Differences in the Distribution of Poly(A) Size Classes in Individual Messenger RNAs from Neuroblastoma Cells*

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Neuroblastoma polysomal mRNAs have been fractionated by a combination of oligo(dT)-cellulose and Millipore filter techniques into four classes, each containing a different average length of poly(A). These classes are present in mRNAs isolated using different extraction techniques and they probably represent the steady state mRNA distribution in neuroblastoma cells. Quantitation of the biological activity of each fraction in a wheat germ translation system shows that typically 30% of the mRNAs contain no oligo(A) longer than 6; 4% have poly(A)s between 6 and 20 nucleotides, 22% have between 20 and 50 nucleotides, and 42% have greater than 50 nucleotides. Two-dimensional gel electrophoresis of the proteins synthesized by the unfractionated and fractionated mRNAs showed there was no mRNA degradation during the fractionation procedures. Analysis of the most abundant peptides synthesized by each RNA fraction shows that most mRNAs are found in each of the four populations. However, the distribution of some individual mRNAs varies widely. The α- and β-tubulin mRNAs are examples of those which are more abundant in the population with long poly(A)s. In contrast, a significant proportion of the mRNAs coding for actin and the R1 and R2 cAMP-binding proteins are nonadenylated. The histone mRNAs are unique in that they are more abundant in the nonadenylated mRNAs than in the mRNAs containing short stretches of poly(A). These results are consistent with a model wherein the poly(A) termini in some mRNA species are more rapidly cleaved than in others.

Intercellular mRNAs have been fractionated by affinity chromatography on oligo(dT)-cellulose or poly(U)-sepharose (1-3) into poly(A)+ mRNAs, containing a hybridizable length of poly(A) at their 3' ends, and poly(A)− mRNAs, which have no hybridizable poly(A) region (4-6). Some relatively abundant mRNAs, such as those coding for the histone mRNAs, are predominantly nonadenylated (7) and others, including the β actin mRNA, have a large poly(A)+ component (8, 9). In some cells, the only abundant nonadenylated mRNAs seem to be those for β actin and the histones (9) whereas in others there are reports of a considerable overlap between the proteins synthesized by both fractions (8, 10). However, comparison of mRNAs present at low frequencies showed that approximately 30% were nonadenylated and, in contrast to the abundant mRNA population, the nonadenylated RNAs had no sequences in common with the adenylated RNAs (8, 11). The nonadenylated RNAs comprised 30 to 40% of the sequence diversity of the total mRNA population (11). However, it has not yet been demonstrated that these RNAs are indeed functional mRNAs.

Both the origin and the function of the heterogeneity in poly(A) content remain unclear. One possibility is that the abundant poly(A)+ mRNAs found in vivo are derived from the adenylated mRNAs by cleavage of their poly(A) regions (12-16). Several studies show that deadenylated globin and histone mRNAs are degraded more rapidly than are their adenylated counterparts (17-19) and it has been proposed that adenylation is important in determining mRNA half-life. This would suggest that the rate of poly(A) processing in a particular mRNA would determine its half-life and such a relationship has indeed been shown for two viral mRNAs (20). The stochastic nature of this decay would predict, in the simplest model, that all nonadenylated mRNAs are equally unstable and that the proportion of each mRNA which is nonadenylated should be constant (20). Previous estimates of the poly(A)+ size distribution in individual mRNAs and their poly(A)− mRNA content may have been complicated by possible artefacts such as poly(A) cleavage during isolation (21-23). Further, complete separation of the poly(A)+ and poly(A)− mRNA species is necessary for a meaningful comparison of their content of individual mRNAs. In order to study the adenylated and nonadenylated mRNA populations in neuroblastoma cells, we have therefore (a) compared different isolation procedures to ensure that the poly(A)+ mRNAs are indeed present in vivo, and (b) used isolation techniques designed to decrease the cross-contamination of the adenylated and nonadenylated mRNA populations.

EXPERIMENTAL PROCEDURES

Tissue Culture of Mouse Neuroblastoma S-20 Cells—Mouse neuroblastoma S-20 cells were cultured as previously described (24). They were harvested in the mid to late logarithmic phase of growth (Day 3).

Isolation of Neuroblastoma RNAs—Solutions and glassware were autoclaved prior to use.

1) Proteins were extracted with Proteinase K and phenol-extracted as previously described (24).

2) Guanidinium chloride extraction of cytoplasmic RNAs. Cytoplasmic RNAs were isolated from the postnuclear supernatant fraction of the rat liver homogenate by guanidinium chloride extraction.
tion by three successive extractions with 6 M guanidinium chloride. 20 mM sodium acetate, and 1 mM dithiothreitol, pH 5.0 (25, 26).

3) Guanidinium chloride extraction of total cellular RNAs. Cells were lysed on the plates by the addition of 6 M guanidinium chloride. 20 mM sodium acetate, and 1 mM dithiothreitol, pH 5.0, and RNA was extracted as in Procedure 2.

**Isolation of Poly(A)-containing RNAs**—The RNAs were ethanol-precipitated and resuspended in 1 ml of 0.4 M NaCl/1% sodium dodecyl sulfate/0.01 M Tris, pH 7.4. The RNA was hybridized to oligo(dT)-cellulose and the poly(A)-containing RNA was eluted with water. This was then made 0.4 M in NaCl and applied to a 4 ml column. The poly(A)-containing RNA was again eluted with water then heated at 65°C for 5 min, quick-cooled on ice, made 0.4 M in NaCl, and repassed over oligo(dT)-cellulose (24). The 1st HS and 3rd HS fractions were rehybridized to the oligo(dT)-cellulose columns to remove contamination with residual poly(A)-containing mRNAs.

**Millipore Binding of RNAs**—Poly(A)-containing mRNAs were fractionated on Millipore filters as previously described (13, 27).

**In Vitro Translation**—RNA samples were translated in the wheat germ cell-free system as previously described (24) except that 0.3 mM spermidine and varying inputs of [55S]methionine (50 Ci/mmol, New England Nuclear) were used. 3rd HzO poly(A)+ mRNA (0.5 pg), 1 pg each of 1st HS poly(A)− mRNA or unfractionated RNA were used in a final volume of 50 ml. These were sub saturating levels for each of the mRNA samples. The mRNA-dependent wheat germ lysate was prepared similarly to the reticulocyte lysate (28). Aliquots of the wheat germ lysate were made 1 mM in CaCl₂ and were digested with 10 μg/ml micrococcal nuclease for 15 min at 20°C. The digested lysates were made 4 mM in EGTA and translation of exogenous mRNA was carried out as previously described (24). The final Mg²⁺ concentration was 1.8 mM in these cell-free systems.

**Protein Gel Electrophoresis**—Two-dimensional gels were run as previously described (29, 30). Equal amounts of trichloroacetic acid-precipitable counts and unlabelled wheat germ cell-free system up to a maximum volume of 40 μl were loaded on each gel. The sample volume was made to 100 μl with lysis buffer. Unlabelled whole cell neuroblastoma proteins were prepared as described by Prashad et al. (30). Non-equilibrium two-dimensional gels were run as previously described (31). Electrophoresis in the first dimension was for 4 h at 400 mV. Gels were stained, fluorographed, dried down, and exposed to Kodak X-Omat R films for varying lengths of time (39).

**Isolation of Radiolabeled RNAs**—Neuroblastoma S-20 cells were labeled for 18 h with 3 μCi/ml [3H]adenosine (35.19 Ci/mmol; New England Nuclear). Polysomal RNAs were isolated by the Proteinase K procedure and fractionation of the RNAs was as for the unlabeled RNAs.

**Analysis of Poly(A) Regions**—RNAs were digested with T₁ and pancreatic ribonucleases as previously described (13). The poly(A) regions were isolated by hybridization to oligo(dT)-cellulose either at room temperature or at 4°C and their size distribution was determined by electrophoresis on sodium dodecyl sulfate-12% polyacrylamide gels, using poly(A)s of defined sizes as internal molecular weight standards (25). The gels were scanned at 280 nm then sliced. Slides were incubated in 0.4 ml of 5% p-aminosalicylic acid (Native Gel); 1 part H₂O; 10 parts toluene for 90 min at 40°C, frozen for 1 h, then counted in 10 ml of toluene-based scintillation fluid (34).

**Materials**—Pancreatic and micrococcal nucleases were obtained from Worthington Biochemical and the T₁ RNAse from Sigma Chemical Company. Proteinase K was obtained from Beckman Chemicals and the oligo(dT)-cellulose (T-3) was from Collaborative Research, Inc. All other biochemical standards and (A)₀, (A)₁, (A)₂, (A)₃ and (A)₄ were obtained from Miles Biochemicals. The oligo(a) standards, (A)₁, (A)₂, (A)₃ and (A)₄ were obtained from Collaborative Research. Histone standards H₁, H₂, and H₄ were obtained from Boehringer Mannheim.

**RESULTS**

**Isolation, Fractionation, and Quantitation of Underdegraded mRNAs**—RNA isolated by Procedure 1 was initially used for fractionation into poly(A⁺) and poly(A⁻) RNA populations. Two passages over oligo(dT)-cellulose were insufficient to remove a final 30% contamination of nonadenylated mRNAs in the poly(A⁺)-enriched RNA fraction and this was not removed by a third oligo(dT)-cellulose column. However, a heating step prior to the 3rd oligo(dT)-cellulose purification removed most of the poly(A⁻) RNA (Ref. 24 and Table 1).

The poly(A⁻) RNA fraction is at least 10-fold-enriched in translatable mRNAs compared with the first high salt fraction, showing that poly(A⁻) mRNAs are selectively retained during the hybridization procedure compared to the ribosomal RNAs. The mRNAs have no hybridizable poly(A) regions as judged by hybridization to [3H]poly(U) or by RNase digestion of [3H]adenosine-labeled RNAs (results not shown).

We routinely observed a loss of 10 to 30% of the biological activity when the activity of the oligo(dT)-fractionated RNA was compared with the activity of the un fractionated RNAs. This was partly due to a 40 to 70% potentiation of overall mRNA translation by the ribosomal RNAs (35, 36). This potentiation did not change the relative translation of different mRNAs. Comparison of the total counts synthesized by each column fraction showed that between 25 and 36% of the total biological activity was contributed by the 1st HS and 3rd HS nonadenylated mRNA fractions (Table 1).

In order to prove that the poly(A⁺) mRNAs were not derived from the poly(A⁻) mRNAs by specific cleavage of the poly(A) regions either during the isolation of polysomes or during the phenol extraction steps, we compared the Proteinase K-isolated mRNA with a guanidinium chloride protocol. This concurrently inactivates ribonucleases and separates the RNA from proteins and DNA, thus making it possible to isolate intact mRNAs even from tissues high in ribonuclease (25). The recovery of poly(A⁺) cytoplasmic neuroblastoma mRNAs was identical to that found in the Proteinase K procedure and between 25 and 36% of the biological activity was still present in the poly(A⁻) fraction (result not shown). The nonadenylated mRNAs, therefore, were not derived from the polyadenylated RNAs as a function of the Proteinase K isolation procedure. As a further check on putative conversion of poly(A⁺) mRNAs to poly(A⁻) mRNAs, [3H]adenosine-labeled poly(A) containing mRNAs were isolated, added to unlabeled cells, and the cytoplasmic RNAs were isolated by extraction with guanidinium chloride. The poly(A⁺) mRNAs were then isolated on oligo(dT)-cellulose columns and the recovery of counts was monitored in the various fractions.

Five per cent of the counts were lost during the first hybridization and 2% were lost on each of the subsequent hybridizations, thus giving an overall loss of 9%. There is therefore little, if any, conversion of polyadenylated to nonadenylated mRNAs during the isolation of cytoplasmic RNAs with gua-

### Table I

| RNA fraction | Eₙ₀ units/ fraction | Biological specificity (cpm/μg) | Biological activity/ fraction |
|--------------|---------------------|-------------------|-----------------|
| Unfractionated RNA | 100 | 3,937 | 100 |
| 1st HS + 2nd HS | 96.7 | 775 | 10 |
| 3rd HS | 1.1 | 23,223 | 6 |
| 3rd H₂O | 2.2 | 117,020 | 64 |

* M. R. Morrison, unpublished results.
Comparison of Proteins Synthesized by Poly(A)$^+$ and Poly(A)$^-$ mRNAs—Translation of the poly(A)$^+$ and poly(A)$^-$ mRNAs in a wheat germ in vitro protein-synthesizing system was used to compare the more abundant mRNAs in each population. Although the cell-free system has relatively low endogenous mRNA activity, some proteins synthesized by the wheat germ mRNAs could be detected on one- and two-dimensional gels after longer exposure times. This was especially true for the proteins synthesized by neuroblastoma mRNAs with relatively low specific activity such as the 1st and 3rd high salt poly(A)$^-$ fractions. Nuclease treatment of the wheat germ extracts prior to addition of exogenous RNAs lowered the background to almost zero while only reducing the efficiency of mRNA translation by 10 to 30%. Most of the remaining background counts were not due to synthesized proteins as (a) they were present even at zero time, and (b) all the endogenous proteins labeled in the undigested blanks were no longer present in the digested samples.

In order to compare the most abundant mRNAs present in each fraction, we resolved the proteins they synthesized in
proteins from two-dimensional gels showed that identical
many proteins which co-migrate with authentic unlabeled
the unfractionated and the fractionated mRNAs synthesized
of the unfractionated mRNA fractions. Both
unfractionated and the fractionated mRNAs synthesized
many proteins which co-migrate with authentic unlabeled
fractions. These proteins are numbered in Fig. 1A with proteins
increased in the poly(A)+ fraction being underlined.
The 3rd HS poly(A)+ mRNAs might be a distinct subclass of the poly(A)+ mRNA population with a particular affinity for the poly(A)+ mRNAs. It does have some quantitative and qualitative differences from the 1st HS poly(A)+ population and might, for example, contain some of the poly(A)+ mRNAs with short poly(U) regions (37). It is also possible that it results merely from nonspecific co-aggregation of RNAs during the isolation procedure (38).

Proteins Synthesized from mRNAs with Different Lengths of Poly(A) Tail—If the abundant poly(A)+ mRNAs were derived from poly(A)+ mRNAs by gradual cleavage of their poly(A) regions, it might be expected that those with short poly(A) regions would be enriched in the mRNAs for actin, the cAMP-binding proteins, and the other mRNAs which were most abundant in the poly(A)+ mRNA fractions. In order to investigate this possibility, we fractionated the adenylated mRNAs by passing both a radiolabeled and nonradiolabeled poly(A)+ mRNA sample separately over Millipore filters under conditions where mRNAs with long poly(A) regions were bound to the filters and those with short poly(A)s pass through. Table II shows that 25 to 27% of both mRNA samples did not bind to the filters. The radiolabeled Millipore-unbound mRNAs had a lower percentage of counts in their poly(A) regions. Suggesting that the filters had fractionated the mRNAs according to their content of poly(A). This was confirmed by hybridization of the unbound mRNAs to [H]-poly(U) (the M-B RNAs hybridized twice as much [H]-poly(U) as did the M-UB RNAs) and by gel electrophoresis of the radiolabeled poly(A)s (Fig. 2). The migration of the radiolabeled poly(A) regions on the gels was compared to poly(A) standards containing 6, 16, 33, 54, and 120 nucleotides. As was previously reported, the poly(A)s migrate anomalously relative to 4 S and 5 S RNAs on these nonnondenaturing gels but the migration of the poly(A) standards was linear with respect to another (33). The molecular weight profiles were corrected for moles of poly(A) in each size range and also for their logarithmic migration on the gels (16) and the results were expressed as a percentage of the total moles of poly(A) in each mRNA fraction. Fig. 2A shows the size distribution of the Millipore-bound poly(A)s. Eighty-eight per cent had a poly(A) size greater than 50, with an average size of 85 to 95 nucleotides. Fig. 2B shows the size distribution of the Millipore-unbound poly(A)s. Seventy-six per cent were smaller than (A)30, with an average size of 40 nucleotides.

Fig. 3 shows the two-dimensional profiles of proteins synthesized by the Millipore-bound mRNAs (A) and by the Millipore-unbound mRNAs (B). The pattern of proteins synthesized from mRNAs with different lengths of poly(A) tails was analyzed by the method described under "Experimental Procedures." The radiolabeled mRNAs were isolated from cells labeled with 3 μCi/ml (3H)adenosine for 18 h. Both the labeled and unlabeled 3rd H2O poly(A)+ mRNAs were fractionated on Millipore filters as described under "Experimental Procedures." The unlabeled mRNAs were translated in the wheat germ system as described under "Experimental Procedures" using 28 μCi [35S]methionine in a 50-μl cell-free system. The labeled mRNAs were digested and the poly(A) regions isolated as described under "Experimental Procedures."
the water poly(A) in each size class: and for the logarithmic relationship between molecular weight and "Experimental Procedures." The molar size distribution was found by they were sliced into 2-mm slices, and counted as described under "Experimental Procedures." The gels were electrophoresed for 20 min at 1 mA/gel, then for 2 h at 4 mA/gel. After scanning the gels at 290 nm, they were sliced into 2-mm slices, and counted as described under "Experimental Procedures." The molar size distribution was found by correcting for the average poly(A) length in each group of gel slices and for the logarithmic relationship between molecular weight and mobility (10). The results are expressed as percentage of the total poly(A) in each size class: A, Millipore-bound mRNAs; B, Millipore-unbound mRNAs; C, oligo(dT)-cellulose 4°C H2O mRNAs.

thesized by both mRNA fractions was similar to those synthesized by the total poly(A)+ mRNA population (compare to Fig. 1B). More than 97% of the proteins synthesized by the mRNAs with longer poly(A)s were also synthesized by the Millipore-unbound mRNAs (Fig. 3, A and B) and most were also quantitatively similar. However, 13 of the major proteins were relatively increased in the Millipore-bound fraction (numbered in Fig. 3A) and 18 were relatively increased in the Millipore-unbound fraction (numbered in Fig. 3B). Proteins also increased in the total poly(A)+ mRNA fraction (Fig. 1A) are underlined. A quantitative analysis of whether these changes could be correlated with the proteins synthesized in different amounts by the poly(A)+ and poly(A)− mRNA populations is shown in Table III. Only seven of the mRNAs which were increased in the mRNA fraction with short poly(A)s (M-UB) were also more abundant in the poly(A)+ fraction compared to the total poly(A)+ mRNA fraction. Only four of the mRNAs which were increased in the mRNA fraction with long poly(A)s (M-B) were also more abundant in the total poly(A)+ mRNA fraction compared to the poly(A)− mRNA fraction. It would therefore seem that there is little correlation between the relative proportions of different mRNAs in the mRNA fraction with short poly(A) tails and the mRNA fraction which has no detectable poly(A) region.

There were variable estimates of the minimum poly(A) length necessary for polyadenylated mRNAs to be retained on oligo(dT)-cellulose (18, 39). Under our experimental conditions, poly(A)s containing 16 nucleotides are quantitatively retained at room temperature and those with 6 nucleotides at 4°C. These differential fractionations permitted the isolation of an mRNA fraction containing short poly(A) regions from the 1st HS poly(A)− mRNAs by further hybridizing these RNAs to oligo(dT)-cellulose at 4°C. Table IV shows that about 10% of the activity of the 1st HS fraction was removed after its passage over oligo(dT)-cellulose at 4°C. Eight-tenths per cent of the RNA was hybridized and this contained 5% of the biological activity of the original samples. The hybridized RNA showed a 10-fold enrichment for biologically active mRNAs. In order to determine the size of the RNase-resistant region in the mRNAs hybridizing to oligo(dT)-cellulose at 4°C, the 1st HS RNA fraction was isolated from cells labeled with [3H]adenosine for 18 h. It was passed a second time over oligo(dT)-cellulose at room temperature then over oligo(dT)-cellulose at 4°C. The fractions at room temperature, 4°C H2O, and 4°C HS were digested as described under "Experimental Procedures" and the resistant regions were isolated by hybridization to oligo(dT)-cellulose at 4°C. The poly(A) regions isolated at room temperature showed the usual heterogeneous profile. However, there were no counts migrating below (A)25

![Fig. 2. Size distribution of poly(A)s isolated from mRNAs labeled in vivo for 18 h with 3 μCi/ml [3H]adenosine and fractionated according to poly(A) length. The radiolabeled Millipore-bound and Millipore-unbound mRNAs (Table II) and the water fraction recovered from passing a radiolabeled 1st HS fraction over oligo(dT)-cellulose at 4°C (Table IV) were digested as described under "Experimental Procedures." The ribonuclease-resistant counts from the Millipore fractions were isolated on oligo(dT)-cellulose at room temperature; the ribonuclease-resistant counts from the 4°C H2O fraction were isolated on oligo(dT)-cellulose at 4°C. The poly(A)s were co-electrophoresed on 12% polyacrylamide gels with unlabeled poly(A) standards and 4 S and 5 S RNAs as described under "Experimental Procedures." The gels were electrophoresed for 20 min at 1 mA/gel, then for 2 h at 4 mA/gel. After scanning the gels at 290 nm, they were sliced into 2-mm slices, and counted as described under "Experimental Procedures." The molar size distribution was found by correcting for the average poly(A) length in each group of gel slices and for the logarithmic relationship between molecular weight and mobility (10). The results are expressed as percentage of the total poly(A) in each size class: A, Millipore-bound mRNAs; B, Millipore-unbound mRNAs; C, oligo(dT)-cellulose 4°C H2O mRNAs.

![Fig. 3. Fluorographs of two-dimensional gel electrophoresis of [35S]methionine-labeled proteins synthesized by Millipore-bound (A) and Millipore-unbound (B) mRNAs in predigested wheat germ cell-free synthesizing systems. Six hundred thousand trichloroacetic acid-precipitable counts per min in 40 μl of the wheat germ cell-free system were loaded on each gel. Conditions were as in the legend to Fig. 2. The gels were exposed for 2 days. Numbered proteins are those relatively increased in amount in the Millipore-bound (A), or Millipore-unbound (B), populations. Those underlined co-migrate with those increased in amount in the 3rd H2O poly(A)+ fraction in Fig. 2B. Numbers corresponding to those in Fig. 2B are those proteins relatively increased in amount in the poly(A)− fraction in Fig. 2B. A, proteins synthesized by Millipore-bound mRNAs; B, proteins synthesized by Millipore-unbound mRNAs.

**Table III**

| Correlation between proteins synthesized in different amounts by the different poly(A)+ mRNA populations and the poly(A)− mRNAs. | | |
| --- | --- | |
| The relative amounts of proteins synthesized by the M-B and M-UB fractions were scored using Fig. 4, A and B, respectively, and one other two-dimensional separation. Those relatively increased or decreased in poly(A)+ and poly(A)− mRNA fractions were taken from Fig. 2. | | |
| mRNA fraction | Number of proteins |
| --- | --- |
| Proteins ↑ in M-B relative to M-UB | 13 |
| Equal synthesis in poly(A)+ and poly(A)− | 7 |
| ↑ Synthesis in poly(A)+ | 2 |
| ↓ Synthesis in poly(A)+ | 4 |
| Protein ↓ in M-B relative to M-UB | 18 |
| Equal synthesis in poly(A)+ and poly(A)− | 8 |
| ↑ Synthesis in poly(A)+ | 7 |
| ↓ Synthesis in poly(A)+ | 3 |
on the oligo(dT)-cellulose at this temperature (results not shown). Fig. 2C shows that the RNAs originally isolated in the 4°C H2O-fraction had RNase-resistant regions migrating between (A)6 and (A)30 on the gels. These had an average size of 12 nucleotides and may be pure oligo(A)s or have other bases interspersed (16). They could not have originated from the ribosomal RNAs as the 4°C HS-fraction had 10-fold less RNase resistance than did the 4°C H2O-fraction (0.3% compared to 3.3% resistance) and the counts were distributed evenly over the gel.

Fluorograms of the two-dimensional profiles of proteins synthesized by these RNAs are shown in Fig. 4. The 4°C HS fraction synthesizes proteins with pI values between 4.5 and 7, and also low molecular weight basic proteins which do not enter the isoelectric focusing gel (Fig. 4B). These proteins comigrate with unlabeled histone Fractions H2 and H4 on basic, nonequilibrium isoelectric focusing gels (Fig. 4D). There is relatively less synthesis of other proteins, including actin, than in the total 1st HS sample (Fig. 1C). The 4°C H2O fraction contains a large amount of actin, and a greater proportion of the other nonhistone proteins, including tubulin, usually synthesized by the total 1st HS fraction (Fig. 4A). There are much smaller amounts of the low molecular weight basic proteins co-migrating with histones H2 and H4 (Fig. 4C). The histone mRNAs are, therefore, the only abundant mRNAs found almost exclusively in the mRNA fraction with oligo(A)s shorter than 6.

Table V shows the approximate distribution of total biolog-
The percentage biological activity in mRNAs with different lengths of poly(A)

| mRNA Fraction          | Approximate poly(A) size | Total biological activity |
|------------------------|--------------------------|---------------------------|
| Millipore bound        | 50-150                   | 42                        |
| Millipore unbound      | 20-50                    | 22                        |
| 1st HS 4°C H2O         | 6-20                     | 4                         |
| 1st HS 4°C HS          | <6                       | 32                        |

FIG. 5. Percentage synthesis of proteins synthesized by each of the 4 mRNA populations. A,△,△, Histone H4; ○, ○, Protein 6; ●, ●, Protein 1; ○, ○, Protein 14. B,△,△, Protein 1. C,△,△, Tα; ○, ○, Tβ. D,△,△, Protein 25; ○, ○, Protein 31. Fluorograms were aligned with the original gels and the parts of the gels corresponding to the selected protein spots were cut out and eluted as described under "Experimental Procedures." The counts were corrected for decay in [35S]methionine and for 100,000 trichloroacetic acid-precipitable cpm loaded on the gels. Two to three gels were used for each determination. The results are expressed as percentage of total counts present in each mRNA fraction in each sample.

Polished activity between the four mRNA classes with different lengths of poly(A). In order to better quantitate the relative proportions of some representative mRNAs in the mRNA fractions with different poly(A) contents and also their distribution between the different fractions, the protein spots from several gel runs were eluted and counted directly. Figure 5, A to D plots the percentage of nine selected proteins synthesized by each of the four mRNA populations. Histone H4 is the only one of these that is synthesized almost exclusively by mRNAs with oligo(A) regions shorter than 6 (Fig. 5A). Two other proteins, actin and Protein 14, have increasing proportions of their mRNAs in the mRNA with shorter (A)6. However, unlike the histone mRNAs, they have proportionally less in the poly(A)+ mRNA fraction compared to the (A)12 fraction (Fig. 5C). The mRNAs for Proteins 1, 2, and 6 are approximately equally distributed in the (A)6 mRNA population and in the poly(A)+ mRNA fraction although their levels are higher in the (A)12 fraction (Fig. 5B). Both α- and β-tubulins are examples of proteins whose mRNAs are more abundant in the (A)50 mRNA fraction than in the poly(A)− mRNA populations; however, α-tubulin has a higher proportion of mRNA in the (A)12 mRNA fraction than does β-tubulin (Fig. 5C). Lastly, there is a class of proteins, represented by Proteins 25 and 31, which are more abundant in the (A)50 fraction than in the (A)12 fraction and which have a much lower abundance in both the (A)12 and the poly(A)− mRNA populations (Fig. 5D).

In order to find the percentage of each mRNA translated in each fraction, the average counts incorporated into a particular protein were multiplied by the percentage of the total mRNA in that fraction (from Table V). The results are expressed as the percentage of each particular mRNA present in each fraction, we find that 97% of the histone H4 mRNAs and 50% of the actin mRNAs are poly(A)−. The actin mRNAs also have an unusually high proportion in the (A)12 fraction (16%). Thirty-five and 38% of Proteins 1 and 14 are synthesized by the poly(A)+ mRNAs, whereas only 4% of Proteins 25 and 31 are synthesized by this fraction.

DISCUSSION

Estimates of the relative number and kinds of proteins synthesized by the abundant poly(A)+ mRNAs vary widely. This may be due in part to intrinsic differences between different cell types or between different physiological states (40-43). Some of the variation, however, may be a function of experimental conditions. Regardless of the method used for isolating the RNA, we find that 20% and 30% of the biologically active neuroblastoma mRNAs have poly(A) regions of six nucleotides or less. We therefore believe that these nonadenylated mRNAs are present in vivo. Hybridization with [3H]poly(U) shows there is no contamination of the neuroblastoma nonadenylated RNA fraction by poly(A)+ mRNAs. Poly(A)+ mRNA contamination of the adenylated mRNA fraction is more difficult to detect, especially as we have shown that the absence of rRNA is no index of poly(A)+ mRNA purity. However, we efficiently remove the contaminating poly(A)+ mRNAs by the heat dissociation step. Further, comparison of less abundant proteins synthesized by different mRNA classes is made possible in our studies by loading large numbers of counts on the gels and by exposing the fluorograms for longer times.

Several groups have shown that the nonadenylated mRNA fraction is synthesized with approximately the same kinetics as is the adenylated fraction (4, 5, 44, 45). Moreover, hybridization analysis shows that most of the steady state, low frequency, nonadenylated mRNA population have different sequences from the adenylated mRNAs (8, 9). These data therefore suggest either that the transcripts of a large fraction of the infrequently transcribed DNA cistrons are never adenylated or that they are rapidly and quantitatively deadenylated. In contrast to the infrequently transcribed mRNAs, Kaufman et al. (8) have shown that some relatively abundant functional mRNAs in HeLa cells are found in both adenylated and nonadenylated forms.

Our comparison of the neuroblastoma poly(A)+ and poly(A)− mRNA fractions is limited to relatively abundant mRNAs which are efficiently translated in the wheat germ in vitro protein synthesizing system. We find that most of these proteins are synthesized by all of the mRNA fractions, although the relative synthesis of some of them varies considerably. Resolution of a further 50 to 100 proteins with PI values greater than 7.0 on nonequilibrium isoelectric focusing gels similarly shows that most are also synthesized by all the
mRNA fractions. These comparisons, therefore, show that few of these abundant neuroblastoma mRNAs are synthesized exclusively by either mRNA population. This would suggest either that most of these nonadenylated mRNAs are derived from their adenylated counterparts by poly(A) cleavage or that a varying proportion of their primary transcripts are not adenylated.

Recent kinetic analysis of the stochastic degradation of two polyadenylated viral mRNAs is indeed consistent both with their nonadenylated products being derived from adenylated precursors and with these nonadenylated mRNAs being unstable (20). The simplest model which would link poly(A) shortening and mRNA turnover predicts that at steady state there would be an equal distribution of mRNAs between the poly(A)+ and poly(A)- fractions (20). However, our results show that the steady state distribution between these fractions varies for about 13% of the neuroblastoma mRNAs, thus showing that the simplest model does not hold for these messengers.

It has been suggested that mRNAs such as those for β actin, which have a large nonadenylated component, might have a relatively short poly(A) tail added to their primary transcripts (9). However, our analysis of mRNAs with an average poly(A) size of 130 nucleotides, which are only dissociated from Millipore filters with a second water wash, shows that all of the nonhistone-translated mRNAs are present in this mRNA fraction. At least a proportion of the mRNAs coding for actin are therefore synthesized with as long a poly(A) tail as the bulk of the adenylated mRNAs.

Our analysis of the proteins synthesized by mRNAs containing different lengths of poly(A) shows that each mRNA has a heterogeneous poly(A) size distribution of between 20 and 150 nucleotides. This is qualitatively similar to that of mRNAs coding for specialized proteins such as globin and ovalbumin (13, 38). Many mRNAs such as those for actin, the R, cAMP-binding protein (45) and Proteins R, CAMP-binding protein (45) and Proteins and 6 are

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