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Synthesis and assembly of native myosin on muscle polyribosomes

Jacques Gagnon[+], Thomas T. Kurowski[+] and Radovan Zak[+*]

[+] *Department of Medicine* and [*] *Pharmacological and Physiological Sciences, The University of Chicago, 5841 South Maryland Ave, Chicago, IL 60637, USA*

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We have used the overload-induced growth of avian muscles to study the assembly of the newly synthetized myosins which were separated by non-denaturing pyrophosphate-polyacrylamide gel electrophoresis. Using this model, we have observed the appearance of fast-like isomyosins in polyribosomes prepared from slow anterior latissimus dorsi muscle after 72 h of overload. These new isoforms comigrating with native myosin from fast posterior latissimus dorsi muscle were not yet present in cellular extracts from the same muscle. The in vitro translation system utilizing muscle specific polyribosomes directs the synthesis of the corresponding myosin isoforms. Under denaturing conditions, myosin heavy chains and light chains dissociate to the expected subunit composition of each specific isoform. The synthesis and assembly of native myosin on polyribosomes indicate that myosin exists as a single mature protein prior to the incorporation in the thick filament.

Myosin isoform; Myosin heavy chain; Myosin light chain; Overload hypertrophy; (Chicken skeletal muscle)

1. INTRODUCTION

Native myosin is a complex of six polypeptides: two heavy chains and two pairs of light chains. The development of the non-denaturing pyrophosphate-polyacrylamide gel electrophoresis system has revealed the presence of multiple myosin isoforms [1–4]. In the chicken slow tonic ALD and fast twitch PLD, 2 and 3 isoforms, respectively, were found that differ in their subunit composition [3,4] (fig.1).

We have studied the myosin assembly using a weight-overload induced muscle growth. In this model, the chicken ALD undergoes rapid enlargement accompanied by a change in isomyosin composition. The SM1 myosin isoform becomes gradually replaced by the SM2 band and three fast-like myosin bands, normally not present in this muscle, become transiently expressed during the early phase of the overload [5,6]. Using this model, we have found that the nascent fast-like myosin isoforms are first present in association with the polyribosomes and only later appear in the muscle extracts.

Correspondence address: J. Gagnon, Dept of Medicine, The University of Chicago, 5841 South Maryland Ave, Chicago, IL 60637, USA

Abbreviations: ALD, anterior latissimus dorsi muscle; PLD, posterior latissimus dorsi muscle; SM1, SM2, slow isomyosin 1, slow isomyosin 2; FM1, FM2, FM3, fast isomyosin 1, fast isomyosin 2, fast isomyosin 3; MHC, myosin heavy chain; MLC, myosin light chain; PP-PAGE, non-denaturing pyrophosphate-polyacrylamide gel electrophoresis; SDS-PAGE, denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. EXPERIMENTAL

Overload hypertrophy was induced in 5 week-old male White Leghorn chicken by the application of a weight equivalent to 10% of the body mass to one wing as described previously [6]. The contralateral wing served as a control. Myosin was extracted with high-salt Guba-Straub buffer [7] and intact polyribosomes were isolated from the hypertrophied and control ALD and PLD at 24, 48 and 72 h following initiation of treatment according to the procedure described previously [8]. High-salt muscle extracts and polyribosome suspensions were loaded onto individual tube gels and PP-PAGE was carried out at 1°C for 24 h at 15 V/cm according to the procedure of Hoh et al. [3]. Native myosin bands were visualized by staining with Coomassie brilliant blue (CBB) G-250 in 3.5% perchloric acid [9]. Immunotransfer [10] of parallel tube gels was performed

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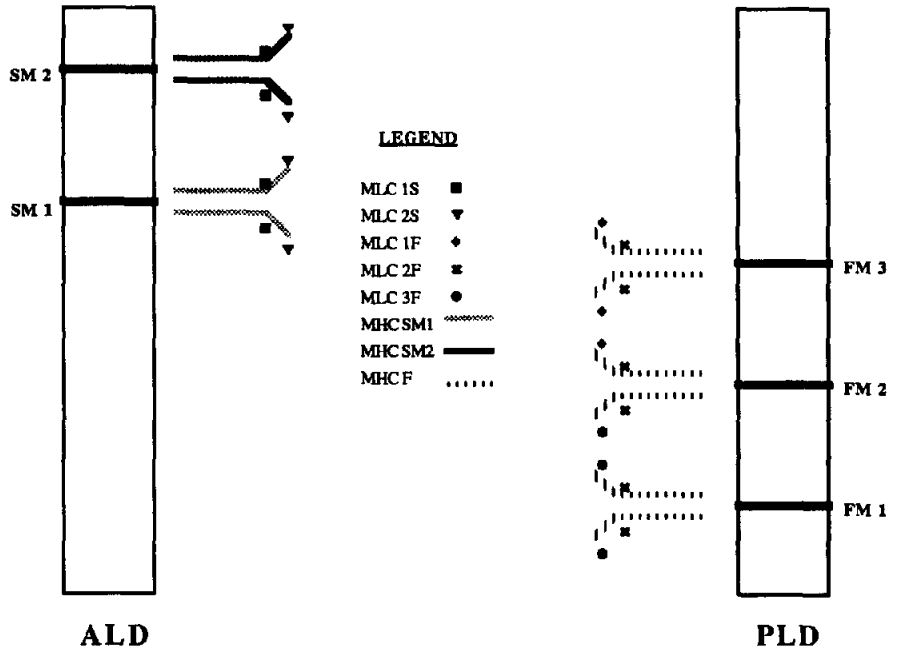

Fig.1. Schematic representation of the subunit structure of two slow myosin isoforms (SM1, SM2) in ALD and three fast myosin isozymes (FM1, FM2, FM3) in PLD present in chicken skeletal muscle. These multiple isoforms are resolved by PP-PAGE based on differences detected either in MHC or MLC composition [3,4]. Refer to the text for abbreviations.

following PP-PAGE and protein bands were stained with Amido-Black (AB) [10] or reacted with monoclonal Ab S-46 (gift from Dr F. Stockdale) or EB-165 (gift from Dr E. Bandman) as described [11] to detect the presence of slow and fast myosin, respectively.

Alternatively, an equal amount of polyribosomes (normalized by hybridization to a specific ribosomal 28 S cDNA probe (gift from Dr J. Gorsk)) was added to 15 μl of mRNA-dependent rabbit reticulocyte lysate containing 60 μCi [^{35}S]methionine (spec. act. 1100 Ci/mmol). Translation was carried out as previously described [8] except that pancreatic ribonuclease A (RNase) was added to duplicate samples either before (+) or after (−) incubation in order to block translation. RNase added before the translation served as a control to detect any non-specific incorporation of labeled [^{35}S]methionine. The reaction mixture was chromatographed on a Biogel P-6 column to remove unincorporated [^{35}S]methionine. The eluate was loaded on a tube gel and resolved by PP-PAGE as described above, transferred to nitrocellulose, stained with AB [10] and prepared for fluorography [12].

Finally, the same reaction mixture was also electrophoresed on a tube gel by PP-PAGE and briefly stained with CBB G-250 in 3.5% perchloric acid [9]. The native isoform contained in each band was excised from the gel and denatured in the presence of SDS by placing the sliced band with 2 times concentrated Laemmli buffer [13] in each well of a stacking gel (4%). After 45 min of equilibration, MHC and MLC were separated on a 5–18% linear gradient or 15% slab gel by SDS-PAGE [13]. To compare the actual subunit composition with the radiolabeled subunits, the gels were either silver stained [14] or prepared for fluorography as previously described [12].

3. RESULTS

Application of the weight to the wing produced a rapid increase in the wet weight of the overloaded muscle when compared to the corresponding control muscle. By 72 h, the hypertrophy of the ALD and PLD was 70 and 13%, respectively. The body weights of the animals were not significantly different during the experimental period (data not shown). The growth of the overloaded ALD was accompanied by a shift in the relative decrease of the slow myosin isozyme SM1 while no significant changes in the relative distribution of fast isomyosins in the overloaded PLD were detected (fig.2). These data are consistent with a previous report [6].

When polyribosomes isolated from the same muscles were analysed by PP-PAGE, we observed a similar pattern concerning the loss of SM1 as shown in fig.2. We also detected three additional myosin isoforms consistently associated with the

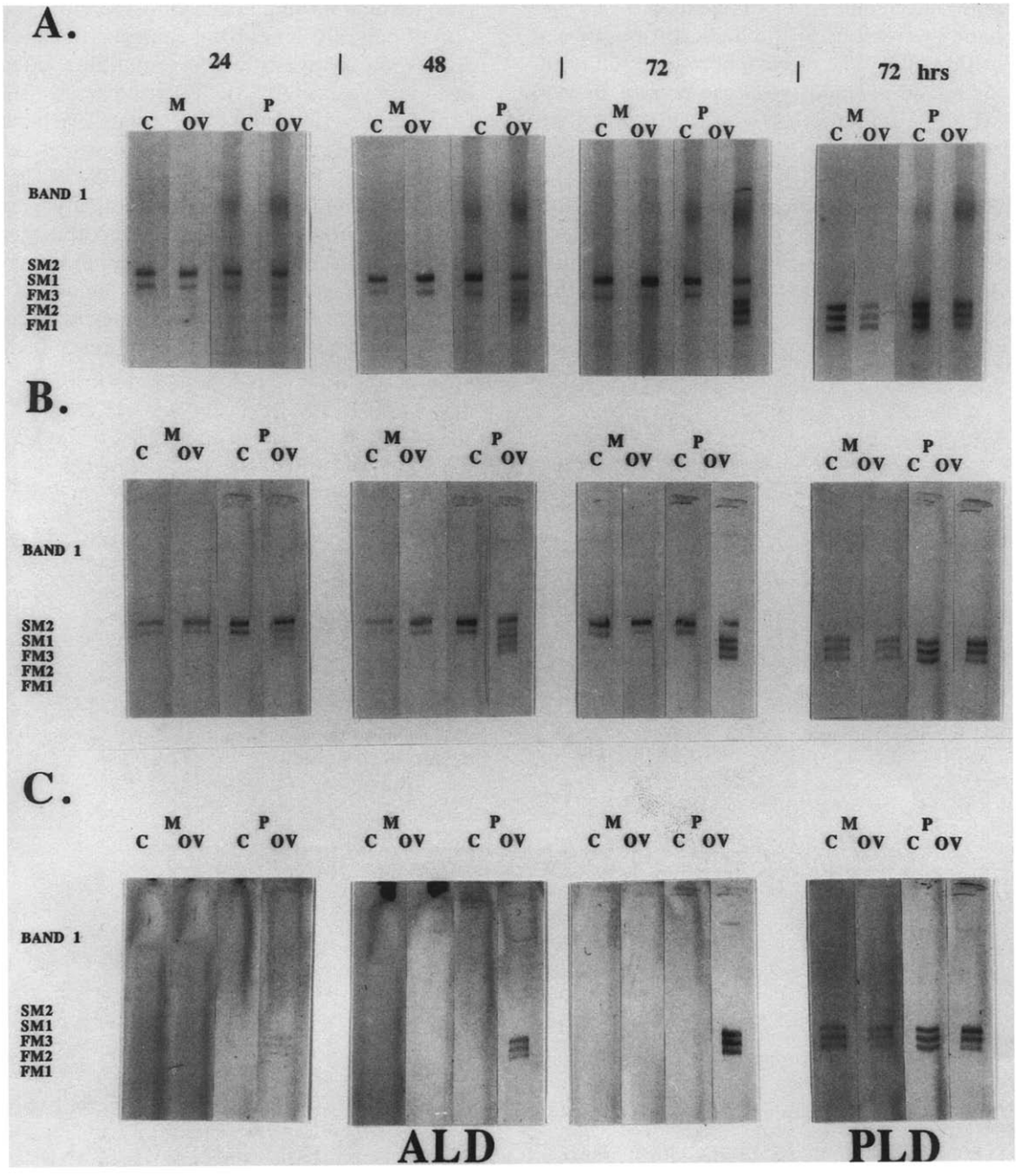

Fig.2. Electropherogram resolved by PP-PAGE of high-salt muscle extracts (M) or polyribosomes (P) from control ALD (C) and from ALD overloaded for 24, 48 and 72 h (OV). C and OV PLD after 72 h are also represented. (A) Amido-Black (AB) stained gels of transferred native myosins. An unidentified band 1 associated with polyribosomes is also detected. (B) Immunoreaction with combined monoclonal antibodies S-46 and EB-165 directed against slow and fast MHC, respectively. (C) Immunoreaction with monoclonal antibody EB-165 only. Refer to text for additional abbreviations.

polyribosomes from ALD overloaded 72 h. No such bands were seen in the high-salt muscle extract at this time. The electrophoretic mobility of these bands corresponds to those present in fast PLD (fig.2). Immunoreactivity with Ab S-46 specific for slow-MHC and with Ab EB-165 specific for fast-MHC (fig.2B) demonstrates the five isoforms found in the polyribosomes of 72 h overloaded ALD. The immunoblots for Ab EB-165 alone (fig.2C) show 3 fast isoforms in the polyribosomes from ALD at 48 and 72 h after overload, respectively.

Using rabbit reticulocyte lysate and labeled [^{35}S]methionine in the reaction mixture, translation of polyribosomes from control ALD and PLD directs the synthesis of corresponding slow and fast isomyosins (fig.3). Polyribosomes isolated from 72 h overloaded ALD translate five bands including 2 slow and 3 fast-like myosin isozymes. Addition of RNase in the translation system abolishes completely the synthesis of native myosin thus ruling out any non-specific incorporation of labeled [^{35}S]methionine. Band 2, comigrating with one of the reticulocyte lysate bands, is also translated but shares no homology with native myosin as demonstrated by the absence of MHC

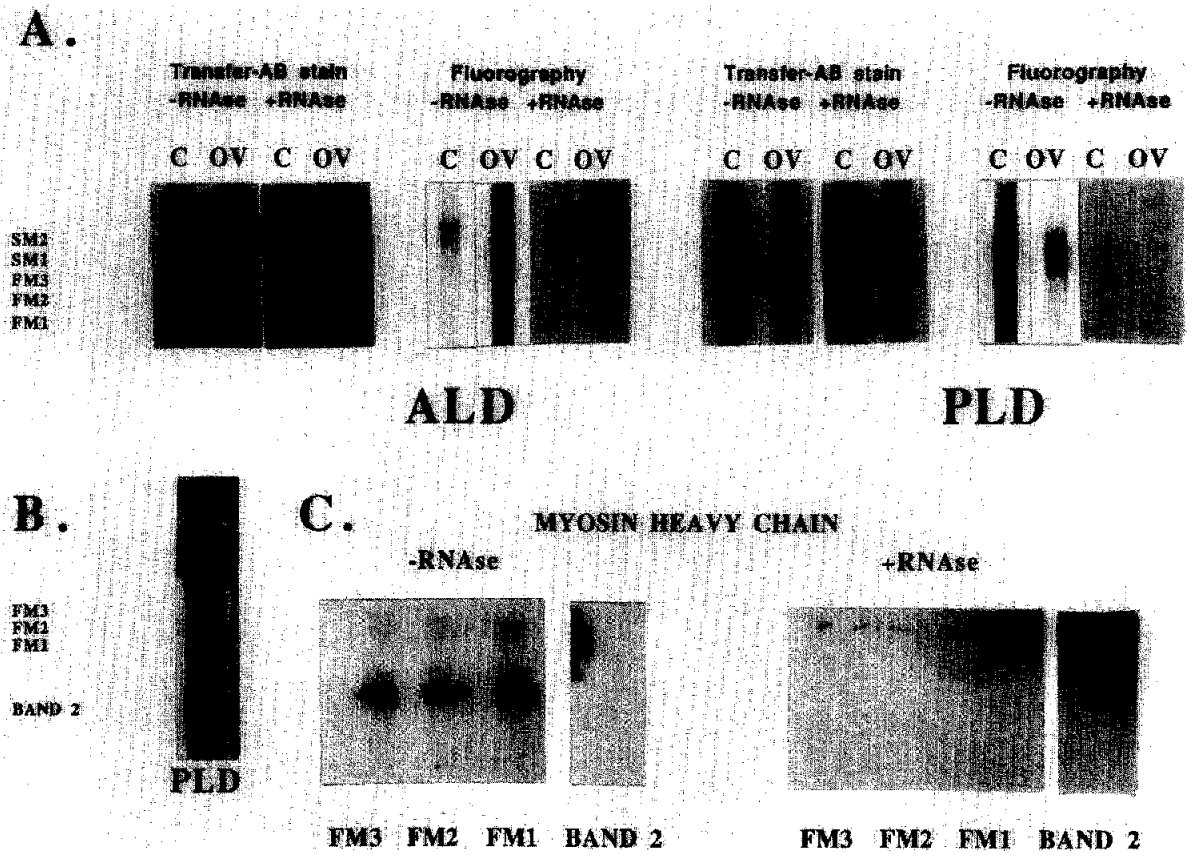

Fig.3. Electropherogram resolved by PP-PAGE of native myosin using polyribosomes translated from ALD and PLD overloaded for 72 h (OV). Contralateral muscles served as a control (C). (A) Amido-Black (AB) stained nitrocellulose after transfer of the corresponding gel was used to demonstrate the efficiency of the transfer from the tube gel. The same transfers were prepared for fluorography after translation in the absence (−) or presence (+) of RNase. RNase was added before incubation to block the translation reaction and was used as a control to detect any non-specific incorporation of labeled [^{35}S]methionine. (B) Fluorogram of native gel from PLD transferred after a shorter run than A showing 3 fast isoforms and an unidentified band 2. (C) Fluorogram of MHC separated by SDS-PAGE (5%). Following the translation reaction in the absence (−) or presence (+) of RNase, the native myosin bands and band 2 were excised from the gel shown in B and dissociated by SDS to analyse the MHC composition. The unidentified radiolabeled band (band 2) contained no MHC subunit.

subunit after denaturation by SDS and electrophoresis on a 5% SDS gel (fig.3C).

The subunit composition of nascent myosin isoforms is shown in fig.4. The native myosin protein bands associated with polyribosomes, either non-radiolabeled or radiolabeled by [^{35}S]methionine incorporation following the translation reaction, were excised and dissociated by SDS treatment into their respective MHC and MLC subunits. By comparing the MLC composition of each native myosin isoform associated with the polyribosomes (fig.4A) after silver stain with that of the newly synthesized isomyosins (fig.4B) after fluorography, we observed that the pattern of radiolabeled translation products resembles the subunit structure of the non-radiolabeled isomyosins.

4. DISCUSSION

The objective of this study was to address the question where and when MHC and MLC associate, and to examine the role of the polyribosome in myosin assembly. Since myosin spontaneously aggregates into thick filament under prevailing cellular ionic conditions [15], it is necessary to reconcile this observation with the temporal frame and topography of synthesis and assembly. One model visualizes the nascent protein as entering the free cytoplasmic pool which is in rapid equilibrium with corresponding molecules within the myofilaments [16]. Radiolabeling kinetics demonstrated that the two myosin subunits turn over at different rates which is consistent with this model. However, the same techni-

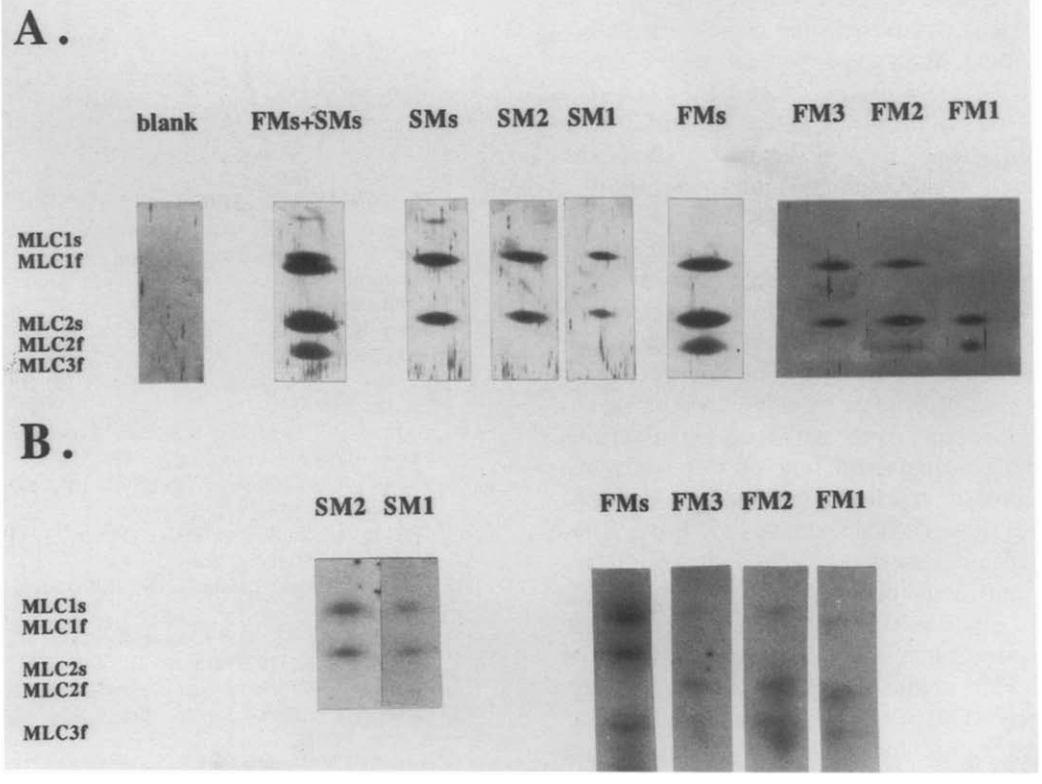

Fig.4. Electropherogram resolved by SDS-PAGE for MLC subunits of native myosin associated with polyribosomes. After electrophoresis by PP-PAGE and brief staining with CBB G-250, each native isozyme band was carefully cut out and denatured in the presence of SDS. (A) Silver stained 5–18% linear gradient gel. Blank corresponds to a piece of gel with no myosin. FMs + SMs mixture, SMs and FMs were obtained from polyribosomes from ALD (OV), ALD (C) and PLD (C), respectively, by the excision of gel pieces containing the myosin bands separated by 5 h run of PP-PAGE. (B) Fluorogram of 15% gel resolved by SDS-PAGE following the translation reaction. The isomyosin bands were dissociated by SDS to analyse the MLC composition of radiolabeled native myosin. Refer to fig.1 and text for additional abbreviations.

que detected the free cytoplasmic pool only for one of the MLCs but not for MHC [17]. A second model postulates co-translational assembly of MHC and MLC on polyribosomes [18]. Since the pioneer work of Heywood et al. [19] on the identification of a class of very large polyribosomes containing 50–60 ribosomes as those which synthesize myosin [19], it has been suggested that completed myosin remains non-specifically associated with polyribosomes [20] and was possibly a myofibrillar contaminant.

Our study is based on PP-PAGE that allows the separation of multiple myosin isoforms based on different electrophoretic mobility mainly due to the differences in MHC and MLC composition [1–4]. Using this system, we were able to detect the appearance of fast-like isomyosins on polyribosomes isolated from the slow ALD following 72 h of overload that are not yet detectable in the cellular extract from the same muscle. In support of this finding, analysis of the isomyosin composition by PP-PAGE of the overload ALD over a period of 80 days did not reveal a significant increase in fast myosin content, but immunocytochemical staining did not show a transient appearance of fast-MHC epitope [21].

Furthermore, when polyribosomes isolated from chicken skeletal muscles were used as the template and the in vitro translational products were analysed by PP-PAGE, we detected the synthesis of the various isoforms of myosin by fluorography of the native isoforms after transfer. Comparison of the subunit content of the native myosin associated with polyribosomes with that of the newly synthesized myosin following [^{35}S]methionine incorporation, showed their similarity. From these results, we conclude that the native myosin is synthesized and assembled at the polyribosome site and hence exists as a single mature protein prior to the incorporation in the thick filament. In addition to these biochemical studies, immunoelectron microscopy [22] in conjunction with rotary shadowing techniques [23] would provide morphological information concerning the nature of the interaction of the polyribosomes with native myosin in vivo.

Our system offers a eukaryotic model to study the assembly of the multiple isoforms of myosin in addition to the recently reported co-expression and assembly of MHC and MLC in *Escherichia coli* [24]. Furthermore, native myosin assembly may serve as a model for studies of the contribution of ribosomes to protein folding [25].

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