HIGHLY PURIFIED mRNA FOR MYOSIN HEAVY CHAIN: SIZE AND POLYADENYLIC ACID CONTENT*

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SUMMARY. The polyadenylic acid segment of highly purified preparations of myosin heavy chain mRNA consists of about 170 nucleotides-about 2.92% of the intact mRNA. The molecular weight of the mRNA is calculated from these values as 2.05 x 10^6. This is in good agreement with the value of 2.23 x 10^6 obtained by formamide-gel electrophoresis. These values are 10-20% higher than the estimated minimum size of the mRNA suggesting that some "non-translatable" segment(s) may be present. No apparent correlation was observed between the sizes of mRNAs present in muscle polysomes of different sizes and their poly(A) contents.

According to the currently accepted view the heavy and light polypeptide chains which together constitute the myofibrillar protein myosin, are translated independently from different monocistronic messages (1,2). Furthermore, different isozymic forms of myosin may result from the selective expression of different sets of genes coding for myosin subunits in the various types of differentiated muscle fibers (3,4). In embryonic chicken muscle a fraction of polysomes containing 50-60 monoribosomes (referred to as polysome fraction I) has been shown to contain a 25-27 S messenger RNA (mRNA) (5). In vitro translation of this mRNA gave a product identifiable as the myosin heavy chain (5,6). The bulk of the preparations of myosin heavy chain mRNA described in the literature consisted of ribosomal RNAs(rRNA) (5,6). It is currently believed that all or almost all mRNAs from eukaryotic cells, with the exception of histone mRNA, contain long stretches of adenylic acid residues at the 3'-terminal (referred to as poly(A) fragment; for a review see ref. 7 and the references cited therein). We have recently been successful in isolating highly purified myosin heavy chain mRNA, free of ribosomal and other cellular RNAs, by utilizing unique properties of the

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poly(A) segment of the mRNA(8-10). In this report we have characterized the myosin heavy chain mRNA with respect to its size and poly(A) content.

METHODS AND MATERIALS. The procedures for obtaining undegraded preparation of chick embryonic polysome fraction I that synthesizes the myosin heavy chain (1,2, 18) and for the isolation of the mRNA free from rRNAs have been recently described by us (9). The poly(A)-rich mRNA was separated from rRNAs that lack the poly(A) segment by a two step process involving binding to millipore filters (11) and chromatography of the millipore-bound RNA fraction on a cellulose column (12). In vitro translation of the purified mRNA was carried out with the use of two types of cell-free systems: a homologous system consisting of salt-washed muscle ribosomes and muscle initiation factors (5,6) and a heterologous rabbit reticulolocyte lysate (13). The products of in vitro translation were identified as the myosin heavy chain as previously described (1). The labeling of the 3'-end of the mRNA was done according to the method of Raj Bhandari (14) as modified by De Wachter and Fiers (15). This method involves oxidation with $NaIO_4$ of the ribose 2' and 3'-OH groups of the 3'-terminal nucleotide followed by tritiation with [^{3}H]$NaBH_4$. After removal of excess [^{3}H]$NaBH_4$ (15), the mRNA was treated with pancreatic RNase and the tritiated poly(A) fragment was isolated essentially as described for the isolation of myosin heavy chain mRNA (9,10). Details of these methods are described in the legends.

RESULTS AND DISCUSSION. Gel Electrophoresis of Purified Myosin Heavy Chain mRNA: Two RNA peaks were obtained from chromatography of the millipore-bound RNA fractions on a cellulose column (for details see Fig. 2 in reference 9). The densitometric scans of polyacrylamide gel runs of RNA samples obtained from these two peaks are shown in Fig. 1. The first peak consists of 28 S rRNA as the major species and a small amount of 18 S rRNA (dotted line). The gel scan of RNA in the second column peak, whose chromatographic properties are like those of a poly(A)-rich mRNA (9), showed a sharp single peak in the gel moving more slowly than 28 S rRNA. No detectable amounts of other cellular RNAs were found in this

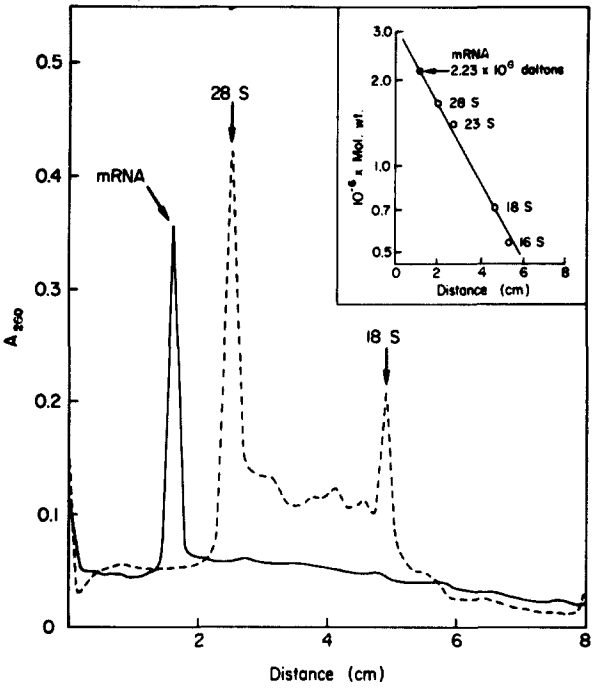

Figure 1. Electrophoresis of RNA samples eluted from cellulose column on 2.4% polyacrylamide gels. For details see text. _____, 26 µg RNA from adsorbed column fraction; ----, 32 µg RNA from unadsorbed fraction (9). Insert: Molecular weight of mRNA. Samples were run on 2.5% gels in the presence of formamide (16) and gels were scanned at 260 nm (9).

RNA indicating that a homogenous preparation had been obtained.

In Vitro Translation of mRNA: The products of in vitro translation of mRNA were purified by coprecipitation at low ionic strength and chromatography on a DEAE-cellulose column (1). Column fractions corresponding to the myosin peak were pooled and concentrated with Aquacide (Cal Biochem). On electrophoresis in 4% SDS-polyacrylamide gels (3) about 55-70% of the radioactivity applied to the gel migrated with the band of the heavy chain marker (Fig. 2). A minor peak containing 5-10% of the total counts migrated more slowly than the 200,000 dalton heavy chain band. As judged by its relative mobility, this peak is presumably due to dimers of the heavy chain which are occasionally observed during the gel runs(3). In order to show that the reticulocyte lysate alone did not synthesize the myosin

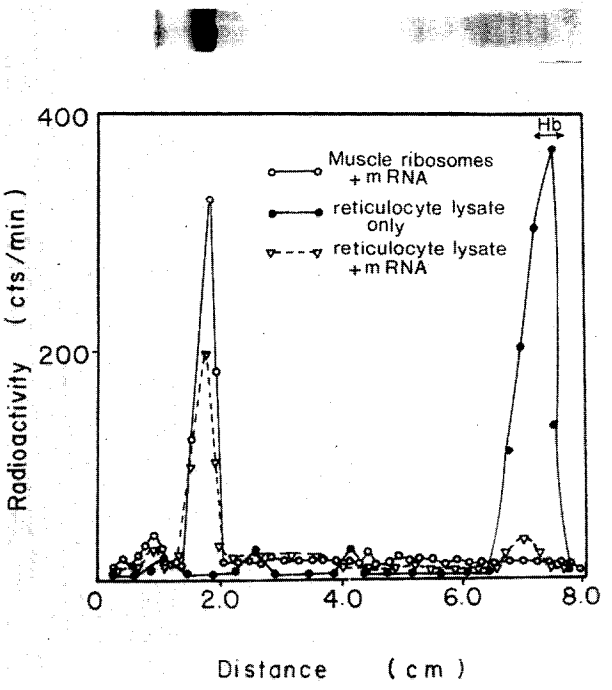

Figure 2. In vitro translation of purified myosin heavy chain mRNA. Products were copurified with 3-4 mg chicken leg myosin (see text) and subjected to electrophoresis on 4% polyacrylamide gels (3). Samples containing 900-1200 cpm (specific activity 4200-5200 cpm/mg) were applied to the gels. The gels were stained, sliced and counted as previously described (1). Top: Gel containing 200 μg myosin run as a marker.

heavy chain, a sample of the total TCA-insoluble protein obtained from the incubation in which the mRNA was omitted, was also examined by electrophoresis. No significant amount of radioactivity above the background level in the myosin heavy chain band was detected, and about 80% of the total radioactivity migrated with a band of hemoglobin run as a marker (Fig.2). When RNA samples present in the millipore filtrate and the unadsorbed peak from the cellulose column (9) were tested for mRNA activity, very little or no activity was found as judged by coprecipitation at low ionic strength, and DEAE-cellulose chromatography.

Size of the Myosin Heavy Chain mRNA: The purified mRNA was analyzed by polyacrylamide gel electrophoresis according to the method of Staynov et al. (16) with the use of samples of chicken 28 S, 18 S and E. coli 23 S and 16 S rRNAs as markers. Using this gel system which employs formamide to unfold RNA molecules, a

linear relationship between the electrophoretic mobilities and log molecular weight was obtained (Insert, Fig. 1). From such a plot the molecular weight of mRNA is estimated as about 2.23×10^6. This value would correspond to a total length of about 6500 nucleotides.

Poly(A) Content of the Myosin Heavy Chain mRNA: The radioactivity in the poly(A) fragment of the mRNA that had been tritiated with [^{3}H]$NaBH_4$ (see Methods) was compared with that of the intact mRNA. About 97% of the label was found in the millipore-bound poly(A) (Table I). After treatment with SDS about 82% of the

TABLE I

Labeling of Poly(A) Fragment of Myosin Heavy Chain mRNA

Condition	Total radioactivity in sample (H^3-cpm)
(a) Intact mRNA	120,900
(b) Millipore-bound Poly(A) after RNase treatment of labeled mRNA	116,000
(c) Poly(A) extracted from millipore filters	95,400
(d) Poly(A) precipitated with ethanol	86,800

Eighty μg of purified mRNA was tritiated (15) with 25 mCi of [^{3}H]$NaBH_4$ (specific activity of 2.8 Ci/m mole; New England Nuclear Corporation). The tritiated RNA was dissolved in 1 ml of 20 mM Tris-HCl, pH 7.5 containing 1 mM EDTA. Key: (a) A portion (0.1 ml) was mixed with 20 volumes of "binding buffer" (11) and filtered through a millipore filter (9). (b) After incubation with 2 μg/ml of RNase at 35° for 30 min, 0.2 ml portion of the remaining sample was diluted with 1 ml of binding buffer containing 2 A_{260} units of 4 S RNA and was filtered through a second millipore filter. (c) The remaining sample was passed through a third filter and the adsorbed poly(A) fragment was eluted twice with SDS (9). (d) The poly(A) fragment eluted from the filters plus 3 A_{260} units of 4 S RNA added as carrier, were precipitated with ethanol (9). The filters were washed, dried and counted (9).

bound radioactivity was eluted, 91% of which were recovered after precipitation with alcohol. These results indicate that the label was primarily restricted to the 3'-terminus of the mRNA. The tritiated poly(A) fragment was examined by gel

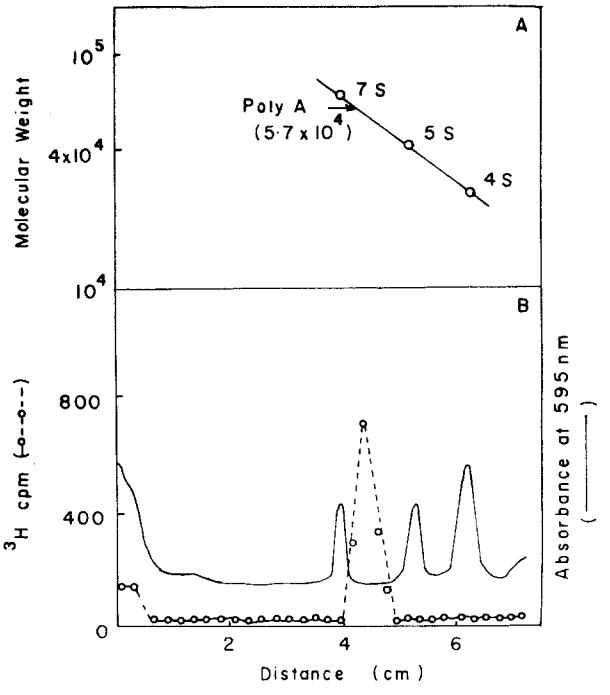

Figure 3. Electrophoresis of tritiated poly(A) fragment of myosin heavy chain mRNA on 6% polyacrylamide gels. For details see text. Panel B: Profiles of radioactivity and absorbance of gels stained with Toluidine blue. Panel A: Plot of log molecular weight vs. migration (see text for markers used).

electrophoresis using 7 S, 5 S and 4 S RNAs as markers. The radioactive poly(A) fragment migrated between 5 S and 7 S RNAs as a distinct sharp peak indicating a high degree of homogeneity in size (Fig.3, panel B). From a plot of log molecular weight vs. migration (panel A) the size of the poly(A) fragment was calculated as 5.7×10^4 daltons or about 170 adenylic acid residues. The size of poly(A) fragments calculated from electrophoretic mobilities has been previously shown to be in good agreement with those obtained from *in vivo* labeling techniques (17). The size of the poly(A) fragment was then used to estimate the molecular weight of the mRNA. The mRNA was purified from embryos which had been pulse-labeled *in ovo* with $^{32}PO_4$ and the poly(A) fragment was isolated as described above. The amount of radioactivity in the poly(A) segment was found to be 2.92% of that in the intact mRNA (Table II). Since this 2.92% represents a fragment consisting of 170 nucleo-

TABLE II

Poly(A) Content of ^{32}P-labeled Myosin Heavy Chain mRNA

Sample	Total	Percent Radioactivity
Intact mRNA	13,400	100
Millipore-bound Poly(A) fragment after RNase treatment of ^{32}P-labeled mRNA	372	2.92

Ten 14-day old chicken embryos were injected in ovo with 5 mCi each of carrier free $^{32}PO_4$ (19). Myosin heavy chain mRNA was isolated (9) after incubation of the embryos at 37° for 2 hr, and the labeled mRNA was diluted with non-radioactive mRNA to a specific activity of 270 cpm/μg. Fifty μg of mRNA was then incubated with RNase, and the poly(A) fragment was adsorbed on a millipore filter.

tides, it is estimated that the mRNA should consist of about 5830 nucleotides with a molecular weight of about 2.05×10^6. The mRNA coding for the myosin heavy chain should have a minimum molecular weight of about 1.88×10^6. The estimates obtained by the two approaches reported here, although 10-20% higher, are in good agreement with the expected molecular weight. These results raise the possibility that some "non-translatable" segment(s), which may include polynucleotide sequences at the 5'-end in addition to the poly(A) segment at the 3'-end, may be present in the mRNA.

Poly(A) fragments of mRNAs of muscle polysomes of different sizes: Embryonic chicken leg muscles contain groups of polysomes of different sizes which synthesize polypeptides of different sizes (1,2,18). Thus, myosin heavy chain (mol. wt. about 200,000) is synthesized on fraction 1 polysomes (50-60 ribosomes); actin (mol. wt. about 42,000) and presumably polypeptides of about 50,000 daltons are synthesized on fraction III polysomes (4-10 ribosomes) (for a description of polysome profiles see Fig. 2 in ref. 1). The method of 3'-end labeling was used to estimate the size of the poly(A) fragment of the mRNAs present in these three classes of polysomes. As shown in Fig. 4, mRNA present in fraction I polysomes

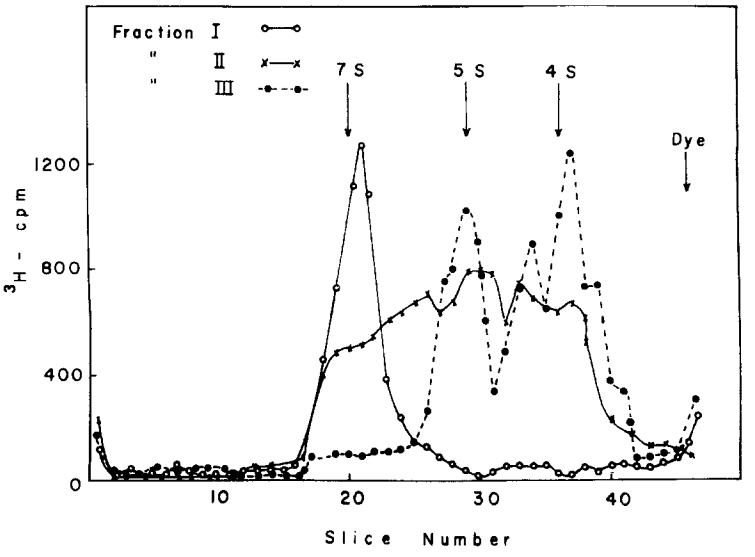

Figure 4. The size of poly(A) fragments derived from mRNAs of different classes of chick embryonic polysome fractions. For details see text. The arrows indicate the position of the peaks of the markers.

gave a sharp peak of poly(A) which moved slightly faster than 7 S. In contrast, the poly(A) fragments obtained from mRNAs of fraction II and III polysomes gave a heterogeneous distribution although the major part of the radioactivity migrated between 7 S and 4 S RNAs. A small but significant amount of counts migrated even faster than 4 S RNA. This indicates that the poly(A) contents of different messages in embryonic muscles are highly variable, and there appears to be no correlation between the size of the mRNA and its poly(A) content. The precise biological role of the poly(A) segment whose size varies from 150-200 nucleotides in most eukaryotic mRNAs (for a review see ref. 7) is not known. Whether the number of adenylic acid residues in the poly(A) fragments of mRNAs in muscle polysomes of different sizes is related to the differences in stability of mRNAs or differences in the rates of processing of the various mRNA species remains to be tested.

The results presented here show that we have isolated highly purified myosin heavy chain mRNA in electrophoretically homogeneous and undegraded form

in quantities suitable for biochemical studies. The characterization of the myosin heavy chain mRNA should be useful in understanding at the molecular level metabolic events such as transcription and translation of the mRNA during various stages of muscle cell differentiation.

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