Shortening of the Poly(A) Region of Mouse Globin Messenger RNA*

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Nucleated erythroid cells isolated from the spleens of anemic mice were used to investigate the processing of the polyadenylic acid region of globin mRNA. Cells were labeled in medium containing [3H]adenosine and transferred to media containing no radioactive precursor and incubated further in the presence or absence of actinomycin D. After various times following the transfer of the cells, globin mRNA was isolated using a combination of oligo(dT)-cellulose affinity chromatography, sucrose density centrifugation, and globin cDNA (the complementary DNA copy of globin mRNA)-cellulose affinity chromatography. The size of the poly(A) region was determined by polyacrylamide gel electrophoresis of the T1 and pancreatic RNase-resistant fragments. The prelabeled poly(A) region which initially comprises approximately 150 adenylate residues was found to become shorter with time, both in cells incubated in medium containing no radioactive precursor and in the presence of actinomycin D. After 9 h of incubation in the presence of actinomycin D, two major size classes of poly(A) were observed, one containing 35 to 45 adenylic acid residues and the other containing 55 to 65 residues. These two size classes are similar to those found in circulating reticulocytes suggesting that the poly(A) shortening observed in these cell incubation studies is similar to that which occurs in vivo.

Two protein synthesis inhibitors, emetine and cycloheximide, were investigated with respect to their effect on poly(A) shortening. Neither drug inhibited the shortening of the poly(A) region of globin mRNA, suggesting that protein synthesis is not required for this process to occur.

Most eukaryotic mRNAs contain a poly(A) region at the 3' terminus, whose function, mechanism of addition, and removal is not well understood. Shortening of the poly(A) region of mRNA has been reported in mouse sarcoma 180 ascites cells (1), L-cells (2, 3), and HeLa cells (4, 5). In addition, Lim and Canellakis (6) observed that the poly(A) region of rabbit globin mRNA was shorter on smaller polysomes than on larger ones. They assumed that the smaller polysomes contained older mRNA and postulated that shortening of the poly(A) region of globin mRNA was occurring with time.

Recently, we reported the presence of several size classes of poly(A) in mouse reticulocyte globin mRNA. Two major size classes containing 35 to 45 and 55 to 65 residues were observed along with a minor component containing 75 to 120 adenylate residues (7). Because reticulocytes contain no nuclei and do not synthesize RNA, all of the RNA isolated from these cells was made at an earlier erythroid stage. Utilizing nucleated erythroid cells from the spleens of anemic mice, which actively synthesize globin mRNA, the poly(A) region of newly synthesized globin mRNA was found to contain 150 adenylate residues (8). Based on these observations, it was proposed that at least 150 adenylate residues are added to the globin mRNA soon after its synthesis and the length of the poly(A) region is shortened with time.

In the present study we confirm this hypothesis by following the fate of the poly(A) region of prelabeled globin mRNA using a nucleated erythroid cell incubation system. In addition, the effect on the poly(A) shortening process of inhibiting protein synthesis is examined.

**EXPERIMENTAL PROCEDURE**

Isolation of Globin mRNA from Nucleated Erythroid Cells—Mouse 9 S poly(A)-containing RNA was isolated from nucleated erythroid cells obtained from the spleens of anemic mice as previously described (8). The nucleated erythroid cells were suspended at $5 \times 10^7$ cells/ml in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum, 2.0 mM glutamine, 1.0 mM sodium pyruvate, 100 units/ml of penicillin, and 100 $\mu$g/ml of streptomycin. In addition, 10 ml of 100 mM nonessential amino acid solution (Gibco) was added to each liter of medium. The cells were incubated for 1 h in the presence of 10 $\mu$Ci/ml of [3H]adenosine at 37° in 5% CO2 in air. The cells were collected by centrifugation and resuspended in the same medium without label. In some instances, actinomycin D (40 $\mu$g/ml), emetine (25 $\mu$g/ml), or cycloheximide (25 $\mu$g/ml) was added and the incubation continued for various times. After incubation, total RNA was isolated by phenol:chloroform:isoamyl alcohol extraction as described (8), and the 9 S poly(A)-containing RNA was isolated by a combination of oligo(dT)-cellulose affinity chromatography and sucrose gradient centrifugation (8). Globin mRNA was separated from the non-globin 9 S poly(A) containing RNA...
The running buffer was 0.2% SDS, 1 by globin cDNA'-cellulose affinity chromatography (Ref. 9 and Footnote 2). Poly(A)-containing 9 S RNA was hybridized to 0.2 ml of globin cDNA-cellulose in a column at 65° for 30 min in Tris/NaCl/EDTA/ SDS at 65° and the bound fraction eluted with H2O at the same temperature.

Ribonuclease Digestion—Globin mRNA was dissolved in 0.3 ml of 0.3 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 2 mM EDTA containing 2 units each of RNase A and T1 (5). The digestion was carried out for 30 min at 37°. The resistant poly(A) fragments were isolated from the total digestion mixture using oligo(dT)-cellulose affinity chromatography. The RNase digestion mixture was passed over an oligo(dT)-cellulose column which had been equilibrated with 0.1 M NaCl and 0.01 M Tris-HCl (pH 7.5). The unbound non-poly(A) fragments and nucleotides were then eluted with 0.25 M NaHCO3, following which the bound poly(A) fragments were eluted with H2O. The solution containing the bound fragments was then lyophilized.

Polyacrylamide Gel Electrophoresis—Twelve percent polyacrylamide gel electrophoresis was carried out according to Morrison et al. (10). The running buffer was 0.2% SDS, 1 mM EDTA, 30 mM NaHPO4, and 30 mM Tris-HCl (pH 8.0). Samples were applied in 1% SDS and run initially for 15 min at 1 mA/gel and then at 5 mA/gel for 5 h. After electrophoresis the gels were sliced, treated with 10% piperidine, and dried at 55° overnight. Samples were counted in 4 ml of H2O and 10 mM of 3H0 (Research Products International). Mouse reticulocyte 4 S and 5 S RNAs were run as markers on parallel gels.

RESULTS

Poly(A) Shortening in Absence of RNA Synthesis Inhibitors—To examine the processing of the poly(A) region of globin mRNA, cells were incubated for 1 h in the presence of [3H]adenosine and then transferred to media containing no label. As previously reported, after a 1-h incubation the poly(A) region of globin mRNA was 150 residues long (Fig. 1a1) (8). Following further incubation for either 6 or 9 h the globin mRNA was isolated and the size of the poly(A) region determined. As shown in Fig. 1, a2 and a3, the poly(A) region after 6 or 9 h was smaller and more heterogeneous.

Although these data were consistent with the initial synthesis of a relatively long and homogeneous poly(A) region which becomes shorter and more heterogeneous with time, it was possible that after nucleated erythroid cells have been cultured for several hours, shorter poly(A) regions were synthesized. In order to determine if this were the case, cells were incubated in unlabeled media for 5 h and then resuspended in media containing [3H]adenosine and incubated an additional hour before the RNA was isolated. The poly(A) size of the radioactive globin mRNA was determined. The results are shown in Fig. 2. It is apparent that the initial size of the poly(A) region is independent of the time the cells have been in culture. Thus, the results shown in Fig. 1a suggest that shortening of the poly(A) region occurs with time.

Shortening of Poly(A) Region in Presence of Actinomycin—Another method for demonstrating that short poly(A) regions arise as a result of degradation of longer poly(A) sequences was to follow the fate of labeled poly(A) in the absence of de novo RNA synthesis. Experiments were performed in which cells labeled for 1 h with [3H]adenosine were resuspended in unlabeled media containing 40 μg/ml of actinomycin D. This concentration of actinomycin D was found to inhibit 95% of RNA synthesis in the nucleated erythroid cells. The cells were then incubated for either 6 or 9 h, the globin mRNA isolated, and the size of the poly(A) region analyzed. The results of this experiment are shown in Fig. 1b. The data clearly show a decrease in the poly(A) size in the absence of RNA synthesis. A comparison of the size of the poly(A) region of globin mRNA isolated from cells incubated either in the presence or absence of actinomycin D (for example, compare Figs. 1, a2 and b2, and 1, a3 and b3) shows that less heterogeneity occurs in the presence of the inhibitor. In the absence of actinomycin, the incorporation of radioactive adenosine into globin mRNA continued until the labeled nucleotide pool was depleted (11). Therefore, the presence of both more and longer poly(A) regions from cells incubated in the absence of actinomycin is expected. This prediction is confirmed with the 6-h incubation (Fig. 1a2) when compared to Fig. 1b2 which shows the poly(A) profile obtained after incubation in the presence of actinomycin.

It is important to note that the poly(A) size classes observed after 9 h in actinomycin D (Fig. 1b3) were similar to those we reported for globin mRNA isolated from reticulocytes (7). This further suggests that the poly(A) region of globin mRNA is shortened to give discrete size classes as previously suggested (12).

The Effect of Inhibition of Protein Synthesis on Poly(A) Shortening—It has been suggested that the size of the poly(A) region of mRNA controls messenger stability (13, 14). This raises the possibility that the half-life of a specific mRNA might be controlled by the number of times that it is translated. It was, therefore, of interest to determine if poly(A) shortening continued in the absence of protein synthesis.

The data of Table I show that protein synthesis in nucleated erythrocytes was inhibited greater than 93% at concentrations of cycloheximide or emetine above 5 μg/ml. Spleen cells were incubated in media containing [3H]adenosine for 1 h and then resuspended in unlabeled media containing either no drug (Fig. 3a), cycloheximide (Fig. 3b), or emetine (Fig. 3c). After 3, 6, and 9 h of incubation the cells were collected and the globin mRNA isolated. No significant difference in poly(A) size was

1 The abbreviations used are: SDS, sodium dodecyl sulfate; globin cDNA, complementary DNA copy of globin mRNA.
2 T. C. Wood, manuscript in preparation.

Fig. 1. SDS-polyacrylamide gel analysis of the poly(A) fragments of globin mRNA. Nucleated erythrocytes were labeled in culture for 1 h with 10 μCi/ml of [3H]adenosine and resuspended in unlabeled medium either with or without actinomycin D and the incubation continued for the times indicated. The globin mRNA was purified and the poly(A) region isolated. Approximately 40,000 cpm of poly(A) were loaded onto each gel. After electrophoresis the radioactive profile were normalized so that each pattern represents the poly(A) from an equal number of cells. Poly(A) size after 1 h in radioactive precursor (a1 and b1); poly(A) size after 1 h in radioactive precursor and 6 (a2) and 9 (a3) h in unlabeled media; poly(A) size after 1 h in radioactive precursor and 6 (b2) and 9 (b3) h in unlabeled media containing 40 μg/ml of actinomycin D.
of protein synthesis inhibited poly(A) shortening, a size similar to the initial poly(A) size, i.e. that observed after a 1-h incubation, whether the cells were incubated in the presence or absence of the protein synthesis inhibitors. Had the inhibition incubation should have been found.

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\begin{array}{cccc}
\text{Fraction} & 15 & 30 & 45 \\
\text{counts per minute} & 15 & 30 & 45 \\
\text{difference} & 15 & 30 & 45 \\
\text{percent inhibition} & 56 & 93 & 97
\end{array}
\]

The ability to study the synthesis and the fate of a particular mRNA requires a system which actively synthesizes mRNA, can be easily manipulated, and from which the mRNA being studied can be prepared in a highly purified form. These criteria are met by the erythroid cells isolated from the epiplasm of anemic mice. They actively synthesize globin mRNA when incubated in tissue culture media and globin mRNA can be isolated in highly purified form using globin cDNA-cellulose affinity chromatography. Globin mRNA labeled for 1 h contains a poly(A) region of approximately 150 adenylate residues. Upon further incubation in unlabeled media or in the presence of actinomycin D, the poly(A) region becomes shorter. Although shortening is observed without the use of actinomycin D, continued incorporation of labeled adenine from the cellular pool does not formally allow one to rule out the possibility that shorter poly(A)s are being synthesized after the cells have been cultured for several hours. However, this does not appear to be the case as only large poly(A) regions are synthesized after 5 h of preincubation in medium containing no radioactive precursor. In addition, when further RNA synthesis is inhibited by actinomycin D, the same shortening process is observed. It is important to note that the size distribution of poly(A) observed after incubation in the presence of actinomycin D is similar to that previously reported for the globin mRNA isolated from mouse reticulocytes. In both cases, two major size classes of poly(A) exist, one approximately 35 to 45 nucleotides long and the other 55 to 65 nucleotides long (Fig. 1b2).

The studies described here confirm the shortening process proposed earlier using in vivo labeling of globin mRNA (12). Although smaller size classes of poly(A) appeared with increasing times of in vivo labeling, these studies were limited because of the time required for reticulocyte maturation. Following the injection of \[^{32}P\] into anemic animals, at least 5 h are required before reticulocytes containing radioactive globin mRNA appear in the circulation (7). Therefore, neither the initial size of the poly(A) region nor shortening at early times after synthesis could be followed.

Shehness et al. (5) have reported that the poly(A) shortening of mRNA from HeLa cells is diminished by the inhibition of protein biosynthesis in these cells. Our studies do not confirm...
this finding for the poly(A) region of globin mRNA. The shortening was the same regardless of whether the cells were incubated in the presence or absence of cycloheximide or emetine.

Poly(A) shortening has been implicated in mRNA turnover by the finding that mRNAs with shorter poly(A) regions do not continue to be translated as long a time in cell-free systems (13, 14) or when injected into oocytes (15) as mRNAs with longer poly(A) regions. Although the studies reported here provide little information on the role of poly(A) in the regulation of globin mRNA half-life, they do indicate that translation is not obligatory for poly(A) shortening. A corollary to this is that if poly(A) shortening regulates or controls mRNA turnover, then translation does not.

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