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Biochemistry

Presence of a putative 15S precursor to β-globin mRNA but not to α-globin mRNA in Friend cells

(globin mRNA precursor/mouse globin cDNA plasmid)

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ABSTRACT **Dimethyl sulfoxide-induced Friend cells were labeled for periods of 5–60 min. The denatured RNA was fractionated by sucrose gradient centrifugation and the distribution of α- and β-globin-specific [^{3}H]RNA was determined by hybridization to hybrid plasmids containing mouse α- and β-globin DNA, respectively. After 5 min of labeling, a 15S peak of β-globin-specific (but not α-globin-specific) [^{3}H]RNA was detected, next to an equal amount of 10S β-globin [^{3}H]RNA. With increasing periods of labeling, the amount of 15S β-globin [^{3}H]-RNA remained constant but the amount 10S β-globin [^{3}H]RNA increased steadily. α-Globin-specific [^{3}H]RNA sedimented at 11 S after 5 min of labeling and at 9.5 S after longer labeling periods. Analysis of 15S globin-specific [^{3}H]RNA purified by the poly(dC)-cDNA method [Curtis, P. J. & Weissmann, C. (1976) *J. Mol. Biol.* 106, 1061–1075] showed oligonucleotides characteristic of β-globin mRNA but not of α-globin mRNA, as well as about 20 new oligonucleotides. Our results suggest that 10S β-globin mRNA arises via a 15S precursor that has a half-life of 5 min or less; 9.5S α-globin mRNA may be derived from an 11S precursor.**

The question as to whether mature eukaryotic mRNA corresponds in length to a primary transcript or is derived from a longer precursor molecule has still not been resolved. Several workers (1, 2) proposed that mRNA, and in particular globin mRNA (3, 4), was derived from high-molecular-weight RNA of chain length greater than 10,000, whereas McKnight and Schimke (5) concluded that ovalbumin mRNA did not undergo a significant change in molecular weight from its initial transcription to its incorporation into polyribosomes. Macnaughton *et al.* (6) provided tentative evidence for globin-specific 14S RNA in nuclei of immature duck erythrocytes. All those studies were performed on unlabeled RNA with labeled cDNA probes. Clearly, if the steady-state level of a precursor is low compared to that of the mature molecule, it is advantageous to examine RNA from cells labeled for periods that are short compared to the half-life of the precursor (7). Using the method of Coffin *et al.* (8), we showed that Friend cells labeled for 20 min with [^{32}P]phosphate contained globin-specific [^{32}P]RNA sedimenting at 15 S as well as at 10 S, the position of mature globin mRNA (9). Similar results were obtained independently by others (10, 11).

In this paper we report on the kinetics of labeling as well as on the isolation of radioactive globin-specific 15S RNA and 10S RNA from short-term labeled Friend cells. Analysis of these RNAs by fingerprinting techniques and by hybridization to cloned mouse α- and β-globin cDNA showed that the 15S peak contained β-globin-specific RNA but not α-globin-specific RNA, as well as additional sequences not found in mature globin mRNAs. After short labeling periods, α-globin-specific RNA of slightly higher molecular weight than the corresponding mature mRNA was detected.

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MATERIALS AND METHODS

Growth and dimethyl sulfoxide–induction of Friend cells, RNA extraction, labeling procedures, and fingerprinting and sequencing were as reported previously (9). The analytical and preparative applications of hybrid isolation by poly(I)–Sephadex chromatography also have been described (9).

Hybrid plasmids consisting of PCRI (12) and mouse α- or β-globin DNA were prepared essentially by the approach of Maniatis *et al.* (13). Poly(dA)–globin cDNA was annealed with poly(dT)–PCRI DNA and transfected into streptomycin-dependent *Escherichia coli* N543 (14, 15). Colonies containing globin DNA were identified by *in situ* hybridization (16) to ^{125}I-labeled mouse α- and β-globin mRNA, isolated by hybridization to rabbit β-globin hybrid DNA PβG (12) and rabbit α-globin hybrid DNA ZRαG (P. Curtis and J. van den Berg, unpublished results). The procedures were carried out in a P3 facility as defined by the NIH guidelines (17). The preparation of plasmid DNA has been described (15). Excision of the insert by S_1 nuclease (18) yielded globin-specific fragments of about 370 (α) and 530 (β) base pairs. The α- and β-globin cDNA inserts had one *Bam*H1 cleavage site each but no *Eco*R1 site.

Fixation of α- or β-globin hybrid plasmid to Millipore filters and hybridization assays were as described (19). α- and β-globin [^{32}P]RNA were purified from 10S [^{32}P]RNA from dimethyl sulfoxide-induced Friend cells (9) by using hybridization to filter-bound α- or β-globin hybrid DNA as described by Weinberg *et al.* (20).

RESULTS

Purification and Characterization of Mature Mouse α- and β-Globin [^{32}P]mRNA. The 8–12S RNA from dimethyl sulfoxide-induced Friend cells labeled with [^{32}P]phosphate for 16 hr was hybridized to Millipore filters to which cloned α- and β-globin hybrid DNA, respectively, had been fixed. The purified preparations were digested with RNase T_1 and the products were separated by the minifingerprint method of Volckaert *et al.* (21) (Fig. 1 *a–c*). The large oligonucleotides were characterized by digestion with pancreatic RNase and identified with the oligonucleotides isolated earlier from a mixture of mouse α- and β-globin mRNA; the numbering used in Fig. 1 is the same as in the previous work (9). The identification of oligonucleotides as being derived from mouse α- or β-globin mRNA had earlier been made by matching partial nucleotide sequences with the known amino acid sequences; the present work confirms all but one of these assignments.

Ratio of 15S and 10S Globin-specific [^{3}H]RNA after Different Times of Labeling; Hybridization Analysis with α- and β-Globin-Specific Probes. Friend cells were labeled with

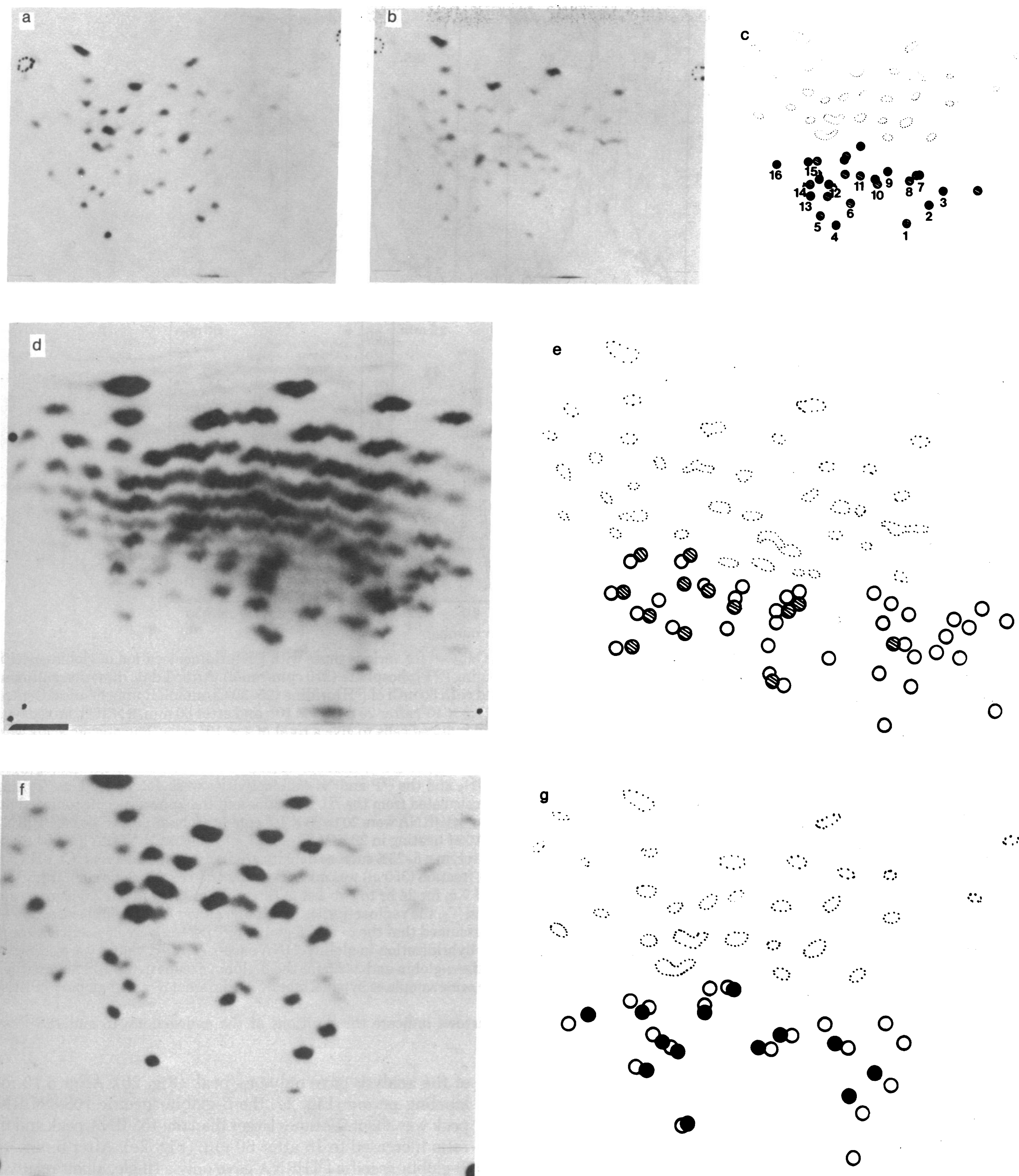

FIG. 1. Characterization of globin-specific [^{32}P]RNAs by T_1 fingerprinting. Labeled and carrier RNA were mixed (total, about 20 μg) and digested with 3 units of RNase T_1 in 3 μl of 20 mM Tris·HCl pH 7.5/1 mM EDTA at 37° for 30 min. The products were fractionated by cellulose acetate electrophoresis at pH 3.5, followed by homochromatography on PEI plates (21). (*a*) α-Globin [^{32}P]mRNA (2500 cpm) purified by hybridization to α-globin hybrid DNA fixed to a Millipore filter. (*b*) β-Globin [^{32}P]mRNA (2500 cpm) purified by hybridization to β-globin hybrid DNA fixed to a Millipore filter. (*c*) Composite tracing of panels *a* and *b*; characteristic α-globin-specific oligonucleotides are shown solid; β-globin-specific ones are hatched. The numbering is as in ref. 9. (*d*) 15S globin-specific [^{32}P]RNA (10^4 cpm) purified from induced cells labeled for 20 min with [^{32}P]phosphate by two cycles of hybridization to poly(dC)–(α+β)-globin cDNA and poly(I)–Sephadex chromatography (9). (*e*) Composite tracing of the T_1 fingerprints of purified 15S globin-specific [^{32}P]RNA from (*d*) (open circles) and purified 10S β-globin [^{32}P]RNA from (*b*) (hatched). (*f*) α-Globin [^{32}P]RNA (5600 cpm) from the 10S to 12S RNA fraction of induced cells labeled with [^{32}P]phosphate for 20 min, purified by hybridization to α-globin hybrid DNA fixed to Millipore filters. (*g*) Composite tracing of the T_1 fingerprints of 10–12S α-globin [^{32}P]RNA from *f* (open circles) and 10S α-globin [^{32}P]mRNA from *a* (solid circles).

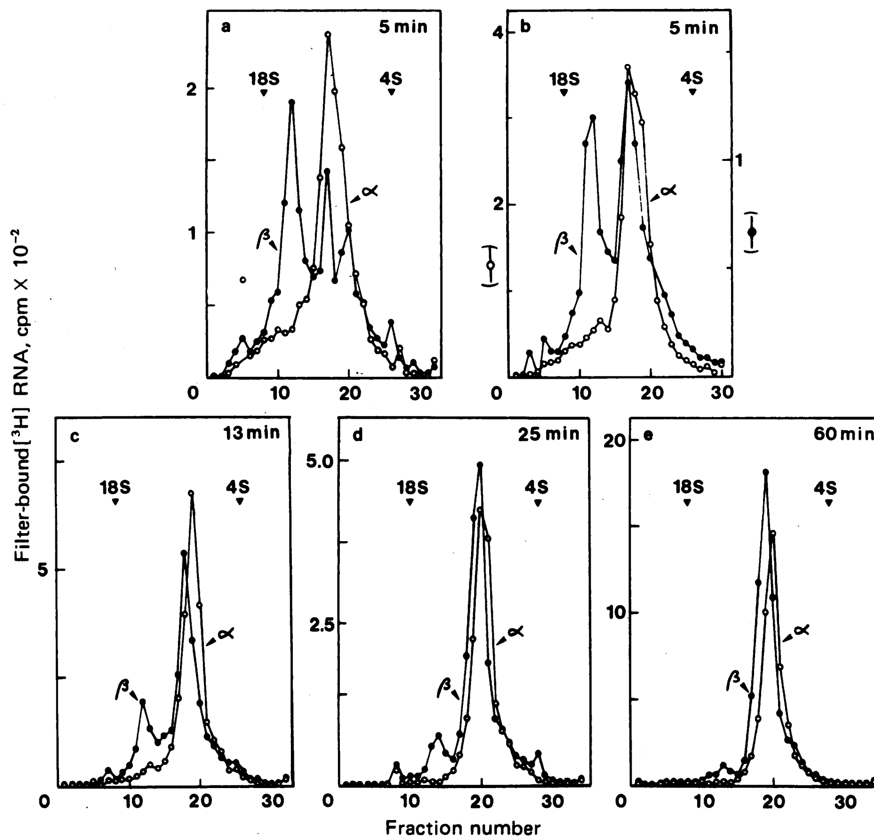

FIG. 2. Sucrose gradient centrifugation of [^{3}H]RNA from Friend cells labeled for various times with [^{3}H]uridine: location of globin-specific sequences by hybridization. Friend cells were grown in medium (9) containing [^{32}P]phosphate (210 cpm/nmol). After 1 day, dimethyl sulfoxide was added to 1.5%; and 4 days later the cells (7×10^7 in 3.5 ml) were labeled with 30 mCi of [^{3}H]uridine (25–30 Ci/mmol, Radiochemical Centre, Amersham) at 37°. Cells were removed (after 5 min, 4×10^7; after 13 min, 1.8×10^7; after 25 min, 7×10^6; and after 60 min, 6×10^6), mixed with frozen medium, and pelleted. Each pellet was resuspended with unlabeled induced cells to give a total of 4×10^7 cells. The nucleic acids were extracted and precipitated with ethanol (9). Nucleoside triphosphates were precipitated from the ethanol supernatant with 16 mM barium acetate in 4 mM $NaHCO_3$, recovered by treatment of the barium salts with Dowex 50 (H^+), and separated by two-dimensional chromatography (22). The appropriate areas of the chromatogram were eluted with 1 M NH_3 and the ^{32}P and ^{3}H radioactivities were determined in a Triton/toluene-based scintillator cocktail (23). The specific ^{3}H radioactivities were calculated from the ^{3}H/^{32}P ratios and the known specific radioactivity of the [^{32}P]phosphate. After DNase digestion as described (9), the recoveries of [^{3}H]RNA were 20 to 30×10^6 cpm in each sample; those of [^{32}P]RNA varied from 150,000 in the 5-min sample to 18,000 in the 60-min sample. After heating in 20 mM Tris·HCl, pH 7.5/1 mM EDTA at 100° for 45 sec, the RNA was centrifuged for 16 hr at 15° and 32,000 rpm in a SW41 rotor, in a 5–23% sucrose density gradient in 50 mM Tris·HCl, pH 7.5/5 mM EDTA/0.1% (wt/vol) sodium dodecyl sulfate. One-half of each gradient fraction (350 μl) was mixed with purified α- and β-globin [^{32}P]mRNA (340 cpm each) and hybridized at 66° in 0.5 M NaCl/10 mM Tris·HCl, pH 7.5, for 24 hr to two 4-mm filters, containing about 3.6 μg of α- and β-globin hybrid DNA, respectively (19). After treatment with RNase A and T_1, the radioactivities were determined (9). The hybridization efficiencies were calculated from the filter-bound ^{32}P radioactivities; it was assumed that the α- and β-globin [^{32}P]RNAs were 65% and 80% pure, respectively, the maximum values of RNase resistance found after liquid hybridization in globin cDNA excess. The ^{3}H values were corrected for the hybridization efficiencies. The correction factors were about 2 for the α-globin and 2–2.5 for the β-globin except in the 10S region where they reached 4–5. Panel *b* shows a repeat of the hybridization analysis on the same sample as in panel *a* but with different α- and β-globin [^{32}P]RNA standards and a different set of filters.

○, α-Globin-specific [^{3}H]RNA; ●, β-globin-specific [^{3}H]RNA. The arrows indicate the positions of the endogenous 4S and 18S [^{32}P]-RNAs.

[^{3}H]uridine for 5, 13, 25, and 60 min. The specific radioactivities of the intracellular ribonucleoside triphosphates were determined for each sample. The RNA was extracted, heated, and fractionated by sucrose density gradient centrifugation. Part of each fraction was mixed with a constant amount of ^{32}P-labeled α- and β-globin mRNA as internal standard and hybridized to two filters, one with α-globin and the other with β- globin hybrid plasmid fixed to it. The filter-bound ^{3}H and ^{32}P radioactivities were determined, the relative hybridization efficiencies were calculated from the ^{32}P values, and the ^{3}H values were corrected accordingly. After 5 min of labeling, more than half of the β-globin-specific [^{3}H]RNA was found in a sharp peak at 15 S and most of the remainder was in the 10S region (Fig. 2*a*). The occurrence of two β-specific peaks in the 10S region is probably an analytical artifact because repetition of the analysis gave only one peak (Fig. 2*b*). After a 13-min labeling period (Fig. 2*c*) the β-globin-specific 10S [^{3}H]RNA peak was about 2.7 times larger than the 15S RNA peak and this ratio increased to 15 after 60 min (Fig. 2*e*). After 5 min, the α-globin-specific [^{3}H]RNA gave only a single, albeit relatively broad, peak at 11 S, with some material trailing to the heavy side. After 13 min of labeling, the peak of α-globin-specific [^{3}H]RNA became narrower and shifted to about 9.5 S. Thus, the 15S RNA consists mainly if not exclusively of β-globin-specific RNA.

The accumulation of 10S and 15S β-globin [^{3}H]RNA (Fig. 3) was estimated by dividing the radioactivity in each peak by the specific ^{3}H radioactivity of the nucleoside triphosphates averaged over the preceding time interval. This is justified if the U:C ratio is close to 1 and if the change of specific radio-

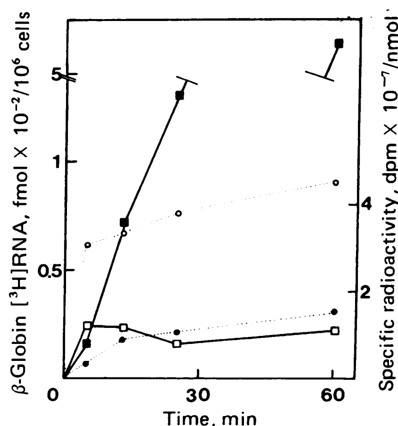

FIG. 3. Accumulation of 15S and 10S β-globin [^{3}H]RNA in induced Friend cells. The radioactivity in 15S and 10S β-globin [^{3}H]RNA was calculated from the data in Fig. 2, taking into account aliquot size and assuming that the recovery of [^{3}H]RNA was 80% for each sample. The total radioactivity in each peak was divided by the specific ^{3}H radioactivities of the UTP and CTP (the ^{3}H radioactivities in ATP and GTP were negligible) averaged over the preceding time interval. ○ - - - ○, Specific ^{3}H radioactivity of UTP; ● - - - ●, specific ^{3}H radioactivity of CTP; □—□, 15S β-globin [^{3}H]RNA; ■—■, 10S β-globin [^{3}H]RNA.

activity is small; the latter is not true for the 5-min sample. Mature 10S β-globin [^{3}H]RNA accumulated steadily over the entire observation period, whereas the 15S β-globin [^{3}H]RNA remained at a constant value after 5 min.

Purification and Analysis of Globin-Specific 15S [^{32}P]RNA. Induced Friend cells were labeled for 20 min with [^{32}P]phosphate. Then the RNA was extracted, heated, and centrifuged through an aqueous sucrose gradient. The distribution of globin-specific [^{32}P]RNA was determined by hybridization to poly(dC)-elongated (α+β) globin cDNA as described (9). The fractions corresponding to the 15S peak were pooled and the globin-specific RNA was purified by two cycles of hybridization to poly(dC)-elongated (α+β) globin cDNA and chromatography on poly(I)–Sephadex (9). Comparison of the fingerprint of the purified [^{32}P]RNA (Fig. 1*d*) with the patterns of purified α- and β-globin mRNAs showed that all typical β-globin-specific oligonucleotides could be accounted for in the pattern of the 15S RNA (as judged by relative mobilities; there was not enough material for further characterization); however, no characteristic α-globin-specific oligonucleotides could be detected. Because the poly(dC)–(α+β) cDNA preparation used in this experiment allowed the purification of almost an equimolar mixture of α- and β-globin mRNAs from long-term ^{32}P-labeled Friend cells (9), these results confirm that there is little if any α-globin-specific RNA in the 15S RNA fraction.

Purified 15S [^{32}P]RNA yielded about 20 T_1 oligonucleotides not found in the case of α- or β-globin mRNA; 8 of these showed the high mobility in the first dimension characteristic of high UMP content. Some or all of the extra fragments could be derived from the 15S globin-specific RNA; however, it is striking that many spots were more intense than the ones attributed to β-globin mRNA. The unequal intensities could be due to different oligonucleotide length, to the coincidence of two or more oligonucleotides, or to unequal specific activities of the four nucleotides. On the other hand, the extra oligonucleotides could be derived from one or a few RNA species copurifying with the 15S β-globin-specific RNA. We consider an unspecific contamination unlikely because (*i*) the fingerprint of 15S [^{32}P]RNA purified by hybridization to β-globin hybrid DNA fixed to Millipore filters or to poly(dC)–rabbit globin cDNA was the same as that of Fig. 1*d* (data not shown) and (*ii*) no comparable spots were found when globin-specific RNA was purified from long-term labeled Friend cells by the same procedure (9) or from 15S short-term labeled RNA by using α-globin hybrid DNA fixed to Millipore filters. Because the purification techniques, in contrast to the analytical procedures, do not involve RNase digestion, specific copurification could occur if β-globin mRNA had some sequences in common with other RNA species; these could then hybridize to the cDNA used in the purification procedures. Alternatively, RNA could attach to 15S globin-specific RNA because of partial complementarity to the latter and, in addition, form concatenates if it contained extended hairpin loops (cf. 24).

Purification and Analysis of α-Globin-Specific [^{32}P]RNA from Cells Labeled for 20 Min. [^{32}P]RNA from Friend cells labeled for 20 min as above was fractionated by sucrose gradient centrifugation. The RNA from the heavy side of the 10S peak was purified by hybridization to Millipore filter-bound α-globin hybrid DNA and fingerprinted as before. All oligonucleotides found in the α-globin mRNA fingerprint were present, with one possible exception (Fig. 1 *f* and *g*). In addition, about six oligonucleotides not found in the α-globin mRNA fingerprint were present, albeit in less than molar yield. These results, along with the sedimentation data in Fig. 2*a*, suggest the presence of an α-globin-specific RNA about 30% longer than the mature globin mRNA.

DISCUSSION

Our findings demonstrate the existence of an RNA, of about 1500 nucleotides in length, containing a segment which by our analysis is indistinguishable from β-globin mRNA. Preliminary experiments (data not shown) suggest that about 50% of the 15S RNA carries a poly(A) tail. Moreover, we have tentative evidence for an α-globin-specific RNA about 200 nucleotides longer than mature α-globin mRNA.

The data of Fig. 3 show that the amount of 10S β-globin [^{3}H]RNA per cell increases steadily over the entire observation period of 1 hr; the amount of 15S β-globin [^{3}H]RNA remains constant after about 5 min. The 10S RNA thus behaves as though it were a steadily accumulating species, either stable or with a half-life long compared to the observation period, whereas the 15S RNA shows the labeling kinetics of an unstable species with a comparatively short half-life. If the labeling procedures did not affect the rates of synthesis and turnover and there was no significant cell growth, then the time required for replacing half the molecules in the 15S globin RNA pool by newly synthesized labeled 15S globin RNA is equal to the half-life of the RNA in this pool. The data of Fig. 3 suggest that this half-life is 5 min or less.

The question as to whether the 15S β-globin RNA is an obligatory precursor of the β-globin mRNA or is a side product of synthesis and is eventually degraded cannot yet be answered conclusively. However, we have calculated that the rate of synthesis of the 15S β-globin RNA (>24 fmol of nucleotide in RNA/10^6 cells per 5 min, estimated from the initial rate of [^{3}H]UMP and [^{3}H]CMP incorporation into the β-globin-specific sequences of 15S RNA) is of the same order of magnitude as the rate of synthesis of 10S β-globin RNA (~27 fmol of nucleotide in RNA/10^6 cells per 5 min) in the middle of the observation period. Because the estimate of the rate of 15S RNA synthesis is a lower one, a precursor-product relationship is possible.

It is remarkable that the 15S peak contains β-globin-specific RNA but little or no α-globin-specific RNA. If the 15S RNA is a precursor, then clearly messenger RNAs do not all originate in a similar fashion; either the α-globin mRNA does not have

a precursor of similar length or, if it does, the precursor is processed far more rapidly. Perhaps some mRNAs are generated without occurrence of a precursor (5) and others are derived from short, long, or very long precursors. The genesis of a particular mRNA may differ from one organism to another or perhaps even from one tissue to another within the same organism.

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