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Segregated Assembly of Muscle Myosin Expressed in Nonmuscle Cells

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Skeletal muscle myosin cDNAs were expressed in a simian kidney cell line (COS) and a mouse myogenic cell line to investigate the mechanisms controlling early stages of myosin filament assembly. An embryonic chicken muscle myosin heavy chain (MHC) cDNA was linked to constitutive promoters from adenovirus or SV40 and transiently expressed in COS cells. These cells accumulate hybrid myosin molecules composed of muscle MHCs and endogenous, nonmuscle, myosin light chains. The muscle myosin is found associated with a Triton insoluble fraction from extracts of the COS cells by immunoprecipitation and is detected in 2.4 ± 0.8-μm-long filamentous structures distributed throughout the cytoplasm by immunofluorescence microscopy. These structures are shown by immunoelectron microscopy to correspond to loosely organized bundles of 12–16-nm-diameter myosin filaments. The muscle and nonmuscle MHCs are segregated in the transfected cells; the endogenous nonmuscle myosin displays a normal distribution pattern along stress fibers and does not colocalize with the muscle myosin filament bundles. A similar assembly pattern and distribution are observed for expression of the muscle MHC in a myogenic cell line. The myosin assembles into filament bundles, 1.5 ± 0.6 μm in length, that are distributed throughout the cytoplasm of the undifferentiated myoblasts and segregated from the endogenous nonmuscle myosin. In both cell lines, formation of the myosin filament bundles is dependent on the accumulation of the protein. In contrast to these results, the expression of a truncated MHC that lacks much of the rod domain produces an assembly deficient molecule. The truncated MHC is diffusely distributed throughout the cytoplasm and not associated with cellular stress fibers. These results establish that the information necessary for the segregation of myosin isotypes into distinct cellular structures is contained within the primary structure of the MHC and that other factors are not required to establish this distribution.

INTRODUCTION

Skeletal muscle myosin is a member of a large multigene family that includes cytoplasmic nonmuscle myosin and smooth muscle myosin (for review see Warrick and Spudich, 1987). The expression of these genes is regulated in a developmental and tissue-specific manner. Myosins within this family exhibit a high degree of sequence conservation and structural similarity but differ in their subunit composition, assembly characteristics, and enzymatic activity (Spudich, 1989). Myosin is composed of two heavy chains (220 kDa) and two pairs of light chains (17–22 kDa) that associate into a highly asymmetric molecule having two globular heads (S1) and a rod-like tail (rod). In vivo, myosin assembles into bipolar filaments through interactions involving the rod. In vertebrate-striated muscle, these assemblies are part of a highly ordered stable structure, the myofibril.

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Myosin, in the absence of accessory proteins, forms filaments in vitro that resemble native myosin filaments. Synthetic myosin filaments generally display a broad range of filament lengths and often lack bare zones. Biochemical, immunochemical, and electron microscopy techniques have shown that the myosin rod contains the assembly function of the molecule (Lowey et al., 1969), and myosin S1 is responsible for the ATPase activity, actin binding, and movement (Toyoshima et al., 1987). In vitro assembly assays using mixtures of different myosin isotypes have shown that muscle and nonmuscle myosins can be polymerized into the same filament (Citi et al., 1987; Waschberger and Pepe, 1980). Microinjection studies using fluorochrome-labeled myosin isotypes support these results (Sanger et al., 1984; Mittal et al., 1987).

Muscle and nonmuscle myosins are both present during the remodeling of the cytoskeleton at the onset of myogenesis (Wang et al., 1988). During the early stages, muscle-specific protein synthesis is turned on, and there is a downregulation of the nonmuscle cytoskeletal proteins. Immunocytochemical studies of early myofibrillogenesis indicate that skeletal muscle myosin segregates from the cytoplasmic myosin forming distinct cytoskeletal structures (Dlugosz et al., 1984; Antin et al., 1986; Wang et al., 1988). The nascent myosin filaments are found in the central portion of the cell aligned with stress fiber-like structures, whereas the nonmuscle myosin is associated with the stress fibers in the periphery of the cells. Many of the myofibrillar accessory proteins, which stabilize and regulate the structure, also associate with the nascent thick and thin filaments at this time. These events culminate in the formation of 1.6-μm-long thick filaments interdigitated with 1.0-μm thin filaments in the complex structure of the myofibril. A number of thick and thin filament associated proteins have been implicated in the regulation of filament length and the segregation of the developing myofibrils into the central portion of the cell by serving as scaffolding or docking proteins (Antin et al., 1986; Handel et al., 1989; Colley et al., 1990). However, the factors that regulate the formation of this complex structure during myogenesis remain elusive.

To investigate the mechanisms controlling early stages of myosin filament assembly, we have expressed an embryonic chicken skeletal muscle myosin heavy chain[1] (MHC) cDNA in the background of nonmuscle cytoskeletons in COS cells and C2 myoblasts. These cells accumulate a hybrid myosin molecule composed of a full length muscle MHC and the endogenous nonmuscle myosin light chains (MLCs). The muscle MHC protein is found assembled into filamentous structures 1–8 μm in length and 12–16 nm in diameter distributed throughout the cytoplasm of these cells. The nonmuscle and muscle MHCs do not colocalize; the nonmuscle MHC is associated with the stress fibers in a normal pattern, and the muscle MHC is assembled into isolated filaments dispersed throughout the cells. These results indicate that the information necessary for the segregation of myosin isotypes into distinct cellular structures is contained within the primary structure of MHC and that other factors are not required to establish this distribution.

[1] Abbreviations used: G418, geneticin; HeBS, HEPES-buffered saline; mAb, monoclonal antibody; MHC, myosin heavy chain; MLC, myosin light chain; NaPi, NaH_2PO_4.

MATERIALS AND METHODS

Tissue Culture

The simian kidney cell lines, COS-7 (CRL 1651) (American Type Culture Collection, Rockville, MD) and COS-1 (CRL 1650), were provided by Dr. Jean Schwarzbauer (Princeton University, Princeton, NJ). Both COS cell lines are maintained in 10% fetal bovine serum (FBS) (GIBCO, Gaithersburg, MD), 90% Dulbecco's modified Eagle's medium (DME) (GIBCO) at 37°C in 5.0% CO_2. The myogenic cell line C2C12 (Yaffe and Saxel, 1977) was purchased from American Type Culture Collection (CRL 1772). All of the transient assays were done with both COS-1 and COS-7 cells; however, the presented data are from experiments with COS-7 cells. C2C12 myoblasts are maintained in 20% bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT), 80% DME at 37°C in 5.0% CO_2 and passaged at 60–70% confluent every 3–4 d. Cells that have been transfected with pSV2Neo are maintained in 20% BCS, 0.5 mg/ml Geneticin (G418, Gibco), and 80% DME. Transfected and nontransfected C2C12 myoblasts were treated with the DNA synthesis inhibitor, cytidine arabinoside (Ara-C) by incubation with 1 μM Ara-C in 20% BCS, and 80% DME for 24–48 h.

Plasmids and Vectors

All of the MHC expression vectors are derived from a full-length chicken embryonic fast skeletal muscle MHC cDNA that has been described previously (Molina et al., 1987). Completion of the cDNA by addition of missing 5′ coding sequences, an alfalfa mosaic virus 5′-untranslated region and unique flanking *Eco*RI and *Bam*HI restriction sites, has been reported (Rindt et al., 1993). The complete MHC region was cloned into the *Eco*RI site of the expression vector pMT2 (Kaufman et al., 1989) after the restriction sites had been blunted to produce the vector pMT2MHC (Rindt et al., 1993). Similarly, a 2.9-kilobase (kb) *Eco*RI to *Hpa* I fragment of the MHC cDNA encoding the S1 region and 118 residues of rod (residues 1–964) was inserted into pMT2 to produce $pMT2tMHC_{964}$.

The selection vector pSV2Neo contains a neomycin phosphotransferase gene linked to the SV40 early promoter region and SV40 late sequences containing splicing and polyadenylation signals (Southern and Berg, 1982). The neomycin phosphotransferase gene in pSV2Neo was replaced with the full length MHC cDNA to construct the expression vector pSV2MHC. Plasmids are carried in *Escherichia coli* strain HB101 and isolated using an alkaline lysis procedure (Birnboim and Doly, 1979) followed by two cesium chloride gradients (Sambrook et al., 1990). Only plasmid DNAs >70% supercoiled as judged by gel electrophoresis are used for transfection experiments.

Transfections

Calcium phosphate and diethylaminoethyl (DEAE) dextran-mediated gene transfer procedures were used for most transient transfection experiments in COS cells. COS cells are plated at 5×10^6 cells per 100 mm culture dish 24 h before transfections, and the media is changed 4 h before transfection. Transfection mixtures contained 30

μg of DNA in 0.5 ml *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered saline (HeBS) plus 124 mM $CaCl_2$ (Parker and Stark, 1979). Mock transfections have pSV2neo DNA substituted for the MHC expression vectors. The cells are incubated with the precipitate in the absence of media for 20 min at room temperature. Media is added back to the plates, and the cells were incubated at 37°C for 5 h before glycerol shock. The media is replaced with 2.5 ml of 15% glycerol in HeBS, and the cells are incubated at 37°C for 3.5 min. The glycerol is removed and the media refreshed. The DEAE dextran procedure is done essentially according to Sambrook *et al.* (1990).

Stable transfections of C2C12 cells and some transient assays on COS cells were performed using a liposome mediated procedure. Lipofectin reagent, N-(1-(2,3-diolelyoxy)propyl)-N,N,N-trimethyl ammonium chloride and dioleoyl phosphatidylethanolamine (GIBCO), was used as outlined by Felgner et al. (1987) after optimization for each cell line. The conditions for transfection of COS cells with lipofectin are as follows: 50 μg of Lipofectin and 1 μg of DNA. The transfection mixture for C2C12 cells contains 35 μg of Lipofectin reagent and 5 μg of DNA (total DNA in reaction). The molar ratio of MHC expression vector to selection vector (pSV2Neo) is varied from 10:1 to 200:1. Mock transfections include a plasmid carrying the full-length MHC cDNA linked to a prokaryotic promoter (pSP64MHC) and selection vector.

C2 myoblasts are plated at 3×10^3 cells/cm^2 in 6 well cluster plates and incubated at 37°C, 5% CO_2 for 24–48 h (cells were 50–60% confluent at the time of transfection). Cells are given fresh media 4 h before transfection. The cells are rinsed twice with HeBS before transfection then the DNA Lipofectin mixture (1.5 ml/well) is added, and the plates are incubated at 37°C for 5 h. The transfection is stopped by the addition of 3 ml of 20% BCS in DME. The cells are allowed to recover for 12–16 h before the media is changed. After this time, the debris is removed by replacing the media and the cells are incubated at 37°C for 24 h. For stable transfections, each well is split into a 100-mm culture dish 36–48 h posttransfection with selection media containing 1 mg/ml G418. The selection media is changed every 3 d to remove debris. Isolated colonies are visible 7–10 d post-transfection. The G418 resistant colonies are combined and analyzed as mixed clonal pools.

Isolation of Clonal Cell Lines

Cloned cell lines from pMT2MHC transfected cultures are isolated by an immuno-replica filter procedure (Raetz et al., 1982) as modified for C2 myogenic cells (Black and Hall, 1985). For constructs containing constitutive promoters, the filters and original plates are grown for 24 h at 37°C after the removal of the filter. The colonies on the filters are fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked as described for immunofluorescence microscopy (see below). Chicken specific anti-myosin monoclonal antibodies (mAbs) are applied to the filters at 1 μg/ml in 1% bovine serum albumin (BSA), 0.05% Tween-20, and phosphate-buffered saline (PBS) and incubated at room temperature for 1–2 h. Excess antibody is removed by washing the filters three times with 0.05% Tween-20 in PBS. The secondary antibody, rabbit anti-mouse IgG (Pierce, Rockford, IL), is applied to the filters at 4 μg/ml in 1% BSA, 0.05% Tween-20, and PBS and incubated for 1 h at room temperature. Excess antibody is removed by washing the filters as above. Bound antibody is detected with ^{125}I-Protein A. Positive colonies are identified by aligning the original clonal plate with the film. The colonies are transferred from the plate to 24-well cluster plates, expanded, and taken through a secondary screening by immunofluorescence.

Isolation and Analysis of DNA and RNA

Genomic DNA is isolated from 80–100% confluent 100-mm plates of cells by disruption of the cells in 100 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) HCl pH 8.0, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml proteinase K (Sambrook et al., 1990). Generally, 500 μg of genomic DNA is isolated from one 100-mm culture plate. Total RNA is isolated by the method of Chirgwin et al. (1979).

Northern and Southern analysis are performed according to standard procedures (Sambrook et al., 1990). Total RNA (5–40 μg) is electrophoresed on 1.0% agarose gels in a formaldehyde-Borate-EDTA buffer system and transferred to Genescreen (New England Nuclear, Boston, MA) by capillary action with 25 mM NaP_i pH 6.5. For dot blot procedures, Genescreen filters are washed with 25 mM NaP_i and 1–10 μg of RNA in 200 μl of 25 mM NaP_i pH 6.5 is applied to each well. The samples are allowed to soak through the manifold before rinsing the wells twice with 25 mM NaP_i. The RNA is crosslinked to the nylon membrane by UV irradiation. For Southern analysis of genomic DNA, 5–10 μg of material is digested with an appropriate restriction enzyme, and the entire digest is electrophoresed on a 0.7% agarose gel in Tris-Borate-EDTA buffer containing 0.5 μg/ml ethidium bromide and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The DNA is stabilized on the filter by baking in vacuo at 80°C for 2 h.

Filter hybridization of nucleic acids is done according to Amasino (1986). The filters are prehybridized and hybridized in 50% deionized formamide, 250 mM NaP_i pH 7.2, 250 mM NaCl, 1.0 mM EDTA, 1.0 mg/ml sonicated salmon sperm DNA, and 7.0% SDS at 43°C for ≥1 h before addition of the probe. Radioactive probes are made using the oligonucleotide random primer procedure essentially according to Feinberg and Vogelstein (1983) with an oligonucleotide labeling kit (Pharmacia, Piscataway, NJ) and ^{32}P-dCTP (3000 Ci/mmol, New England Nuclear). Labeled probes (10^6 cpm/ml) are hybridized at 43°C for 8–16 h. Posthybridization washes are done at 50°C using 0.1× SSC, 0.1% SDS.

Preparation of mAbs

A library of mAbs that react with skeletal muscle MHC was prepared and characterized as previously described (Winkelmann et al., 1983; Winkelmann and Lowey, 1986). The IgG class mAbs are purified on Protein A sepharose (Ey et al., 1978) from ascites fluid obtained by passage of hybridoma lines through CAF1/J mice (Winkelmann et al., 1983). mAbs F18 and F59 were prepared and characterized by Miller et al. (1989) and kindly provided by Dr. Frank Stockdale (Stanford University, Stanford, CA). A rabbit polyclonal sera that reacts specifically with nonmuscle MHC was a gift from Dr. Jim Sellers (National Institutes of Health [NIH]). mAb MY-21 (Sigma, St. Louis, MO), reacts with the 20-kDa light chain of cytoplasmic and smooth muscle myosin and crossreacts with the MLCs of COS cells. The patterns of crossreactivity for each antibody was determined by Western blots of whole cell lysates, enzyme-linked immunoadsorbent assay (ELISA) of isolated proteins, immunofluorescent microscopy, and immunoprecipitations of radiolabeled cell extracts (Table 1). For clarity in the text, mAbs F18, 10F12.3, and 7B11.4 are referred to as chicken specific anti-S1, anti-S2, and anti-LMM, respectively. The mAb 13F6.5 crossreacts with the sarcomeric MHC expressed in C2C12 myotubes and is used to detect the expression of the mouse muscle specific cytoskeleton.

mAbs are biotinylated according to Bayer and Wilchek (1979). N-hydroxysuccinimido-biotin was dissolved in N,N-dimethyl formamide (Sigma) and incubated at 5:1 molar ratio with the antibody (10 mg/ml) for 60 min at 25°C. Excess biotin is removed by gel filtration chromatography on Econopak 10 DG columns (BioRad, Cambridge, MA).

Metabolic Labeling of Proteins and Immunoprecipitations

Transfected cells are metabolically labeled with ^{35}S-methionine. Cells are incubated with 50 μCi/ml of ^{35}S-methionine (3000 Ci/mmol, New England Nuclear) in 10–20% serum and DME for 2–16 h at 37°C. For short labeling times, the cells are methionine starved by incubation

in 10–20% dialyzed FBS in methionine-free DME (GIBCO) for 30 min before labeling. For longer labeling times, normal media is used.

Preparation of cell extracts and immunoprecipitations are done essentially according to Isaacs and Fulton (1987). Anti-myosin mAbs (25 μg/reaction) are used as the primary antibody. Immunoprecipitates are formed by the addition of 100 μg rabbit anti-mouse IgG (Pierce) and 100 μl of 10% (wt/vol) formalin-fixed *Staphylococcus aureus* (Immunoprecipitin, GIBCO). The precipitates are analyzed by SDS-polyacrylamide gel electrophoresis.

Immunofluorescence Microscopy

Cells are grown on glass coverslips for immunofluorescence microscopy. The coverslips are sterilized in 100% ethanol and coated with 1% porcine gelatin (Sigma) for C2C12 cells or 10 μg/ml poly-L-lysine for COS cells. Paraformaldehyde fixation of the cells is used with minimal loss of immunoreactivity. The cells are rinsed with cold PBS, covered with 4% paraformaldehyde in PBS, and incubated for 15–30 min at 0°C. The cells are rinsed with PBS three times, permeabilized with 1% Triton X-100 in PBS for 1 min, rinsed with PBS and free aldehydes reduced with 0.05% $NaBH_4$ in PBS for 30–60 min at 4°C (Doane and Birk, 1991).

Coverslips are blocked with 0.02% normal donkey serum in PBS for 2–16 h in humidified chambers. The blocking solution is replaced with primary antibody (1 μg/ml in 1% BSA, 0.05% Tween-20 in PBS) and incubated for 4–8 h. Mouse mAbs are detected with minimal cross-reacting donkey anti-mouse antisera (Jackson Immunological Lab, West Grove, PA) conjugated with tetramethyl rhodamine (TRITC). Rabbit polyclonal sera is detected with minimal cross-reacting donkey anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC). The anti-cytoplasmic MLC mAb MY-21 is an IgM and is detected with a rabbit anti-mouse IgM heavy chain specific antisera (Pierce) followed by FITC donkey anti-rabbit antisera. Antibodies that have been directly conjugated with biotin are detected with TRITC-streptavidin conjugate (Molecular Probes, Eugene, OR). Stress fibers are labeled with FITC or TRITC conjugated to phalloidin (Molecular Probes). The coverslips are mounted with FITC-guard mounting media (Testog, Chicago, IL).

Photomicrographs are recorded with a Nikon Optiphot 2 (Nikon, Tokyo, Japan) epifluorescence photomicroscope using a 1.4 NA ×60 objective. Measurements of the myosin filament length distribution are made on digitized photomicrographs sampled on a raster of 0.3 μm/pixel. Images are enhanced, and filament boundaries are defined, thresholded, and measured using the interactive program NIH Image (National Institutes of Health Research Service Branch, Bethesda, MD).

Immunoelectron Microscopy

Immuno-gold labeling and embedding are done essentially according to Birk et al. (1988). Twenty-four h posttransfection COS-7 cells are lifted from tissue culture flasks using trypsin and plated on 60-mm Permanox plates (Electron Microscopy Sciences, Fort Washington, PA). Forty-eight h post transfection, the cells are fixed and permeabilized with paraformaldehyde/Triton as described for immunofluorescence microscopy. Relatively mild paraformaldehyde fixation is necessary for retention of immunoreactivity; however, this mild fixation is not optimal for maintaining cellular morphology (Pommeroy et al., 1991). The cells are incubated with primary antibody (1.0 μg/ml) in 1% BSA, 0.05% Tween-20, and PBS for 6 h at 4°C. Excess antibody is removed by gently washing the samples. The secondary antibody, goat anti-mouse IgG conjugated with 5-nm gold particles, was a generous gift of Dr. David Birk (Tufts University, Boston, MA). The gold-conjugated antibody is incubated with the samples for 12 h at 4°C. Excess antibody is removed by washing as above followed by three washes with PBS and rinsed with 0.1 M cacodylate buffer pH 7.4.

The samples are dehydrated and embedded in epon (Birk et al., 1988). Permanox dishes are peeled from the epon-embedded cells. Selected areas are cut from the epon discs and attached to epon-filled beem capsules using 5 min epoxy and trimmed for microtomy. For transmission electron microscopy, gold sections are cut with a diamond knife, picked up on formvar coated grids, and stained with 2% uranyl acetate followed by 1% phosphotungstic acid pH 3.2. Sections are examined and photographed using a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

RESULTS

Muscle Myosin Expression in COS Cells

To analyze the assembly of a muscle myosin in the absence of muscle specific accessory proteins, an embryonic chicken skeletal muscle MHC was expressed in the background of a nonmuscle cytoskeleton. This MHC corresponds to the earliest isoform expressed during the differentiation of the chicken pectoralis muscle (Moncman, 1993). The full-length chicken muscle MHC cDNA (Molina et al., 1987) and a truncated cDNA corresponding to the S1 fragment plus 118 residues of rod are linked to viral promoters in the vectors pMT2 or pSV2 (pMT2MHC, pSVMHC, and $pMT2tMHC_{964}$). The SV40 origin of replication is included in these vectors; consequently, the COS cells replicate the foreign DNA to very high copy numbers resulting in high levels of expression. The vector pMT2 is specifically designed for use in COS cells and includes signals necessary for message stability as well as 5′ and 3′ splicing (Kaufman et al., 1989).

When the MHC expression vectors are introduced into COS-7 cells using calcium phosphate–mediated procedures, the muscle MHC transcripts are detected in total RNA 24 h post transfection (Figure 1A). The transfected cells express transcripts of the appropriate size for each construct: 7.4 kb for pMT2MHC, 4.3 kb for $pMT2tMHC_{964}$, and 6.0 kb for pSVMHC. Cells transfected with pMT2MHC and $pMT2tMHC_{964}$ have steady-state levels of the chicken MHC transcript at 50–75% of the level detected for the embryonic MHC message in developing pectoralis muscle. The third vector pSVMHC is expressed less efficiently in these cells at levels of 0.1–0.5% of the embryonic MHC message. No cross-hybridization is detected in the mock-transfected cells.

These transcripts direct the synthesis of full-length myosin polypeptides. COS-7 cells transfected with each of the MHC constructs were metabolically labeled with ^{35}S-methionine 24 h posttransfection. The transfected cells were extracted with Triton X-100, and the Triton soluble extracts and insoluble fractions were analyzed by immunoprecipitation with chicken specific anti-myosin mAbs (Figure 1B). The anti-LMM and anti-S1 mAbs precipitated a 220-kDa polypeptide from the Triton insoluble fraction of pMT2MHC- and pSVMHC-transfected cells. The majority of the full-length chicken MHC (90–95%) is associated with the Triton insoluble material of the COS-7 cells. Extraction of the Triton insoluble material with 500 mM NaCl and 5 mM ATP

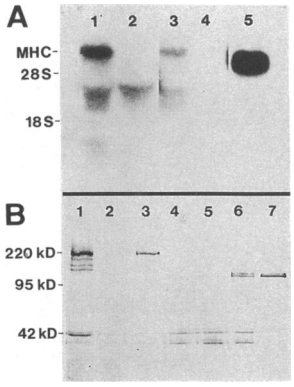


Figure 1. Chicken skeletal muscle MHC expression in COS-7 cells is detected at the mRNA and protein levels. (A) Northern blot of RNA isolated from COS-7 cells 24 h after transfection with pMT2MHC (lane 1), pMT2tMHC$_{964}$ (lane 2), pSV2MHC (lane 3) and mock-transfected cells (lane 4). The Northern was probed with radiolabeled full-length MHC cDNA. Chicken pectoralis muscle RNA was used as a positive control for hybridization (lane 5). Lanes 3 and 4 were exposed 18 times longer than the other lanes. Transcripts expressed from the pMT2 vector are polycistronic with a dihydrofolate reductase cDNA inserted 3′ to the MHC and, therefore, are 1.4 kb larger than the insert cDNA. (B) Immunoprecipitation of metabolically radiolabeled myosin from Triton extracts of transfected COS-7 cells. The immunoprecipitates from the Triton insoluble fraction of cells transfected with pMT2MHC (lanes 1–3) and pMT2tMHC$_{964}$ (lanes 4–6) with anti-LMM (lanes 1 and 4), nonimmune mAb (lanes 2 and 5), and anti-S1 (lanes 3, 6, and 7). The immunoprecipitate from the Triton soluble supernatant of pMT2tMHC$_{964}$-transfected cells is in lane 7. Chicken MHC was not detected in the mock-transfected and pSVMHC-transfected COS-7 cells. Approximately 90% of the chicken muscle MHC synthesized by these cells is in the Triton insoluble fraction. In contrast, 65% of the S1-like myosin fragment is found in the Triton soluble extracts. A 42-kDa protein was found in all of the immunoprecipitates including the nonimmune mAb indicating that this was a nonspecific reaction.

released >60% of the MHC from the pMT2MHC-transfected cells and all of the MHC from the pSVMHC-transfected cells. These extraction properties are characteristic of native myosin (Pollard, 1982). The truncated MHC polypeptide is precipitated only with the anti-S1 mAb and not with the chicken specific anti-LMM mAb. Triton extraction of cells expressing tMHC$_{964}$ released ~65% of the 105-kDa myosin fragment. The remaining fraction of the truncated MHC is found in the Triton insoluble cytoskeletal extract and is not released with the high salt/ATP extraction. Radiolabeled protein migrating with myosin is not found in the immunoprecipitates from the mock transfected cells.

Assembly of Expressed Muscle Myosin

The insoluble nature of the chicken muscle MHC after Triton extraction suggests that it is associated with the cytoskeleton. To investigate the distribution of the muscle MHC, COS-7 cells were transfected with each of the MHC constructs, and the location of the muscle MHC was analyzed as a function of time post transfection by immunofluorescence microscopy. The full-length muscle MHC is found assembled in elongated structures distributed throughout the cytoplasm of pMT2MHC- and pSVMHC-transfected cells (Figure 2, A and C). The size and shape of the structures formed by the muscle MHC is suggestive of myosin filament bundles. The lengths of these structures range from 1 to 8 μm; however, the distribution is heavily weighted to 2.4 ± 0.8 μm (n = 1230) long structures. Both the filament length distribution and the images of the filaments suggest that the larger structures are derived from the overlap of shorter, 1–2 μm-long units (see inset Figure 2A).

The formation of the muscle MHC assemblies is dependent on the accumulation of the protein. At 12–24 h post transfection, the muscle MHC is concentrated in the perinuclear region, but as the amount of protein increases (36–48 h post transfection), the muscle MHC is found almost exclusively in the short filamentous structures. At longer times post transfection (>60 h), the cytoplasm becomes densely packed with the exogenous MHC, and the cytoskeletal architecture is severely disrupted. In contrast, the truncated MHC that terminates at residue 964 was diffusely distributed throughout the cytoplasm at all times and shows no evidence of the formation of higher ordered structures (Figure 2B). This protein does not colocalize with the microfilaments. These results suggest that the COS cells have a limited capacity to accommodate the expression of the full-length skeletal muscle MHC; thus, further analysis was limited to 12–48 h post transfection.

The association of the nonmuscle MLCs with the exogenous muscle MHC in transfected COS-7 cells was examined by double-label immunofluorescence microscopy. Cells expressing the muscle MHC are identified using a biotinylated chicken specific anti-S2 mAb and streptavidin-rhodamine. The nonmuscle MLC is detected using an anti-MLC mAb (MY-21) that does not cross-react with muscle or nonmuscle MHC. All of the cells expressing the muscle MHC clearly have nonmuscle MLCs associated with the muscle myosin filamentous structures (Figure 3, A and B). The nonmuscle MLCs in these cells are also found distributed along stress fibers in a punctate manner presumably with the nonmuscle MHC. In addition, the fluorescence signal for MLC was significantly brighter in the cells containing the muscle myosin filaments than in nontransfected cells in the same field. This increased intensity may reflect both the association of the MLCs with discrete cellular structures and an increase in the amount of the endogenous MLC expressed by these cells. The colocalization of the nonmuscle MLC with the nonmuscle and muscle MHCs is also evident after brief Triton extraction before fixation. Thus, it appears that the nonmuscle MLCs as-

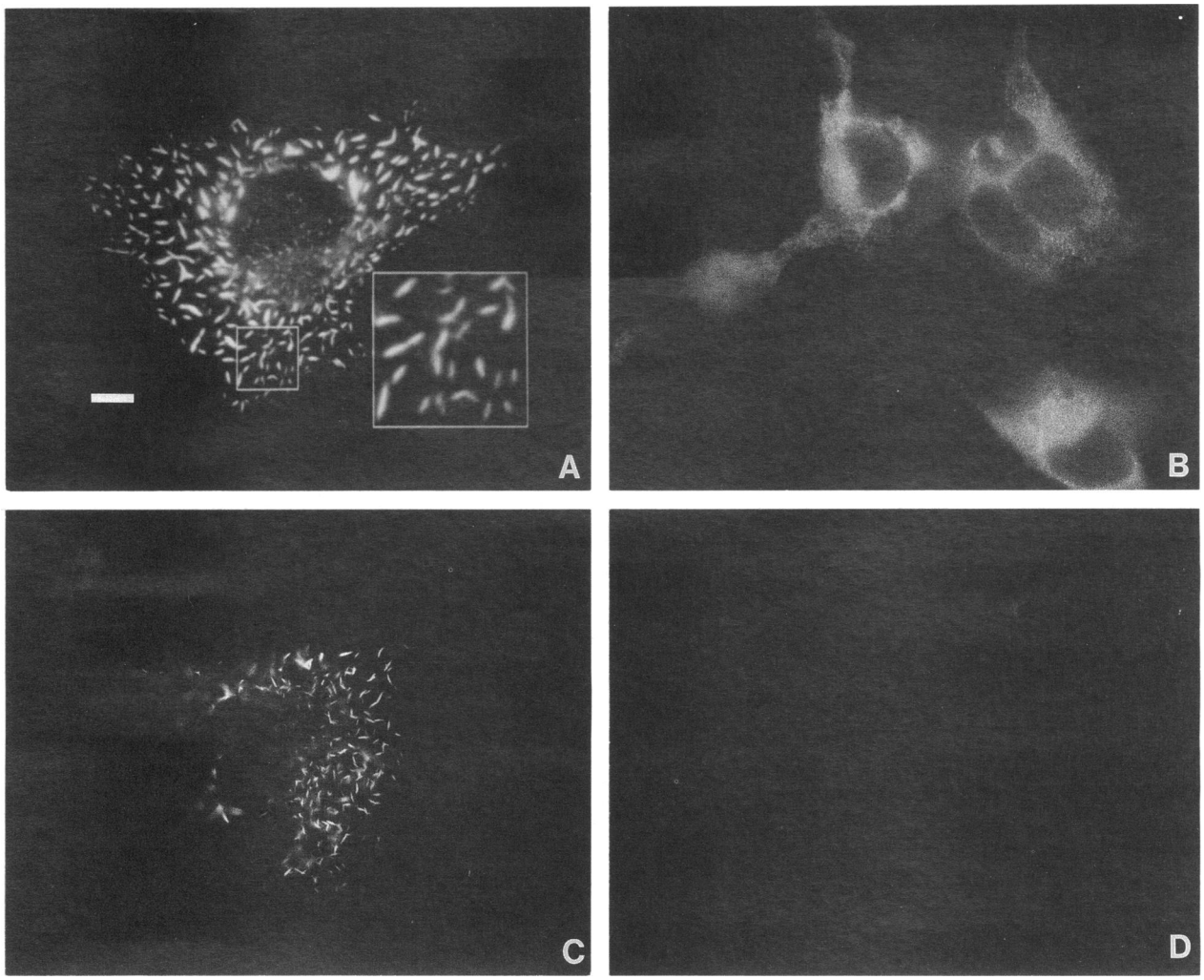


Figure 2. The chicken MHC is distributed throughout the cytoplasm of transfected COS-7 cells. COS-7 cells were fixed 48 h post transfection and probed with chicken specific anti-S1 mAbs. The chicken MHC expressed by pMT2MHC (A) and pSVMHC (C) is found in filamentous structures throughout the cytoplasm, whereas the protein expressed by pMT2tMHC$_{964}$ (B) is more highly dispersed throughout the cytoplasm. The inset in A is a 2.5-fold magnification of the boxed area in the cell. The longer filamentous structures are derived from overlap of short structural units. (D) Control experiments demonstrate that the chicken specific anti-MHC mAbs do not cross-react with any cytoskeletal elements of the mock-transfected cells. Bar, 10 μm.

sociate with both MHCs expressed in transfected COS cells.

The muscle myosin structures are readily detected when the cells are labeled only with the anti-MLC mAb or double-labeled with the anti-MLC mAb and fluorescein-phalloidin (Figure 3, C and D). The anti-MLC mAb again detects two distinct distributions of MLCs: one associated with the muscle myosin filamentous structures that does not correlate with the distribution of actin and the other associated with the nonmuscle MHC distributed along the actin-containing stress fibers. These results emphasize an important feature of these experiments: the distribution of the muscle myosin does not overlap the major stress fiber of the phalloidin-stained actin cytoskeleton.

These data also indicate that the staining of the muscle myosin assemblies by the anti-MLC mAb is not because of spectral overlap between the fluorescein and rhodamine detection systems in this double-label immunofluorescence experiment or to the cross-reaction of the anti-IgM detection reagents with the chicken specific anti-S2 IgG mAb. Furthermore, in mock-transfected cells the distribution of the nonmuscle MLCs only correlates with the staining pattern of the nonmuscle MHC and the phalloidin-stained stress fibers.

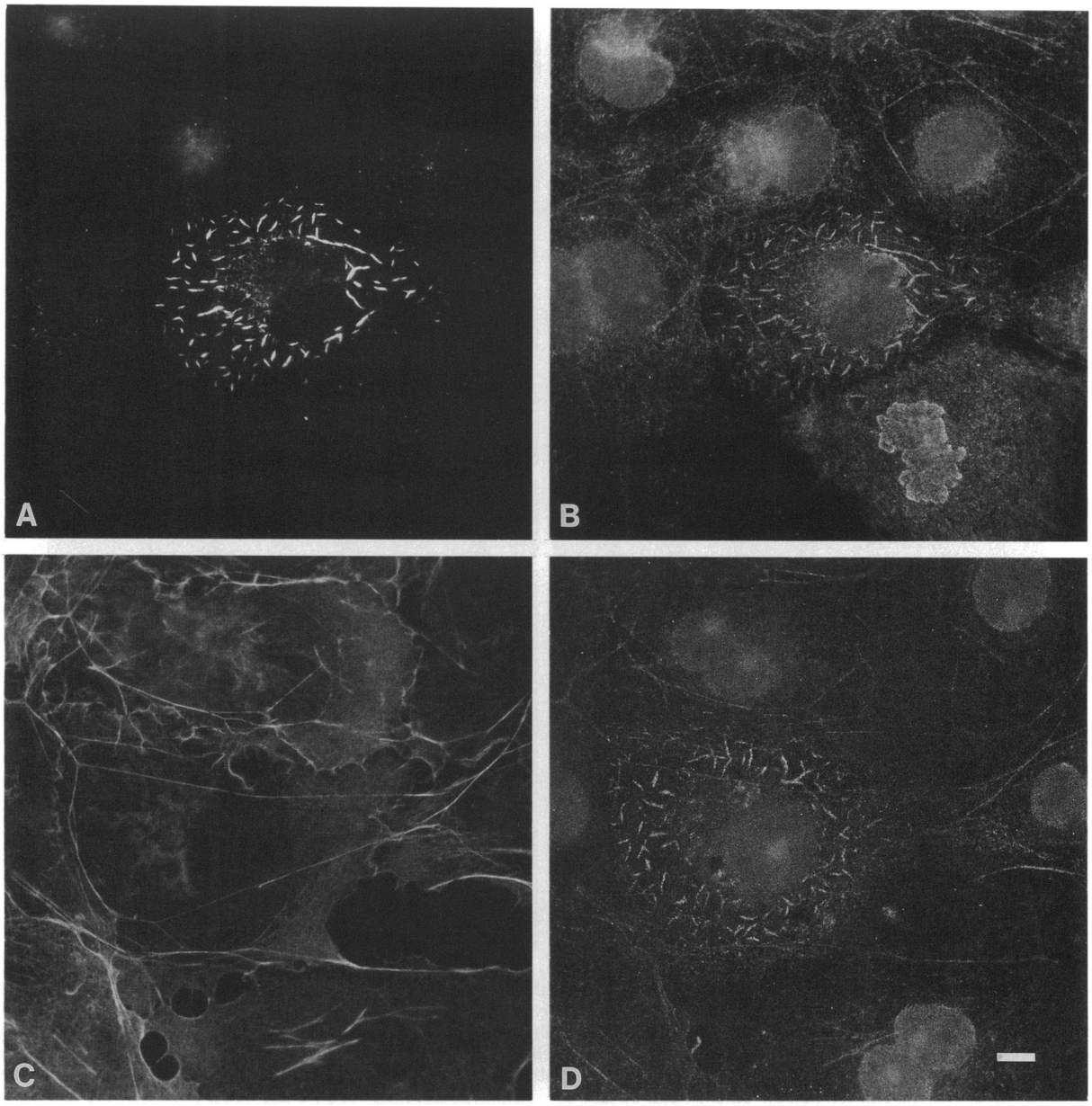


Figure 3. The endogenous nonmuscle MLCs coassemble with the chicken skeletal muscle MHC into short filamentous structures. COS-7 cells were transfected with pMT2MHC and processed 48 h post transfection for double-label immunofluorescence microscopy. (A and B) The same field of cells probed with a biotinylated, chicken myosin specific anti-S2 mAb (A), and an anti-MLC mAb, My-21 (B). The biotinylated anti-S2 mAb is detected with streptavidin-rhodamine, and the anti-MLC mAb was detected using affinity purified rabbit anti-mouse IgM antisera followed by fluorescein-labeled donkey anti-rabbit antibodies. (C and D) Cells from the same transfection are also double-labeled with rhodamine-phalloidin (C) and the anti-MLC mAb (D). The fluorescein-labeled donkey anti-rabbit detection system shows some nonspecific staining of the nuclei of COS-7 cells. Bar, 10 μm.

To confirm that the nonmuscle MLCs are associated with the heterologous muscle MHC rather than with nonmuscle myosin coassembled with the muscle myosin, the distribution of the muscle and nonmuscle MHCs was examined by double-label immunofluorescence microscopy (Figure 4). The nonmuscle myosin is detected with a rabbit polyclonal anti-platelet myosin antisera that does not cross-react with chicken skeletal muscle MHC (see Table 1). Transfected cells were probed with the anti-platelet myosin antisera in combination with the muscle specific anti-MHC mAbs (Figure 4, A and B), the anti-MLC mAb

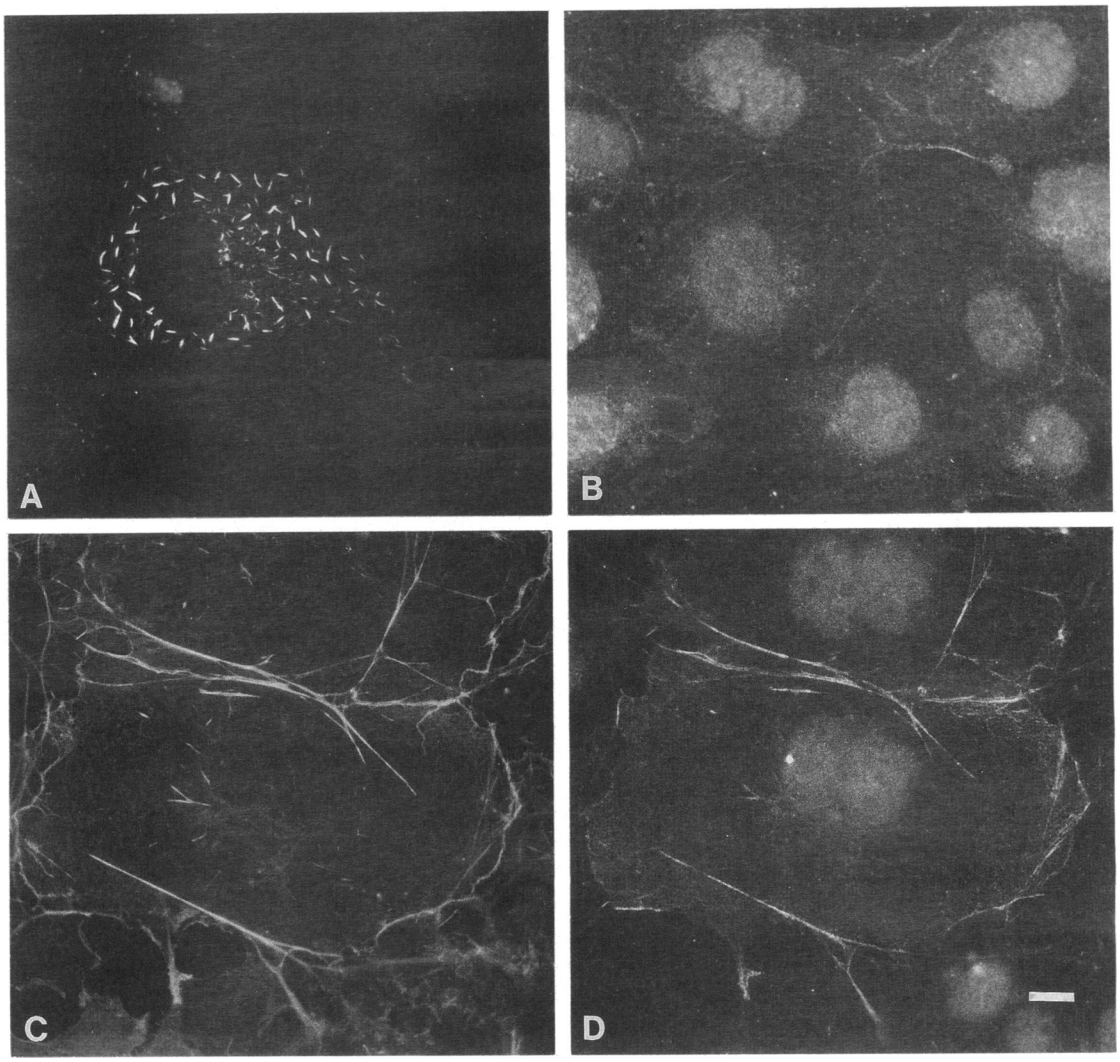


Figure 4. The endogenous nonmuscle MHC does not colocalize with the chicken MHC filaments. (A and B) COS-7 cells that had been transfected with pMT2MHC were processed for immunofluorescent microscopy 48 h post transfection and double-labeled with chicken specific anti-S2 mAb (A) and an anti-platelet myosin rabbit antisera (B). The different antibodies were detected with rhodamine-labeled donkey anti-mouse IgG and fluorescein-labeled donkey anti-rabbit IgG. (C and D) Cells from the same transfection were also double-labeled with rhodamine-phalloidin (C) and anti-platelet myosin antisera (D). The nonmuscle MHC is found to colocalize with stress fibers of the transfected cells but not to be associated with the chicken MHC filaments. Bar, 10 μm.

and fluorescein phalloidin (Figure 4, C and D). The nonmuscle MHC is found in a punctate staining pattern distributed along the stress fibers of the transfected COS cells and is not found coassembled with the muscle myosin structures. Therefore, it is concluded that the nonmuscle MLCs associate independently with both MHCs expressed in transfected COS cells and the muscle myosin and the nonmuscle myosin assemble independently into distinct structures.

Muscle Myosin Forms Filaments in COS-7 Cells

Immunoelectron microscopy of pMT2MHC, pSVMHC, and mock-transfected COS-7 cells was used to examine the muscle myosin containing filamentous structures detected by immunofluorescence microscopy. The cells were probed with chicken specific anti-MHC mAbs and colloidal gold-labeled anti-mouse IgG. The immunogold–labeled muscle myosin is found in 12–16 nm diameter cytoplasmic filaments that were loosely organized into bundles (Figure 5, A and B). The gold-labeled myosin filaments are distinguished easily from the unlabeled intermediate filaments (Figure 5A) and actin filaments. The lengths of the myosin filament bundles in these micrographs are ~2 μm (Figure 5, B and C). In addition to these filaments, immuno-gold label also is found associated with cytoplasmic polysomes.

Table 1. Specificity of antibodies

Antibody	Specificity	Pattern of crossreactivity			
		Embryonic chicken myosin	COS nonmuscle myosin	C2C12 myoblast myosin	C2C12 myotube myosin
7B11.4 (IgG_1)	anti-LMM	++	−	−	−
10F12.3 (IgG_1)	anti-S2	++	−	−	−
F18 (IgG_1)	anti-S1	++	−	−	+/−
13F6.5 (IgG_{2a})	anti-S1	−	−	−	++
12C5.3 (IgG_1)	anti-S1[a]	−	−	−	−
MY21 (IgM)	anti-MLC	−	++	−	−
anti-Platelet MHC (polyclonal)	Nonmuscle MHC	−	++	++	−

The pattern of crossreactivity of the anti-myosin antibodies used in these experiments was determined by Western blot assays, immunoprecipitations, ELISA, and immunofluorescence microscopy (see MATERIALS AND METHODS). ++, strong reaction; +/−, weak reaction; −, no reaction.

[a] mAb 12C5.3 does not cross-react with any of the proteins analyzed in this study, so this antibody is included in most experiments as a nonimmune control mAb.

These results demonstrate that a skeletal muscle MHC expressed in COS cells associates with the nonmuscle MLCs and assembles into 12–16 nm diameter myosin filaments that are segregated from the nonmuscle myosin. The segregated assembly of the muscle myosin filaments appears to be an inherent attribute of the full length MHC; however, it is possible that this behavior is an anomaly of the expression of a muscle specific protein in the background of a COS cytoskeleton. To address this question, myosin expression vectors were introduced into a myogenic cell line (C2C12 cells) by cotransfection with a selection vector carrying a drug resistance marker (pSV2Neo).

Constitutive Expression of Muscle Myosin in C2C12 Myoblasts

To produce stable cell lines expressing the chicken muscle myosin, cotransfections were done by varying the molar ratio of MHC vector to selection vector from 10:1 to 200:1. This strategy ensures that cells that have taken up the selectable marker have also taken up the gene of interest. Preliminary experiments indicated that the liposome-mediated transfection procedure was 100–300-fold more effective with C2C12 cells than calcium phosphate precipitation procedures. Nonetheless, the transfection efficiencies for Geneticin (G418)-resistant C2C12 cells carrying the full-length chicken muscle MHC (pMT2MHC) are still low, ranging between 1–2% for all molar ratios examined. In contrast, cells transfected with $pMT2tMHC_{964}$ or pSP64MHC, a vector lacking a eukaryotic promoter, have ≤25-fold higher transfection efficiencies, suggesting that the low efficiency is related to the introduction of a constitutively expressed muscle MHC. The drug resistant colonies, resulting from the transfections with pMT2MHC at high molar ratios (>100:1), contain a large percentage of fragile multinucleated cells with abnormal morphology. These cells are not aligned myotubes; they appear to be syncytia that may have arisen from membrane fusion or from an inability to complete cytokinesis. These syncytia are lost after three to four passages of the drug resistant cells. The low transfection efficiencies and the alteration in morphology are not seen with $pMT2tMHC_{964}$- or pSP64MHC-transfected cultures, suggesting that the inappropriate expression of the skeletal muscle MHC caused the deleterious effects.

C2 myoblasts expressing the muscle MHC from a constitutive viral promoter (designated C2CM cells) were identified by immunofluorescence microscopy. The muscle MHC protein again is found in 1–5-μm-long filamentous structures distributed throughout the cytoplasm in 5% of the C2CM myoblasts (Figure 6A). These structures are similar in appearance and distribution to the short muscle myosin filaments formed in the transfected COS-7 cells; however, they display a narrower length distribution (Figure 6A, inset). Analysis of the MHC filament length distribution indicates a mean length of 1.5 ± 0.6 μm (n = 630). Here again, the truncated MHC does not assemble and is distributed diffusely throughout the cytoplasm in 25% of the $pMT2tMHC_{964}$-transfected cells (Figure 6B). The expression of the muscle MHC in C2CM cell lines and the truncated MHC is stable over 30 passages when the lines are carried in G418 selection medium.

The distributions of the endogenous nonmuscle MHC and stress fibers were examined by immunofluorescence microscopy. The nonmuscle MHC is found in a punctate staining pattern aligned with the stress fibers. The levels and distribution of the nonmuscle actin and myosin does not appear to be altered by the presence of the muscle MHC filaments or the truncated MHC protein. The

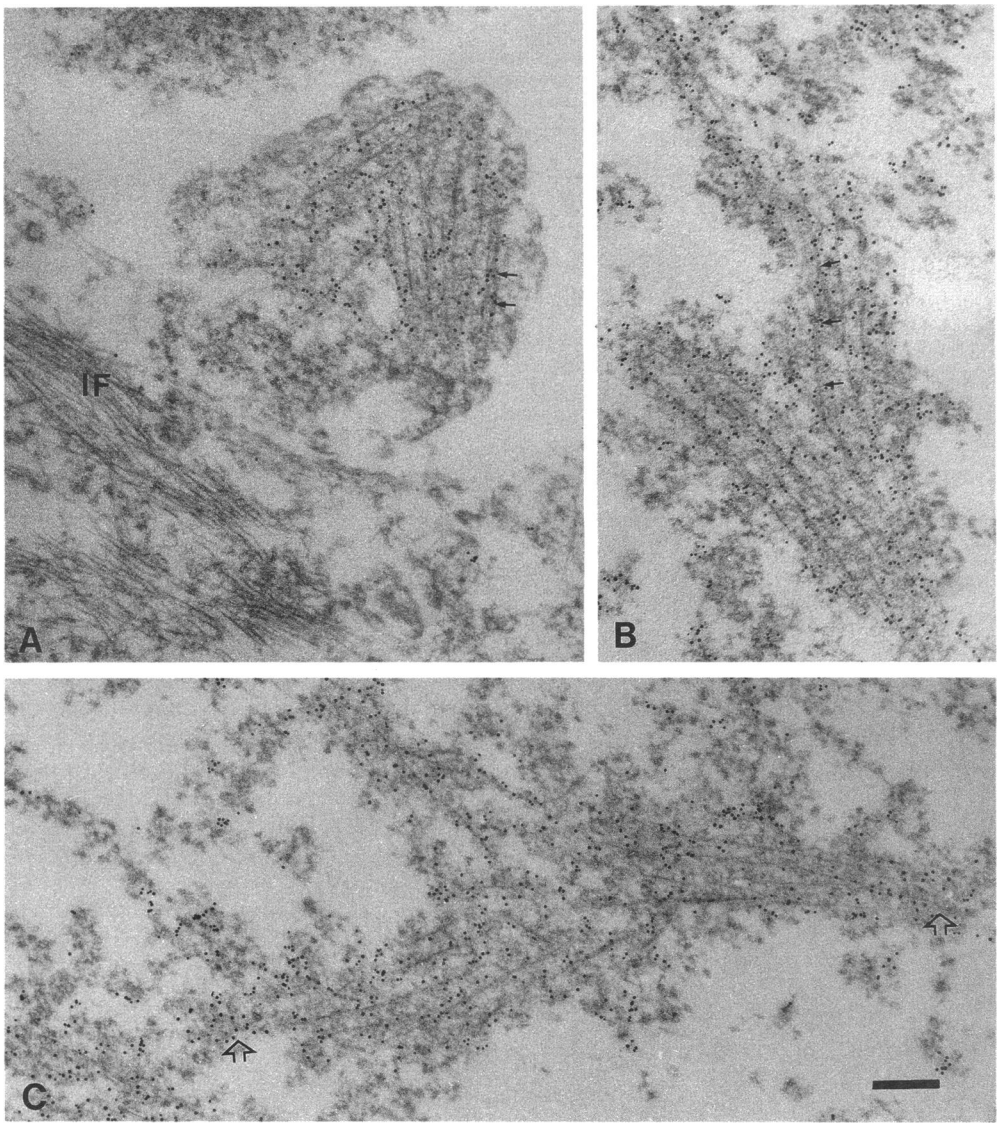


Figure 5. Immunoelectron microscopy was used to determine the ultrastructure of the chicken myosin filaments in the transfected cells. COS-7 cells were transfected with pSVMHC by liposome-mediated procedures and fixed with paraformaldehyde for immunogold labeling. (A) Colloidal gold labeling is found associated with filaments that measure 12–16 nm in diameter. These filaments are loosely organized into bundles that are distinct from the intermediate filaments. (B and C) The muscle MHC filament bundles are often Y- or V-shaped in appearance. The small arrows in A and B identify single filaments within the loosely organized bundles. The MHC filament bundles range from 1 to 5 μm in length. The open arrows in (C) mark the ends of one bundle that is 2 μm in length. The measured diameter may include a contribution from the immunolabeling with the mAb. Bar, 200 nm.

muscle MHC filaments assembled in the C2CM lines are distributed throughout the cytoplasm with no apparent correlation with the existing cytoskeletal architecture; thus, stable constitutive expression of the full-length muscle MHC in the undifferentiated C2CM cells results in the same assembly and segregation patterns observed with transient expression in COS cells. The distribution of the nonmuscle MLC was not determined, because the anti-MLC mAb does not cross-react with the nonmuscle MLC of C2C12 myoblasts.

The C2CM cell lines were subcloned by an immunoreplica filter technique (Raetz et al., 1982; Black and Hall, 1985). In one cloning experiment, 29 of 641 colonies were strongly positive for the expression of the muscle MHC protein (4.5%). Fifteen of the positive colonies were picked and expanded. The cloned lines were put through secondary screening by immunofluorescent microscopy (Figure 6C). All of the cells in the cloned cell lines display a weak, but over background, immunofluorescent signal; however, the fraction of cells containing well-defined, muscle myosin filaments varied among the clones, ranging from 1–50% of the cells.

Five of the clones ($C2CM_{1-5}$) were further characterized at the molecular level. Southern analysis of genomic DNA from C2CM clones reveals that the genomic copy number of the transfected gene is between 3–15 (Figure 7A). Although the muscle MHC transcript is not detectable in RNA extracted from mixed pools of drug resistant cells, dot blot analysis of total RNA from clones $C2CM_{1-5}$ detects low levels of transcript expressed at 0.1–1.0% of the level seen for the MHC transcript expressed in developing pectoralis muscle (Figure 7B).

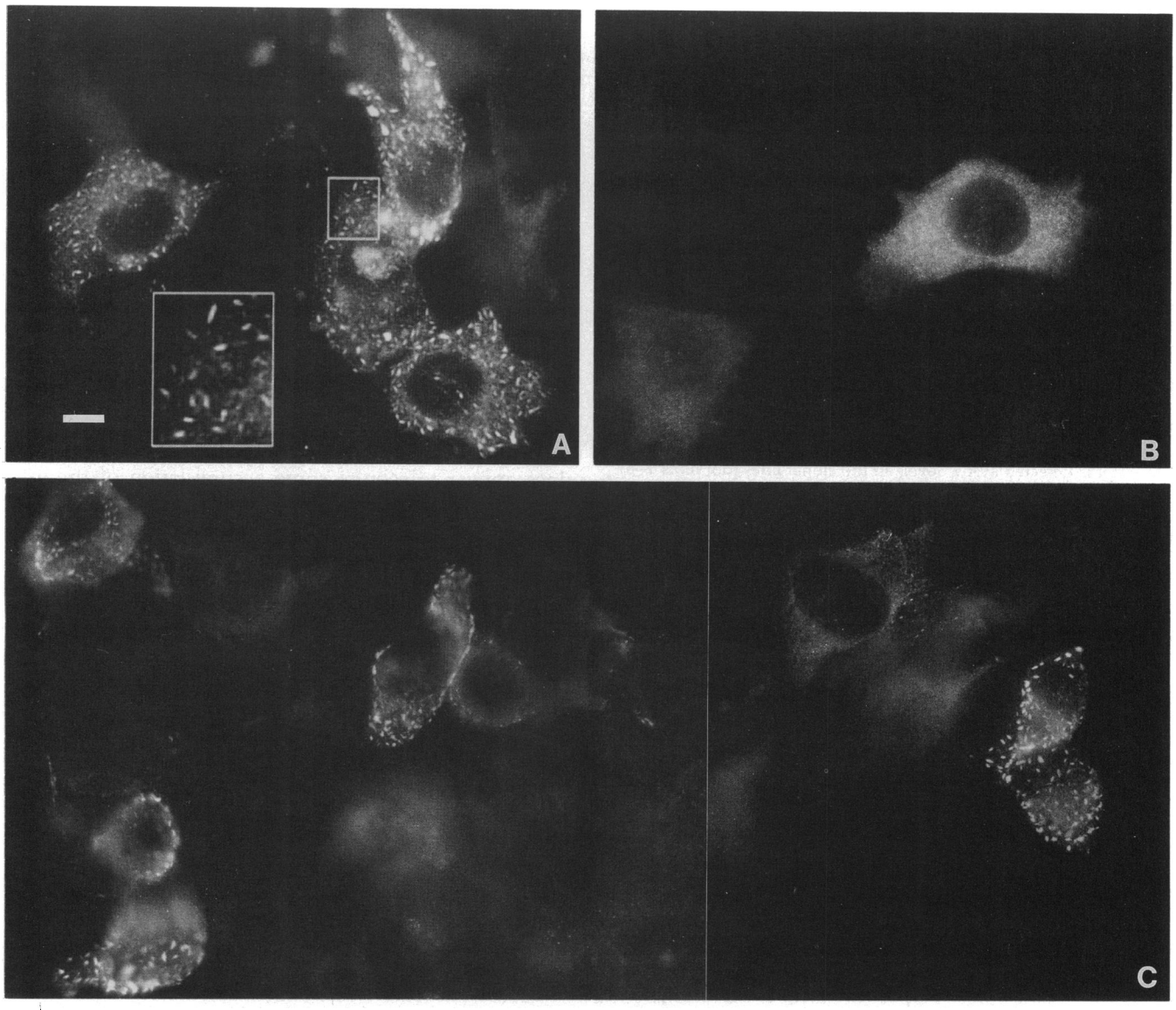


Figure 6. The chicken MHC expressed in transfected C2 myoblasts forms filamentous structures throughout the cytoplasm that can be detected by immunofluorescence microscopy with a chicken specific anti-S2 mAb. (A) Transfected C2 myoblasts with brightly staining, elongated assemblies detected with the anti-S2 mAb. These structures can be found in 5% of the G418 resistant cells after transfection with pMT2MHC. These filaments appear similar to the chicken MHC filaments formed in transfected COS cells but shorter in length. The inset is a 2.5-fold magnification of the boxed area of the cell and clearly shows the short filaments. (B) The stable expression of the truncated MHC that lacks much of the rod results in the same diffuse staining pattern that was seen with transient expression in COS. (C) A replica filter screening technique was used to isolate clonal lines that constitutively express the chicken skeletal muscle MHC. Secondary screening of a C2CM clonal line using immunofluorescence reveals a higher frequency of positive cells. C is a composite micrograph from $C2CM_5$ probed with the anti-S2 antibody. Bar, 10 μm.

Based on the Northern, it is unlikely that all of these genes are active in the C2CM clones, and the level of transcript is apparently independent of genomic copy number. However, there is a correlation between the fraction of cells from a clone that accumulate the muscle myosin filaments and the amount of transcript detected.

The solubility properties of the chicken muscle myosin expressed in C2CM cells have been characterized by immunoprecipitations of radiolabeled cell extracts with the chicken specific anti-myosin mAbs. The chicken myosin copurifies with the endogenous nonmuscle myosin through extraction with Triton and high salt/ATP-containing buffers. Greater than 90% of the chicken myosin is associated with the Triton insoluble fraction, and all of this myosin is released by extractions with 500 mM NaCl/5mM ATP. These data indicate that

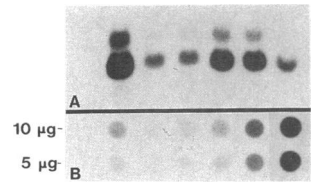


Figure 7. Quantitation of the chicken MHC genomic DNA and RNA transcript in the C2CM clonal lines. (A) Genomic DNA was isolated from five C2CM clonal lines and from the untransfected parental cell line C2C12. The DNA (10 μg) was digested with the restriction enzyme *Pst* I and analyzed by Southern blot. Lane 1 contains DNA isolated from mock-transfected C2C12 cells. Lanes 2–6 are DNAs isolated from C2CM clones 1–5, and lane 7 contains five genomic equivalents of the MHC gene. The copy number for the integrated chicken MHC DNA ranges from 3 to 15 for the cloned cell lines. Radiolabeled full-length MHC DNA was used for the probe. (B) Samples of total RNA (5 μg and 10 μg) isolated from the same clonal lines were analyzed by dot blot hybridizations for the presence of the chicken transcript using a unique 3′ untranslated region as a probe. The foreign MHC transcript is detected at low levels in the clonal lines (dot blots 2–6). This transcript is not detected in RNA from the parental cell line, C2 (dot blot 1). Total RNA from day 14 in ovo chick pectoralis muscle is shown for comparison (dot blot 7). Dot blots 1–6 were exposed for 80 h, and dot blot 7 was exposed for 15 h.

the exogenous muscle myosin has physical properties characteristic of a native myosin.

The constitutive expression of the skeletal muscle MHC in C2 myoblasts has several unusual effects. Cells transfected with pMT2MHC lose their ability to differentiate and do not spread on a substrate as well as the normal cells; these effects are not seen in cells transfected with a nonfunctional myosin expression vector (pSP64MHC) or the truncated MHC expression vector (pMT2tMHC$_{964}$). In cultures of C2CM cells that have been induced to differentiate by treatment with a DNA synthesis inhibitor or depletion of media, the cells migrate, line-up for fusion, but few myotubes are formed. Instead, the cells roundup and detach from the plate. These phenotypic changes suggest that the inappropriate timing of expression of a muscle MHC is deleterious to these cells.

Interestingly, the muscle myosin filaments are not found in dividing cells, and the fraction of cells containing the myosin filaments is somewhat dependent on the density of the culture. These observations suggest that assembly of the myosin filaments might require the accumulation of the expressed protein. Forced withdrawal of C2CM cell lines from the cell cycle by treatment with the DNA synthesis inhibitor cytidine arabinoside (Ara-C) results in a large increase in both the percentage of the cells expressing the chicken muscle myosin filaments and the size of the cells, consistent with this suggestion (Figure 8, C and D). Closer examination of the Ara-C–treated C2CM cells reveals numerous muscle myosin filaments dispersed throughout the cytoplasm (Figure 8E). Normal C2 myoblasts treated under identical conditions begin to differentiate and express the endogenous sarcomeric MHC (Figure 8, A and B). However, C2CM cells do not differentiate during this treatment, and the expression of the endogenous sarcomeric MHC is not detected.

These data show that the skeletal muscle MHC gene has stably incorporated into the genome of the C2 cells and that the level of expression of the exogenous gene is significantly lower in these cells than in the COS-7 cells. The segregated assembly of the muscle myosin filaments demonstrated in both the COS-7 cells and the C2C12 myoblasts argues that this is an inherent attribute of the constitutive expression of the full-length muscle myosin. It does not appear to be dependent on the nature of the nonmuscle cytoskeleton or on the high levels of expression attained with transient expression in COS cells; however, the assembly of muscle myosin filaments does appear to require some accumulation of the expressed protein.

DISCUSSION

The formation of the myofibrillar thick filaments requires assembly events to occur at three levels: the assembly of the myosin molecule, the assembly of the thick filaments, and the incorporation of the filaments into the striated myofibril. It is not clear whether these events occur as sequential steps in the formation of the myofibril or as a simultaneous process. By expressing a skeletal muscle MHC in the background of a nonmuscle cytoskeleton, we have begun to dissect these assembly events in the formation of the thick filaments.

We have introduced chicken embryonic skeletal muscle MHC gene constructs into simian kidney cell lines, COS, and the murine myogenic cell line, C2C12. The expression vectors pMT2MHC and pSVMHC direct the synthesis of full-length MHC polypeptides that assemble into filamentous structures distributed throughout the cytoplasm of the cells. Ultrastructural studies of the transfected cells reveal that the chicken skeletal muscle MHC forms filaments in the absence of other myofibrillar proteins. The endogenous nonmuscle MLCs are associated with the muscle MHC filaments, and these filaments are segregated from the rest of the endogenous cytoskeleton. A third construct, pMT2tMHC$_{964}$, encodes a portion of the MHC corresponding to the globular head of the myosin molecule and 118 residues of the rod domain. In contrast to the distribution of the full length heavy chain, the truncated MHC is distributed diffusely throughout the cytoplasm of the transfected COS and C2 cells. We see no evidence of incorporation of this truncated protein into higher order structures.

Recently, Rindt et al. (1993) have expressed pMT2MHC and pMT2tMHC$_{964}$ in COS-1 cells and par-

tially purified the corresponding expressed proteins. The full-length and truncated MHCs possessed ATPase activities and actin binding properties comparable to purified muscle myosin. These results together with our findings indicate that this heterologous expression system produces both enzymatically active and assembly competent myosin molecules.

Assembly of the Myosin Molecule

We have shown an association between the endogenous nonmuscle MLCs and the chicken skeletal muscle MHC in transfected COS cells, suggesting limited selectivity in the interaction of these subunits. Using a complimentary strategy, Soldati and Perriard (1991) have shown that nonmuscle MLC3 does not discriminate in binding between muscle and nonmuscle MHC isotypes. Our results are in agreement with this work and support the findings of in vitro studies on MLC association with denuded MHCs and MHCs expressed in bacteria that suggest that MHCs are quite promiscuous in their interactions with heterologous light chains (Chantler and Szent-Gyorgyi, 1980; Reinach et al., 1986; McNally et al., 1988).

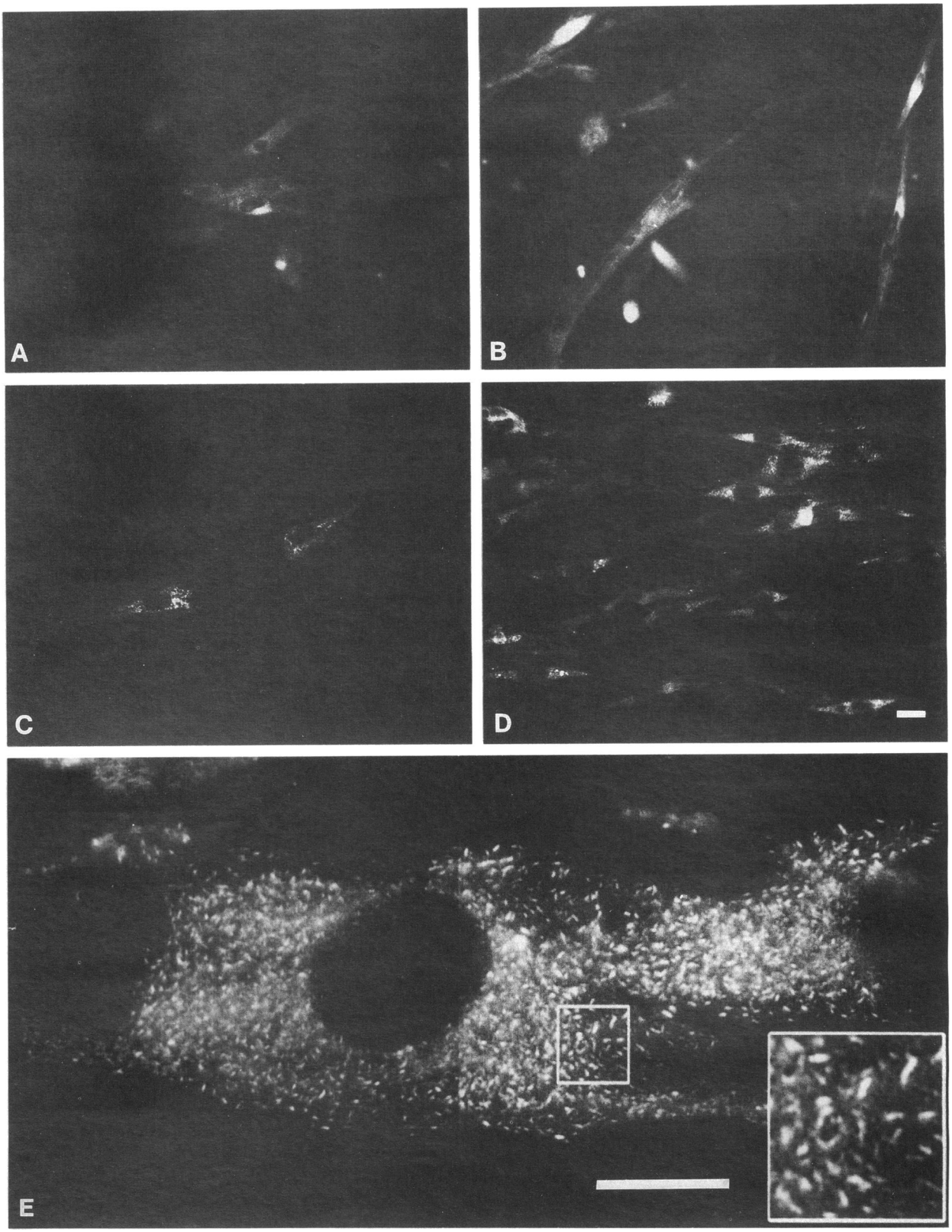
The association of the nonmuscle MLCs with the endogenous nonmuscle MHC does not appear to be disturbed by the presence of the exogenous MHC. Similarly, the association of the endogenous MLCs with a MHC fragment expressed in *Dictyostelium discoideum* does not interfere with the subunit interaction of the endogenous full-length MHC (Manstein et al., 1989). In skeletal muscle, MHCs and MLCs turnover at different rates (Zak et al., 1979; Silver and Etlinger, 1985; Moss et al., 1986); however, there is no consensus on the evidence for free cytoplasmic pools of myosin subunits (Devlin and Emerson, 1978; Zak et al., 1979; Sameral et al., 1987; Gagnon et al., 1989). In our experiments, the fluorescence signal for the nonmuscle MLC shows a significant increase in the cells containing the muscle myosin filaments. This may be because of an upregulation of the nonmuscle MLC synthesis or to the change in the amount of assembled MLC found in the cells. Further work is needed to assess whether nonmuscle MLC synthesis has been altered in response to expression of an extra MHC or if the MHC is associating with MLCs found in a free cytoplasmic pool of subunits.

The full-length muscle myosin expressed in both cell lines exhibits extraction properties characteristic of native protein and copurifies with the endogenous myosin. We have noted differences in the amount of muscle myosin that can be extracted from the Triton insoluble material using high salt/ATP buffers that correlates with the level of expression. Transfection of COS cells with pMT2-based vectors produces high levels of expression of the muscle myosin; however, only 60–65% of this myosin is native based on extraction properties. In experiments with vectors producing significantly lower levels of expression (i.e., pSVMHC transiently expressed in COS cells and the C2CM cloned cell lines), all of the muscle myosin was found to be native based on this criteria. These results suggest that the cells have a limited capacity to accommodate the exogenous myosin. This limitation was also noted in immunofluorescence assays.

Assembly of Myosin Filaments

Thick filament formation has been extensively studied in vitro. It has been demonstrated that myosins from a wide variety of species can form bipolar thick filaments, and that this concentration dependent polymerization does not require accessory proteins. In vitro, myosin isotypes can form hybrid thick filaments (Citi et al., 1987; Waschberger and Pepe, 1980). However, in the developing myotube, myosin isotypes are segregated into different cellular structures and compartments. It has been hypothesized that the segregation of the myosin isotypes is because of cellular sorting or targeting of the isotypes into distinct cellular compartments during synthesis of the proteins (Dlugosz et al., 1984; Antin et al., 1986; Isaacs and Fulton, 1987; Wang et al., 1988). We have found that the chicken skeletal muscle MHC segregates into structures that are distinct from the endogenous nonmuscle MHC in COS cells and C2C12 myoblasts. The segregation of the myosin isotypes into different cytoskeletal structures in these cell lines does not require the expression of other myofibrillar proteins.

In vitro assembly studies using proteolytic fragments of myosin have shown that the rod, specifically the carboxyl terminal portion of the heavy chain, is essential to the solubility and assembly properties of the myosin molecule (Nyitray et al., 1983; Atkinson and Stewart, 1991). We have found that expression of a truncated MHC that lacks most of the rod domain of the myosin molecule does not form the segregated assemblies seen with the full-length heavy chain. The expression of a truncated heavy chain in myosin null mutants of *D. discoideum* also results in the formation of an assembly deficient hybrid myosin molecule and a diffuse distribution of the exogenous MHC (DeLozanne and Spudich, 1987; Manstein et al., 1989). The expression of the truncated MHC and the full-length MHC in COS cells produces equivalent amounts of protein. The localization of the full-length MHC in filament bundles compared to the diffuse cytoplasmic distribution of the truncated MHC suggests that the segregated assembly of the muscle myosin is a function of the protein and not an artifact resulting from aggregation of the overexpressed exogenous protein. The differences in the distribution of the full-length and truncated MHCs localize the signals for segregation to the rod assembly domain of the protein. Furthermore, dramatically different amounts of protein are produced with expression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vectors, pMT2MHC and pSVMHC, in the transient assays. Expression of the full-length MHC from both of these vectors results in the formation of the filament bundles, again indicating that the filament bundles are not an aggregation artifact because of overexpression. These results indicate that the information necessary for segregation of the skeletal muscle myosin isotype is programmed into the primary structure of the skeletal muscle MHC, specifically within the rod domain of the molecule.

The thick filaments contained within the myofibril lattice of vertebrate-striated muscle are of a unique and determinant length. Although the filaments formed by expression of the muscle MHC in these experiments show a broader range, the distributions of filament length in both the COS and C2 cells are heavily weighted to 1–2 μm filaments. In addition, the longer filaments appear to be because of overlap of multiple short (1–2 μm) filaments. These results suggest that filament size is also an inherent function of the full-length MHC. The formation of the filaments in both expression systems is dependent on the accumulation of the protein. Forced withdrawal of C2CM cells from mitosis results in increased muscle myosin filament formation suggesting that accumulation of a critical amount of protein was necessary. The shift in distribution of the chicken MHC from concentrated perinuclear regions to filamentous structures dispersed throughout the cytoplasm of the COS cells as the protein levels increase also supports this idea. Similar shifts in myofibril protein distributions have been noted in rounded postmitotic myocytes during differentiation in culture (Colley et al., 1990).

Filament length may be regulated by the environment in which the filaments are formed. The cytoskeletons of COS and C2CM cells are very different. COS cells have extensive cortical actin fibers and few internal stress fibers; in contrast, C2CM cells have an intricate actin-based cytoskeleton with numerous stress fibers distributed throughout the cytoplasm. Nonetheless, we see similar types of filaments formed in both cell lines. During myofibril assembly, formation of myosin filaments may be influenced by the presence of other cytoskeletal structures. This may additionally involve the interaction with accessory proteins, such as titin and C-protein.

Studies of genetic mutations of cytoskeletal proteins and drug-induced perturbations of the cytoskeleton have suggested the presence of two organizing centers during myofibrillogenesis. Null actin mutants in *Drosophila* have been shown to form normal thick filament assemblies that are poorly organized (Beall et al., 1989). Similarly, chicken pectoralis myocytes treated with taxol or colcemid display normal myosin filaments and I-Z-I complexes without forming organized myofibrils (Hill et al., 1986). Thick filament assembly in both of these systems occurs in the presence of other myofibril components. The segregated assembly of the chicken myosin filaments in the background of a nonmuscle cytoskeleton that we have observed supports the concept of thick filament assembly independent of the thin filament organizing center. The random distribution of the chicken myosin thick filaments in the transfected cells indicates that factors other than the MHC are necessary for the incorporation of these filaments into the lattice of the myofibril.

The limited differentiation potential of the C2CM lines and their inability to form striated myofibrils is further evidence to suggest that the coordinate expression of other muscle specific proteins is a prerequisite for this maturation to occur. The phenotypic changes resulting from the constitutive expression of the muscle myosin in the stable C2CM cell lines are similar to the changes resulting from altering the ratio of actin isotypes expressed in the nonmuscle cells (Schevzov et al., 1992). This suggests a fine balance must be maintained in expression of different isotypes of cytoskeletal proteins.

Myofibril assembly has been studied by a variety of techniques, including in vitro assembly experiments with isolated proteins, microinjection of fluorochrome labeled myofibrillar proteins, and immunocytochemical analysis of developing myocytes. Mammalian expression systems offer distinct advantages over in vitro assays because they enable the examination of function in a cellular context. Microinjection of isolated myofibril proteins also allows for the examination of ongoing cellular processes; however, microinjection uses terminally folded proteins that may be missing information necessary for the sorting of newly synthesized proteins as they incorporate into cellular structures. The use of mammalian expression systems in combination with immunocytochemical and ultrastructural analysis of the

Figure 8. Forced withdrawal from the cell cycle caused by treatment of the cells with the DNA synthesis inhibitor Ara-C promotes fusion and differentiation of normal C2 cells. (A) C2 cells grown to confluence at 5 d in culture and probed for the distribution of the endogenous sarcomeric MHC. (B) C2 cells grown for 5 d in culture but exposed to 1.0 μM Ara-C for the last 24 h period show an increase in the amount of sarcomeric MHC present and in the number of multinucleated cells. (C) C2CM cells grown for 5 d in culture and probed with chicken specific anti-myosin mAb show ~10% of cells contain the chicken sarcomeric MHC assemblies. (D) When these cells are treated with the Ara-C 24 h before fixation, the percentage of cells containing the chicken sarcomeric MHC assemblies increases to >80% of the cells. Under these conditions, C2CM cells do not differentiate, and the endogenous sarcomeric MHC cannot be detected. (E) Composite image of C2CM cells treated with Ara-C for 24 h shows an abundance of 1.6-μm-long myosin filament bundles. Inset in E is 2.5-fold magnification of boxed region. Bars, 25 μm.

cells offers new approaches for addressing questions concerning myofibril assembly.

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