

# Biosynthesis of Titin in Cultured Skeletal Muscle Cells

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**Abstract.** Although significant progress has been made regarding the structure and function of titin, little data exist on the biosynthesis of this large protein in developing muscle. Using pulse-labeling with [<sup>35</sup>S]methionine and immunoprecipitation with an anti-titin mAb, we have examined the biosynthesis of titin in synchronized cultures of skeletal muscle cells derived from day 12 chicken embryos. We find that: (a) titin synthesis increases >4-fold during the first week

in culture and during this same time period, synthesis of muscle-specific myosin heavy chain increases >12-fold; (b) newly synthesized titin has a  $t_{1/2}$  of ~70 h; (c) titin is resistant to extraction with Triton X-100 both during and immediately after its synthesis. These observations suggest that newly synthesized titin molecules are stable proteins that rapidly associate with the cytoskeleton of developing myotubes.

**C**ARDIAC and skeletal muscle are unique in possessing sarcomeres, the highly ordered and periodic arrays of actin and myosin that generate the contractile force of muscle. Although sarcomeres are unique to striated muscle, most contractile proteins, such as actin and myosin, are not; nonmuscle or smooth muscle isoforms are found in most cells. One of the few proteins preferentially found in striated muscle is titin, a recently described myofibril protein with a molecular mass exceeding 1,000,000 D (reviewed in Wang, 1982, 1984, 1985; Ohtsuki et al., 1986). Titin (also called connectin; Maruyama et al., 1984) is relatively abundant, making up ~10% of myofibril mass (Wang et al., 1984; Trinick et al., 1984). It is usually detected as a doublet of proteins, termed TI and TII,<sup>1</sup> on low porosity gels. The presence of titin in all striated muscles (Locker and Wild, 1986; Osborn et al., 1986; Gassner, 1986; Hill and Weber, 1986; Wang and Greaser, 1985; Hu et al., 1986; Wang et al., 1985) has sharpened interest in the function of this protein.

Titin has been detected in the myofibril by immunofluorescence (Osborn et al., 1986; Gassner, 1986; Hill and Weber, 1984; Wang and Greaser, 1985; Wang et al., 1985) and immunoelectron (Gassner, 1986; Hill and Weber, 1986; Maruyama et al., 1984, 1985) microscopy. mAbs to different epitopes of titin show that titin lies symmetrically relative to the M line (Wang and Greaser, 1985; Wang and Ramirez-Mitchell, 1983) and that single titin molecules stretch from the Z line to within 0.2  $\mu$ m of the M line (Furst et al., 1988). This latter study by Furst et al. further demonstrated that the epitopes of titin near the Z line are present on the TI form of titin, but not the smaller TII form.

At the ultrastructural level, titin has been detected immunologically on "gap" filaments that are visible in overextended muscle, and associated with purified thick filaments (Gassner, 1986; Hill and Weber, 1986; Maruyama et al., 1984, 1985). Thus, the overall image presented by light microscopy, that titin is a myofibril protein with a specific distribution, has been confirmed. The patterns seen so far led Wang (1984) and Wang et al. (1984) and others (Gassner, 1986; Maruyama et al., 1984) to interpret titin as a major component of the "third filament" or "gap filament" system of the myofibril.

The existence of a third filament system in the myofibril that provides elasticity has been proposed in several forms, and titin appears to be one major component of such a third filament system. Direct evidence of an elastic role was detected by using ionizing radiation to ablate preferentially titin and nebulin from isolated myofibrils (Horowitz et al., 1986). Such myofibrils are much less elastic and become misaligned axially, providing direct evidence for titin's role in elastic recoil and sarcomere stability. These results are consistent with the demonstration by Trinick et al. (1984) that the secondary structure of titin consists almost entirely of random coil, characteristic of elastic proteins. Further support for a general role in striated muscle elasticity comes from the wide distribution of titin (Locker and Wild, 1986; Osborn et al., 1986; Gassner, 1986; Hu et al., 1986; Maruyama et al., 1984).

A mechanical role for titin thus is reasonably well established. A few observations suggest that titin may also participate in the morphogenesis of the myofibril. Titin is present and periodic when the first periodic arrays of myosin are seen, in both skeletal (Hill et al., 1986) and cardiac (Wang et al., 1984) cells. Titin may be required for such periodic arrays because it is one of the few proteins unique to and ubiquitous in striated muscle.

At present, there is very little known about either the syn-

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1. *Abbreviations used in this paper:*  $\beta$ ME,  $\beta$ -mercaptoethanol; MHC, myosin heavy chain; TI, TII, titin.

thesis or assembly of titin in developing muscle cells. These questions are particularly interesting in view of the molecular dimensions of this protein. Given its tremendous size, the question arises of how titin is properly oriented in the developing sarcomere. Before questions pertaining to the assembly of titin can be properly addressed, however, characterization of the biosynthesis of this protein is necessary. In this study, we describe the immunoprecipitation of newly synthesized titin from cultures of developing myotubes. We have used this immunoprecipitation protocol to quantitate the synthesis, accumulation, and stability of titin during the early stages of myofibrillogenesis.

## Materials and Methods

### Cell Culture

Primary cultures of chicken leg myoblasts, synchronized by a cycle of divalent cation depletion and repletion, were prepared from day 12 embryos as previously described (Konieczny et al., 1982; Denning et al., 1988). Equal numbers of cells, as determined by counting with a hemocytometer, were plated in collagen-coated 35-mm dishes to initiate each experiment.

### Radiolabeling of Cultures

For pulse labeling (<1 h), 35-mm plates were rinsed twice and incubated at 37°C with Met-free DME containing dialyzed horse serum and chick embryo extract. After 5 min, this medium was replaced with 200  $\mu$ l of the same medium containing 1 mCi/ml [<sup>35</sup>S]methionine (1,000 Ci/mmol; ICN Radiochemicals, Irvine, CA), the medium was covered with a coverslip to spread it, and plates were incubated at 37°C for various periods, as stated in the text. For longer labeling periods (e.g., 16 h), [<sup>35</sup>S]methionine was added directly to the complete growth medium at a concentration of 150  $\mu$ Ci/ml. After labeling, cultures were either extracted immediately (see below), or chased by adding complete medium supplemented with 2 mM unlabeled methionine and incubating at 37°C for various periods before extraction. In some pulse-labeling experiments, cultures were labeled in complete medium without any starvation period. This labeling protocol yields the same results except that the incorporation of radioactivity is reduced over sevenfold.

For labeling cells with [<sup>3</sup>H]puromycin, 35-mm dishes were incubated at 37°C with 10  $\mu$ Ci of (8<*n*>-<sup>3</sup>H) puromycin dihydrochloride (5–10 Ci/mmol; Amersham Corp., Arlington Heights, IL) in 100  $\mu$ l (final concentration of puromycin, 20  $\mu$ M) of complete culture medium. After 10 min, 1 ml of complete medium containing 0.4 mM unlabeled puromycin was added and the incubation was continued for an additional minute. The cultures were then rinsed and extracted as described below. The concentration of puromycin used is sufficient to release >95% of [<sup>35</sup>S]methionine-labeled nascent chains from polysomes when analyzed by sedimentation through sucrose gradients (see Grollman, 1968; Moldave and Sadnik, 1979).

### Extraction of Cultures

After labeling, plates were rinsed twice with PBS containing 2 mM MgCl<sub>2</sub> and 1 mM EGTA, and extracted on ice with 0.5 ml of extraction buffer (100 mM KCl, 10 mM Pipes, pH 6.8, 300 mM sucrose, 2 mM MgCl<sub>2</sub>, and 1 mM EGTA) containing 0.5% Triton X-100 and protease inhibitors (PMSF and leupeptin, at concentrations of 1 mM and 100  $\mu$ M, respectively). After 10 min, this extractable fraction was removed from the dish and prepared immediately for immunoprecipitation by the addition of SDS (see below). Triton-resistant material that remained after the removal of the extraction buffer (cytoskeletal fraction) was scraped into extraction buffer containing 2% SDS, 1%  $\beta$ -mercaptoethanol ( $\beta$ ME), and 100 mM NaCl instead of KCl. To reduce trapping of titin, the DNA present in the cells was degraded either by (a) treatment with DNase during the Triton X-100 extraction step, or (b) gentle shearing by repeated pipetting through a 1-ml blue pipette tip. Equivalent results were obtained by either method as long as the shearing was not excessive.

### Immunoprecipitation

Titin was immunoprecipitated using a mouse mAb, AMF-1, which we

generated using embryonic myofibril as the immunogen. This antibody was judged to be monospecific for titin by the criteria described in Fig. 1. Hybridoma cells producing this antibody were cloned twice in soft agar. Muscle-specific myosin heavy chain was immunoprecipitated using MF-20 (Fischman and Masaki, 1982), a gift of D. Fischman (Cornell University, New York).

SDS and  $\beta$ ME were added to samples to be immunoprecipitated to final concentrations of 2 and 1%, respectively. Samples were heated at 70°C for 10 min, centrifuged for 1 min at 10,000 g, and the supernatants were diluted with 4 vol of 2.5% Triton buffer containing 50 mM Tris, pH 7.4, 190 mM NaCl, and 6 mM EDTA as described by Goldman and Blobel (1978). 200  $\mu$ l of conditioned AMF-1 tissue culture supernatant or 200  $\mu$ l of unconditioned medium containing nonspecific mouse IgG were added to samples that were then incubated for 16 h at 4°C. 50  $\mu$ l of a 10% solution (wt/vol) of fixed *Staphylococcus aureus* cells (washed in boiling 2% SDS, 1%  $\beta$ ME before use; Boehringer-Mannheim Biochemicals, Indianapolis, IN) were added to samples, and the samples were incubated 15 min at room temperature; Staph A cells were recovered by a 20-s centrifugation (10,000 g) and washed four times in buffers containing 0.5 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 15 mM  $\beta$ ME, 5 mM EDTA, and 50 mM Tris, pH 7.4. Washed pellets were heated for 5 min at 70°C in SDS sample buffer and centrifuged; the supernatants were then subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, soaked in 1 M sodium salicylate, dried, and exposed to preflashed Kodak XAR-5 film at -70°C. Quantitation of incorporated radioactivity was performed by excision of the appropriate region of the dried gels and counting in 3a70 scintillation fluid (RPI). Quantitation of immunoprecipitated protein was performed by quantitation of Coomassie brilliant blue staining by scanning densitometry at 600 nm before fluorography.

### Electrophoresis and Immunoblot Analysis

SDS-PAGE was performed using either 9% acrylamide gels as described by Laemmli (1970) or 3.2% acrylamide gels as described by Fairbanks et al. (1971) and modified by Wang (1982). We have further modified this protocol by the inclusion of 1% agarose in the gel mixture to increase mechanical strength. Where noted (i.e. Fig. 1), gradient gels containing 2–11% acrylamide were employed to increase resolution; these gels, however, are difficult to reproducibly prepare because of insertion of the well-forming comb into the gradient and thus were not routinely used. Immunoblotting was performed as described by Towbin et al. (1979) using a biotinylated second antibody and avidin- $\beta$ -galactosidase detection system with blue-gal as substrate.

### Immunofluorescence

Cultures were fixed for 10 min with absolute ethanol at -20°C. After rinsing with PBS, the cultures were incubated with the primary antibody for 30 min at 37°C. The antibodies used were same described above for immunoprecipitation with the exception of the antibody used to stain muscle-specific myosin heavy chain, MF-18, was used for immunofluorescence, a gift of D. Fischman. After washing with PBS, the cultures were incubated with fluorescein-conjugated sheep anti-mouse IgG (Cappel Laboratories, Malvern, PA), rinsed, and mounted in PBS/glycerol (1:10) containing 1 mg/ml phenylene diamine.

## Results

### Muscle Cultures

The cultures used throughout this study were derived from the thigh muscle of day 12 chicken embryos as previously described (Konieczny et al., 1982; Denning et al., 1988). These cultures are synchronized by a limited divalent cation depletion and repletion cycle. Briefly, 18 h after plating, Mg<sup>2+</sup> and Ca<sup>2+</sup> levels are reduced to 100 and 25  $\mu$ M, respectively. 48 h later, the cations are repleted. After repletion, development proceeds rapidly, with the number of cells possessing sarcomeres increasing from <5 to >90% within 72 h. Results similar to those described below have been obtained with nondepleted cultures.

### Titin Is Very Labile

Previous investigators have noted the peculiar fragility of titin (Wang, 1982; Maruyama et al., 1981). We confirm these findings, and have used a number of precautions to better preserve this protein in the studies described here. When extracting cultures in the absence of protein denaturants, the integrity of titin is strictly dependent on the presence of protease inhibitors, specifically, leupeptin. A variety of other protease inhibitors (e.g., PMSF, iodoacetamide, EGTA, benzamide,  $\epsilon$ -aminocaproic acid, pepstatin, or 1,10-phenanthroline) will not substitute for leupeptin.

Titin can be effectively extracted from cultured muscle cells with SDS as long as two conditions are met: (a) adequate reducing agent is present, e.g., >100 mM  $\beta$ ME; and (b) trapping in nucleic acid is minimized by shearing or previous digestion with DNase, with the latter being preferable, since excessive shearing decreases titin recovery. As previously noted (Wang, 1982), boiling of titin in SDS leads to its rapid degradation.

### Characterization of Antititin Antibody

From a population of hybridomas generated against chicken thigh muscle myofibril, we obtained an mAb that by immunofluorescence stains the A-I junction and that reacts with an 1.0–1.2-MD<sup>2</sup> doublet on immunoblots of isolated myofibrils (Fig. 1). These criteria indicate that this mAb reacts with titin, both the high- and low-molecular weight forms (TI and TII, respectively). The antibody binds to no components of cultured fibroblasts, nor does it recognize any proteins in nonmuscle cells in immunoblots (data not shown).

### Newly Synthesized Titin Can Be Immunoprecipitated from Extracts of Cultured Myotubes

To study synthesis of titin in developing muscle, we have developed a protocol to immunoprecipitate titin specifically and quantitatively from cultures of [<sup>35</sup>S]methionine-labeled myotubes (see Materials and Methods). Fig. 2 shows titin immunoprecipitated from a muscle culture after a 16-h labeling period. Titin is recovered as a major species comigrating with TI. The presence of a distinct band corresponding to TII is much less conspicuous in titin obtained from cultured cells when compared with titin obtained from tissue, but is occasionally observed, particularly in older cultures (see Fig. 3, *l*, lane *e*). Fractionation of labeled cultures with Triton X-100 before immunoprecipitation reveals that the titin antibody recovers a number of lower molecular weight bands ( $0.6\text{--}0.8 \times 10^6$  D<sup>2</sup>) from the extractable fraction. These bands are substantially smaller than the TII form of titin, and probably represent titin degradation products because they are more abundant when leupeptin is omitted from the extraction buffer (data not shown). However, it should be noted that these bands are immunoprecipitable from unextracted cultures which have been solubilized directly with 2% SDS after labeling. Virtually none of the full-sized titin (TI or TII) is extractable with Triton X-100 alone (Fig. 2 *d*), but a

2. This molecular weight estimation is based on cross-linked myosin heavy chain as standards (Wang, 1982). Under the conditions described, titin comigrates with hexamers and heptamers of myosin heavy chain. Recently, the molecular weight of titin has been reported to be  $2.6\text{--}2.8 \times 10^6$  (Kurban and Wang, 1988; Hainfeld et al., 1988).

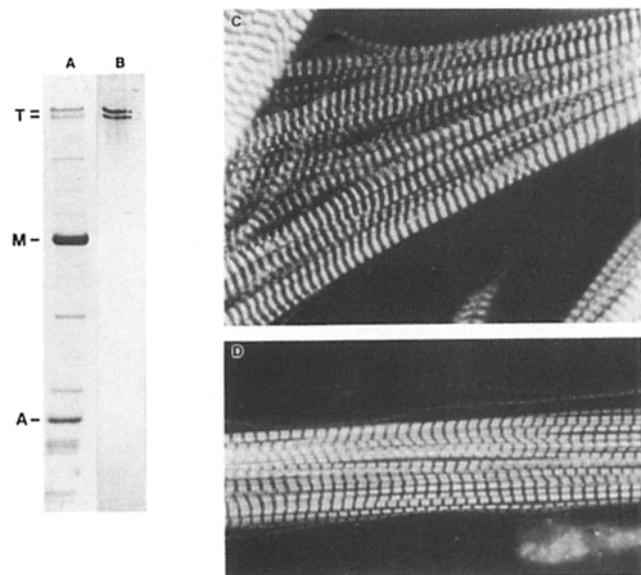


Figure 1. Characterization of AMF-1, an mAb specific for titin. Lane A, myofibrillar proteins obtained from 17-d embryonic thigh muscle, separated on 2–11% acrylamide gradient gel and stained with Coomassie brilliant blue. The positions of titin (both TI and TII), MHC, and actin are marked by T, M, and A, respectively. Lane B, immunoblot of a sample identical to the one in lane A, stained with AMF-1. Note that this antibody recognizes both TI and TII forms of titin. (C) Immunofluorescent localization of titin in cultured myotubes using AMF-1. (D) Immunofluorescent localization of muscle-specific myosin heavy chain using MF-18. Double-labeling analysis using both these antibodies demonstrates that the epitope recognized by AMF-1 is located at the A-I junction (data not shown).

significant fraction of TII can be released by the inclusion of 50 mM pyrophosphate in the extraction buffer (data not shown).

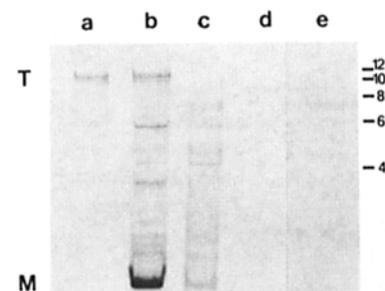
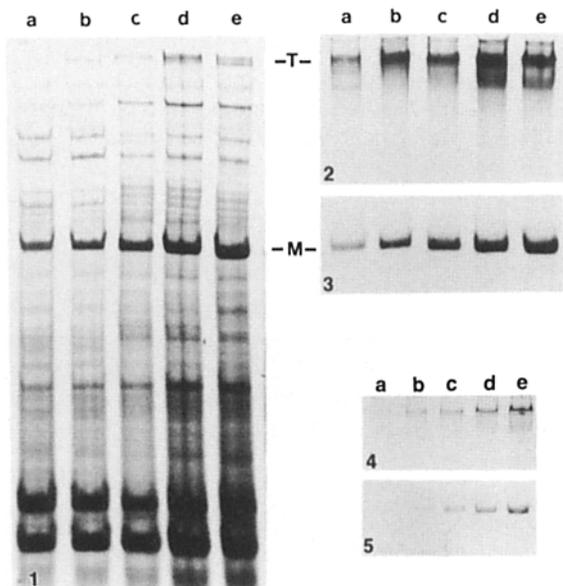


Figure 2. Immunoprecipitation of titin from [<sup>35</sup>S]methionine-labeled muscle cultures. Muscle cultures were labeled for 16 h and extracted with 0.5% Triton X-100 as described in Materials and Methods. Titin present in the extractable and cytoskeletal fractions was recovered by immunoprecipitation. The immunoprecipitates as well as the fractions from which they were obtained were run on 3.2% acrylamide/1% agarose gels, and subjected to autoradiography. Lane a, titin immunoprecipitated from the cytoskeletal fraction. Lane b, cytoskeletal fraction. Lane c, Triton-extractable fraction. Lane d, titin immunoprecipitated from the extractable fraction. Lane e, lane d exposed four times longer than lanes a–d (4 vs. 1 d). T and M mark the positions of titin and MHC, respectively. The numbers on the left side mark the molecular weights of cross-linked myosin standards ( $\times 10^5$ ).

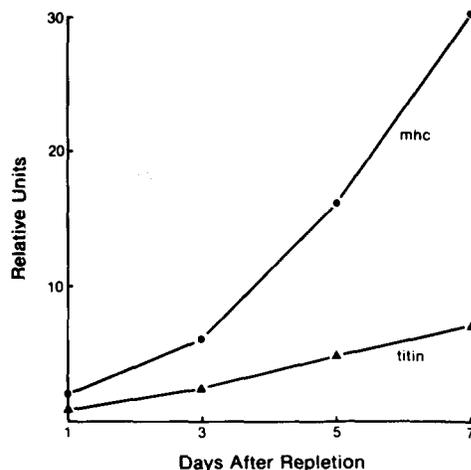
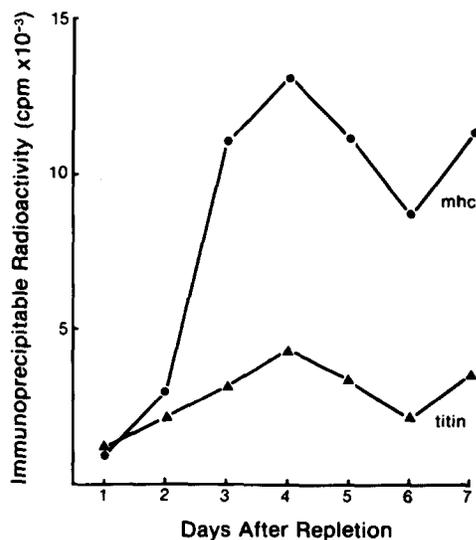


**Figure 3.** Titin and MHC synthesis and accumulation in cultured myotubes. (1) Autoradiography of muscle cultures labeled for 16 h with [ $^{35}$ S]methionine on successive days of culture. Lanes a–e, labeling period terminated 24, 48, 72, 96, and 120 h, respectively, after replation of divalent cations to the cultures. (2) Autoradiography of titin immunoprecipitates obtained from the samples shown in 1, using the same lane designations. (3) Autoradiography of MHC immunoprecipitates from these samples. (4 and 5) Coomassie brilliant blue staining of the immunoprecipitates shown in 2 and 3, respectively. In each case, the amount of cell extract immunoprecipitated with antititin antibody was fourfold greater than the amount immunoprecipitated with anti-MHC antibody, i.e., each titin immunoprecipitate has been recovered from one-third of a 35-mm culture dish, whereas for MHC this value is one-twelfth.

### **Titin Accumulation in Cultured Myotubes**

Experiments were performed to characterize the synthesis and accumulation of titin as a function of time in culture. During successive days in culture, muscle cultures were pulse-labeled for 16 h, and the amount of titin (and muscle-specific myosin heavy chain [MHC]) present in the cultures was quantitated by immunoprecipitation. Fig. 3 shows that at the earliest time point examined (labeled from 8–24 h after divalent cation replation), detectable amounts of newly synthesized titin are present. At this stage of development *in vitro*, fusion is occurring rapidly and <5% of the cells contain sarcomeres (Denning et al., 1988). The amount of titin synthesis in the cultures increases approximately fourfold by day 4 (i.e. 4 d after replation). Comparison of the amount of labeled titin to the amount of labeled MHC reveals that the ratio of these proteins changes with time (Fig. 4, *top graph*), as MHC synthesis increases >12-fold by day 4. These changing rates of titin and MHC syntheses are reflected in the accumulation of these proteins as detected by staining with Coomassie brilliant blue (Fig. 4, *bottom graph*). The amount of titin present in the cultures gradually increases fourfold by 7 d after replation. During this same time period, the amount of MHC has increased >15-fold.

To examine the stability of newly synthesized titin in culture, muscle cultures (2 d after replation) were labeled for 16 h and then chased with unlabeled methionine for various

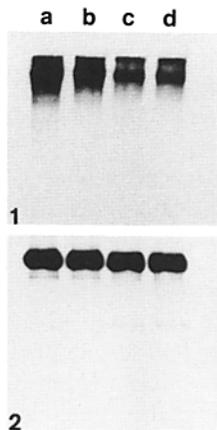


**Figure 4.** Quantitation of titin and MHC synthesis and accumulation. An experiment identical to the one described in Fig. 3 was performed and analyzed by immunoprecipitation and autoradiography. The amounts of newly synthesized titin and MHC present in the cultures were determined by cutting the regions containing these immunoprecipitated proteins from the gels and counting the radioactivity present (*top*). The accumulation of these proteins was measured by scanning densitometry (*bottom*). The culture medium was changed on days 3 and 6.

periods. From the rate of disappearance of labeled titin, a  $t_{1/2}$  of  $\sim 70$  h can be calculated. This is slightly less than the value obtained for MHC in the same cultures (96 h, Fig. 5).

### **Newly Synthesized Titin Molecules Are Resistant to Extraction with Triton X-100**

To determine whether newly synthesized titin molecules exist as soluble, posttranslational intermediates (similar to what has been observed for another highly insoluble filamentous protein, vimentin (Blikstad and Lazarides, 1983; Isaacs et al., 1989) cultures were pulse labeled for 10 min with  $^{35}$ S methionine and fractionated by extraction with Triton X-100. Fig. 6 (lanes a, *left* and *right*) shows the titin present in the extractable and cytoskeletal fractions recovered by immunoprecipitation. Virtually all full-sized titin is in the cytoskele-



**Figure 5.** Newly synthesized titin and MHC are stable proteins. Cultures were labeled for 16 h on day 3 after repletion. The cultures were then washed three times, chased with fresh culture medium containing 1 mM unlabeled methionine. At various times, cultures were extracted with 0.5% Triton X-100, and the titin and MHC present in the cytoskeletons was recovered by immunoprecipitation. Autoradiographs of titin (1) and MHC (2) immunoprecipitated after no chase (lane a), 24-h chase (lane b), 72-h chase (lane c), and 120-h chase (lane d). The radioactivity in each of these bands (plus an additional time point at 96 h of chase) was quantitated and the half-life of these proteins was determined: for titin, 70 h; for MHC, 96 h.

tal fraction. The extractable fraction contains <20% of the total immunoprecipitable radioactivity, present in the 0.6–0.8 million-D proteins described above. Chasing a pulse-labeled culture with unlabeled methionine (Fig. 6, lanes b–f) results in little change in the fractionation pattern. These results suggest that at no time after synthesis do full-sized titin molecules exist in a freely diffusing extractable form. It should be noticed in Fig. 6 that as the chase proceeds, the highest molecular weight band (TI) can be observed to increase in intensity, particularly between 1 and 1.5 h of chase (compare lane d with lanes a–c, Fig. 6, left). This process is blocked if protein synthesis is inhibited by cycloheximide (Fig. 6, lane g), and thus, presumably represents accumulation of completed TI molecules.

#### **Nascent Titin Molecules Are Also Resistant to Extraction with Triton X-100**

We next wished to determine whether titin molecules develop resistance to Triton X-100 extraction during translation. To do so, muscle cultures were incubated with [<sup>3</sup>H]puromycin to simultaneously label (and thereby identify) and release nascent chains from ribosomes (Grollman, 1968; Moldave and Sadnik, 1979; Allen and Zamecnik, 1962). After extraction and immunoprecipitation, 74% ( $\pm$  8%,  $n = 5$ ) of the [<sup>3</sup>H]puromycin radioactivity recovered by the titin

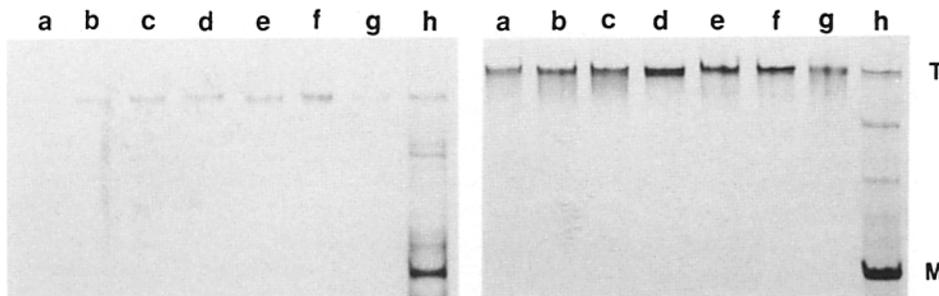
antibody is present in the cytoskeletal fraction. For comparison, <20% of the total, acid-precipitable, [<sup>3</sup>H]puromycin-labeled peptides are found in this fraction (data not shown). Analysis of the labeled titin immunoprecipitates by SDS-PAGE and fluorography is presented in Fig. 7. These results indicate that most titin molecules become resistant to Triton extraction during translation and that this resistance is not due to ribosomal attachment.

#### **Discussion**

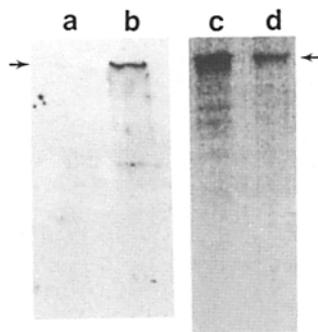
We have developed a protocol to specifically immunoprecipitate titin from cultured muscle cells. The ability to rapidly recover largely undegraded titin from cultures of developing muscle cells is an important step toward the elucidation of the role of this protein in myofibrillogenesis.

Several factors are important in the identification and quantification of titin in cultured cells: (a) inhibition of proteolytic activity with leupeptin; (b) maintenance of a reducing environment with sulfhydryl compounds; and (c) prevention of entrapment of titin in cellular DNA. The first factor is critical when nondenaturing buffers are used, whereas the last two pertain to the use of denaturing buffers. Previous workers have made similar observations pertaining to the isolation of titin from skeletal muscle tissue (Wang, 1982; King and Kurth, 1980). The identification of a leupeptin-sensitive, titin-degrading activity, however, has not been previously noted.

We have compared the synthesis rates of titin and MHC as a function of time in culture. At the earliest times examined, approximately the same amount of [<sup>35</sup>S]methionine is incorporated into each protein during the labeling period. Assuming MHC has threefold more methionine residues than does titin per unit mass (Maruyama et al., 1981), these cultures are synthesizing about threefold more titin by mass than MHC. This corresponds to  $\approx$ 4 mol of MHC per mol of titin (assuming respective molecular weights of 200 and 2,600 kD). After 7 d of development, the synthesis ratio has increased to  $\approx$ 20 MHC/titin. In adult muscle the molar ratio of MHC to titin can be estimated at between 40 and 60, assuming these molecular weights. We observe that newly synthesized titin has a shorter  $t_{1/2}$  than MHC (70 and 96 h, respectively), and this obviously is a factor in determining the steady-state molar ratio of these proteins. It is interesting to speculate, however, that during the initial stages of sarcomere formation, structures form that are enriched in titin relative to mature sarcomeres, and that these structures act as



**Figure 6.** Newly synthesized titin resists extraction with Triton X-100. Muscle cultures were pulse-labeled for 10 min on day 3 after repletion and subsequently extracted with 0.5% Triton X-100, or chased for various periods with unlabeled methionine and then extracted. Titin in the extractable (left) and cytoskeletal (right) fractions was recovered by immunoprecipitation. Lanes a, no chase; lanes b, 30-min chase; lanes c, 60-min chase; lanes d, 90-min chase; lanes e, 120-min chase; lanes f, 180-min chase; lanes g, 120-min chase plus cycloheximide (0.4 mM) added at the beginning of the chase; lanes h, whole fractions from cultures chased for 180 min (left, extractable; right, cytoskeletal). T and M mark the positions of titin and MHC, respectively.



**Figure 7.** [ $^3\text{H}$ ]Puromycin labeling of titin nascent chains. 4 d after replating, muscle cultures were labeled with [ $^3\text{H}$ ]puromycin as described in Materials and Methods. The cultures were then extracted with 0.5% Triton X-100, and titin (and MHC for comparison) in each fraction was recovered by immunoprecipitation. The immunoprecipitates were run on 9% acrylamide

gels, with a 4.6% acrylamide stacking gel. Under these conditions, molecules with molecular weights of  $\sim 0.5$  million and greater accumulate at the stacking gel interface (arrowhead, left of lane a). Lanes a and b, titin immunoprecipitates from extractable (a) and cytoskeletal (b) fractions. Exposure time, 1 mo. Lanes c and d, MHC immunoprecipitates from the same fractions used for lanes a and b, respectively. Arrowhead on the right side of lane d marks the position of MHC (200 kD). Exposure time, 5 wk.

scaffolds or templates upon which additional thick filaments accumulate as development proceeds. Stoichiometry measurements of titin and MHC present in purified myofibrils of different ages would be needed to test this possibility. The idea that titin-containing structures act as organizing templates has been previously proposed by a number of investigators (Wang, 1985; Wang et al., 1985; Hill et al., 1986).

The precise molecular weight of titin has been the subject of some controversy and has been reported to be in the range from 1,000,000–3,000,000 (Wang, 1982; Ohtsuki et al., 1986). Recently, a value of  $2.6\text{--}2.8 \times 10^6$ , obtained by equilibrium sedimentation studies (Kurzban and Wang, 1988) has been reported. In the biosynthetic studies presented here, it can be estimated that the time required for a titin molecule to be synthesized is between 1 and 1.5 h (see Fig. 6). Assuming a rate of polymerization of five amino acids per second (Lodish and Jacobsen, 1972), this would be enough time to synthesize a protein of molecular weight between 2,000,000 and 3,000,000. It is also interesting to note that, after a pulse with labeled methionine, we observe no discrete lower molecular weight species (i.e., precursor) that disappear during chase with unlabeled methionine. These results are consistent with the assumption that titin is synthesized as a single polypeptide chain. This assumption is further supported by the previous work of Furst et al. (1988) demonstrating the nonrepetitive nature of 10 distinct epitopes observed on titin molecules residing in the half-sarcomere.

Using pulse-chase labeling analysis, we provide evidence to suggest that newly synthesized titin rapidly associates with the cytoskeleton; labeling of nascent titin molecules with tritiated puromycin suggests that this association forms during translation and is independent of ribosomal attachment. Association during translation (i.e., cotranslational assembly) has been previously demonstrated for MHC in developing muscle cultures (Isaacs and Fulton, 1987). It is quite possible, however, that titin molecules, both nascent and complete, resist release from the myotube cytoskeleton not because they are cotranslationally assembled but rather because of (a) trapping due to their large size or (b) intrinsic

insolubility in Triton X-100. With regard to the latter, the limited solubility of titin, particularly the TI form, has been repeatedly observed (Wang, 1982; Maruyama et al., 1981; Trinick et al., 1984). It should be mentioned, however, that is not necessary for a cellular component to be soluble in a given buffer in order to be extracted by it. For example, single ribosomes are readily extracted from tissue culture cells by Triton X-100 (Lenk et al., 1977), but these organelles are "insoluble" in this detergent as evidenced by their sedimentation behavior, i.e., they are not dissociated into subunits and sediment as intact complexes. With regard to trapping, note that if titin is restrained from diffusion out of the cell by its size, its diffusion within the cell is most likely similarly restrained. Indeed, it would seem unlikely that a protein that is at least  $1.2 \mu\text{m}$  long (Wang et al., 1984; Furst et al., 1988; Hainfeld et al., 1988) is unrestricted in its diffusion about the cytoplasm (Luby-Phelps et al., 1986, 1987). This would impose restrictions upon the distance an assembly site might be from the synthesis site for titin.

It is obvious that more work will be needed to determine the precise relationship between the synthesis and assembly of titin during myofibrillogenesis. We are currently using the system described here, in conjunction with chemical cross-linking agents, to characterize the initial sites of interaction between newly synthesized titin and the nascent myofibril.

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