Proc. Natl. Acad. Sci. USA
Vol. 74, No. 1, pp. 178–182, January 1977
Biochemistry

Purification of a putative precursor of globin messenger RNA from mouse nucleated erythroid cells

(heterogeneous nuclear RNA/globin cDNA cellulose/mRNA processing)

SAU-PING KWAN, T. GORDON WOOD, AND JERRY B. LINGREL

Department of Biological Chemistry, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Communicated by James Bonner, November 4, 1976

ABSTRACT **Nucleated erythroid cells were incubated for 10 min in the presence of [5-^{3}H]uridine, and the total RNA was isolated by three different extraction procedures. RNA containing globin messenger RNA sequences was purified from other cellular RNAs by selective hybridization to globin complementary DNA cellulose. Depending upon the extraction procedure employed, 0.4–0.6% of the radioactively-labeled total cellular RNA applied to the column annealed to globin complementary DNA cellulose. The annealed RNA was treated with formaldehyde and analyzed by formaldehyde/polyacrylamide gel electrophoresis. Mature globin mRNA and an RNA migrating at approximately 15 S were observed. No globin mRNA containing sequences larger than 20 S were present. The 15S RNA was partially resolved from mature globin mRNA by neutral sucrose density gradient centrifugation. The RNA isolated from the heavy region of this gradient migrated as 15 S in the formaldehyde/polyacrylamide gels and retained its ability to quantitatively anneal to globin complementary DNA cellulose. On the basis of these observations, we conclude that nucleated erythroid cells obtained from the spleens of anemic mice have a 15S RNA which contains globin mRNA sequences. The 15S RNA is not an aggregate and is a good candidate for a globin mRNA precursor.**

Although it has been postulated that the cytoplasmic mRNAs of eukaryotic cells are derived from higher molecular weight precursors (1–3), attempts to identify and isolate precursors for specific mRNAs have met with considerable difficulty. There are several reasons for this; first, the precursor for a single mRNA may occur in only low amounts within the cell, depending upon its rate of synthesis and processing time, and second, mature mRNA sequences may form aggregates and appear in larger structures which are interpreted as precursors. Nevertheless, based on biological activity and hybridization by using complementary DNA (cDNA) probes, the presence of globin mRNA sequences in molecules larger than mature cytoplasmic globin mRNA has been reported (4–10).

Because of the potential problems associated with the study of mRNA precursors, we have used a new approach to identify and isolate such molecules. RNA from pulse-labeled nucleated erythroid cells was fractionated by using globin cDNA-cellulose and the purified RNA analyzed by polyacrylamide gel electrophoresis after treatment with formaldehyde. In this communication, we describe the isolation of a 15S RNA which contains globin mRNA sequences and, therefore, is a candidate for the globin mRNA precursor.

MATERIALS AND METHODS

Incubation of nucleated erythroid cells

Nucleated erythroid cells were obtained from the spleens of anemic mice (11) and incubated as follows: 0.75 ml of packed cells ($\simeq 4.0 \times 10^8$ cells) was suspended in 3.75 ml of prewarmed (37°) tissue culture medium, RPMI 1640, containing 10% fetal calf serum and 2 mM glutamine (obtained from Gibco). The cells were preincubated for 5 min at 37° and then 12 ml of the tissue culture medium, containing 12.4 mCi of [5-^{3}H]uridine (27 Ci/mmol), were added and the incubation continued for an additional 10 min. After the incubation, the cells were chilled on ice, collected by centrifugation, washed once with 10 ml of cold (4°) isotonic saline, and resuspended in 2 ml of cold saline.

Isolation of RNA

Proteinase K Procedure. The cell suspension (0.9 ml) was added dropwise with stirring to 50 ml of a solution (12) containing 30 mM Tris·HCl, (pH 7.4), 100 mM NaCl, 5 mM EDTA, 3% sodium dodecyl sulfate ($NaDodSO_4$), heparin (150 μg/ml) dextran sulfate (100 μg/ml), 3 mM each of 2′ and 3′ AMP, CMP, and UMP, and 300 μg/ml of proteinase K. The proteinase K was autodigested in the above buffer, without $NaDodSO_4$, for 10 min at 35° prior to use. The cell suspension was incubated for 30 min at 35°. After the incubation, 50 ml of the above buffer including $NaDodSO_4$, but without proteinase K were added, followed by 100 ml of buffer-saturated, redistilled phenol and chloroform (1:1, vol/vol). The mixture was stirred at room temperature for 15 min. The aqueous phase was collected by centrifugation at 5000 × *g* for 5 min at 20°. The interphase was re-extracted with extraction buffer minus proteinase K and the combined aqueous phases were re-extracted twice with phenol/chloroform and once with chloroform alone. The aqueous solution was adjusted to 0.3 M NaCl and the nucleic acids were precipitated with 2 volumes of absolute ethanol at −20°. After allowing the mixture to stand overnight at −20°, the nucleic acids were collected by centrifugation and washed three times with 70% ethanol. The nucleic acids were immediately dissolved with stirring at 3°, in 10 ml of a solution containing 10 mM sodium acetate (pH 5.2), 3 mM $MgCl_2$, heparin (200 μg/ml), polyvinyl sulfate (30 μg/ml), dextran sulfate (200 μg/ml), 3 mM each of 2′ and 3′ AMP, CMP, and UMP and 40 μg/ml of DNase (Worthington RNase-free) which had been treated with iodoacetate (13). The mixture was stirred at 10° for 20–25 min during which time the viscosity became markedly reduced. The solution was then made 2% in $NaDodSO_4$, 5 mM in EDTA, and 300 μg/ml of proteinase K was added to digest the DNase. After a 10 min incubation at 30°, sodium acetate (pH 5.2) was added to give a final concentration of 50 mM and an equal volume of phenol/chloroform was then added and the mixture stirred for 5 min at 25°. The aqueous layer was collected, re-extracted first with phenol/chloroform and then twice with chloroform, and the RNA precipitated as described above. The RNA pellet was washed with 70% ethanol, dissolved in water and stored at −70°. The recovery of the radioactive RNA was determined according to the method of Lee *et al.* (14).

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary DNA.

Table 1. Comparison of RNA extraction methods

Extraction method	% RNA recovered from cells	cpm × 10^7 applied to cDNA cellulose	% cpm bound
Hot phenol	72	2.12	0.4
Holmes-Bonner	93	2.7	0.6
Proteinase K	97	2.9	0.6

Nucleated erythroid cells were incubated in the presence of [^{3}H]uridine for 10 min and the RNA was extracted by the three procedures described under *Materials and Methods*. The isolated RNA was applied to a globin cDNA cellulose column and the material which hybridized was eluted and reapplied to the column. The percent of radioactivity which bound to the cDNA cellulose column is based on the material which hybridized upon reapplication to the column.

Holmes-Bonner Procedure. The nucleated erythroid cell suspension (0.9 ml) was added to 100 ml of lysing solution (15), which contained the nuclease inhibitors used in the proteinase K procedure. Phenol/chloroform (100 ml) was added immediately and subsequent isolation steps were the same as described by Holmes and Bonner (15) except that the DNA digestion was performed as in the proteinase K procedure.

Hot Phenol Procedure. The hot phenol procedure as described by Scherrer and Darnell (16) was used to isolate RNA. The extraction buffer also contained the nuclease inhibitors used in the proteinase K procedure.

Polyacrylamide gel electrophoresis

RNA samples were treated with formaldehyde and analyzed by 2.7% polyacrylamide and 0.13% *N,N'*-methylene bisacrylamide gels according to the procedure of Ojala and Attardi (17). The 10 cm gels were run for 6½ hr at 5 mA per gel. The gels were sliced and the radioactivity determined as previously described (11).

Purification of globin mRNA-containing RNA sequences using cDNA cellulose

The cDNA cellulose was prepared as described elsewhere (18) by a method similar to that of Venetianer and Leder (19). Globin mRNA isolated from the polysomes of mouse reticulocytes (20) was copied by using reverse transcriptase (RNA-dependent-DNA nucleotidyltransferase) and the oligo(dT) covalently attached to a cellulose matrix as primer. Total cellular RNA from mouse nucleated erythroid cells was incubated with globin cDNA cellulose containing 65 μg of globin cDNA per ml of packed cellulose at 65° for 30 min in hybridization buffer [10 mM Tris·HCl, (pH 7.5) 100 mM NaCl, 0.1 mM EDTA, and 0.2% $NaDodSO_4$]. The unbound RNA fraction was eluted with hybridization buffer at 65° and the bound fraction was eluted with H_2O at the same temperature. These conditions do not allow ribosomal RNA, poly(A), or significant amounts of L-cell RNA (<0.09%) to anneal to globin cDNA cellulose (18). Recovery of RNA from the globin cDNA cellulose column was >90% based on the input radioactivity.

RESULTS

Experimental Design. The isolation of the globin mRNA precursor requires that the precursor be quantitatively recovered from the cells by the RNA extraction procedure and that a method be available for separating globin mRNA containing molecules from other RNAs. In addition, one must be able to show that some of the globin mRNA containing molecules are larger than mature globin mRNA. For these reasons, our approach has been to isolate the RNA by three different extraction procedures, to use a highly specific globin cDNA cellulose column for purifying small amounts of globin mRNA containing molecules, and to determine the size of these molecules by polyacrylamide gel electrophoresis performed under denaturing conditions.

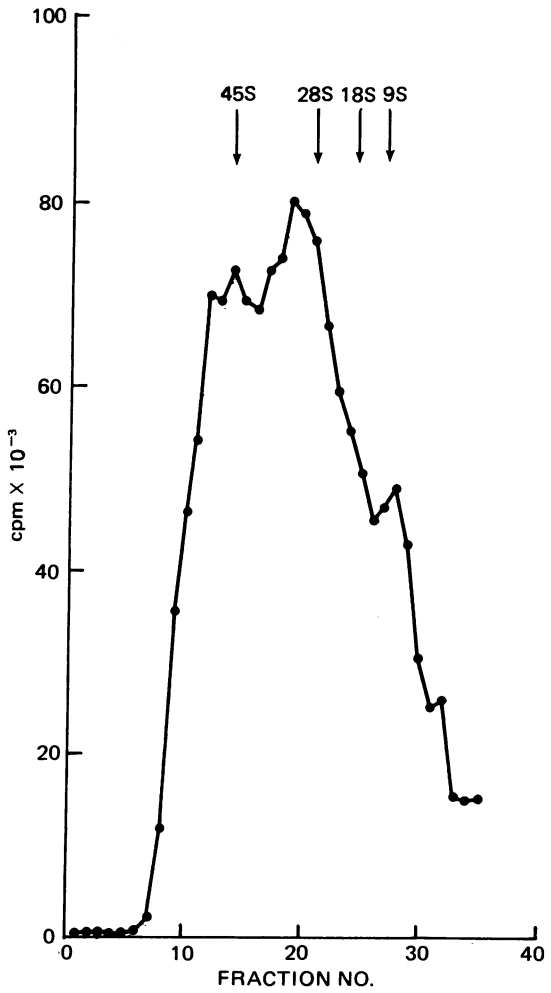

FIG. 1. Denaturing sucrose density gradient centrifugation of total cellular RNA. RNA was isolated from cells exposed for 10 min to [^{3}H]uridine by the Holmes-Bonner procedure. The total cellular RNA (60 μg) was dissolved in 200 μl of 85% formamide made 4 M in urea, 1 mM EDTA, 10 mM Tris·HCl (pH 7.2), and heated to 65° for 10 min prior to centrifugation. The RNA was layered on a 13 ml, 2–10% linear sucrose gradient (wt/wt) containing urea and formamide as described above. The gradients were centrifuged at 40,000 rpm for 27 hr at 25° in an SW40 rotor. Fractions (350 μl each) were collected and the trichloroacetic acid precipitable counts determined.

Comparison of Methods for the Extraction of RNA. Nucleated erythroid cells were pulse-labeled for 10 min in the presence of [^{3}H]uridine and the RNA isolated by three independent methods. These were: (*i*) the proteinase K procedure in which the protein is degraded prior to phenol extraction, thus eliminating protein RNA complexes which may be retained at the interphase. (*ii*) The Holmes-Bonner procedure, which utilizes 7 M urea to dissociate RNA protein complexes. (*iii*) The hot phenol procedure. Because the nucleated erythroid cells from the spleens of anemic mice were found to contain considerable RNase activity, the RNA was extracted from whole cells rather than from isolated nuclei. Nevertheless, most of the radioactive RNA present after a 10-min pulse is presumed to

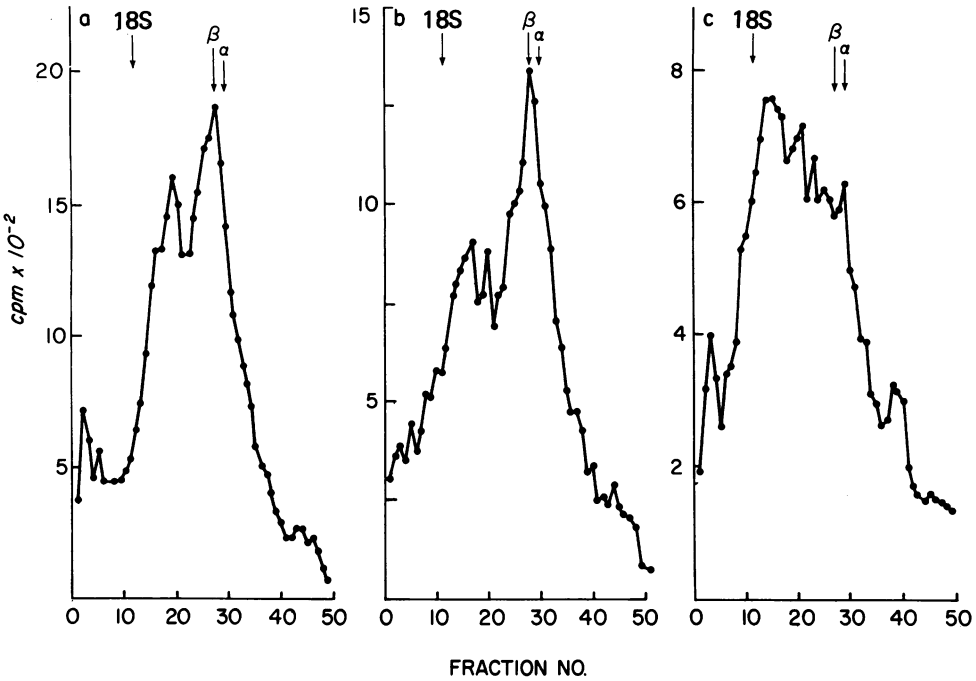

FIG. 2. Polyacrylamide gel electrophoresis of the RNA bound to globin cDNA cellulose. The RNA which hybridized to the globin cDNA cellulose column was dissolved in 10 mM sodium phosphate buffer (pH 7.5), containing 3% formaldehyde and heated for 15 min at 63° before applying it to the gels. The gels were run as described under *Materials and Methods*. (a) RNA isolated by the proteinase K procedure; (b) RNA isolated by the Holmes-Bonner procedure; (c) RNA isolated by the hot phenol procedure. Globin mRNA and rRNAs served as markers and were analyzed in parallel gels.

be confined to the nucleus. Essentially all the RNA labeled during the pulse is recovered using the proteinase K and Holmes-Bonner procedures, while only 72% is recovered with the hot phenol procedure (Table 1). The RNA extracted by the three methods was dissolved in 85% formamide, made 4 M in urea, heated to 65° for 10 min, and analyzed by urea/formamide sucrose gradients. The radioactive profile of the RNA isolated by the Holmes-Bonner procedure is shown in Fig. 1. Radioactivity is found throughout the gradient with peaks at 32 S, and 45 S; some molecules as large as 55 S are observed. Presumably, much of the radioactivity is present in ribosomal RNA precursors. The radioactive profiles of the RNA isolated by the proteinase K and hot phenol procedures are similar (not shown).

Isolation of Globin mRNA Containing RNA Sequences by Globin cDNA Cellulose. The ability to prepare globin cDNA covalently attached to cellulose provides the opportunity for separating those RNA molecules which contain globin mRNA sequences from the other cellular RNAs.

Pulse-labeled total cellular RNA was applied to the globin cDNA cellulose column and incubated at 65° by using conditions which permitted the selective annealing of globin mRNA. The globin cDNA cellulose was then washed with hybridization buffer at 65° to remove those sequences which had not annealed to the immobilized globin cDNA. The hybridized RNA was removed by washing the cDNA cellulose with water at 65°. This RNA fraction was reapplied to the cDNA cellulose column and the bound RNA isolated in an identical manner. To insure the quantitative recovery of globin mRNA sequences, we performed all hybridization reactions by using an excess of globin cDNA. Total RNA isolated by either the Holmes-Bonner or the proteinase K procedures was shown to contain 0.6% globin RNA sequences by hybridization to globin cDNA cellulose, while only 0.4% of the radioactive RNA isolated by the hot phenol procedure was retained by globin cDNA cellulose.

Formaldehyde-Polyacrylamide Gel Electrophoresis of the RNA Retained by Globin cDNA Cellulose. Although the RNA retained by the globin cDNA cellulose column must contain globin mRNA sequences, it was not known whether this RNA represented only mature globin mRNA or whether some of the globin sequences were present in larger molecules. The RNA which hybridized to globin cDNA cellulose was treated with formaldehyde to eliminate possible aggregates between mature globin mRNA and itself or other RNAs, and analyzed by polyacrylamide gel electrophoresis. The resulting pattern of radioactivity contained a peak which comigrated with a mature globin mRNA marker and an additional peak migrating at 14–16 S. This was true for the RNA isolated by all three procedures (Fig. 2). The RNA isolated by the proteinase K (Fig. 2a) and Holmes-Bonner (Fig. 2b) procedures showed a definite peak at approximately 15 S. Although more than 95% of the radioactivity entered the gels, one could not rule out the possibility that RNAs larger than 20 S were present in the RNA preparation. To eliminate this possibility, we analyzed the RNA bound to the globin cDNA cellulose by sucrose density gradient centrifugation with the denaturing conditions described in Fig. 1. No radioactivity migrating larger than approximately 25 S was observed (Fig. 3).

Fractionation of cDNA Bound RNA by Sucrose-NaDodSO$_4$ Gradient Centrifugation. Although mature globin mRNA and the larger globin mRNA containing molecules are resolved by the denaturing polyacrylamide gels, the RNAs could not be readily recovered from such gels. Also, the urea/formamide containing sucrose gradients did not give a good separation of these two RNAs. Therefore, the globin cDNA bound RNA was examined using nondenaturing sucrose gradients (Fig. 4). The pulse-labeled cDNA bound RNA contained a population of RNA molecules which migrated as a heavier fraction than the mature globin mRNA marker. Fractions from the pulse-labeled RNA gradient designated by the bars shown on Fig. 4 were pooled, treated with formaldehyde, and analyzed by polyac-

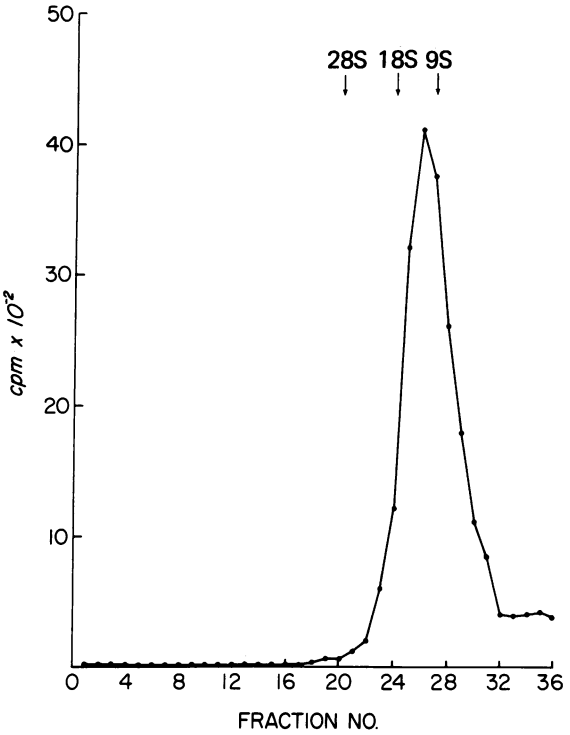

FIG. 3. Denaturing sucrose density gradient analysis of RNA bound to globin cDNA. The RNA from the proteinase K isolation which bound to the cDNA cellulose column was sedimented on a sucrose density gradient containing urea and formamide as described in the legend of Fig. 1.

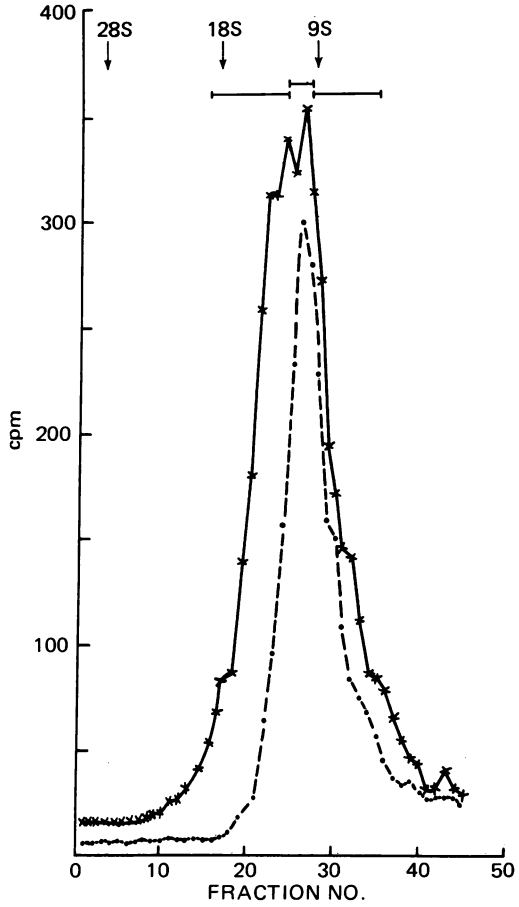

FIG. 4. Nondenaturing sucrose density gradient analysis of RNA bound to globin cDNA. The RNA was dissolved in 200 μl of distilled water containing 0.2% $NaDodSO_4$ and heated at 85° for 15 min. The sample was cooled rapidly and layered on a 13 ml, 15–30% linear sucrose gradient (wt/wt) containing 10 mM Tris·HCl (pH 7.4), 0.1% $NaDodSO_4$, and 1 mM EDTA, and centrifuged at 40,000 rpm for 16.5 hr at 18° in an SW40 rotor. Fractions (300 μl) were collected and 20 μl aliquots were used for measurement of radioactivity. The solid line (×—×) shows the radioactive profile of cDNA bound RNA isolated by the proteinase K procedure while the dashed line (●- — -●) represents newly synthesized mature globin mRNA (11) analyzed in a parallel gradient.

rylamide gel electrophoresis. The globin cDNA bound material, which cosediments with globin mRNA in the sucrose gradient, also comigrated with this RNA on the gels (Fig. 5a), while the heavier material migrates slower with a peak of radioactivity at approximately 15 S (Fig. 5c). The material which sediments between these two fractions on sucrose gradients also migrates at an intermediate position on the gels (Fig. 5b). The results from this experiment rule out the possibility that formaldehyde is crosslinking RNAs and producing a larger globin mRNA containing molecule. If this were the case, then the RNA which cosedimented with globin mRNA in the sucrose gradient would have contained much more high-molecular-weight material after formaldehyde treatment. The three fractions obtained from the sucrose density gradient (Fig. 4) were also reapplied to the globin cDNA cellulose column. More than 90% of each fraction reannealed. This indicates that the larger RNA molecules indeed contain globin mRNA sequences and, therefore, this RNA is a good candidate for a globin mRNA precursor.

DISCUSSION

A precursor–product relationship between heterogeneous nuclear RNA and cytoplasmic mRNA has been suggested. To support this hypothesis, several reports have appeared indicating that the two RNAs have sequences in common (3, 21–23), and in fact, globin mRNA sequences have been reported in RNA sedimenting faster than mature globin mRNA in both nondenaturing (4, 6, 10) and denaturing sucrose density gradients (5, 7–9). The work described in this paper utilizes a new approach for identifying globin mRNA containing molecules. The RNA was isolated from nucleated erythroid cells after a 10-min pulse and the globin containing RNA sequences isolated by hybridization to a globin cDNA cellulose column. Regardless of the method used for extracting the RNA, radioactivity was retained by the cDNA cellulose column. When the bound RNA was analyzed by polyacrylamide gels by using denaturing conditions, molecules migrating slower than mature globin mRNA were observed. The larger RNA could be separated from mature globin mRNA by sucrose density gradient centrifugation. This RNA retained its migration properties on denaturing polyacrylamidegels and its ability to hybridize to globin cDNA cellulose. These results indicate that the larger molecules contain globin mRNA sequences and are not the result of aggregation. After the completion of this work a report appeared describing a similar RNA in erythroleukemia cells (24). It is interesting to note that the putative globin mRNA precursor is smaller than the majority of the pulse-labeled RNA present in erythroid cells.

Although precursor-product studies will be required to establish whether these larger mRNA containing molecules are truly precursors to cytoplasmic mRNA, such studies will be difficult because of the small amount of this RNA present in cells.

A molecular weight of 6×10^5 can be estimated for the 15S putative precursor of globin mRNA based on its migration in polyacrylamide gels. This is three times larger than mature

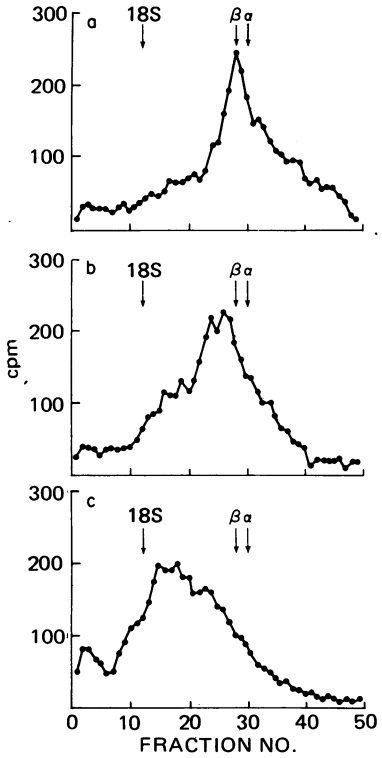

FIG. 5. Formaldehyde/polyacrylamide gel electrophoresis of RNA fractions obtained by sucrose density gradient centrifugation. Fractions designated by the bars in Fig. 4 were pooled, concentrated by ethanol precipitation, and analyzed by the formaldehyde/polyacrylamide gel technique described in the legend of Fig. 2. (a) Analysis of the 6–10S RNA; (b) analysis of the 10–11S RNA; (c) analysis of the RNA greater than 11 S.

globin mRNA and it will be interesting to determine whether the globin mRNA sequences are located near the 5′ terminus, the 3′ terminus, or at an internal position. It is also possible that a single precursor could contain two α- or two β-globin mRNA sequences as there is evidence that these genes are duplicated in mice (25, 26). The isolation of a globin mRNA containing molecule which is larger than mature globin mRNA provides the opportunity to obtain answers to these questions and will also allow us to determine if the nonconserved portion is transcribed from repetitive DNA sequences. The presence or absence of a triphosphate at the 5′ terminus may help to decide if this RNA is a primary transcript.

Note Added in Proof. A 14S RNA which contains globin mRNA sequences has recently been detected in mouse fetal liver cells by liquid cDNA hybridization. This RNA is kinetically related to mature globin mRNA [Ross, J. (1976) *J. Mol. Biol.* **106**, 403–420].

This work was supported by Grants GM-10999 from the USPHS, BMS 74-01783 A01 from the National Science Foundation, and NP-57F from the American Cancer Society. T.G.W. is a postdoctoral fellow of the National Cancer Institute. The reverse transcriptase used in these studies was obtained from the Office of Program Resources and Logistics Viral Cancer Program, Viral Oncology, National Cancer Institute. This work was done by one of us (S.P.K.) in partial fulfillment of the requirements for the Doctor of Philosophy degree.

1. Darnell, J. E. (1974) in *The Harvey Lecture Series 69* (Academic Press, New York), pp. 1–47.
2. Lewin, B. (1975) *Cell* **4**, 11–20.
3. Lewin, B. (1975) *Cell* **4**, 77–93.
4. Williamson, R., Drewienkiewicz, C. & Paul, J. (1973) *Nature New Biol.* **241**, 66–68.
5. Knöchel, W. & Tiedemann, H. (1975) *Biochim. Biophys. Acta* **378**, 383–393.
6. Melli, M. & Pemberton, R. E. (1972) *Nature New Biol.* **236**, 172–173.
7. Scherrer, K. (1973) in *Karolinska Symposia on Research Methods in Reproductive Endocrinology*, 6th Symposium, ed. Diczfalusy, D. (Karolinska Institute, Stockholm), pp. 95–129.
8. Ruiz-Carrillo, A., Beato, M., Schutz, G., Feigelson, P. & Allfrey, V. G. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3641–3645.
9. Macnaughton, M., Freeman, K. B. & Bishop, J. O. (1974) *Cell* **1**, 117–125.
10. Ostertag, W., Gaedicke, G., Kuge, N., Melderis, H., Weimann, B. & Dube, S. K. (1973) *FEBS Lett.* **32**, 218–222.
11. Merkel, C. G., Kwan, S.-P. & Lingrel, J. B. (1975) *J. Biol. Chem.* **250**, 3725–3728.
12. Suzuki, Y. & Brown, D. D. (1972) *J. Mol. Biol.* **63**, 409–429.
13. Zimmerman, S. B. & Sandeen, G. (1966) *Anal. Biochem.* **14**, 269–277.
14. Lee, S. Y., Krsmanovic, V. & Brawerman, G. (1971) *J. Cell Biol.* **49**, 683–691.
15. Holmes, D. S. & Bonner, J. (1973) *Biochemistry* **12**, 2330–2338.
16. Scherrer, K. & Darnell, J. E. (1962) *Biochem. Biophys. Res. Commun.* **7**, 486–490.
17. Ojala, D. & Attardi, G. (1974) *J. Mol. Biol.* **88**, 205–219.
18. Wood, T. G. & Lingrel, J. B. (1976) *J. Biol. Chem.*, in press.
19. Venetianer, P. & Leder, P. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3892–3895.
20. Gorski, J., Morrison, M. R., Merkel, C. G. & Lingrel, J. B. (1974) *J. Mol. Biol.* **86**, 363–372.
21. Perry, R. P., Kelley, D. E., Friderici, K. & Rottman, F. (1975) *Cell* **6**, 13–19.
22. Saldett-Georgieff, M., Jelinek, W., Darnell, J. E., Furuichi, Y., Morgan, M. & Shatkin, A. J. (1976) *Cell* **7**, 227–237.
23. Lengyel, J. & Penman, S. (1975) *Cell* **5**, 281–290.
24. Curtis, P. J. & Weissmann, C. (1976) *J. Mol. Biol.* **106**, 1061–1075.
25. Russell, E. S. & McFarland, E. C. (1974) *Ann. N.Y. Acad. Sci.* **241**, 25–38.
26. Kitchen, H. (1974) *Ann. N.Y. Acad. Sci.* **241**, 12–24.