Translation of Mouse Globin Messenger Ribonucleic Acid from Which the Poly(adenylic acid) Sequence Has Been Removed†

Robert Williamson,* Jennifer Crossley, and Stephen Humphries

ABSTRACT: Mouse globin mRNA was isolated using oligo(dT)-cellulose and treated with *Micrococcus* polynucleotide phosphorylase to remove nucleotides from the 3′ end. The material which no longer bound to oligo(dT)-cellulose was then separated from that which still bound. The molecular weight of the major peak of the nonbinding mRNA was reduced from 220,000 to 185,000, as calculated from mobility in polyacrylamide gel electrophoresis. The treated mRNA which still bound to oligo(dT)-cellulose migrated at the same mobility as untreated globin mRNA. The treated mRNA did not function as a template for reverse transcriptase. Two-dimensional fingerprint analysis of phosphokinase-labeled fragments from a combined pancreatic T1 ribonuclease digest demonstrated the absence of any large oligo(A) fragments from the nonbound mRNA; at least 90% of the poly(A) region had been removed. The treated nonbound mRNA stimulated globin synthesis in the Krebs ascites cell system, but the nonbound mRNA was only approximately 50% as efficient as treated bound mRNA as judged by product analysis on carboxymethylcellulose columns. In the case of mouse globin mRNA the presence of a long poly(A) sequence is not required for successful translation.

Poly(adenylic acid) sequences have been demonstrated to occur at the 3′ termini of all mRNAs from higher cells (Edmonds *et al.*, 1971; Darnell *et al.*, 1971; Lee *et al.*, 1971) with the exception of histone mRNAs (Adesnik *et al.*, 1972; Adesnik and Darnell, 1972; Schochetman and Perry, 1972). Various roles may be suggested for these sequences: (a) markers for the processing of heterogeneous nuclear RNA (HnRNA) to messenger RNA (mRNA) (Edmonds *et al.*, 1971; Philipson *et al.*, 1971; Jelinek *et al.*, 1973; Darnell *et al.*, 1972); (b) transport from nucleus to cytoplasm (Philipson *et al.*, 1971); (c) regulation of mRNA half-life (Sheiness and Darnell, 1973; Sussman, 1970); and (d) binding of poly(A)-specific proteins (Kwan and Brawerman, 1972; Blobel, 1973).

Histone mRNAs contain no poly(A) sequences yet migrate to the cytoplasm and are translated (Adesnik *et al.*, 1972; Adesnik and Darnell, 1972; Schochetman and Perry, 1972; Jacobs-Lorena *et al.*, 1972). On the other hand, viral mRNAs may contain poly(A) sequences although synthesized in the cytoplasm (Yogo and Wimmer, 1972; Johnston and Bose, 1972). We have used polynucleotide phosphorylase to remove the poly(A) sequence from mouse globin mRNA and tested the product in the Krebs ascites protein synthesis system for its ability to direct mouse globin synthesis.

Materials and Methods

Mouse reticulocyte polysomal RNA was prepared as previously described (Williamson *et al.*, 1971) and the globin mRNA was isolated using oligo(dT)-cellulose (Aviv and Leder, 1972). Oligo(dT)-cellulose was purchased from Collaborative Research, Waltham, Mass. The purity of the mRNA preparation was confirmed by 2.6% polyacrylamide gel electrophoresis and by its ability to direct mouse globin synthesis in a duck reticulocyte lysate cell-free system, both as previously described (Williamson *et al.*, 1971; Lingrel *et al.*, 1971; Lanyon *et al.*, 1972). [^{3}H]- and [^{14}C]leucine and [γ-^{32}P]-ATP were purchased from the Radiochemical Centre, Amersham.

Micrococcus lysodiekticus polynucleotide phosphorylase (EC 2.7.7.8) was purchased from Worthington Biochemicals. Mouse globin mRNA (100 μg/ml) was incubated with 500 μg/ml of polynucleotide phosphorylase in 50 mM Tris (pH 7.5)–15 mM $MgCl_2$–15 mM potassium phosphate at 37° for 7 min (Grunberg-Manago, 1963). The reaction was stopped by adding one-tenth volume of 10% sodium dodecyl sulfate and the reaction mixture was deproteinized by shaking with a half-volume of phenol–chloroform. The RNA was precipitated with ethanol, dissolved in 0.5 M NaCl–0.01 M Tris (pH 7.5)–0.1% sodium laurylsarcosine, and passed through a dT-cellulose column. The material which was not retained was collected and ethanol precipitated; the proportion of unretained RNA varied from preparation to preparation but was of the order of 80%. The size of the treated mRNA was compared with that of the original mRNA on 6% polyacrylamide gels, with added marker 4S and 5S RNA from mouse reticulocytes.

The ability of the treated nonbound mRNA to be copied by reverse transcriptase with an oligo(dT) primer was compared with that of untreated mouse globin mRNA. The enzyme was prepared and the assay carried out as described by Harrison *et al.* (1972).

Two-dimensional fingerprint analysis of treated and untreated mRNA was performed after digestion for 45 min at 37° with pancreatic and T1 ribonucleases each at 0.05 mg/ml in 10 μl of 0.01 M Tris (pH 7.4). The digestion was terminated by addition of an equal volume of water-saturated phenol–chloroform (1:1) and the aqueous phase was dried down after deproteinization. The oligonucleotide mixture was dissolved in 1% sodium dodecyl sulfate containing 0.1 mg/ml of bacterial alkaline phosphatase and incubated for 30 min at 37°. The reaction mixture was deproteinized, dried down, and then taken up in 1 μl of 0.1 M Tris, 11 mM mercaptoethanol, 11 mM $MgCl_2$, 0.27 mM [γ-^{32}P]ATP, and 2 units of polynucleotide kinase (a gift from Dr. Ken Murray, University of Edinburgh). Incubation was carried out at 37° for 45 min,

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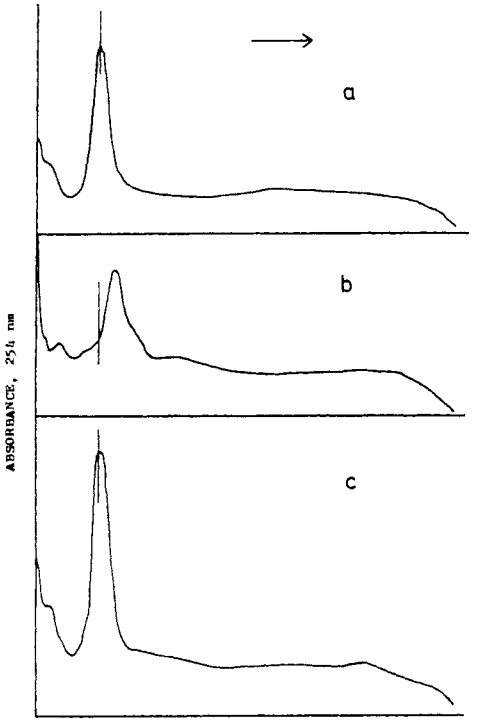

FIGURE 1: 6% polyacrylamide gel electrophoresis of globin mRNAs following incubation with polynucleotide phosphorylase. Migration from left to right; gels 12 cm long and 0.7 cm diameter; electrophoresis at 2.5 mA/gel for 30 min and then 10 mA/gel for 4 hr (Williamson *et al.*, 1971): (a) 13 μg of untreated 9S globin mRNA; (b) 13 μg of phosphorylase-treated nonbound mRNA; (c) 15 μg of phosphorylase-treated bound mRNA.

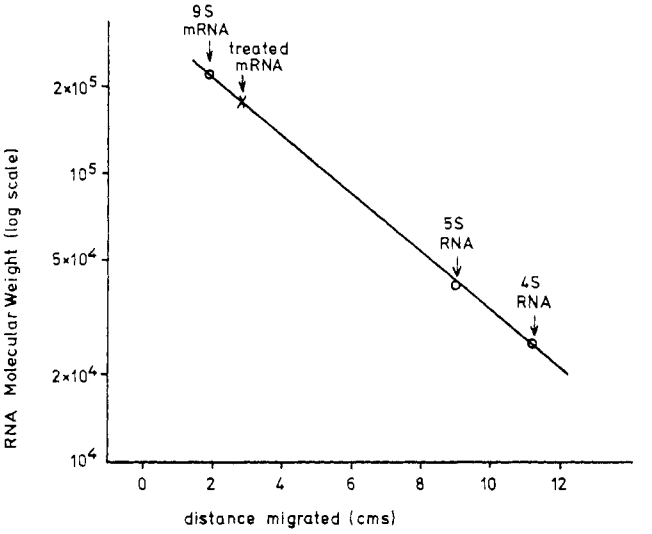

FIGURE 2: Determination of molecular weight of treated globin mRNA. The treated nonbound mRNA was run on 6% polyacrylamide gels with markers of untreated 9S globin mRNA (mol wt 2.20×10^5, Williamson *et al.*, 1971), mouse 5S RNA (mol wt 4.1×10^4, Williamson and Brownlee, 1969), and mouse 4S RNA (mol wt 2.6×10^4, Staehelin *et al.*, 1968).

the mixture was dried, and the ^{32}P-labeled oligonucleotides were taken up in 0.7 μl of dye mixture and separated by electrophoresis on cellulose acetate strips in the first dimension and by chromatography on PEI-cellulose thin-layer plates in the second dimension, as described elsewhere (Southern and Mitchell, 1971; Crossley *et al.*, 1974).

The ability of the treated mRNA to direct protein synthesis was assayed in a preincubated Krebs ascites cell-free system (Mathews and Korner, 1970; Mathews *et al.*, 1972; Mathews, 1972). The dependence of incorporation on added globin mRNA was determined by incubation for 60 min at 37° followed by 10-min digestion with alkali. Protein was precipitated with 10% CCl_3COOH after addition of 50 μg of bovine serum albumin and the precipitates were collected onto Whatman GFC glass fiber filters, washed, dried, and counted in toluene-based scintillator. Product analysis was performed on 100-μl incubation mixtures each containing 5 μg/ml of mRNA, incubated for 60 min at 37°. Carrier mouse globin was added and the protein was extracted with acid acetone at −20°, pelleted, and washed twice with acetone. The globin was then chromatographed on carboxymethylcellulose (Whatman, CM-52) using a gradient of 0.01–0.1 M disodium phosphate (pH 6.8) in 8 M urea–0.05 M mercaptoethanol. Fractions of 5 ml were collected, precipitated onto glass fiber disks, solubilized with NCS (Nuclear-Chicago Corp.), and counted in toluene-based scintillator.

Results

The profiles obtained after electrophoresis on 6% acrylamide gels for untreated mRNA, treated mRNA which does not bind to oligo(dT)-cellulose, and treated mRNA which still binds to oligo(dT)-cellulose are shown in Figure 1. The molecular weight determination for the treated unbound mRNA is shown in Figure 2.

The molecular weight of the peak of unbound phosphorylase-treated mRNA varied in five preparations between 185,000 and 200,000 (av mol wt 190,000) taking the molecular weight of the untreated mRNA as 220,000. The treated bound mRNA migrates to the same position as untreated mRNA. A second smaller peak (of av mol wt 175,000) was usually seen in the treated unbound preparations, as was small amounts of material migrating in the 4S–5S region.

9S mRNA, incubated for 7 min under phosphorolysis conditions but in the absence of polynuceotide phosphorylase, was found to be 100% retained by a dT-cellulose column. This material behaves in an exactly similar manner to the original 9S mRNA in gel and sequence analyses.

Two-dimensional fingerprints of T1 plus pancreatic RNase-treated 9S RNA, phosphorylase-treated nonbound mRNA and poly(A) are shown in Figure 3. Since pancreatic RNase hydrolyzes cyclic purine nucleotides to 3′-phosphate only slowly if at all (Markham, 1957), two series of oligonucleotide spots are seen for poly(A), corresponding to $(pA)_n$-p and $(pA)_n$-OH. For 9S globin mRNA, the proportion of the total radioactivity found in these two oligonucleotide isopliths derived from poly(A) (but not including pAp, which is also found in fingerprints of rRNA) is approximately 20 ± 3%. (A full quantitative analysis of these data for untreated 9S mRNA is in preparation and will appear elsewhere.) The proportion of radioactivity in the oligo(A) tracts for the treated nonbound mRNA is 1.4 ± 0.2%, and the great majority is found in the smallest oligonucleotides.

In an experiment in which both untreated and treated nonstuck globin mRNA were copied by reverse transcriptase, the incorporation obtained is shown in Table I.

The dependence of amino acid incorporation on added treated bound and treated nonbound globin mRNA is shown in Figure 4. In both cases the stimulation of incorporation is linear and similar up to 4 pmol of mRNA added per 50 μl of incubation mixture. The amount of added mRNA was cal-

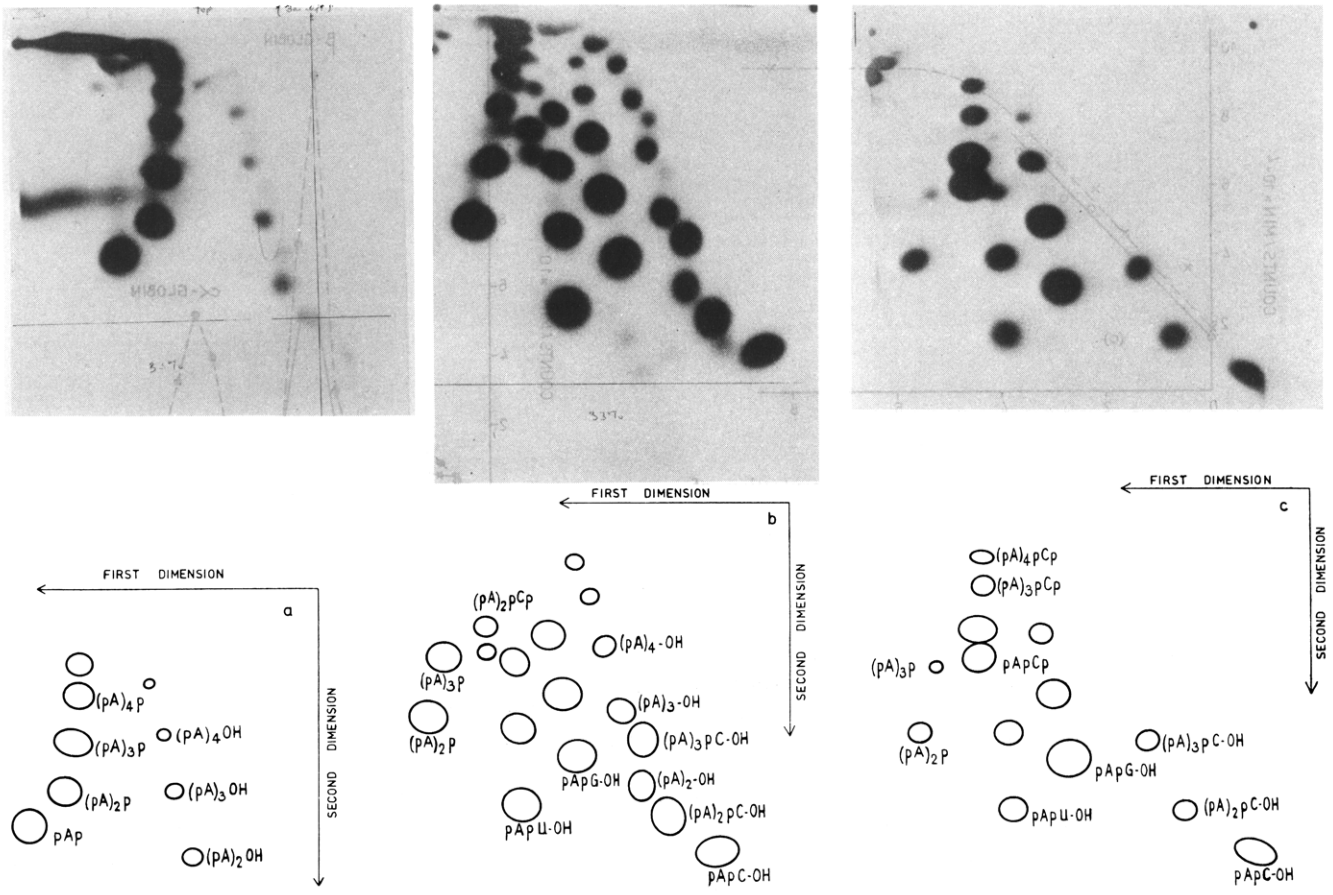

FIGURE 3: Fingerprints of commercial poly(A), untreated 9S mRNA, and phosphorylase-treated nonbound mRNA: (a) commercial poly(A) treated with pancreatic RNase and labeled with phosphokinase; (b) 9S globin mRNA; (c) phosphorylase-treated nonbound mRNA–treated with Tl plus pancreatic ribonucleases followed by phosphatase and then labeled at the 5′ end with phosphokinase. Note the complete absence in (c) of the isoplith $(pA)_n$-OH and the absence of any spots larger than $(pA)_3$-p in the isoplith $(pA)_n$-p.

TABLE I: Transcription of Untreated and Treated Nonbound Globin mRNA with Reverse Transcriptase in the Presence of Oligo(T) Primer.

	Cpm/μg of Template
Control globin mRNA	7.4×10^6
Treated globin mRNA (nonbound)	4.9×10^4

culated using molecular weights of 220,000 (control) and 185,000 (treated, nonbound).

The elution profile of globin chains synthesized after addition of treated bound and nonbound mRNAs in this linear range to the Krebs ascites system is shown in Figure 5. The counts recovered in each globin peak and in the breakthrough peak are given in Table II. The nonbound mRNA is approximately half as active as the control mRNA judged by product analysis, and there is a greater depression of α-chain synthesis than β-chain synthesis.

Discussion

The isolation of the treated mRNA used in these experiments depends upon its nonbinding to oligo(dT)-cellulose. This material migrates at a maximum molecular weight of 200,000 and an average molecular weight of 185,000, at least

TABLE II: Amino Acid Incorporation into Separated Globin Chains.

CCl_3COOH-Precipitable Counts in Peak	Bound 9 S	Nonbound 9 S	Nonbound: Bound (%)
α-Globin	15,370	3,890	25
β-Globin	24,435	15,513	63
Breakthrough	60,000	70,756	
α + β	39,805	19,203	48
Total	100,552	89,959	89.5

20,000 below the molecular weight of intact mouse globin mRNA. The uncertainty as to the exact molecular weight of mouse globin mRNA does not affect the calculation of the difference in molecular weights, which depends upon the relative mobility of the intact and treated 9S RNA.

Only small amounts of absorbing material migrate other than in the major component of mol wt 185,000 after phosphorylase treatment. We estimate that at least 80% of the RNA is in this peak, and there is little if any material migrating in the position of intact 9S mRNA,

The poly(A) sequence of mouse globin mRNA is approximately 50–70 residues in length (Lim and Canellakis, 1970; Morrison *et al.*, 1973), and therefore the molecular weight reduction is calculated to be sufficient to remove all of the

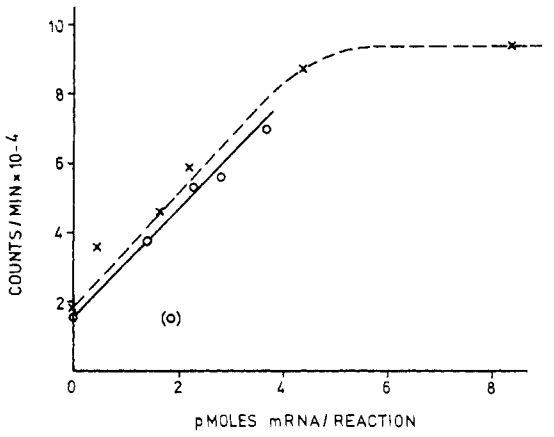

FIGURE 4: Dependence of incorporation upon concentration of added globin mRNA: (O—O) bound mRNA; (×—×) nonbound mRNA. Regression analysis gives slopes of 14,900 (nonbound) and 14,700 (bound, omitting one low point).

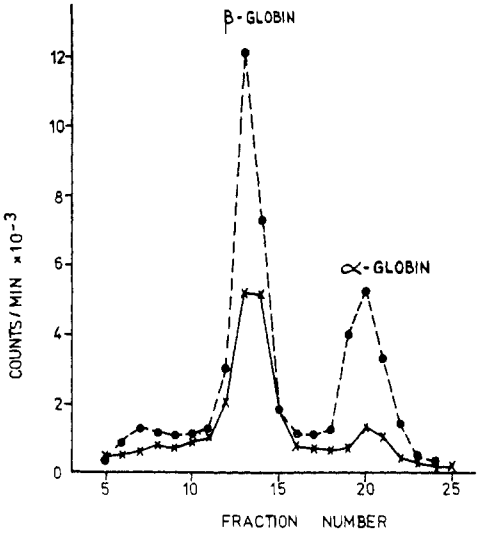

FIGURE 5: Product analysis, Krebs ascites cell-free system: (O—O) bound mRNA; (×—×) nonbound mRNA.

poly(A) sequence. It is of interest that after fractionation of the treated mRNA the bound material still migrates at a position identical with untreated mRNA. This may be due to a total lack of degradation by the enzyme, or to a secondary structure which is dependent for its maintenance on the existence of at least a short poly(A) sequence. When the entire poly(A) sequence is removed, the secondary structure and migration properties may change considerably. Further analysis of the poly(A) sequence and its interaction with the rest of the mRNA molecule will be necessary to resolve this point.

The phosphokinase fingerprint of the pancreatic RNase digest demonstrates the absence of at least 90% of the oligo(A) sequences derived from the poly(A) tract in intact globin mRNA. Therefore we conclude from the lack of binding to oligo(dT)-cellulose, the reduction in molecular weight, the lack of template activity with reverse transcriptase and the absence of oligo(A) isopliths that the treated preparation is essentially free of poly(A) sequences on the 3′ end.

Since human globin mRNA, which migrates to the same position as mouse globin mRNA on gels, is known to contain long untranslated nucleotide sequences between the normal termination codon and the poly(A) sequence of both α- and β-globins mRNAs (Clegg *et al.*, 1971; Flatz *et al.*, 1971), similar sequences may occur in other mammals. The reduction in size obtained after enzyme treatment would not be sufficient to affect the globin coding sequence of minimum mol wt 143,000 (α chain) and 148,000 (β chain).

Polynucleotide phosphorylase attacks RNA from the 3′ end and is particularly active in digesting homopolymers, including poly(A) (Grunberg-Manago, 1963). The phosphorylase-treated mRNA migrates in 6% acrylamide gels as a sharp peak, with a shoulder moving more rapidly. Under our conditions, digestion by the enzyme does not proceed rapidly beyond the first hundred nucleotides or so. This may be because of the rapid rate of digestion of homopolymer as compared with RNA, or due to secondary structure of the RNA molecule. Globin mRNA is known to have a high hyperchromicity, indicating considerable secondary structure (Williamson *et al.*, 1971; Lingrel *et al.*, 1971).

In the Krebs ascites cell-free system the incorporation directed by the treated non-bound mRNA is somewhat less than that for the control mRNA, but is still very much more than could be accounted for by contamination with intact globin mRNA. The fingerprint data demonstrate that the treated mRNA contains less than 10% of the oligo(A) fragments derived from poly(A) in intact globin mRNA. This small amount of material which could be derived from poly(A) sequences in the mRNA is very short in fragment length and shows a different distribution from that obtained with untreated mRNA, making it unlikely that it is obtained from a proportion of the intact mRNA molecules.

The incorporation experiment shown in Figure 5, and similar experiments, were carried out in a response range where amino acid incorporation is linear with added mRNA. Even 10% contamination with intact globin mRNA would at most account for 20% of the observed incorporation into globin chains.

There is a puzzling discrepancy between the fact that CCl_3COOH-precipitable counts for treated and control mRNAs are equal but globin chain synthesis is less for the treated messenger. This may be due to an increased number of incomplete polypeptide chains; analysis of the "breakthrough" peak of the CM-cellulose column showed more counts for the treated than control mRNA, which increase the measured incorporation to an approximately equal level (Table II). However, although this peak contains polypeptide material (Mathews and Osborn, 1971), which may represent incomplete globin chains, it is not clear if it is globin mRNA directed, and further work is required to elucidate the reason for this difference.

The Krebs cell-free protein synthesis system used to assay for globin synthesis in these experiments is much less efficient than *in vivo* protein synthesis. In the Krebs system used it is unlikely that each exogenous mRNA molecule is translated more than once (Mathews *et al.*, 1972). Our data cannot therefore exclude the possible role of poly(A) in reinitiation, and experiments are in progress to investigate this. The difference in ratio of α- to β-globin chains for the treated mRNA may be due to the characteristics of the cell-free system, in which a factor required for the specific synthesis of α-globin has been demonstrated (Wigle and Smith, 1972).

The results reported above demonstrate that globin chain synthesis as characterized by chromatography on CM-cellulose is primed in a heterologous system by deadenylated globin mRNA, although in reduced amounts as compared with mRNA which still contains an intact poly(A) sequence.

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