A unique subspecies of histone H4 mRNA from rat myoblasts contains poly(A)
(polyadenylated mRNA/histone variants/myogenesis/ribonucleoprotein)

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Communicated by Robert P. Perry, June 17, 1985

ABSTRACT
Fractionation of rat L6 myoblast histone H4 mRNA into its three component subspecies revealed that one of the major subspecies (H4-1) contained poly(A). The unique poly(A)+ H4 mRNA makes up about 8% of the total polyosomal H4 mRNA population detected. Unlike the poly(A)- subspecies, whose levels are reduced by >95% when myoblasts differenate into myotubes, the poly(A)+ subspecies is reduced by only 70%. The poly(A)+ H4 mRNA from myotubes incubated with actinomycin D decays with a half-life of 37–42 min, which is similar to that obtained for the poly(A)+ H4 mRNAs in myoblasts. Both the poly(A)+ and poly(A)+ subspecies decay at an increased rate after inhibition of RNA synthesis. In myoblasts the poly(A)+ H4 mRNA exists almost exclusively in the polyosomal compartment (>95%) with little (<5%) in the free ribonucleoprotein (mRNA–protein or mRNP) complex compartment of the cell. Poly(A)+ histone H4 mRNA subspecies, on the other hand, are distributed with approximately 80% in the polyosomal compartment and 20% in the free mRNP complex compartment. The unique poly(A)+ H4 mRNA is unusual, not only in that it contains poly(A) but also in its behavior compared to poly(A)+ H4 mRNAs during terminal differentiation.

The majority of eukaryotic mRNAs are polyadenylated at their 3′ end shortly after the termination of transcription (1). Histone mRNAs, in contrast, are commonly cited as the best examples of those messages lacking poly(A) tails (cf. reviews in refs. 2–4). There are, however, exceptions. Evidence for poly(A)-containing histone mRNAs has been obtained in: (i) a variety of organisms in which a portion of all subclasses of histone mRNAs contain poly(A) (5–8); (ii) yeast cells in which all histone mRNA contain poly(A) (9); and (iii) Tetrahymena in which H3 and H4 mRNAs are largely polyadenylated (10). In none of the above cases, however, was the distribution of poly(A) among subspecies of histone mRNA determined.

While it is not known what function polyadenylation of mRNA serves within eukaryotic cells, there are suggestions that it may improve the stability of individual mRNAs and affect their efficiency of function (1, 11). Here we report that one of the three major subspecies of histone H4 mRNA found in rat L6 myoblasts is unique in that it contains poly(A). The following is a characterization of the behavior of this unusual histone mRNA during terminal myoblast differentiation.

MATERIALS AND METHODS
Cell Culture and RNA Preparation. Rat L6-5 myoblasts (12) were grown in α minimal essential medium (Flow Laboratories) supplemented with 10% fetal bovine serum. Proliferating myoblasts were induced to differentiate into myotubes at 60% confluency with α minimal essential medium supplemented with 2.5% donor horse serum. More than 95% of the cells were syncytial within 3 days, and polysomes were prepared on day 4. In some cases cells were treated with actinomycin D (3 μg/ml; Calbiochem) or cytosine arabinoside (40 μg/ml; Sigma). The cells were washed once in 10 mM Hepes, pH 7.6/0.9% NaCl on ice, lysed in 25 mM Tris-HCl, pH 8.5/0.5% Nonidet P-40/5 mM MgCl2/0.25 M NaCl/2.5 mM CaCl2/0.2 mg of emetine per ml/0.005% phenylmethylsulfonyl fluoride/10 μM aminocarboxylic acid, homogenized, and centrifuged at 12,000 × g (20 min at 2°C).

Polysomes were prepared from the supernatant by centrifugation through a 25% (wt/vol) sucrose cushion at 178,000 × g (1 hr at 2°C). The supernatants were mixed and subsequently pelleted by centrifugation at 178,000 × g (16 hr at 2°C) to produce the postpolysomal or free ribonucleoprotein (RNP) pellet. The pellets were resuspended and digested in 10 mM Tris-HCl, pH 7.5/1 mM Na2EDTA/0.5% NaDodSO4/0.5 mg of protease K (EC 3.4.21.14) per ml (1 hr at 37°C), followed by phenol/chloroform extraction (13). The RNA in the aqueous phase was ethanol-precipitated and stored at −20°C.

Oligo(dT)-Cellulose Chromatography. RNA was separated into poly(A)+ and poly(A)− populations by fractionation on oligo(dT)-cellulose (Collaborative Research, Waltham, MA) (14). 0.2 g was suspended in binding buffer (10 mM Tris-HCl, 7.6/1 mM Na2EDTA/0.5% NaDodSO4/0.5 mM NaCl) and poured into a 0.7 × 10 cm column. The RNA (1–2 μg) was suspended in binding buffer without salt, heated (1 min at 65°C), made 0.5 M in NaCl, rapidly cooled to room temperature, and applied to the column. The column was washed with 25 ml of binding buffer, the first 2 ml of which was retained as the poly(A)+ fraction. The poly(A)+ fraction was eluted with 3 ml of elution buffer (1 mM Na2EDTA, pH 8/0.1% NaDodSO4). The poly(A)+ RNA bound to the oligo(dT)-cellulose was eluted and rechromatographed three times. The poly(A)+ fraction was passed over the column twice to ensure against cross-contamination with poly(A)+ RNA. In some cases equal amounts of (5 μg) of poly(U) and poly(A)+ RNA were combined in 1 ml of 50 mM NaCl/10 mM Tris-HCl, pH 7.6/1 mM Na2EDTA/0.5% NaDodSO4, heated to 65°C for 1 min, and allowed to cool to room temperature over 1 hr. The solution was made 0.5 M in NaCl, and the prehybridized sample was applied to the oligo(dT)-cellulose column as described above.

RNA Electrophoresis. RNA fractions were analyzed on a 6% polyacrylamide gel containing 8.3 M urea/50 mM Tris borate, pH 8.3/1 mM Na2EDTA that had been prerun for 1 hr at 15 W and for 18 hr at 200 V (15). Total polyosomal RNA

Abbreviations: RNP, ribonucleoproteins; mRNA, mRNA-protein complexes.

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or poly(A)\textsuperscript{−} RNA (50 \( \mu \)g) or poly(A)\textsuperscript{+} RNA (5 \( \mu \)g) was dissolved in 10 \( \mu \)l of 8.3 M urea/5 mM Na\textsubscript{2}EDTA, pH 8 and heated to 100°C for 2 min. The gel was washed sequentially in 50 mM NaOH (20 min), 0.2 M KH\textsubscript{2}PO\textsubscript{4} (pH 5, twice for 10 min), and 25 mM KH\textsubscript{2}PO\textsubscript{4} (pH 5, twice for 10 min), electroblotted to DPT paper (16) for 4 hr at 3.6 V/cm at 4°C, and probed for histone H4 sequences. The blot was prehybridized for 6 hr (42°C) in 10 ml of 50\% (vol/vol) formamide/0.75 M NaCl/75 mM sodium citrate, pH 7.0/1\% (wt/vol) glycine/0.02\% Ficoll/0.02\% polyvinylpyrrolidone/0.1\% yeast tRNA per ml/0.1\% NaDodSO\textsubscript{4}. Hybridization to 2 \( \times \) 10\textsuperscript{6} cpm of nick-translated probe (ref. 17; 2 \( \times \) 10\textsuperscript{6} cpm/\( \mu \)g) was performed in 10 ml of prehybridization solution without glycine (3 days at 42°C). Probes were denatured in 0.2 M NaOH (10 min) and neutralized with 1 vol of 1 M Tris-HCl (pH 7.5) prior to addition to hybridization buffer. The blot was washed as described (18) except that each wash lasted 20 min. Autoradiograms were prepared, and quantitation was performed by scanning original, unsaturated autoradiograms. The response was linear with respect to the number of micrograms of RNA.

RESULTS AND DISCUSSION

These studies were designed to characterize the histone H4 mRNA subspecies in rat myoblasts. The myoblast histone H4 mRNAs were separated on polyacrylamide gels into three subspecies designated 1–3 (Fig. 1). Subspecies 1 and 2 (major bands) and 3 (minor band) all hybridized to a cloned human genomic H4 sequence, pF0108A (19). The three subspecies of H4 mRNA migrate in an identical fashion to those from HeLa cells in this gel system (data not shown). We have estimated the length of these H4 mRNAs by comparison to the HeLa cell H4 mRNA subspecies and to other RNAs of known molecular weight that also can be identified. Subspecies 1 was 439 bases long, subspecies 2 was 394 bases long, and subspecies 3 was approximately 364 bases long. These estimates of rat L6 myoblast H4 mRNA size were identical to those previously reported for human H4 mRNAs (15).

mRNAs coding for H4 were observed in both the poly(A)\textsuperscript{−} and poly(A)\textsuperscript{+} fractions. The poly(A)\textsuperscript{−} fraction contained all three H4 subspecies, although subspecies 1 was diminished in intensity after removal of poly(A)\textsuperscript{+} material. The poly(A)\textsuperscript{+} fraction, on the other hand, yielded only one subspecies, which comigrated with the diminished subspecies 1 in the poly(A)\textsuperscript{+} fraction. Removal of the poly(A)\textsuperscript{−} fraction of H4 subspecies 1 left a heterogeneous smear of density in this region in poly(A)\textsuperscript{−} samples. We have grouped all of these remaining subspecies into the poly(A)\textsuperscript{−} H4 subspecies 1 class. No poly(A)\textsuperscript{+} subspecies representing H1, H2a, H2b, or H3 histone mRNAs was detected (data not shown).

To confirm that the poly(A)\textsuperscript{+} H4 mRNA interacted specifically with oligo(dT) residues, a high-stringency wash (binding buffer with 0.1 M NaCl) was interposed between the high-salt wash and elution of the poly(A)\textsuperscript{+} RNA (Fig. 2). This wash was designed to remove nonspecific and less tightly bound RNAs from the column. No H4 mRNA was detected in this wash, although subsequent elution yielded the same H4 mRNA. The oligo(dT)-cellulose-bound H4 mRNA reacted with sufficient strength to withstand a 0.1 M salt wash, consistent with the characteristics of RNA containing poly(A). In addition, prehybridization of the poly(A)\textsuperscript{+} RNA with an equal amount of poly(U) blocked completely the interaction of the H4 mRNA with oligo(dT)-cellulose. The prehybridized H4 mRNA passed through the column and did not bind, whereas subsequent elution demonstrated that no portion of the poly(U)-prehybridized H4 sequences bound to the oligo(dT)-cellulose. In more than 12 repetitions of this experiment, no evidence of heterogeneity was observed within the poly(A)\textsuperscript{+} H4 band, suggesting that there is little variation in the number of adenosine residues added. The poly(A)\textsuperscript{+} H4 mRNA always appeared as a sharp discrete band.

Unlike subspecies 2 and 3, subspecies 1 appeared to be composed of two subfractions—i.e., one with and one without poly(A). The amount of poly(A)\textsuperscript{+} H4 mRNA in band 1 was determined by comparing the amount of H4 probe hybridized to band 1 and band 2 before and after removal of the poly(A)\textsuperscript{−} mRNA subspecies. There were approximately equal amounts of hybridizable material in bands 1 and 2 prior to removal of poly(A)\textsuperscript{+} subspecies. After removal of poly(A)\textsuperscript{+} material, there was a loss of approximately 20\% of the H4 mRNA from band 1. Approximately 20\% (±6\% maximum sample variation) of subspecies 1 or 8–10\% of all of the H4 mRNAs detected contained poly(A).

The relationship between poly(A)\textsuperscript{+} and poly(A)\textsuperscript{−} H4 mRNAs is uncertain. The poly(A)\textsuperscript{+} portion of H4 subspecies 1 may be a polyadenylylated copy of the lower-molecular weight subspecies or may represent a unique subspecies of H4 mRNA. Although the poly(A)\textsuperscript{+} H4 mRNA always appeared as a single band, it still may be composed of a population of mRNAs, each of which contains poly(A). Identification of the poly(A)\textsuperscript{+} H4 mRNA has relied upon its ability to interact with oligo(dT)-cellulose and poly(U). The results do not preclude the possibility that the poly(A) sequence is encoded within the H4 gene; however, no examples have been reported among the histone genes that have been sequenced (2, 3).

![Fig. 1. Analysis of poly(A)\textsuperscript{+} and poly(A)\textsuperscript{−} RNA fractions from L6 myoblasts for histone H4 sequences. Electrophoresis on 6% polyacrylamide gels containing 8.3 M urea was followed by RNA blot analysis and hybridization to pF0108A (containing an H4 sequence). Lanes: Total, total polysomal RNA; A\textsuperscript{−}, poly(A)\textsuperscript{−} RNA; A\textsuperscript{+}, poly(A)\textsuperscript{+} RNA. H4 subtypes 1–3 are indicated.](image)

![Fig. 2. Characterization of poly(A)\textsuperscript{+} H4 mRNA binding to oligo(dT)-cellulose. Lanes: a', total polysomal RNA; a, poly(A)\textsuperscript{−} RNA; b, poly(A)\textsuperscript{+} RNA; c, stringent wash (0.1 M NaCl) of poly(A)\textsuperscript{+} RNA bound to oligo(dT)-cellulose; d, elution of poly(A)\textsuperscript{+} after stringent washing; e, RNA fraction unbound by oligo(dT)-cellulose after prehybridization with poly(U); f, elution of material bound by oligo(dT)-cellulose after prehybridization with poly(U). H4 subspecies 1–3 are indicated.](image)
FIG. 3. Demonstration of linearity of autoradiograms of RNA blot hybridization. Progressive dilutions of homologous plasmid DNA (pFO108A) (B) or L6 myoblast poly(A)+ RNA (C) were loaded onto gels and subjected to RNA blot-hybridization analysis and autoradiography. The x-ray films were scanned, and bands were quantitated by using a microdensitometer. The relative density on the x-ray films was plotted against mass of input nucleic acid (A), showing homologous plasmid DNA (C) and poly(A)+ RNA from myoblasts with a greater (●) or lesser (△) content of H4 mRNA per µg.

To quantitate the levels of histone mRNA from autoradiograms of RNA blots, it was necessary to establish the range over which the density observed was linearly proportional to mass of input nucleic acid. Increasing amounts of homologous plasmid DNA (containing approximately 6% homologous sequence by weight) or total poly(A)+ RNA from L6 cells was analyzed (Fig. 3). Density on the autoradiograms, for both DNA and RNA, was found to be linear up to the equivalent of 30 pg of homologous plasmid and nonlinear above this value as the film became saturated. Lighter exposures, used to quantitate heavy bands, resulted in lower apparent threshold values, observed as a lower intercept value between the curve and the axis. Longer exposures resulted in higher background and higher intercept values.

Only nonsaturated films were used for quantitation. Within any given experiment and for any given exposure producing a nonsaturated image, the density was proportional to the mass of homologous sequence present.

The levels of the poly(A)+ H4 mRNA subspecies during growth and after differentiation to myotubes were determined to assess whether its production was coordinately regulated with all of the histone H4 subspecies found in total polysomal RNA. We previously have shown a coordinate decrease of >95% in the amount of all subspecies of histone mRNA after myoblast differentiation (Fig. 4 and unpublished data). In contrast, the amount of poly(A)+ H4 mRNA found in myotubes was reduced by only 70% (Fig. 4). Since <5% of the nuclei in differentiated myotubes remain unfused and the

FIG. 4. The level of poly(A)+ (Right) and poly(A)- (Left) histone H4 mRNAs in myoblast and myotubes. Poly(A)+ and poly(A)- polysomal RNAs, prepared from myoblasts (MB) and 4-day myotubes (MT), were compared by electrophoresis on a 6% polyacrylamide gel (8.3 M urea), followed by electroblotting and probing for H4 sequences. Histone H4 mRNA subspecies 1–3 are noted. Only H4 subspecies 1 is apparent in poly(A)+ fractions. A 70% (±6% maximum sample variation) reduction in the amount of poly(A)+ H4 mRNA accompanied differentiation, whereas poly(A)- H4 mRNAs were reduced by more than 95% (±3% maximum sample variation).
poly(A)− histone mRNA subspecies were virtually undetectable, the disproportionately large amount of poly(A)− H4 mRNA detected is likely a characteristic of L6 myotubes.

The half-life of the poly(A)− H4 mRNA was measured in myoblasts, myotubes, and after inhibition of DNA synthesis to determine whether this mRNA behaved similarly to poly(A)− histone mRNAs in proliferating myoblasts (Fig. 5). In both cases poly(A)− H4 mRNA exhibited similar half-lives to poly(A)− histone mRNAs (unpublished data). After inhibition of transcription, the half-life of poly(A)− H4 mRNA was about 37–42 min (compared to 38–40 min for poly(A)− histone mRNAs). After inhibition of DNA synthesis, the half-life of poly(A)− H4 mRNA decreased to about 10–15 min after a lag of approximately 15 min (compared to 10–13 min for poly(A)− histone mRNAs) and, thus, displayed the same behavior as other poly(A)− histone mRNAs in the presence of inhibitors of DNA synthesis. The reduction in H4 mRNA half-life observed after inhibition of DNA synthesis has been reported by others for other histone mRNAs (20, 21).

The subcellular distribution of H4 mRNAs was measured in L6 myoblasts and myotubes to determine whether the poly(A)− H4 mRNA was distributed between the polysomal and free (postpolysomal) RNP compartments in ratios comparable to poly(A)− H4 mRNAs (Fig. 6). In myoblasts approximately 20% of the poly(A)− H4 mRNA was found in the free mRNA-protein complex (mRNP) fraction and 80% in the polysomal compartment. All of this detected poly(A)− H4 mRNA subspecies were present in the two subcellular compartments. It is interesting to note, however, that the proportion of poly(A)− H4 subspecies 2, compared to subspecies 1 and 3, was higher in the polysomal fraction than in the free mRNP fraction. In contrast to poly(A)− H4 mRNA, <5% of the poly(A)− H4 mRNA in myoblasts was localized in the free mRNP compartment, and the remaining 95% was localized in the polysomes. In terminally differentiated myotubes, on the other hand, approximately 20% of the poly(A)− H4 mRNA was present in the free mRNP fraction and 80% was present within the polysomal fraction. Thus, the subcellular distribution of poly(A)− H4 mRNA in myotubes was similar to that observed for poly(A)− H4 mRNA in myoblasts.

In a comparative study, the distribution of ribosomal protein mRNAs was determined in the polysomal and free RNP compartment. Approximately 25–30% of the ribosomal protein mRNAs were found in the free RNP compartment, which is comparable to the levels found for poly(A)− H4 mRNAs in myoblasts (22). In myoblasts, therefore, the level of poly(A)− H4 mRNA is unusually high in the polysomal compartment. The reason for this enrichment is unclear but may reflect a more efficient recruitment of this mRNA for translation in L6 myoblasts. Since the relative amount of poly(A)− H4 mRNA in the free RNP fraction increased to approximately 20% in myotubes, the apparent need for more efficient recruitment seems to vanish after differentiation.

The identification of a discreet poly(A)− H4 mRNA was initially surprising because histone mRNAs are among the best examples of poly(A)− mRNAs (2–4, 23, 24). There is evidence for poly(A)− containing histone mRNAs in yeast and other species, including chickens, in which a polyadenylated and highly divergent embryonic histone H2A mRNA has been isolated (25) and in which all of the distantly related (non-cell-cycle-regulated) erythroid-specific histone H5 mRNAs are polyadenylated (26, 27). A human poly(A)− H3.3 mRNA also has been identified recently (28). However, L6 myoblasts are, to our knowledge, the only example of a cell containing a unique subspecies of poly(A)− containing histone H4 mRNA. The most unusual characteristic of this particular mRNA is its degree of independence after differentiation. A substantial amount of residual histone H4 mRNA remains on polysomes in nonproliferating myotubes. The poly(A)− H4 mRNA may be a unique species of mRNA tailored to a specific role during periods when the cells are not growing (and may be related to DNA repair synthesis) or it may be an evolutionary relic without a specific role. In either

**Fig. 5.** The half-life of poly(A)− H4 mRNA in myotubes after the inhibition of transcription by actinomycin D or following the inhibition of DNA synthesis by cytosine arabinoside (araC). a, Total myoblast polysomal RNA from actinomycin D-treated cells; b, poly(A)− RNA from actinomycin D-treated myoblasts; c, total polysomal RNA from myoblasts treated with araC; d, poly(A)− RNA from myoblasts treated with araC. Cells were treated with drug for 15, 30, or 60 min or were untreated (0 min). The dashed line represents the lowest limit of detection. The percentage of H4 mRNA remaining and the mean value of five independent experiments calculated (error bars represent SD) was determined at each time point. The half-life of total histone mRNAs from myoblasts was 36–40 min. The half-life of poly(A)− H4 mRNA in myotubes was 37–42 min unless DNA synthesis was inhibited, in which case it was approximately 15 min (after a 15-min lag). The half-life of total H4 mRNAs in araC-treated myoblasts was 10–13 min.

**Fig. 6.** Subcellular distribution of histone H4 mRNA in L6 myoblasts and myotubes. Polysomal and free (postpolysomal) RNP were isolated from myoblasts (lanes MB) and myotubes (lanes MT). Each was subfractionated into poly(A)− and poly(A)+ fractions and compared by electrophoresis on a denaturing 6% polyacrylamide gel, followed by electroblotting and probing for H4 sequences. Lanes: a, poly(A)− polysomal RNA; b, poly(A)+ RNA isolated from the free RNP compartment; c, poly(A)− polysomal RNA; d, poly(A)+ RNA isolated from the free RNP compartment; e, poly(A)− polysomal RNA; f, poly(A)+ RNA isolated from the free RNP compartment. H4 mRNA subspecies 1–3 are noted. No H4 mRNAs were detectable in the poly(A)+ fractions from myotubes (data not shown). Sample variation was typically less than ±6%. Only nonsaturated x-ray films were used for quantitation.
case it provides a unique tool for studying the function of polyadenylylation of individual mRNAs.

This investigation was supported by grants from the Medical Research Council, National Cancer Institute, and Muscular Dystrophy Association. R.C.B. was a recipient of a Medical Research Council Postdoctoral Fellowship, and F.A.J. was a recipient of a Muscular Dystrophy Association, Canada, Predoctoral Fellowship.