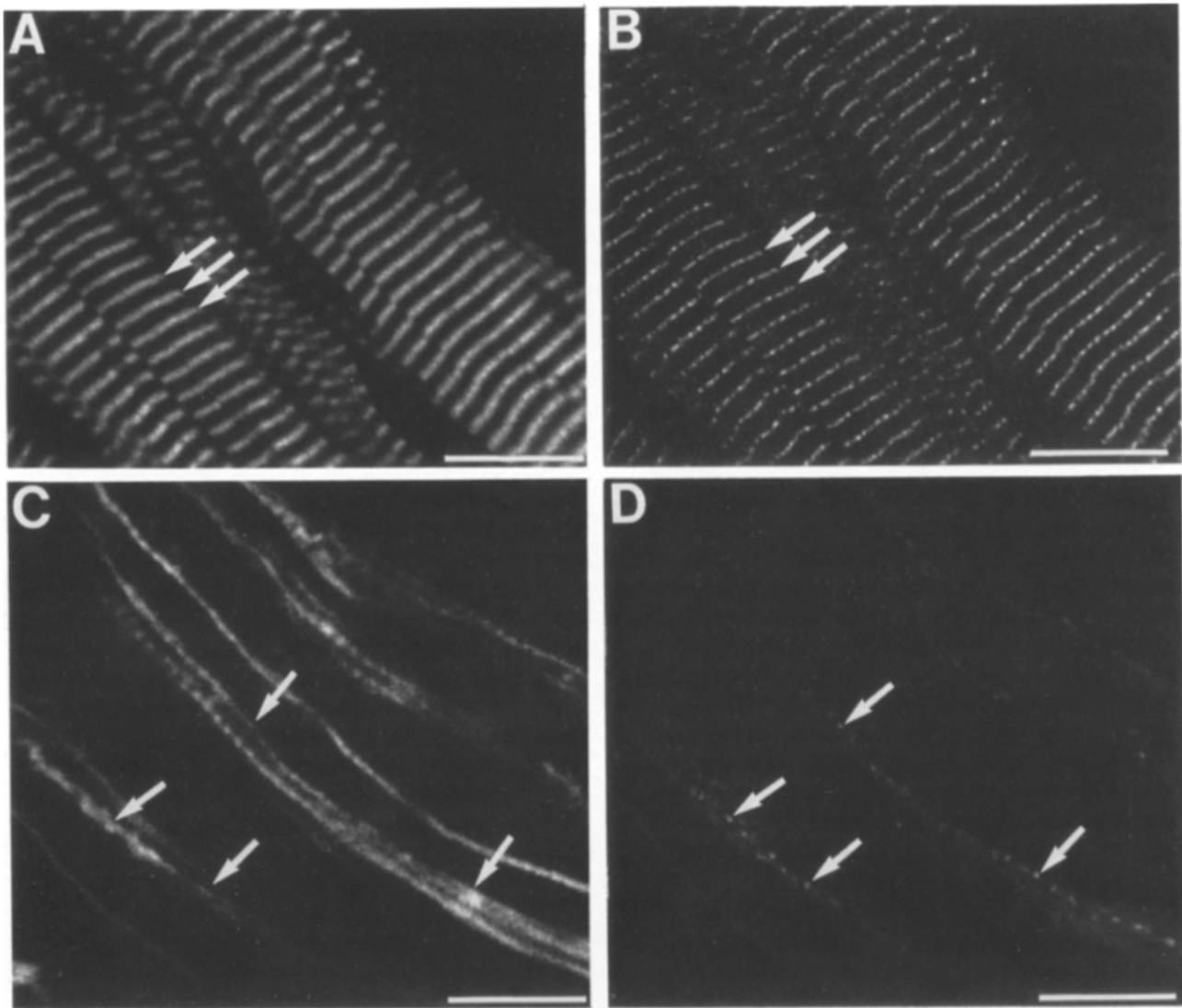
*Figure 8.* Simultaneous localization of f-actin using a rhodamine-phallacidin conjugate (*A* and *C*) and the alpha and beta foot proteins using the antibody, 34C (*B* and *D*) in sections of day N3 neonatal (*A* and *B*) and day E10 embryonic (*C* and *D*) chick breast muscle. The arrowheads indicate identical locations in each pair (*A* and *B*, and *C* and *D*) of images. Bars, 10 µM.

the present studies, relatively less well-developed fibers were selected, to gain insights into the temporal relationships between foot protein expression and other aspects of muscle differentiation.

In day E10 muscle fibers, actin, detected using rhodamine-phallacidin, is assembled in ribbon-like longitudinal filaments that exhibit the initial segmentation associated with sarcomere formation (Fig. 8 *C*). Identical actin distributions were observed when an antiactin antibody was used instead of phallacidin, to localize this protein (data not shown). In the same sections, the alpha foot protein is localized longitudinally as rows of foci that appear to be associated spatially with, and distributed along, the actin filaments (Fig. 8 *D*). In N3 neonatal muscle fibers, actin and the foot protein (Fig. 8, *A* and *B*) have the respective, sarcomeric and peri-Z line distributions typical of mature muscle. In muscle fibers at comparable stages of development, the heavy chain of myosin exhibits distributions similar to those just described for actin (data not shown). In similar studies, the alpha foot protein was found to be localized between adjacent rows of titin

proteins before complete Z disk formation in day E10 muscle fibers (Fig. 9, *C* and *D*), a relationship that is more clearly appreciated from the mature muscle-like distributions of these proteins seen in day N3 muscle (Fig. 9, *A* and *B*). In conclusion, these studies suggest that the alpha foot protein isoform is expressed in developing embryonic chick skeletal muscle at the time of myofibril assembly and Z line formation.

## Discussion

The results of this study demonstrate (*a*) that both the alpha and beta foot proteins increase dramatically in abundance at the time of hatching; (*b*) that the two avian skeletal muscle foot protein isoforms are expressed at different times during embryonic chick skeletal muscle development; and (*c*) the alpha foot protein is expressed at the time of myofibril assembly and Z line formation.

A dramatic increase in specific [$^3$H]ryanodine binding by pectoral muscle homogenates occurs at the time of hatching.
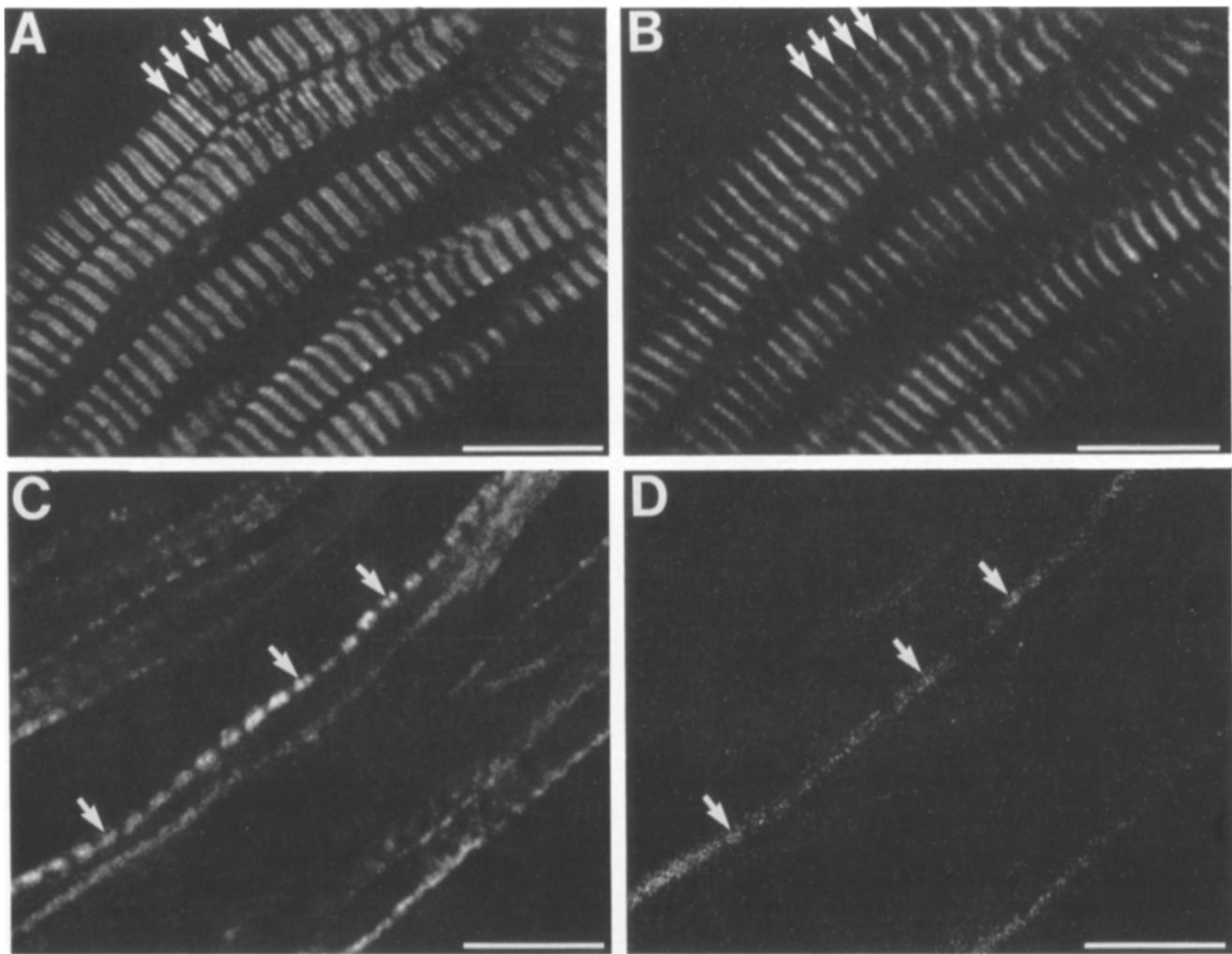
*Figure 9.* Simultaneous localization of titin (*A* and *C*) and the alpha and beta foot proteins (*B* and *D*) using an antititin antibody and the antibody, 34C, respectively, in sections of day N3 neonatal (*A* and *B*) and day E10 embryonic (*C* and *D*) chick breast muscle. The arrowheads indicate identical locations in each pair (*A* and *B*, and *C* and *D*) of images. Bars, 10 $\mu$M.

This increase in binding activity appears primarily, if not exclusively, due to increases in foot protein abundance. The elevated levels of foot protein isoforms are consistent with the increased demand for muscle activity at hatching. The biosynthesis of other proteins involved in muscle cell calcium metabolism, such as the dihydropyridine receptor (22), and the fast twitch fiber–specific calcium ATPase isoform (9, 14, 15) also increases at this time. The time course of the expression of the alpha foot protein isoform (this study) and of [$^3$H]nitrendipine binding (and presumably the dihydropyridine receptor; 22) during the embryonic development of chicken breast and leg muscle, respectively, are similar, suggesting that the expression of these functionally related proteins may be coordinately regulated.

The differential expression of alpha and beta foot protein isoforms in developing chick pectoral muscle observed in the present studies, and the continued expression of these proteins in mature muscle (2) are consistent with these proteins making different contributions to muscle cell structure and function. Multiple experimental protocols were used to ensure that temporal differences in the expression of the isoforms were not due to the low abundance of these proteins and/or to a lack of sensitivity of the methods used. In initial studies, the ratio of the alpha and beta isoforms, determined from densitometric scans of SDS-PAGE gels, were found to decrease as development proceeded. This result is consistent with the differential expression of these proteins. We have not used these ratios to support this argument, since there is no a priori reason to assume that ratios found either to be constant or variable during the stages of development where they can be measured are necessarily the same during earlier stages where they cannot be determined. Moreover, their measurement is still dependent on the sensitivity of the methods used to detect the individual isoforms. The results obtained with the biochemical and immunocytochemical methods used in these studies indicate that the alpha foot protein isoform is expressed in pectoral muscle $\sim$5 d before the beta isoform can be detected.

As noted in the introduction, the foot proteins may subserve structural, signal transduction, and ion channel functions. An isoform specialized for one of these roles may better fit the needs of the developing muscle cell. For example, if the foot protein acts primarily as a cytoskeletal component during the early stages of skeletal muscle cell development, foot protein isoforms specialized for a structural role may be expressed. As intracellular calcium release events become

important, a foot protein isoform capable of mediating calcium release will be required. A tetrameric foot protein appears to be essential for both the ligand binding and ion channel functions (12). Consequently, the observation that the alpha and beta foot proteins appear to exist predominately as tetrameric proteins capable of binding [$^3$H]epiryanodine is consistent with the idea that these proteins are involved in intracellular calcium release. We are currently investigating the time course of the appearance of ryanodine-sensitive calcium release events in embryonic chick muscle.

If ion channel activity and modulator binding are not required early during muscle differentiation, a monomeric foot protein may be physically capable of serving a structural role in the developing fiber. The gamma protein, which is recognized by antifoot protein antibodies, does not bind [$^3$H]ryanodine and is transiently expressed in developing muscle, and could be a foot protein isoform specialized to be a structural element. However, the levels of the gamma polypeptide in days E8-E10 embryonic muscle appear to exceed those required for a protein involved only in the initial formation of the triad junction. Even when the initial fivefold concentration of the solubilized protein is considered, the levels of gamma polypeptide in day E10 embryonic muscle appear to be greater than those of the alpha and beta isoforms in day N7 muscle. Moreover, the levels of gamma are greater still in muscle from day E8 embryos. The gamma protein could represent a transiently expressed embryonic protein containing sufficient sequence homology with the foot proteins to form common epitopes. The gamma polypeptide appears to interact with antifoot protein antibodies less well than either the alpha or beta isoform. For example, as shown in Fig. 4, the antibody 110E, which recognizes the beta and gamma polypeptides, does not stain day E10 embryonic muscle, suggesting either that the gamma polypeptide epitope recognized by this antibody is not available to interact with the antibody in situ, or the epitope is sensitive to the tissue fixation methods used in these studies. We are currently developing gamma-specific antibodies to localize this protein in developing muscle and to gain further insight into the function of this polypeptide in embryonic muscle.

The expression of the alpha foot protein in embryonic breast muscle fibers during myofibril assembly and Z line organization suggests that this protein may participate in events involved in skeletal muscle differentiation. Such a role could conceivably involve two different functional modalities of this protein. The foot protein could participate in embryonic muscle development as an intracellular calcium release channel. Changes in intracellular calcium have been reported to influence muscle differentiation. For example, the activation of embryonic frog skeletal myocytes by the iontophoretic application of acetylcholine or KCl accelerates myofibril assembly (10). In addition, the rate of synthesis of the acetylcholine receptor in cultured chicken skeletal muscle cells is influenced by ryanodine, presumably through its ability to alter sarcoplasmic reticulum calcium release (20). Whether other elements of the excitation pathway in mature skeletal muscle cells, such as the dihydropyridine receptor, and elevations of cytoplasmic calcium are involved in this process remain important questions. The observation by Schmid et al. (22) of measurable [$^3$H]nitrendipine binding in day 10 embryonic chick leg muscle is consistent with the involvement of dihydropyridine receptors. It should be noted, through,

that different muscles in the embryo develop at different times, therefore, the present observations concerning the specific time course of the expression of the foot protein isoforms can at present only be applied to pectoral muscle.

The foot proteins could also act as cytoskeletal elements and participate in organizing of muscle-specific structures, such as the triad junction, myofibrillar arrays, and the Z line. At present, little is known about either the importance of the foot protein as a structural component of the triad junction in mature muscle, or the interactions between the foot protein, or other components of the triad junction and the cellular cytoskeleton. The apparent close association of the alpha foot protein and f-actin seen in day E10 muscle raises the possibility that an interaction between these proteins may be involved in assembly of the triad junction and it may be speculated that the focal localization of the foot protein along actin filaments (see Fig. 8) approximates sites of Z line formation.

In conclusion, the levels of both foot protein isoforms increase dramatically at the time of hatching, consistent with the dramatically increased requirement for skeletal muscle function at this time. The two avian foot protein isoforms are expressed at markedly different times during chick pectoral muscle development, suggesting that they make unique contributions to muscle cell structure and function. The alpha foot protein is expressed relatively early during muscle differentiation during sarcomere organization, indicating that this protein may participate in events involved in muscle differentiation.

## References

1. Abramson, S. N., M. H. Ellisman, T. J. Deerinck, Y. Maulet, M. K. Gentry, B. P. Doctor, and P. Taylor. 1989. Differences in structure and distribution of the molecular forms of acetylcholinesterase. J. Cell Biol. 108:2301-2311.
2. Airey, J. A., C. F. Beck, K. Murakami, S. J. Tanksley, T. J. Deerinck, M. H. Ellisman, and J. L. Sutko. 1990. Identification and localization of two triad junction foot protein isoforms in mature avian fast twitch skeletal muscle. J. Biol. Chem. 265:14187-14194.
3. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. 1983. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. Anal. Biochem. 132:74-81.
4. Caswell, A. H., and J. -P. Brunschwig. 1984. Identification and extraction of proteins that compose the triad junction of skeletal muscle. J. Cell Biol. 99:929-939.
5. Ezerman, E. B., and H. Ishikawa. 1967. Differentiation of the sarcoplasmic

reticulum and T system in developing chick skeletal muscle in vitro. *J. Cell Biol.* 35:405–420.

6. Franzini-Armstrong, C. 1970. Studies of the triad. I. Structure of the junction in frog twitch fibers. *J. Cell Biol.* 47:488–499.

7. Hymel, L., M. Inui, S. Fleischer, and H. Schindler. 1988. Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms $Ca^{2+}$-activated oligomeric $Ca^{2+}$ channels in planar bilayers. *Proc. Natl. Acad. Sci. USA.* 85:441–445.

8. Ishikawa, H. 1968. Formation of elaborate networks of T-system tubules in cultured skeletal muscle with special reference to the T-system formation. *J. Cell Biol.* 38:51–66.

9. Kaprielian, Z., and D. M. Fambrough. 1987. Expression of fast and slow isoforms of the $Ca^{2+}$-ATPase in developing chick skeletal muscle. *Dev. Biol.* 124:490–503.

10. Kidokoro, Y., and M. Saito. 1988. Early cross-striation formation in twitching *Xenopus* myocytes in culture. *Proc. Natl. Acad. Sci. USA.* 85:1978–1982.

11. Lai, F. A., H. P. Erickson, E. Rousseau, Q. -Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (Lond.).* 331:315–319.

12. Lai, F. A., M. Misra, L. Xu, H. A. Smith, and G. Meissner. 1989. The ryanodine receptor $Ca^{2+}$ release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. *J. Biol. Chem.* 264:16776–16785.

13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

14. MacLennan, D. H., and K. P. Campbell. 1979. Structure, function, and biosynthesis of sarcoplasmic reticulum proteins. *Trends Biochem. Sci.* July. 148–151.

15. Martonosi, A. 1975. Membrane transport during development in animals. *Biochim. Biophys. Acta.* 415:311–333.

16. Narvarro, J. 1987. Modulation of [$^3$H]dihydropyridine receptors by activation of protein kinase C in chick muscle cells. *J. Biol. Chem.* 262:4649–4652.

17. Ogata, T. 1988. Morphological and cytochemical features of fiber types in vertebrate skeletal muscle. *CRC Rev. Anat. Cell Biol.* 1:229–275.

18. Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.

19. Pellegrino, C., and C. Franzini. 1963. An electron microscope study of denervation atrophy in red and white skeletal muscle fibers. *J. Cell Biol.* 38:323–338.

20. Pezzementi, L., and J. Schmidt. 1981. Ryanodine alters the rate of acetylcholine receptor synthesis in chick skeletal muscle cell cultures. *J. Biol. Chem.* 256:12652–12654.

21. Reik, L. M., S. L. Maines, D. E. Ryan, W. Levin, S. Bandiera, and P. E. Thomas. 1987. A simple, non-chromatographic purification procedure for monoclonal antibodies against cytochrome P450 isozymes. *J. Immunol. Methods.* 100:123–130.

22. Schmid, A., J. -F. Renaud, M. Fosset, J. -P. Meaux, and M. Lazdunski. 1984. The nitrendipine-sensitive $Ca^{2+}$ channel and chick muscle cells and its appearance during myogenesis *in vitro* and *in vivo*. *J. Biol. Chem.* 259:11366–11372.

23. Schmidt, J., S. Rossie, and W. A. Catterall. 1985. A large intracellular pool of inactive Na channel *alpha* subunits in developing rat brain. *Proc. Natl. Acad. Sci. USA.* 82:4847–4851.

24. Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

25. Sutko, J. L., L. J. Thompson, R. G. Schlatterer, F. A. Lattanzio Jr., A. J. Fairhurst, C. Campbell, S. Martin, L. Ruest, and P. Deslongchamps. 1985. Separation and formation of ryanodine from dehydroryanodine. Preparation of [$^3$H]ryanodine. *J. Labeled. Compd. & Radiopharm.* 23:215–22.

26. Tokuyasu, K. T., and P. A. Maher. 1987. Immunocytochemical studies of cardiac myofibrillogenesis and early chick embryos. I. Presence of immunofluorescent titin spots in premyofibril stages. *J. Cell Biol.* 105:2781–2793.