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Cytoplasmic processing of myosin heavy chain messenger RNA: Evidence provided by using a recombinant DNA plasmid

[myoblast differentiation/myosin heavy chain recombinant clones/poly(A) processing/mRNA metabolism]

RUSSELL M. MEDFORD, ROBERT M. WYDRO, HANH T. NGUYEN, AND BERNARDO NADAL-GINARD*

Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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ABSTRACT **A recombinant DNA plasmid, designated pMHC25, has been constructed that contains structural gene sequences for rat skeletal muscle myosin heavy chain (MHC). The identity of the MHC sequence insert in pMHC25 was determined by muscle-tissue specificity, inhibition of MHC protein synthesis *in vitro* by hybrid-arrested translation, purification of mRNA that directs the synthesis of MHC protein *in vitro*, and hybridization to a 33S cytoplasmic mRNA found only in differentiated muscle cells. pMHC25-DNA-excess filter hybridizations were used to show that more than 90% of the newly synthesized MHC mRNA that appears in the cytoplasm of differentiated L6E9 myotubes contains a long 3′ poly(A) tail. In contrast, 90% of the MHC mRNA that accumulates in the cytoplasm of these same cells during myogenic differentiation lacks this long 3′ poly(A) tail. These results suggest the occurrence of a posttranscriptional event in differentiated L6E9 myotubes that involves the cytoplasmic processing of poly(A)$^+$ MHC mRNA to poly(A)$^-$ or poly(A)-short MHC mRNA.**

In vitro myogenesis offers an attractive model for the study of regulation of gene expression during cellular differentiation. Fusion of mononucleated myoblasts into multinucleated myotubes results in the coordinate appearance of a number of new, muscle-specific, contractile proteins (1–3). The synthesis of one of these proteins, myosin heavy chain (MHC), has been studied extensively. As the major contractile protein in muscle, MHC represents over 20% of the total protein synthesis in the fully differentiated skeletal muscle cell (1). MHC gene expression has been postulated to be regulated at both the transcriptional and posttranscriptional levels (4–6). Multiple isozymic forms of MHC have also been identified and may be under similar regulatory control (7–9).

The validity of many of the conclusions in the above studies has depended to some degree on the methodology used to identify and quantitate the MHC mRNA sequences. A recombinant DNA clone containing the MHC structural gene sequences would provide large amounts of homogeneous probe and facilitate the study of MHC gene expression at both the DNA and RNA level. We report here on the construction, identification, and characterization of a recombinant plasmid, designated pMHC25, that contains structural gene sequences for rat skeletal muscle MHC.

Previous reports from our laboratory (4, 10) have shown that, at the peak of L6E9 cell myogenic differentiation, 90% of the MHC mRNA found in the cytoplasm lacks the long 3′ poly(A) characteristic of most eukaryotic mRNA (11). Using pMHC25, we present evidence suggesting that this poly(A)$^-$ MHC mRNA originally appears in the cytoplasm with a long 3′ poly(A) tail, then undergoes a processing event or events that shorten the 3′ poly(A) tail, resulting in the cytoplasmic accumulation of poly(A)$^-$ or poly(A)-short MHC mRNA.

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MATERIALS AND METHODS

Cells, Tissue Culture, and Labeling Conditions. The L6E9 cell line, a subclone of the L6 rat myogenic cell line (12) isolated in our laboratory (1), was maintained and induced to differentiate as described (1).

Labeling Conditions and Preparation of RNA. Differentiated myotubes (6 days after transfer to differentiation medium) (1, 4), were labeled for 5 hr with [^{3}H]uridine (Amersham, 50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at a concentration of 300 μCi/ml, prior to RNA extraction. [^{3}H]Uridine was incorporated linearly for over 6 hr. Rat skeletal muscle total RNA was isolated from 21-day-old Wistar rats by the hot phenol procedure (13). Total cytoplasmic and polysomal RNA was isolated from L6E9 cells by using guanidine hydrochloride (4). Polysomal RNA was size fractionated by sucrose-gradient density centrifugation [15–30% sucrose in ETS buffer (1 mM EDTA/10 mM Tris·HCl, pH 7.4/0.2% $NaDodSO_4$)]. The pooled 26S region of this gradient was highly enriched for MHC synthesis, as assayed by cell-free translation, and was used for the subsequent cloning procedure. Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Collaborative Research) (14) or poly(U)-Sepharose 4B (Pharmacia) (15). Poly(A)$^-$ RNA was defined as RNA that did not bind to oligo(dT)-cellulose in NETS buffer (100 mM NaCl/1 mM EDTA/10 mM Tris·HCl, pH 7.4/0.2% $NaDodSO_4$) or poly(U)-Sepharose in ETS buffer.

cDNA Synthesis and Construction and Screening of pBR322-cDNA Hybrid Molecules. Complementary DNA molecules (cDNA) were synthesized from highly enriched MHC mRNA by using avian myeloblastosis virus reverse transcriptase (generously supplied by J. W. Beard, Life Sciences, St. Petersburg, FL) according to the procedure of Benoff and Nadal-Ginard (4) except that no actinomycin D was used and the final dCTP concentration was 1.0 mM. The cDNA was prepared for cloning by synthesis of the second strand of cDNA by using *Escherichia coli* DNA polymerase I Klenow fragment followed by S1 nuclease treatment (16). Approximately 10–15 dC residues were added to each 3′-OH terminus by using calf thymus terminal transferase (Enzo Biochemical, New York) in the presence of $CoCl_2$ (17). The EK2 plasmid vector pBR322 (18) was cleaved with restriction endonuclease *Pst* I at the single site within the gene encoding ampicillin resistance. Poly(dG), approximately 10–15 residues in length, was synthesized on each 3′-OH terminus of the plasmid, using terminal transferase (17). Poly(dC)-tailed double-stranded cDNA was hybridized with poly(dG)-tailed plasmid DNA and used to transform the EK2 *E. coli* strain χ1776 (16). Bacterial colonies containing recombinant pBR322 DNA were screened for muscle specificity by the Grunstein–Hogness colony filter hybridization

Abbreviations: MHC, myosin heavy chain; bp, base pair(s).

* To whom reprint requests should be addressed.

procedure (19) with myogenic and nonmyogenic cytoplasmic poly(A)$^+$ RNA that was ^{32}P-end-labeled *in vitro* by using phage T4 polynucleotide kinase (Miles) (20). Before *in vitro* labeling, RNA samples were hydrolyzed for 30 min at 90°C in 50 mM Tris·HCl, pH 9.5/5 mM glycine/10 μM EDTA/100 μM spermine. After labeling, unincorporated nucleotides were removed by chromatography on Bio-Gel P-6 (Bio-Rad). Bacterial plasmids of interest were amplified and purified according to established procedures (21, 22). All experiments were performed according to established National Institutes of Health guidelines for recombinant DNA research.

Identification of Plasmid by Negative and Positive Cell-Free Protein Synthesis. RNA and plasmid DNA were prepared for hybrid-arrest translation (23) and R-loop purification (24) exactly as described. All RNA preparations were translated in a nuclease-treated rabbit reticulocyte cell-free amino acid-incorporating system followed by electrophoresis of the translation products on 10% NaDodSO$_4$/polyacrylamide gels and fluorography for ^{35}S (25).

DNA-Excess Filter Hybridization. Hybridization of labeled RNA to an excess of nitrocellulose filter-bound plasmid DNA was performed in 2 × TESS [0.01 M Tes (2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid), pH 7.4/0.3 M NaCl/0.01 M EDTA/0.2% NaDodSO$_4$] at 65°C for 40 hr. After hybridization, filters were extensively washed and radioactivity was measured as described (16) except that *in vitro* ^{32}P-end-labeled RNA was not treated with pancreatic RNase A. In some cases, the supernatants of the hybridization reactions were rehybridized to fresh DNA-filters to ensure that hybridization was complete (>85% efficiency of binding).

Sizing of RNA. RNA samples were fully denatured by glyoxal treatment (26), separated by electrophoresis on 1.2% agarose horizontal slab gels, transferred to diazobenzyloxymethyl-paper (Schleicher & Schuell) and hybridized to nick-translated (27) ^{32}P-labeled plasmid DNA by the method of Alwine *et al.* (28).

Restriction Endonuclease Mapping of Plasmid DNA. Reaction conditions for restriction endonuclease cleavage of plasmid DNA were essentially as suggested by the supplier. Enzymes were added at 1 unit/μg of DNA substrate and incubated for 5 hr at 37°C. Fragments were electrophoresed on 8% polyacrylamide/TBE (50 mM Tris–borate, pH 8.3/1 mM EDTA) gels and visualized by UV after staining with ethidium bromide at 1 μg/ml. Fragments were sized by comparison with *Hin*cII restriction fragments of phage φX174 DNA run on the same gel. Plasmid DNA restriction fragments were ^{32}P-5′-end-labeled with T4 polynucleotide kinase essentially as described for RNA except that the DNA was treated with bacterial alkaline phosphatase before labeling. Restriction fragments cut from the gel were electroeluted and concentrated by ethanol precipitation.

RESULTS

Identification of Recombinant Plasmids Containing Muscle-Specific DNA Sequences. High molecular weight RNA (>26S) from the heavy polysome fraction of well-differentiated L6E9 myotubes, which contains 50% MHC mRNA as assayed by *in vitro* translation (25), was used to prepare the double-stranded cDNA used for the subsequent bacterial transformation. Fifty transformed colonies were replica plated and hybridized, by colony-filter hybridization (19), to ^{32}P-end-labeled cytoplasmic poly(A)$^+$ RNA from myogenic (L6E9 myotubes) and nonmyogenic (L6E9 myoblasts, GH3 rat pituitary cells) sources. Colonies that hybridized strongly to the L6E9 myotube probe relative to the two nonmyogenic probes were defined as containing muscle-specific sequences. Purified plasmid was prepared from five of these muscle-specific colonies and immobilized on nitrocellulose filters. The relative muscle specificity of each plasmid was quantitated by DNA-filter hybridization to both myogenic and nonmyogenic RNA and cDNA. Of the colonies tested, one plasmid, pMHC25, showed a pattern of hybridization that best correlated with the known muscle-tissue specificity and induction of MHC mRNA during myogenic differentiation. pMHC25 showed a 100-fold greater binding to myogenic than to nonmyogenic probe (Table 1) and was chosen for further characterization.

Table 1. pMHC25-DNA-excess filter hybridization to cytoplasmic poly(A)$^+$ RNA and cDNA from different cell types

Cell type	% hybridization to pMHC25 DNA	
	Poly(A)$^+$ RNA*	cDNA†
Differentiated L6E9 myotube	0.1	0.34
Growing L6E9 myoblast	<0.001	<0.01
Rat pituitary cell line (GH3)	<0.001	<0.01

* Cytoplasmic poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography and end-labeled *in vitro* with ^{32}P by using T4 polynucleotide kinase. The amount hybridized per filter was 2.0 × 10^7 cpm. Nonspecific hybridization to blank, λ phage, or pBR322 DNA filters was less than 0.001%.

† cDNA was synthesized from poly(A)$^+$ RNA, using [^{3}H]dCTP. The amount hybridized per filter was 1.0 × 10^7 cpm. Nonspecific hybridization to blank, λ, or pBR322 DNA filters was less than 0.005%.

Partial Restriction Map of Plasmid pMHC25. The size of the inserted cDNA sequence was determined by digestion of pMHC25 with *Pst* I. Four linear DNA molecules were readily separated on nondenaturing agarose or polyacrylamide gels. As predicted, there was a fragment that corresponded to full-length pBR322 DNA. Three smaller fragments, designated *Pst*-A, -B, and -C, were approximately 360, 210, and 120 base pairs (bp), respectively, and represented the inserted cDNA sequences and the flanking G·C tails as shown in Fig. 1. The three *Pst* I fragments were ^{32}P-end-labeled by using T4 polynucleotide kinase (20) and purified by electroelution from an 8% polyacrylamide gel. Each purified fragment was digested with a battery of restriction enzymes (see legend to Fig. 2).

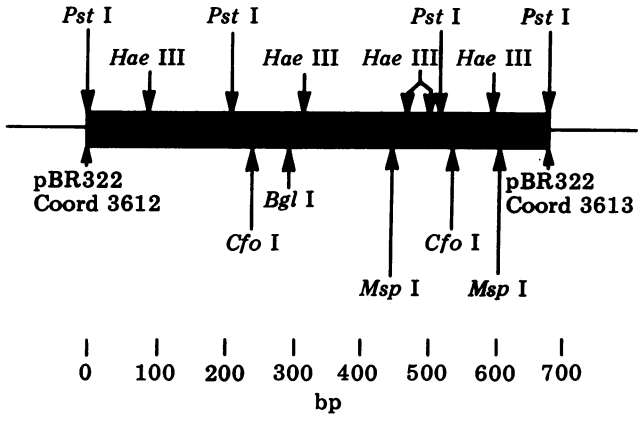

FIG. 1. Partial restriction map of plasmid pMHC25. The black bar shows the inserted cDNA sequence and its orientation in the pBR322 plasmid DNA. Coordinates are as in ref. 29. The cleavage sites for *Bgl* I, *Hae* III, *Cfo* I, *Msp* I, and *Pst* I are indicated. The fragment was inserted in the *Pst* I site of pBR322 by the G-C-tailing method, which reconstructed the *Pst* I sites found at each end of the cDNA fragment. The inserted cDNA fragment did not contain any cleavage sites for *Bam*HI, *Bgl* II, *Eco*RI, *Hin*dIII, *Hpa* I, *Kpn* I, *Sal* I, *Sma* I, *Taq* I, or *Xba* I.

Restriction fragments were identified by ethidium bromide staining and oriented by double digestions and autoradiography of ^{32}P-end-labeled fragments. A partial restriction map of pMHC25 was constructed (Fig. 1).

Identification of MHC mRNA Sequences in Plasmid pMHC25. On the basis of the biological and physical characteristics of MHC mRNA and protein, two criteria were established to test for the identity of the MHC structural gene sequence in pMHC25. The first criterion, muscle-tissue specificity, was tested as part of the initial plasmid selection and has already been described. The second criterion, specific hybridizations of pMHC25 DNA sequences to MHC mRNA, was tested by using three procedures: inhibition of MHC protein synthesis *in vitro* by hybrid-arrested translation, purification of mRNA that directs the synthesis of MHC protein *in vitro*, and hybridization to a cytoplasmic mRNA of the appropriate size found only in muscle cells.

The hybrid-arrested translation technique is based on the observation that an mRNA in hybrid form with complementary DNA sequences will not direct the synthesis of protein in a eukaryotic cell-free system (23). The specific loss of the polypeptide is used to identify the structural gene component of the cDNA. Specific hybridization of pMHC25 sequences to MHC mRNA was assayed by using an *in vitro* rabbit reticulocyte protein-synthesizing system highly efficient for the translation of MHC protein (25). MHC protein synthesized in this *in vitro* system by addition of exogenous muscle mRNA has been shown to be identical to MHC protein synthesized *in vivo* (4, 25). Because it is not known whether the MHC protein synthesized by L6E9 myotubes is, in effect, skeletal MHC, total RNA from rat skeletal muscle was used for the *in vitro* translation experiments. Excess, linearized, denatured DNA was hybridized to total RNA from rat skeletal muscle under conditions that favor the formation of RNA·DNA hybrids. Rat skeletal muscle RNA mock hybridized with pBR322 DNA (Fig. 2*B*) or with no DNA (Fig. 2*A*) efficiently directed the synthesis of MHC polypeptide *in vitro*. Rat skeletal muscle RNA hybridized with pMHC25 DNA was unable to direct the synthesis of MHC polypeptide *in vitro* (Fig. 2 *A* and *C*) but apparently did not affect the synthesis of any other protein. A portion of this same RNA preparation was heat denatured to melt the RNA·DNA hybrids. Translation of this material resulted in the full recovery of MHC protein synthesis (Fig. 2*A*). These results demonstrate that pMHC25 DNA sequences are complementary to MHC mRNA sequences by their ability to specifically inhibit the synthesis of MHC protein *in vitro*.

Plasmid pMHC25 was assayed for its ability to purify MHC mRNA from rat skeletal muscle total RNA by the R-loop purification method of Woolford and Rosbash (24). Linearized pMHC25 DNA was hybridized to rat skeletal muscle total RNA under conditions that favor the formation of R-loops. RNA hybridized to pMHC25 DNA was separated from nonhybridized RNA by gel filtration chromatography, heat denatured, and translated *in vitro* (Fig. 2*D*). The protein product of the R-loop mRNA fraction is predominantly full-length MHC polypeptide and represents a substantial purification of MHC mRNA relative to unfractionated RNA. The lower molecular weight bands most probably represent premature termination of the MHC polypeptide because their synthesis is inhibited when pMHC25 is used in hybrid-arrested translation (Fig. 2*A*). Translationally active rat skeletal muscle mRNA was not observed when pBR322 DNA or no DNA was mock hybridized to rat skeletal muscle total mRNA (data not shown).

To determine the size and tissue specificity of MHC mRNA, total cytoplasmic RNA from several myogenic and nonmyogenic sources was denatured with glyoxal treatment (26), electrophoresed on 1.2% agarose gels, transferred to diazobenzyloxymethyl-paper, and hybridized to ^{32}P-labeled nick-

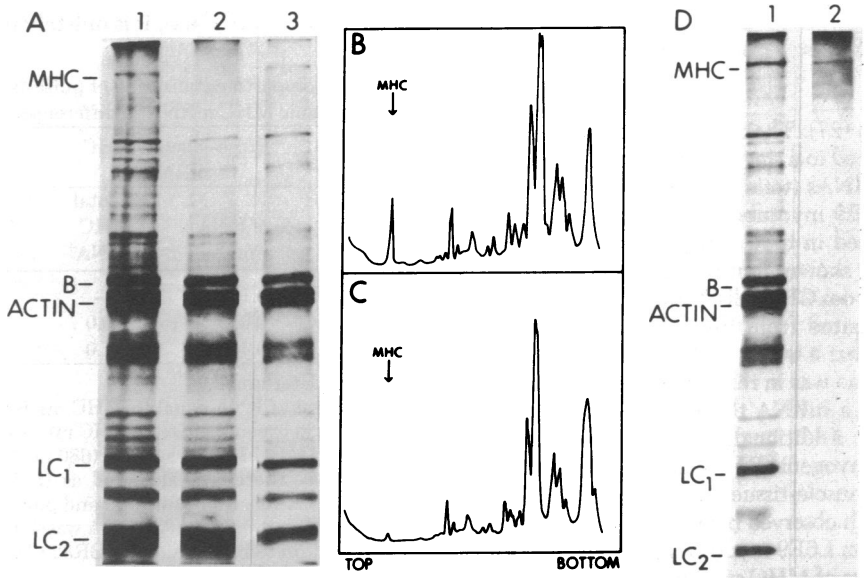

FIG. 2. Identification of MHC mRNA sequences in pMHC25 DNA by negative and positive *in vitro* translation. (*A*, *B*, and *C*) *Hybrid-arrested translation.* Two micrograms of *Pst* I-digested pBR322 or pMHC25 DNA was hybridized to 20 μg of rat skeletal muscle total RNA. Before translation in a cell-free system, half of each hybridization reaction mixture was heated to 100°C for 60 s to dissociate any mRNA·DNA hybrids. The [^{35}S]methionine-labeled translation products were separated on a 10% NaDodSO$_4$/polyacrylamide gel and fluorographed. *B* and *C* are tracings of the resulting fluorograph made with a Joyce–Loebl double-beam recording microdensitometer. (*A*) Lane 1, rat skeletal muscle total RNA; lane 2, rat skeletal muscle total RNA hybridized with *Pst* I-digested pMHC25 DNA; lane 3, rat skeletal muscle total RNA hybridized with *Pst* I-digested pMHC25 DNA but denatured before translation. (*B*) Translation products of rat skeletal muscle total RNA hybridized to *Pst* I-digested pBR322 DNA. (*C*) Translation products of rat skeletal muscle total RNA hybridized to *Pst* I-digested pMHC25 DNA. (*D*) *mRNA purification.* RNA was purified by R-loop hybridization of rat skeletal muscle total RNA to *Eco*RI-digested pMHC25 DNA and translated in a cell-free system (see text). The translation products were electrophoresed on a 10% NaDodSO$_4$/polyacrylamide gel and fluorographed for [^{35}S]methionine label. Lane 1, unfractionated rat skeletal muscle total RNA; lane 2, RNA purified by R-loop hybridization to pMHC25 DNA. B, endogenous protein band; LC$_1$ and LC$_2$, myosin light chains 1 and 2.

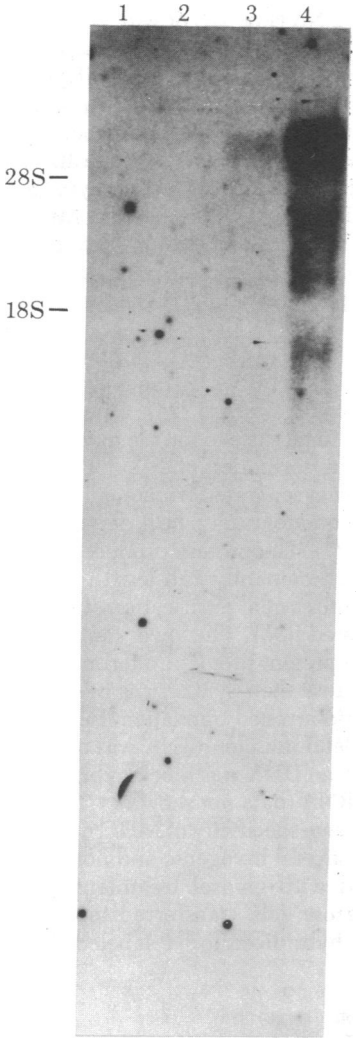

FIG. 3. Hybridization of pMHC25 DNA to size-fractionated RNA from myogenic and nonmyogenic cells. Twenty micrograms of each RNA was treated with glyoxal and electrophoresed in 1.25% agarose gels, transferred to diazobenzyl-oxymethyl-paper, and hybridized to ^{32}P-labeled nick-translated pMHC25 DNA. Twenty-microgram samples of total cytoplasmic RNA from various cell sources were in the following lanes: lane 1, GH3 rat pituitary; lane 2, undifferentiated L6E9 myoblast; lane 3, differentiated L6E9 myotube; lane 4, heavy polysomal RNA from differentiated L6E9 myotubes.

translated pMHC25 DNA (27). The results are shown in Fig. 3. pMHC25 DNA hybridized to a single high molecular weight band in both myogenic RNAs (total cytoplasmic and heavy polysomal RNA from L6E9 myotubes). After long exposure times, a band was observed in total cytoplasmic RNA from L6E9 myoblasts (data not shown), but no band was observed in total cytoplasmic RNA from GH3 cells. Using ribosomal RNA as size markers, we estimated from the migration of MHC mRNA in this gel system that it is 33S, with a calculated size of 7100 nucleotides. This value was in relatively close agreement with the expected size of a mRNA that codes for a 200,000 molecular weight protein. Additionally, the absence of a detectable signal in the nonmyogenic RNA samples confirms the original observation of muscle-tissue specificity. The large difference in signal strength observed between polysomal RNA and cytoplasmic RNA from L6E9 myotubes is consistent with the observed predominance of MHC synthesis *in vivo* in L6E9 myotubes.

MHC mRNA Is Synthesized as a Poly(A)$^+$ mRNA but Accumulates as a Poly(A)$^-$ mRNA in L6E9 Myotubes. Previous reports from our laboratory have shown that although the cytoplasmic MHC mRNA found in L6E9 myoblasts contains a long 3′ poly(A) tail, essentially all of the increased cytoplasmic MHC mRNA concentration observed during myogenic differentiation is due to the accumulation of MHC mRNA that does not contain a long 3′ poly(A) tail (4, 10) and that has been operationally defined as poly(A)$^-$. The question raised by this result was whether the accumulated poly(A)$^-$ MHC mRNA found in myotubes was a product of cytoplasmic processing of the poly(A)$^+$ MHC mRNA or represented the synthesis of a new MHC mRNA, distinct from the poly(A)$^+$ MHC mRNA found in myoblasts.

We approached this question by determining whether the newly synthesized cytoplasmic MHC mRNA in L6E9 myotubes was poly(A)$^+$ or poly(A)$^-$. Newly synthesized cytoplasmic MHC mRNA from [^{3}H]uridine pulse-labeled L6E9 myotubes was separated into poly(A)$^+$ and poly(A)$^-$ fractions by poly(U)-Sepharose chromatography. Each fraction was hybridized to excess filter-bound pMHC25. Table 2 shows that the poly(A)$^+$ fraction contains essentially all of the newly synthesized cytoplasmic MHC mRNA sequences, whereas less than 10% are found in the poly(A)$^-$ fraction. A sample of the same poly(A)$^+$ and poly(A)$^-$ RNA fractions used to determine the compartmentalization of newly synthesized MHC mRNA was used to determine the compartmentalization of the steady-state cytoplasmic MHC mRNA in the L6E9 myotube. Each sample of RNA was end labeled *in vitro* with ^{32}P, using T4 polynucleotide kinase, and hybridized to filter-bound pMHC25 DNA. Contrary to the results obtained with the pulse-labeled RNA, essentially all of the steady-state concentration of MHC mRNA was poly(A)$^-$ (Table 2), which confirmed previously reported results from our laboratory (4, 10). This poly(A)$^-$ MHC mRNA compartment is not an artifact produced by degradation during RNA extraction. If RNA degradation had occurred during extraction, it would be expected that both newly synthesized and steady-state MHC mRNA populations would fail to bind to poly(U)-Sepharose. The fact that in differentiated L6E9 myotubes MHC mRNA is synthesized as a poly(A)$^+$ molecule but accumulates in the cytoplasm as a poly(A)$^-$ molecule suggests a precursor–product relationship between the two mRNA populations. Because MHC mRNA is synthesized as a poly(A)$^+$ molecule throughout L6E9 cell myogenic differentiation (unpublished data), it is unlikely that the accumulation

Table 2. Compartmentalization of pulse-labeled and steady-state cytoplasmic MHC mRNA in differentiated L6E9 myotubes

	Pulse-labeled MHC mRNA		Steady-state MHC mRNA	
RNA fraction	% of RNA input*	% total MHC mRNA†	% of RNA input‡	% total MHC mRNA§
Total	0.139	—	0.247	—
Poly(A)$^-$	0.017	9.0	0.233	90.0
Poly(A)$^+$	0.465	91.0	ND	(10.0)¶

ND, not determined.

* The percent of RNA equal to MHC mRNA was corrected for the difference between the size of MHC mRNA (7100 nucleotides) and the size of MHC DNA fragment (680 nucleotides). The corrected percent was therefore calculated as (bound cpm/input cpm) × (7100/680) × 100. Input for total and poly(A)$^-$ RNA was 1.0×10^7 cpm each. Input for poly(A)$^+$ RNA was 4.0×10^6 cpm. Background hybridization to blank, λ, and pBR322 DNA filters was less than 0.001%.

† The distribution of MHC mRNA between the poly(A)$^-$ and poly(A)$^+$ compartments was corrected for the distribution of total RNA between the two compartments. For example, because 27% of the labeled RNA was poly(A)$^+$ by the criterion of binding to poly(U)-Sepharose, the percent MHC mRNA that is poly(A)$^+$ was calculated as (0.465/0.139) × 0.27 × 100.

‡ Percentages were calculated as for column 2. pMHC25-DNA filters bound 5 to 10 times the background hybridization values. Inputs of total and poly(A)$^-$ RNA were 2.0×10^7 cpm each.

§ Percentage for MHC poly(A)$^-$ mRNA was calculated as in column 3, except poly(A)$^-$ RNA was 96% of total unlabeled RNA.

¶ Value calculated from percent poly(A)$^-$ MHC mRNA.

of poly(A)$^-$ MHC mRNA can be explained by the synthesis of a poly(A)$^-$ MHC mRNA distinct from the poly(A)$^+$ MHC mRNA.

DISCUSSION

We have constructed and characterized a recombinant bacterial plasmid, designated pMHC25, that contains a structural gene sequence for rat skeletal muscle MHC. The identity of pMHC25 was established by several biological and physical criteria. pMHC25 DNA sequences were muscle-tissue specific, complementary to MHC mRNA by both negative and positive *in vitro* translation assays, and hybridized to a 7100-nucleotide mRNA of the expected size for MHC mRNA that was enriched in the heavy polysome fraction of differentiated muscle cells. A partial restriction map of pMHC25 revealed a 680-bp insert in pBR322 that was almost 10% of the full-length MHC mRNA. The cDNA used to construct this insert was synthesized from RNA primed with oligo(dT). Because oligo(dT) priming usually occurs at the 3′ poly(A) tail of the mRNA, we have tentatively assigned the pMHC25 insert sequences to the 3′ terminus of the MHC mRNA molecule.

We have used pMHC25 to obtain evidence for a cytoplasmic event in differentiated L6E9 myotubes that involves the processing of poly(A)$^+$ MHC mRNA to poly(A)$^-$ MHC mRNA. Most eukaryotic mRNAs contain 50–200 residues of poly(A) at their 3′ ends (11), although there are significant exceptions (30). Yet the role of poly(A) in the control of gene expression is essentially unknown. Significant fractions of muscle-specific proteins and isozymes have been shown to be synthesized by poly(A)$^-$ mRNA (25, 31, 32). Previous studies (4, 10) have shown that the concentration of MHC mRNA in the L6E9 cytoplasm undergoes a 200-fold increase from the nondifferentiated myoblast to the fully differentiated myotube. Using a cloned MHC cDNA sequence, we have confirmed that essentially all of this accumulated MHC mRNA lacks the long 3′ poly(A) tail. In contrast, we find that essentially all of the newly synthesized MHC mRNA that appears in the cytoplasm of differentiated L6E9 myotubes contains the long 3′ poly(A) tail. These results suggest that, during myogenic differentiation of L6E9 cells, newly synthesized MHC mRNA appears in the cytoplasm with a long 3′ poly(A) tail and then undergoes a processing event or events that shorten the 3′ poly(A) tail, resulting in the observed accumulation of poly(A)$^-$ MHC mRNA in the cytoplasm.

Certain eukaryotic mRNAs have a significant percentage of their sequences in the poly(A)$^-$ fraction (33, 34). Nevertheless, it has not been proven whether that poly(A)$^-$ fraction has been derived from the poly(A)$^+$ mRNA (35). It has been reported that globin mRNA undergoes progressive shortening of its poly(A) tail as the mRNA molecule ages (36), although not as dramatically as that observed for MHC mRNA. The simplest interpretation for our results is consistent with the notion that MHC mRNA is synthesized as a poly(A)$^+$ mRNA then processed to a poly(A)$^-$ mRNA in the cytoplasm. In order to definitively establish this processing sequence, a precursor–product relationship between the MHC poly(A)$^+$ and poly(A)$^-$ mRNA must be established. Whether or not this posttranscriptional process is a regulatory step in the expression of MHC during myogenesis will require further study to determine the rates of synthesis and changes in half-life of both MHC mRNA populations during myogenesis in L6E9 cells and derived mutant cell lines conditionally defective for MHC protein synthesis.

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1. Nadal-Ginard, B. (1978) *Cell* **15**, 855–864.
2. Devlin, R. B. & Emerson, C. P. (1978) *Cell* **13**, 599–611.
3. Merlie, J. P., Buckingham, M. E. & Whalen, R. G. (1977) *Curr. Top. Dev. Biol.* **11**, 61–114.
4. Benoff, S. & Nadal-Ginard, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1853–1857.
5. Strohman, R. C., Moss, P. S., Micou-Eastwood, J., Spector, D., Przbyla, A. & Paterson, B. (1977) *Cell* **10**, 265–273.
6. Buckingham, M. C., Cohen, A. & Gros, F. (1976) *J. Mol. Biol.* **103**, 611–626.
7. Chi, J. C., Fellini, S. A. & Holtzer, H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4999–5003.
8. Masaki, T. & Yoshizaki, C. (1974) *J. Biochem.* **76**, 123–131.
9. Sreter, F. A., Balient, M. & Gregely, J. (1975) *Dev. Biol.* **46**, 317–325.
10. Benoff, S. & Nadal-Ginard, B. (1980) *J. Mol. Biol.* **140**, 283–298.
11. Brawerman, G. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **17**, 117–148.
12. Yaffe, D. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 477–483.
13. Soeiro, R., Birnboim, H. C. & Darnell, J. E. (1966) *J. Mol. Biol.* **19**, 362–372.
14. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
15. Molloy, G. R., Jelinek, W., Salditt, M. & Darnell, J. E. (1974) *Cell* **1**, 43–53.
16. Harpold, M. M., Dobner, P. R., Evans, R. M. & Bancroft, F. C. (1978) *Nucleic Acids Res.* **5**, 2039–2053.
17. Roychoudhury, R., Jay, E. & Wu, R. (1976) *Nucleic Acids Res.* **3**, 101–116.
18. Bolivar, R., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
19. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961–3965.
20. Richardson, C. C. (1971) *Prog. Nucleic Acid Res. Mol. Biol.* **2**, 815–830.
21. Kupersztock, Y. M. & Helinski, D. R. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1451–1459.
22. Cohen, S. N. & Miller, C. A. (1970) *J. Mol. Biol.* **50**, 671–687.
23. Paterson, B. M., Roberts, B. E. & Kuff, E. L. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4370–4374.
24. Woolford, J. L. & Rosbash, M. (1979) *Nucleic Acids Res.* **6**, 2483–2490.
25. Benoff, S. & Nadal-Ginard, B. (1979) *Biochemistry* **18**, 494–500.
26. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4835–4838.
27. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
28. Alwine, J. C., Kemp, D. J. & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
29. Sutcliffe, J. G. (1978) *Nucleic Acids Res.* **5**, 2721–2728.
30. Revel, M. & Groner, Y. (1978) *Annu. Rev. Biochem.* **47**, 1076–1126.
31. Patrinou-Georgulas, M. & John, H. A. (1977) *Cell* **12**, 491–499.
32. Perriard, J.-C., Perriard, E. R. & Eppenberger, G. (1978) *J. Biol. Chem.* **253**, 6529–6535.
33. Sheiness, D. & Darnell, J. E. (1973) *Nature (London) New Biol.* **241**, 265–268.
34. Perry, R. P. & Kelley, D. E. (1973) *J. Mol. Biol.* **79**, 681–696.
35. Kaufmann, Y., Milcarek, C., Berissi, H. & Penman, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4801–4805.
36. Lowenhaupt, K. & Lingrel, J. B. (1978) *Cell* **14**, 337–344.