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Cotranslational assembly of myosin heavy chain in developing cultured skeletal muscle

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ABSTRACT **To examine how nascent myosin heavy chains associate with the cytoskeletons of developing muscle cells, we used pulse labeling, cell fractionation, and immunoprecipitation. More than 80% of nascent myosin heavy chains associate with the cytoskeleton. More than one-third of these nascent chains are not released by puromycin and/or RNase. The fraction of nascent heavy chains that resists release increases during development of muscle cells in culture. Treatment with cytochalasin D but not nocodazole decreases myosin heavy chain cotranslational assembly. These results indicate that (*i*) cotranslational assembly of myosin heavy chains is developmentally regulated, (*ii*) structures containing actin and not microtubules may mediate initial association of the heavy chains with the cytoskeleton, and (*iii*) the site of translation dictates where a significant fraction of the heavy chains will be inserted into the cytoskeleton.**

Eukaryotic cells contain a complex intracellular network, the cytoskeleton. Most cytoskeletons include the three major filament systems and numerous associated proteins (1–5). Cells have characteristic cytoskeletons with specific protein compositions and a high degree of spatial order (refs. 6 and 7; reviewed in refs. 8 and 9). Complex structures of cells, in which different regions not separated by membranes retain different compositions, present major problems for assembly. Earlier studies (10, 11) showed that many cytoskeletal proteins associate with the Triton-resistant cytoskeleton shortly after or during translation; such proteins were found near polyribosomes and redistributed with time. Cytoskeletal assembly studied in an *in vitro* translational system indicated that over three-quarters of cytoskeletal protein formed puromycin-resistant contacts during translation; these contacts required initiation *in vivo* and low calcium concentration (12). More recent studies (13) show that some cytoskeletal proteins are coordinately expressed. Coordinate synthesis is one simple mechanism for cotranslational assembly.

These studies did not address whether a given protein assembles into the cytoskeleton during translation. In the systems in which association during synthesis was examined, no one specific protein could be identified, since nascent polypeptides differ in isoelectric point and molecular weight from completed proteins. Clearly, one must identify nascent polypeptides of a specific protein to address this question further.

Such identification is possible with monoclonal antibodies (mAbs) that recognize proteins near their amino terminus. A mAb that recognizes muscle myosin heavy chain (MHC) 30 kDa from the amino terminus has permitted us to examine the extent of cotranslational assembly of MHC into the cytoskeleton during muscle development.

MATERIALS AND METHODS

Muscle Culture. Thigh muscle of 12-day-old chicken embryos was cultured as described (ref. 14; further details will be published elsewhere). Twenty-four hours after plating, cultures were subjected to limited divalent cation depletion and subsequent repletion. This yields cultures of muscle with highly synchronous appearance of sarcomeres.

Radiolabeling of Cultures. For pulse labeling, plates (35 mm) were rinsed twice and incubated at 37°C with methionine-free culture medium containing dialyzed horse serum and chicken embryo extract. After 10 min this medium was replaced with 100 μl of the same medium containing [^{35}S]-methionine (1 mCi/ml, 1000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), the medium was covered with a coverslip to spread it, and the plates were incubated at 37°C for 6 min. After labeling, cultures were either extracted immediately (see below) or "chased" by addition of complete medium supplemented with 2 mM methionine and incubation at 37°C for 25 min before extraction.

Extraction of Cultures. After labeling, plates were rinsed twice with isotonic phosphate-buffered saline containing 2 mM $MgCl_2$ and 1 mM EGTA and then were extracted with two aliquots of skeleton buffer (100 mM KCl/10 mM Pipes, pH 6.8/300 mM sucrose/2 mM $MgCl_2$/1 mM EGTA) containing 0.5% Triton X-100 and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, benzamidine, ε-aminocaproic acid, and iodoacetamide at respective concentrations of 1 mM, 100 μM, 5 mM, 5 mM, and 2 mM). The first 10-min extraction was performed on ice, and the second (also 10 min) at room temperature. In most experiments the second extraction buffer contained either RNase A (Sigma, type III-A; 10 μg/ml, pretreated at 100°C for 10 min) and/or puromycin (0.4 mM). When a wash was performed after the room temperature extraction, it never yielded more than 3% of either cellular protein or myosin. Triton-resistant material that remained (cytoskeletal fraction) was scraped into skeleton buffer containing 2% $NaDodSO_4$, 0.5% 2-mercaptoethanol, and 100 mM NaCl instead of KCl.

Immunoprecipitation. Skeletal MHC was immunoprecipitated using a mouse mAb, MF-18 (gift of D. Fischman, Cornell University). $NaDodSO_4$ and 2-mercaptoethanol were added to samples to be immunoprecipitated, to final concentrations of 2% and 0.5%, respectively. Samples were boiled for 2 min and diluted with 4 volumes of 2.5% Triton buffer containing 50 mM Tris (pH 7.4), 190 mM NaCl, and 6 mM EDTA as described (15). Two hundred microliters of MF-18 hybridoma culture supernatant or of unconditioned medium containing nonspecific mouse IgG was added to samples, which were then incubated for 16 hr at 4°C. Thirty microliters of a 10% (wt/vol) suspension of fixed *Staphylococcus aureus* cells (Calbiochem) was added to samples; after incubation for 15 min at room temperature, the *S. aureus* cells were re-

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Abbreviations: MHC, muscle-specific myosin heavy chain(s); mAb, monoclonal antibody.

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covered by centrifugation (10,000 × g) for 20 sec and washed four times in buffer containing 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% $NaDodSO_4$, 15 mM 2-mercaptoethanol, 5 mM EDTA, and 50 mM Tris (pH 7.4). Washed pellets were boiled in $NaDodSO_4$ sample buffer and subjected to PAGE in 8% acrylamide gels (16). After electrophoresis, gels were stained, destained, soaked in 1 M sodium salicylate, dried, and exposed to preflashed Kodak XAR-5 film at −70°C. This procedure recovered more than 95% of muscle myosin in samples (data not shown).

Quantitation of Nascent Chains. After autoradiography, exposed films were used to locate nascent MHC within dried gels—i.e., the regions below full-sized MHC. Appropriate regions were excised and placed into 3a70 liquid scintillation fluid (RPI) for measurement of radioactivity. To correct for label that cannot be chased into full-sized MHC, the amount of radioactivity (cpm) present below full-sized MHC after electrophoresis of a chase sample was subtracted from the corresponding amount obtained from a non-chase sample. Thus, only radioactive material that could be chased into completed MHC was counted as nascent MHC. For several experiments, multiple exposures on preflashed films were quantitated by scanning densitometry; this procedure gave similar results.

Drug Treatments. Where noted, cultures were treated with anticytoskeletal drugs before labeling. Cytochalasin D, taxol, or nocodazole were added at 5, 1, and 10 μg/ml, respectively. Stock solutions were stored at −20°C in dimethyl sulfoxide.

Labeling with [^{3}H]Puromycin. Cultures in 100-mm dishes were rinsed and extracted as described above, except that the skeleton buffer contained 50 μCi of [8(n)-^{3}H]puromycin dihydrochloride (Amersham, 11 Ci/mmol) in a 1-ml volume. After 10 min at room temperature, buffer was removed, the cultures were rinsed once with skeleton buffer, and soluble and cytoskeletal fractions were prepared for immunoprecipitation as described above. To control for nonspecific binding, identical cultures were labeled in the presence of a 100-fold excess of unlabeled puromycin.

RESULTS

Muscle Cell Cultures. Cultures were derived from thigh muscle of 12-day-old chicken embryos. One day after plating, cultures were synchronized by limited Ca^{2+}/Mg^{2+} depletion and repletion; this procedure yields muscle cultures, relatively free of contaminating non-muscle cells, that rapidly fuse and synchronously develop upon cation repletion. Standard (nonsynchronized) cultures gave similar results (data not shown).

Identification of Nascent Chains. For study of cotranslational assembly, nascent polypeptides must be identified. This can be done by specific immunoprecipitation using a mAb that binds an epitope sufficiently close to the amino terminus that most nascent chains will be recognized. mAb MF-18 recognizes an epitope 30 kDa from the amino terminus of muscle-specific MHC (17).

Our immunoprecipitation procedure can recover nascent MHC. Fig. 1 shows immunoprecipitates from homogenates of muscle cultures after a 6-min pulse of [^{35}S]methionine followed by various chase periods. The autoradiogram indicates that large amounts of material smaller than completed MHC are immunoprecipitated. Eighty percent of the material recovered by immunoprecipitation after a pulse (measured either by scanning densitometry or scintillation counting) is less than full-sized. If elongation were proceeding at the rate seen in intact erythrocytes (27), a protein the size of MHC would be expected to be 50% complete in this time. The approximately 60% reduction of elongation rate seen here probably reflects the low concentration of methionine (1% that of growth medium). The smaller polypeptides disappear

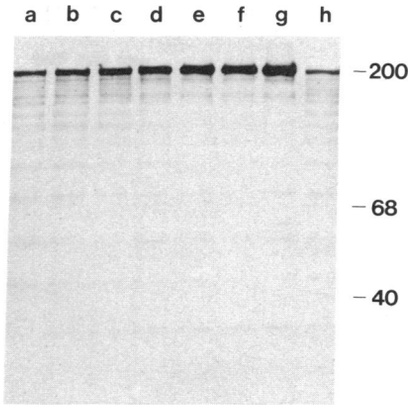

FIG. 1. Recovery of nascent MHC. mAb MF-18 allows immunoprecipitation of polypeptides substantially smaller than completed MHC myosin. All lanes contain immunoprecipitates. Lane a: sample from cells incubated with [^{35}S]methionine for 6 min. Lanes b–g: sample from cells incubated with [^{35}S]methionine for 6 min ("pulse") and then incubated with nonradioactive methionine ("chased") for 2.5, 5, 10, 20, 30, or 60 min, respectively. Lane h: sample from cells chased for 20 min in the presence of cycloheximide. Radioactive polypeptides smaller than completed MHC are recovered after the pulse and can be chased to full-size; this chase does not occur without protein synthesis. Molecular masses (kDa) of marker proteins run in parallel are shown at right.

with time, while radioactivity present in full-sized MHC increases during the chase, consistent with the lower molecular weight bands being nascent MHC. In labeled cultures chased with cycloheximide present, nascent chains are not completed (Fig. 1, lane h).

To test whether the lower molecular weight material contained breakdown products that retained the carboxyl terminus, identical pulse-labeled samples of cultured muscle were immunoprecipitated with two different mAbs, one sample with MF-18 and one with MF-20, a mAb that recognizes an epitope near the carboxyl terminus of MHC (18). Whereas MF-18 recovers both nascent and full-sized MHC, MF-20 recognizes principally full-sized protein (Fig. 2), consistent with the interpretation that MF-18 is recovering nascent proteins and not breakdown products.

Nascent MHC Are Associated with the Cytoskeleton. Expo-

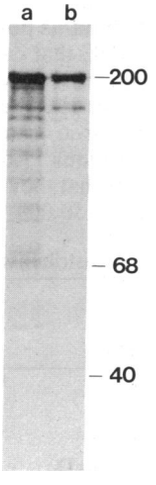

FIG. 2. Comparison of mAbs that recognize different regions of MHC. Cells were incubated with [^{35}S]methionine for 6 min. MF-18 (lane a) recovers full-size and nascent muscle-specific MHC; MF-20 (lane b), which recognizes an epitope near the carboxyl terminus, recovers largely full-size MHC.

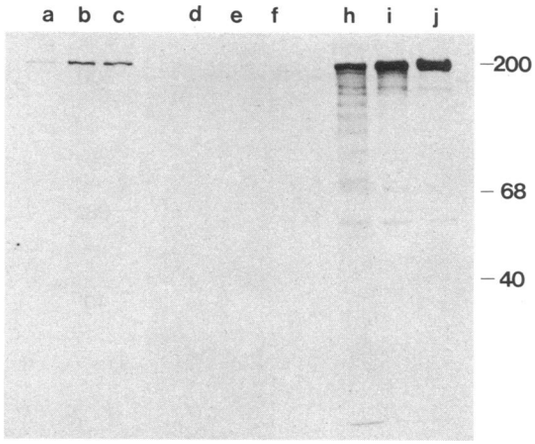

FIG. 3. Nascent MHC are associated with the cytoskeleton. All lanes contain immunoprecipitates. Muscle cultures were pulse-labeled for 6 min and directly extracted (lanes a, d, and h) or chased for either 7.5 min (lanes b, e, and i) or 25 min (lanes c, f, and j) before extraction. Lanes a–c: material released by a 10-min extraction at 0°C with Triton X-100. Lanes d–f: material released by a second 10-min extraction at room temperature with Triton. Lanes h–j: immunoprecipitates of the Triton-resistant cytoskeletons.

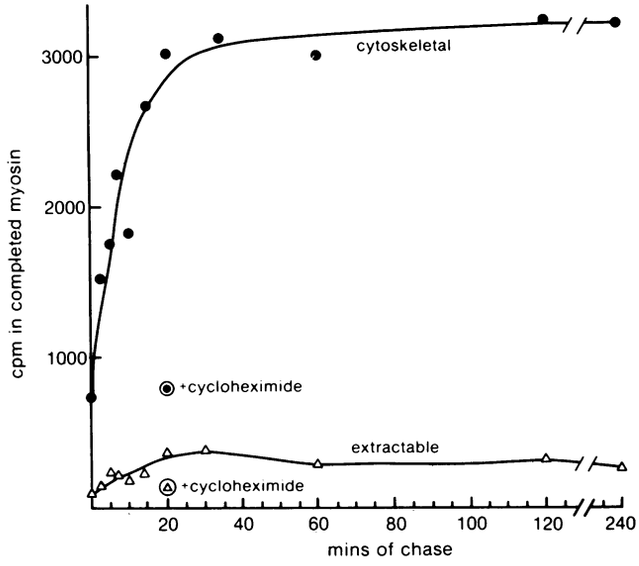

FIG. 4. Assembly of completed MHC during chase. Muscle cultures were pulse-labeled and then chased (as described in the legend to Fig. 3) for 0, 2.5, 5, 7.5, 10, 15, 20, 30, 60, 120, and 240 min. Cultures were extracted, and released and cytoskeletal MHC were immunoprecipitated. After $NaDodSO_4$/PAGE, the amount of completed MHC present was determined by measurement of radioactivity (cpm) comigrating with full-sized MHC (200 kDa). Completed MHC present in the released fraction and in the cytoskeletal fractions is denoted by triangles and circles, respectively. One culture was chased for 20 min in the presence of cycloheximide.

sure of metabolically labeled cultures to 0.5% Triton X-100 at 0°C extracts 57 ± 7% of the radioactivity incorporated into protein. Room temperature extraction with Triton X-100 releases another 21 ± 3% of the acid-precipitable radioactivity, leaving 22 ± 4% in the cytoskeleton. Analysis of these fractions by immunoprecipitation, $NaDodSO_4$/PAGE, and autoradiography (Fig. 3) reveals that most nascent MHC resist release by Triton. By excision of relevant regions of the gel, immunoprecipitated MHC are separated into completed and nascent molecules that can be quantitated by liquid scintillation counting (Table 1). Of nascent MHC polypeptides, 83% are associated with the cytoskeleton at 6 min. Of the completed MHC, 73% are associated with the cytoskeleton at the end of the pulse. After a 25-min chase, all nascent chains are completed to full-sized MHC, of which 85% are bound to the cytoskeleton (Fig. 4). Thus, of those chains which do not associate during translation, most will do so later. Since only 20% of the MHC is full-sized at the end of the 6-min pulse, its extensive association with the cytoskeleton suggests very rapid assembly, possibly occurring at late stages of translation. The absence of a large pool of myosin is also consistent with this possibility. The existence of a discrete fraction of MHC that can be released suggests that resistance to release shown by the remaining MHC reflects interaction with other cytoskeletal components (i.e., assembly), rather than intrinsic insolubility of this protein under the extraction conditions. Additional evidence that released MHC are soluble is that they remain in solution after centrifugation for 30 min at 150,000 × g (data not shown).

One-Third of Nascent MHC Molecules Are Bound to the Cytoskeleton via a Puromycin- and RNase-Resistant Linkage. As described above, almost all nascent MHC associate with the cytoskeleton during translation. However, most if not all polyribosomes are associated with the cytoskeleton (10, 19–21), so this amount is not an estimate of cytoskeletal association of myosin itself. Nascent myosin molecules will be associated at least through mRNA. Therefore, to detect myosin associated directly with the cytoskeleton, cytoskeletons were treated with either puromycin (to release nascent chains from ribosomes) or with RNase (to release ribosomes from the cytoskeleton). Treatment with puromycin and RNase reduced the fraction of nascent chains associated with the cytoskeleton to 60% and 35%, respectively (Table 1 and Fig. 5). Treatment with both together did not remove more nascent MHC from the cytoskeleton. Mg-ATP also did not release additional nascent chains (data not shown).

Full-length MHC were not released from the cytoskeleton by these treatments. Why puromycin released fewer nascent MHC than RNase is not certain; however, these extraction conditions are not optimized for the puromycin reaction (22).

Table 1. Distribution of nascent MHC

Culture condition	% MHC released by Triton alone	Treatment after Triton	% MHC released by treatment	% MHC on cytoskeleton	n
Control	6 ± 5	None	11 ± 2	83 ± 4	6
Control	10 ± 7	Puromycin	30 ± 9	60 ± 8	5
Control	15 ± 12	RNase	50 ± 11	35 ± 6	9
Cytochalasin D	58 ± 13	RNase	30 ± 5	11 ± 8	6
Nocodazole	12 ± 10	RNase	55 ± 7	34 ± 10	3
Taxol	12 ± 13	RNase	58 ± 6	31 ± 14	3
<48 hpr	27 ± 7	RNase	59 ± 8	14 ± 10	4
>8 dpr	12 ± 12	RNase	33 ± 14	54 ± 19	6

hpr, Hours after repletion with divalent cations; dpr, days after repletion with divalent cations.

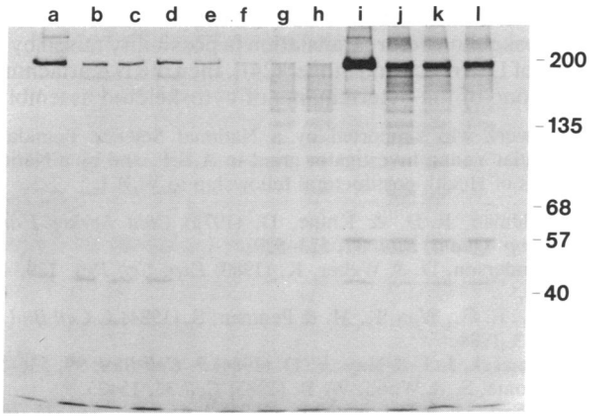

FIG. 5. Retention of nascent MHC on the cytoskeleton after treatment with RNase or puromycin. All lanes contain immunoprecipitates. Material in lanes a–d was released by the first extraction with Triton. Material in lanes e–h was released by the second extraction with Triton. Material in lanes i–l remained with the cytoskeleton. The sample in lanes a, e, and i was taken from cells pulsed for 6 min and chased for 25 min; it contains predominantly completed MHC. All other samples were taken from cells pulsed for 6 min and not chased. The sample in lanes b, f, and j had no treatment to release nascent chains from polyribosomes during the second extraction. Most nascent MHC remained with the cytoskeleton (lane j). The sample in lanes c, g, and k was taken from cells treated with puromycin during the second extraction with Triton; some nascent MHC was removed during the second extraction (lane g). A substantial fraction of nascent MHC remained with the cytoskeleton (lane k). The sample in lanes d, h, and l was taken from cells treated with RNase during the second extraction. Again, although some nascent chains were released (lane h), many remained on the cytoskeleton (lane l).

Under the conditions used here, only the chains that are in the ribosomal peptidyl (P) site should be released. Longer extractions in the presence of either agent do not release more nascent chains.

To estimate nonspecific adsorption and exchange of nascent MHC, metabolically labeled cell extracts were added to unlabeled cytoskeletons and incubated at either room temperature or on ice for 10 min. Labeled extracts were removed and the fraction of labeled MHC bound to skeletons was measured. At either temperature, the skeleton bound only 8% or less of the added nascent MHC. By contrast, during a room temperature incubation, 23% of the added full-sized MHC bound, possibly reflecting exchange as observed by Saad *et al.* (23).

These results were obtained from muscle cultures 4 days after repletion with divalent cations. Similar studies done earlier in development (i.e., during the first 2 days after repletion) reveal differences (Table 1). Now, only 14% of nascent MHC are associated with the cytoskeleton after RNase treatment. Moreover, cultures examined 8 days after repletion have 54% of nascent MHC associated with the cytoskeleton after RNase treatment. These results show that the amount of nascent MHC released by Triton X-100 does not reflect solubility *per se* but rather their relation to the cytoskeleton. The results further suggest that such cytoskeletal relations change during development.

To quantitate cotranslational assembly of MHC by another method, extracted cultures were exposed to [^{3}H]puromycin to simultaneously label and release nascent chains from ribosomes. Radioactivity present in immunoprecipitates of released and cytoskeletal MHC was measured; 32% of the nascent chains remained with the cytoskeleton after such an extraction ($n = 4$). All chains labeled in this way are nascent.

Effects of Cytochalasin D and Other Anticytoskeletal Agents. To investigate the roles of microfilaments and microtubules during MHC assembly, we perturbed these structures with anticytoskeletal drugs before labeling. Cytoskeletal associations of nascent MHC are greatly altered by pretreatment (20 min) with cytochalasin D, but not by pretreatment with either nocodazole or taxol (Table 1). Cells treated with cytochalasin D release many nascent MHC during the 0°C Triton extraction, before RNase treatment (Fig. 6). Furthermore, the number of nascent MHC cotranslationally assembled is reduced by about two-thirds. Increasing or decreasing cell volume by changing medium osmolarity did not change the extent of assembly during translation (data not shown).

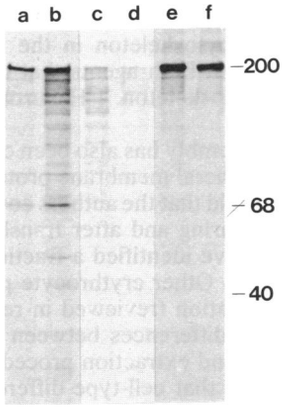

FIG. 6. Cytochalasin D alters the distribution of nascent MHC. Muscle cultures were treated with either dimethyl sulfoxide (vehicle control; lanes a, c, and e) or cytochalasin D (lanes b, d, and f; 5 μg/ml final concentration) for 20 min before metabolic labeling (6 min), fractionation, and immunoprecipitation with MF-18. Lanes a and b: material released by Triton X-100. Lanes c and d: material released by a 10-min incubation with RNase. Lanes e and f: material present in the RNase-resistant cytoskeletal fractions.

DISCUSSION

The cytoskeleton of eukaryotic cells contains on the order of 75–150 different proteins (9). Within the cytoskeleton, numerous microstructures contain complements of cytoskeletal proteins that are distinct from other structures within the cytoplasm of the same cell. A structure of this complexity and order poses serious problems of assembly. Previous models (reviewed in ref. 9) have postulated assembly from solution, which carries with it the implicit assumption that protein molecules contain in themselves enough information to generate structures. However, efforts to date to replicate assembly of comparably complex structures *in vitro* have not been successful, although simpler subassemblies can be generated.

One alternative to assembly from solution is that some cytoskeletal proteins are ordered at synthesis: cotranslational assembly. A similar restriction of fate occurs for membrane-associated proteins; failure to associate during translation often prevents assembly later. In addition, several cytoskeletal proteins have highly localized mRNAs (24); this would be irrelevant for posttranslational assembly, given rapid diffusion.

Our results show that during muscle development an increasing fraction of muscle MHC associates with the cytoskeleton during translation; some myosin forms a contact that neither RNase nor puromycin can disturb in the presence of Triton X-100. Resistance to Triton release is not a property of myosin itself, since early in muscle development most nascent MHC are released from cells. Nascent chains of most proteins are associated with the cytoskeleton through their polyribosomes; however, it seems unlikely that this is the only contact formed by myosin. One-third of

nascent MHC polypeptides at day 4 in culture remain associated with the cytoskeleton in the presence of both RNase and puromycin, both agents that release polysomal material from the cytoskeleton. [^{3}H]Puromycin gives comparable results.

Cotranslational assembly has also been considered (25) for the erythrocyte peripheral membrane protein 4.1. Assembly of protein 4.1 is so rapid that the authors could not distinguish between assembly during and after translation. On muscle cytoskeletons, we have identified a fraction of stably associated nascent MHC. Other erythrocyte proteins appear to assemble after translation (reviewed in ref. 26). There are, however, numerous differences between the two systems, including cell types and extraction procedures. Our preliminary studies suggest that cell-type differences may be important.

Many details of MHC cotranslational assembly are not known. Spatial proximity to adjacent filaments is probably not sufficient, since 2-fold changes in cell volume do not alter the extent of cotranslational assembly. Some components of the cytoskeleton appear to be more important than others for myosin assembly, since cytochalasin D reduces it by two-thirds, and nocodazole does not affect it. This pattern of drug sensitivity corresponds to the drug sensitivity of the aperiodic cable of actin and myosin that gives rise to sarcomeres (G. M. Denning and A.B.F., unpublished observations).

The mode of assembly shown here for some MHC was suggested by three other studies (10, 12, 13). In 3T3 cells, HeLa cells, and IMR-90 fibroblasts, different methods of approach suggested cotranslational assembly of some cytoskeletal proteins. Because MHC become increasingly cotranslational in their assembly during development, cotranslational assembly of cytoskeletal proteins may be under cellular control. The extent to which it occurs will need to be studied individually in different cell lines, using antibodies capable of recognizing nascent proteins, before the overall contribution of this mechanism for assembly can be assessed.

How does cotranslational assembly differ from assembly of completed proteins? It might lead to two classes of cytoskeletal proteins: one cotranslationally assembled and rarely, if ever, exchanging with proteins in solution; and a second class that is in solution for some period after translation and then enters the cytoskeleton (perhaps reversibly). Some evidence for long residence on the cytoskeleton comes from quantitative autoradiography (11); comparable data are not available for muscle. At least, cotranslational assembly leads to more persistent locations for assembly than is possible with assembly from solution; proteins translated from the same polyribosome are more highly correlated in position than are proteins diffusing freely. Finally, if mRNAs are attached to the cytoskeleton before translation [a possibility raised by the results of Lawrence and Singer (24)], then mRNA attachment will be one of the determinants of cytoskeleton assembly.

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