EMBRYONIC CHICK MYOSIN HEAVY CHAIN mRNA IS POLY(A)$^+$

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1. Introduction

The presence of a 3′-OH polyadenylate terminus (poly(A) tail) is a characteristic feature of a large proportion of eukaryotic messenger RNAs [1]. A notable exception to this feature are post-fertilization histone mRNAs [2], while other poly(A)$^-$ RNAs coding for specific proteins have been observed in a large number of cell types [3–5]. The shortening of the poly(A) tail during the utilization of mRNA in the cytoplasm may result in the heterogeneous lengths of poly(A) tracts found on the mRNAs [6]. The function of poly(A) tails in mRNA metabolism or translation is not known, although a number of different hypotheses have been presented which include mRNA stability, mRNA translatability, mRNA storage in mRNPs, and mRNA transport from the nucleus to the cytoplasm [7]. Since poly(A) tails were first discovered [8] their existence has been used for the isolation of mRNAs, utilizing oligo d(T)-cellulose or poly(U) Sepharose as a ligand for the poly(A) tract.

The existence of poly(A) tails on myosin heavy chain mRNA (MHC-mRNA) has been the subject of conflicting reports. In cases where MHC-mRNA has been reported to contain poly(A) tails, the estimates of the poly(A) length have ranged from a very short poly(A) tail [9,10] to a very long poly(A) tail [11]. A number of laboratories have routinely utilized the presence of the poly(A) tail to isolate and quantitate MHC-mRNA as a marker to study molecular controls during myoblast differentiation [12–17]. In the present report we present evidence that in both the embryonic chick and in chick muscle cell culture the MHC-mRNA contains a poly(A) tail of heterogeneous length which, however, is sufficiently long to permit quantitative isolation by phenol extraction and oligo d(T)-cellulose chromatography. Little or no poly(A)$^-$ MHC-mRNA is detectable by translation assay or hybridization to cDNA.

2. Materials and methods

2.1. *Muscle cell culture*

Breast muscle cultures were obtained from 12-day, pathogen-free chick embryos as described by Dym et al. [17]. Cells were plated at a density of 6×10^6 per 100 mm plate. Pre-fusion myoblast cultures were harvested at 40 h, while post-fusion cultures were harvested at 70–75 h, a time at which at least 80% of the nuclei were found in myotubes [15]. The medium was changed every 24 h.

2.2. *Isolation of RNA coding for MHC*

Preparation of the cytoplasmic heavy polysome and free messenger ribonucleoprotein particle (mRNP) fractions in pre- and post-fusion cultures was by the technique of Dym et al. [17]. The polysome and mRNP fractions were phenol extracted [18] and the RNA precipitated with 2.5 vol. of ethanol for at least 3 days at −30°C.

RNA coding for MHC was isolated from 10–30% sucrose gradients of the above precipitates. A typical sucrose density gradient and the area collected for poly(A) length determinations of the RNA are shown in fig.1. RNA fractions were precipitated at −30°C with 2.5 vol. of ethanol and 0.1 vol. of 2.4 M ammonium acetate.

2.3. *Cell-free system and gel electrophoresis*

Separation of poly(A)$^+$ from poly(A)$^-$ RNA was accomplished by oligo d(T)-cellulose chromatography (Collaborative Research Inc.) as described by Aviv and Leder [18]. Both the bound, poly(A)$^+$, RNA and the unbound, poly(A)$^-$, RNA were analyzed on sucrose density gradients and the material sedimenting at 26S was collected.

Cell-free translation of these RNA fractions was performed as previously described [19]. After incubation for 1 h at 35°C, myosin heavy chain was purified

from the reaction mixture by two rounds of ionic precipitation followed by DEAE-cellulose chromatography [19]. Subsequently, the translation products were analyzed by SDS-slab gel electrophoresis as described [20], followed by autoradiography [19].

2.4. *Poly(A) length determination*

Digestion of the RNA fractions (fig.1) was performed essentially according to the procedures described by Darnell et al. [8] in the buffer subsequently used for electrophoresis (0.04 M Tris–HCl, pH 7.4, 0.02 M sodium acetate, 0.0025 M disodium EDTA). After incubation with 2 units of T_1 ribonuclease (Calbiochem) and 1 μg of bovine pancreatic ribonuclease (Worthington 5 X crystallized) for 30 min at 37°C, the reaction mixture was brought to 0°C and SDS added to a 1% concentration. Electrophoresis was performed as described by Loening [21] in 7.5 cm, 15% polyacrylamide gels for 4.5 h at 5 mA per gel. The acrylamide gels were analyzed by slicing into 0.5 mm discs, pooling eight adjacent slices (4 mm) in each test tube, and extracting overnight with 2 X SSC. A portion of the supernatant of each tube was hybridized to 25 000 cmp of [^{3}H]-polyuridylic acid (New England Nuclear) for 35 min at 45°C, brought to 0°C, 2 ml of ice-cold 2 X SSC added, and digested with 50 μg of pancreatic ribonuclease for 20 min. Undigested polynucleotides were precipitated onto glass fiber filters with 5% TCA and the filters dried and counted in a toluene-based scintillation fluid [22].

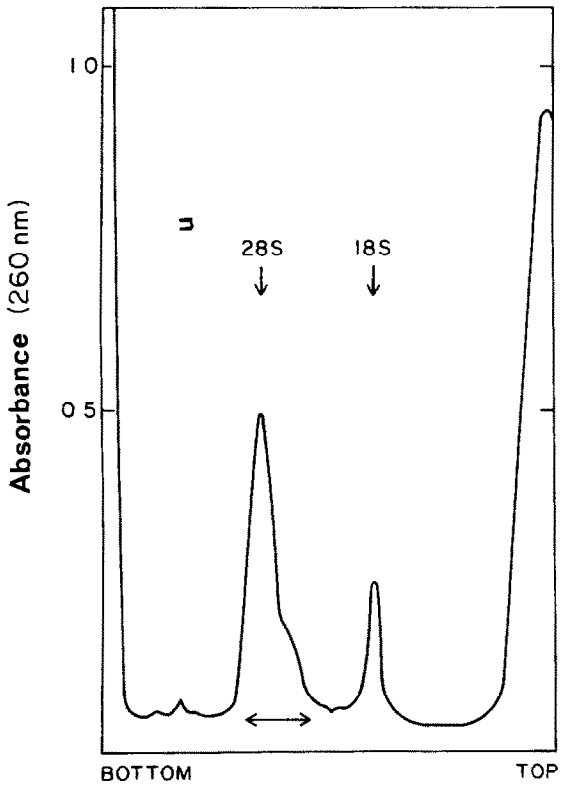

Fig.1. Typical sucrose gradient of RNA from either heavy polysome or mRNP fractions of preparative gradients [17]. Horizontal arrow denotes area collected for oligo d(T)-cellulose chromatography or for poly(A) tail length analysis.

3. Results

3.1. *Cell-free translation of poly(A)$^+$ and poly(A)$^-$ RNA*

The 26S poly(A)$^+$ RNA isolated from embryonic chick muscle has previously been shown to direct the synthesis of a 200 000 dalton protein which has been identified as authentic myosin heavy chain (MHC) [15]. In order to determine if there is a correlation between presence or absence of a poly(A) tail (as defined by oligo d(T)-cellulose chromatography) and translation of MHC-mRNA, 26S poly(A)$^+$ and poly(A)$^-$ RNA from either heavy polysomes or free messenger ribonucleoprotein particles (mRNPs) from both pre-fusion and post-fusion cultures were translated in a reticulocyte cell-free system. As shown in table 1, the amount of protein synthesis varies considerably depending on the state of polyadenylation of the RNA added to the cell-free system. The amount of protein synthesis directed by 26S poly(A)$^-$ RNA is very low compared to the synthesis resulting from the addition of 26S poly(A)$^+$ RNA. The relatively large amounts of poly(A)$^-$ RNA required to cause a significant increase in the incorporation of radioactivity over background suggests that there is little translatable RNA in these fractions. It is likely that 28S rRNA comprises a considerable proportion of the RNA in this fraction. The comparison to 26S MHC-mRNA (poly A$^+$) obtained from embryonic chick muscle tissue is made because of our experience with this mRNA species [12,14,17] and the difficulties in the translation of this mRNA obtained from tissue culture [23].

The extent of MHC synthesis was determined after two rounds of ionic precipitation followed by DEAE-cellulose chromatography [19]. We have found this

Table 1
Translation of 26S RNA from heavy polysomes and mRNPs in vitro

Experiment number	Source of RNA	RNA added (μg)	cpm per μg RNA added
1	endogenous only	0	0
2	prefusion mRNP poly(A)$^+$	2	6320
3	prefusion polysomal poly(A)$^+$	2.3	1810
4	postfusion mRNP poly(A)$^+$	0.9	2374
5	postfusion polysomal poly(A)$^+$	0.3	5126
6	prefusion mRNP poly(A)$^-$	10	247
7	prefusion polysomal poly(A)$^-$	3	364
8	postfusion mRNP poly(A)$^-$	10	423
9	postfusion polysomal poly(A)$^-$	3	852
10	tissue 26S poly(A)$^+$	1	4013

Reticulocyte cell-free system as described by Rourke and Heywood [19]

necessary, even with RNase-treated reticulocyte lysates, due to the occasional appearance of radioactivity migrating as large proteins upon acrylamide gel analysis. The autoradiographic analysis of the electrophoretically separated myosin heavy chain (fig.2) indicates that the poly(A)–26S RNA fractions direct the synthesis of very little myosin heavy chain. The faint bands migrating slightly faster than MHC are likely a result of either premature termination during synthesis of this very long protein or proteolytic degradation after synthesis. Their appearance is variable and is dependent on the preparation of reticulocyte lysate used for the messenger translation. The small amount of MHC synthesized after the addition of poly(A)$^-$ 26S RNA to the cell-free system (fig.2), reflects the low level of MHC-mRNA in those poly(A)$^-$ fractions.

3.2. *Poly(A) length*

The determinations of poly(A) tail lengths were performed on the total RNA fraction (fig.1) without separating poly(A)$^+$ and poly(A)$^-$ RNAs by oligo d(T)-cellulose chromatography. The results are shown in fig.3. Poly(A) from pre-fusion mRNPs and post-fusion heavy polysomes shows considerable heterogeneity. This heterogeneity is consistent with that reported in similar studies using globin mRNA [6,24]. Based on comparison with markers run on parallel gels [24] the sizes of poly(A) in pre-fusion mRNPs range from 20–30 nucleotides to larger than 150 nucleotides, with a major peak at about 70 nucleotides. A similar profile is seen in post-fusion heavy polysomes, with a similar-sized peak present.

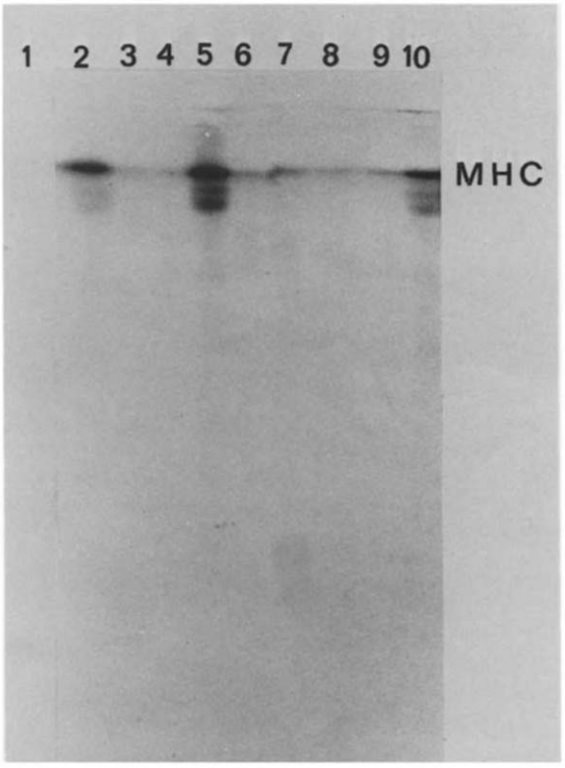

Fig.2. Autoradiography of gel electrophoresis of [^{35}S]methionine-labeled MHC purified from reticulocyte lysate. Well numbers correspond to experiment numbers in table 1. MHC, position of myosin heavy chain markers; Lane 1, blank; Lanes 2–5, poly(A)$^+$ fractions; Lanes 6–9, poly(A)$^-$ fractions; Lane 10, 26S poly(A)$^+$ RNA from tissue.

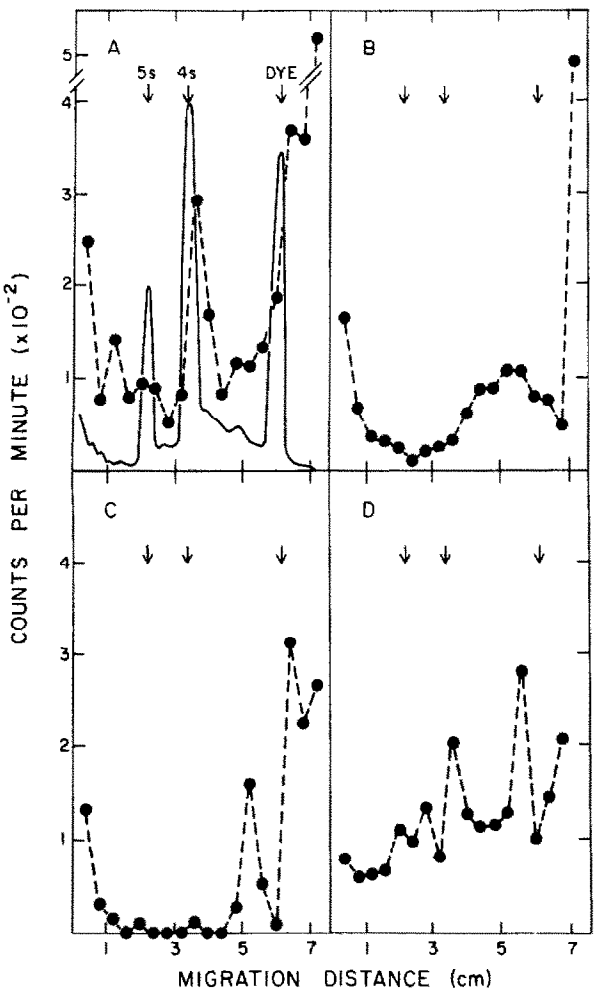

Fig.3. Determination of poly(A) tail length by gel electrophoresis (see section 2). Electrophoresis from left to right. Arrows denote positions of 5S RNA, 4S RNA, and Bromophenol Blue markers. (A) Prefusion mRNPs; (B) Prefusion heavy polysomes; (C) postfusion mRNPs; (D) postfusion heavy polysomes. (——), A_{260} of marker gel (•– – –•), [^{3}H]poly(U).

In contrast, pre-fusion heavy polysome RNA and post-fusion mRNP-RNA show only short poly(A) tails, though long enough to bind to oligo d(T)-cellulose under the conditions used in this study (see section 2) [26]. It may be significant that the similarities in the profiles of poly(A) tails of the MHC mRNA from myoblasts mRNPs and myotube heavy polysomes are consistent with the transfer of stored MHC-mRNA from free cytoplasmic mRNPs to heavy polysomes, which occurs concommitant with fusion and the significant rise in MHC synthesis in cultured chick embryonic muscle [17,27].

4. Discussion

The presense of a poly(A) tail has been used to isolate MHC-mRNA in several laboratories [4,7,31]. The possibility remains, however, that there are two populations of MHC-mRNA, as poly(A)$^+$ and poly(A)$^-$ both able to direct the synthesis of MHC. A number of reports in which MHC-mRNA has been isolated indicates some differences (table 2). Mondal and Sarkar [13] originally reported that MHC mRNA from embryonic muscle tissue was poly(A)$^+$, and subsequently determined the length of the poly(A) tail to be ~170 nucleotides [11]. We have similarly confirmed this approximate length, although we have observed more heterogeneity in the length of poly(A) tail (table 2) (unpublished results). In tissue culture, the presence of poly(A)$^+$ MHC mRNA has been reported in both heavy polysomes and stored mRNPs of embryonic chick muscle [17], while Buckingham et al. [28] found poly(A)$^+$ 26S message in heavy polysomes and mRNPs from pre-fusion and post-fusion fetal calf muscle cultures. Furthermore, they estimated that 90–100% of the presumptive MHC-mRNA in free mRNPs was poly(A)$^+$.

In contrast, Przybyla and Strohman [9] reported that MHC-mRNA obtained from myotubes of chick muscle cell cultures was poly(A)$^-$. However, by using different techniques from their earlier work and by examining total cytoplasmic RNA, Strohman, et al., [29] more recently reported that the majority of MHC mRNA is poly(A)$^+$. Finally, Benoff and Nadal-Ginard [10] using the L6E9 rat muscle cell line reported that the majority of MHC-mRNA is not bound by oligo d(T)-cellulose.

The studies reported here confirm that chick muscle MHC-mRNA possesses a poly(A) tail of sufficient length to bind to oligo d(T)-cellulose under conditions which will not bind a poly(A) tract of less than 20 nucleotides [26]. Furthermore, we show that the poly(A)$^+$ MHC mRNA is present, although at different levels, in both polysomes and free mRNPs, in pre-fusion and post-fusion cultures. While the presence of a small amount of 26S poly(A)$^+$ RNA which is not MHC-mRNA cannot be ruled out, the data obtained by the cell-free synthesis of MHC (fig.2) strongly suggest that virtually all of the MHC-mRNA possesses a poly(A) tail. Recent studies by Dym et al. [17], using a long cDNA probe for MHC-mRNA, showed that virtually all the MHC mRNA in post-fusion polysomes is poly(A)$^+$. Taken together these results sug-

Table 2
Reported sizes of the MHC-mRNA poly(A) tails

MHC mRNA source	Poly(A) length (nucleotides)	Ref.
Embryonic chick breast muscle culture, post fusion polysomes	<25[a]	9
L6E9 rat myogenic cell line, total cytoplasmic (pre-fusion)	<25[a] (90%)	10
Fetal calf leg muscle culture, pre- and post-fusion polysomes	>25[a]	28
Embryonic chick leg muscle, tissue (14-day) polysomes	170	11
Embryonic chick leg muscle, tissue (13-day) polysomes	20–90	31
Embryonic chick leg muscle, tissue (13-day) mRNPs	60–180	31
Embryonic chick breast muscle culture, total cytoplasmic (post-fusion)	>25[a]	29
Embryonic chick breast muscle culture, pre-fusion mRNPs	30–>150	(this report)
pre-fusion polysomes	10–30	
post-fusion mRNPs	10–30	
post-fusion polysomes	30–>150	

[a] Poly(A) size estimated by comparison of isolation techniques used by Nudel et al. [25]

gest that if there is any poly(A)$^-$ MHC mRNA during early chick muscle myogenesis, it is present in extremely small amounts, or it is not translatable, even when deproteinized by phenol extraction. The reason for the variability of the translation of the 26S RNA in terms of cpm/μg of RNA using RNA from different cellular components of different stages (table 1) is not known. This may be related to altered states of the mRNA in different cellular compartments during differentiation or to the presence of the mRNAs for multiple forms of MHC [31].

The lengths of poly(A) tails from both prefusion mRNPs and from post-fusion heavy polysomes from tissue culture are consistent with the heterogeneity in size reported, and confirmed by us (unpublished results), for globin mRNA [6,26]. The similarities in the pattern of poly(A) content between the myoblast mRNP-MHC-mRNA and the myotube polysomal ones are consistent with their being in a precursor-product relationship [27]. The poly(A) tails of the MHC-mRNA from tissue cultures appear to be shorter, on the whole, than those seen in tissue preparations (table 2). However, this would not explain the apparent lack of poly(A) tails seen in the L6E9 rat muscle cell line [10]. These particular cells appear to synthesize only a single species of MHC-mRNA, while preparations from embryonic chick muscle contain the RNAs for two or more forms of MHC [17,31]. A further feature of the MHC mRNA from L6E9 cells may be that it either possesses a very short poly(A) tail or the poly(A) tail is absent. Since the function of poly(A) tails is still unknown, it is difficult to speculate about the advantages of its presence or absence on MHC-mRNA [7,30]. Nevertheless, it appears as if MHC-mRNA isolated from embryonic chick muscle is poly A$^+$ and that those mRNAs with the longer poly A tails are found in those cellular compartments which had been previously shown to contain functional myosin mRNA [17].

Acknowledgements

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