THE ABSENCE OF A PRECURSOR LARGER THAN 16 S TO GLOBIN mRNA

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1. Introduction

Recent studies have established that a 16 S RNA precursor exists for the β-globin mRNA [1–4], but it is unknown whether larger transcripts of this RNA occur or whether a precursor to the α-globin mRNA exists. Using globin cDNA cellulose, a 27 S RNA has been isolated [5] from erythroleukemia cells and evidence presented, based on competition hybridization studies, that it contains globin mRNA sequences [5]. It was suggested to be a precursor to globin mRNA based on pulse-chase studies [5]. Therefore, it was of interest to determine by direct analysis if this RNA contained either α- or β-globin mRNA sequences. In this communication, we describe experiments determining the ability of the 27 S RNA to anneal with the DNA from plasmids containing mouse α- or β-globin cDNA sequences. It was found that the 27 S RNA did not hybridize with the DNA from either plasmid, suggesting that it is not a precursor to either mRNA. No RNA larger than the 16 S RNA hybridized to the DNA of the β-plasmid and only RNA at the size of mature α-globin mRNA hybridized to the DNA of the α-plasmid.

2. Materials and methods

2.1. *Cell culture and labeling*

Growth of Friend erythroleukemia cells and induction of erythroid differentiation was according to [6]. Cells grown for 3 days in the presence of 1.8% dimethylsulfoxide were concentrated by centrifugation and resuspended at 10^7 cells/ml in media containing 100 μCi/ml [^{3}H]uridine for 10–15 min. Isotope incorporation was stopped by the addition of an equal volume of ice cold media.

2.2. *Isolation of globin RNA*

Total RNA was isolated using the proteinase K procedure [1]. RNA containing globin mRNA sequences was purified by two applications to a globin cDNA cellulose column [7].

2.3. *Purification of globin cDNA-containing plasmids*

α- and β-globin cDNA-containing plasmids (pCR1αGM10 and pCR1βGM9) were a gift from B. Mach [8]. *Escherichia coli* strain χ1776 was a gift from R. Curtiss, iii. Transfection of χ1776 with globin cDNA-containing plasmids, growth of transfected bacteria, amplification of plasmids, lysis of bacteria, and methods for clearing the lysate were as in [9]. The growth medium was supplemented with 25 μg/ml kanamycin. Plasmids were isolated from the cleared lysate by making it 0.5% in sodium dodecylsulfate (SDS) and extracting with an equal volume of phenol–chloroform (1:1) for 1 h. Nucleic acids were precipitated from the aqueous phase with ethanol, collected by centrifugation, and dissolved in 10 mM Tris (pH 7.4), 1 mM EDTA. Ethidium bromide was added to final conc. 0.5 mg/ml and cesium chloride was added to reach a specific gravity of 1.55, followed by centrifugation at 39 500 rev./min for 48 h in a Beckman Ti-60 rotor at 23°C. The denser of two DNA bands was collected and extracted 3 times with an equal volume of *n*-butanol, applied to a Biogel A5 gel filtration column, and eluted from the column with 10 mM Tris (pH 8.5), 10 mM EDTA, and 0.5 M NaCl.

Appropriate procedures involving recombinant

DNA were performed under EK-2 and P3 containment conditions in accordance with NIH guidelines.

2.4. *Filter hybridizations*

Plasmid DNA containing either the α- or β-globin sequences was cleaved with the restriction endonuclease *Hin*dIII in 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris (pH 7.4) at 37°C for 1 h. Linearized plasmid DNA was immobilized on Schleicher and Schuell BA85 nitrocellulose filters by the method in [10]. Filter-bound DNA, ~80 μg, was used in each hybridization reaction. Filter hybridization and elution techniques were as in [11], except that the hybridization buffers were modified. Normal stringency hybridization reactions contained 0.3 M NaCl, 10 mM Tris (pH 7.9), 1 mM EDTA, 0.5% SDS, 200 μg/ml tRNA, 200 μg/ml poly(A) and 50% deionized formamide, and were incubated at 45°C for 18 h. Reduced stringency hybridization reactions were the same except that the NaCl was increased to 0.75 M and the temperature reduced to 37°C.

2.5. *Polyacrylamide gel electrophoresis*

Filter-bound and unbound RNA was analyzed on 4% polyacrylamide gels in the presence of 99% formamide as in [12].

3. Results

The experimental design for determining if the 27 S RNA contained globin mRNA sequences involved isolating this RNA using globin cDNA-cellulose and hybridizing it to DNA from plasmids containing either the α- or β-globin sequences. Following 3 days induction with dimethylsulfoxide, Friend erythroleukemia cells were pulse labeled for 10 min with [^{3}H]uridine and the RNA isolated. The radioactive RNA was applied to a globin cDNA cellulose column as in [7], and the retained material analyzed by polyacrylamide gel electrophoresis in the presence of 99% formamide, (fig.1). Three major components were observed; mature globin mRNA, the 16 S β-globin mRNA precursor as in [1–4], and the 27 S RNA as in [5]. To determine if the 27 S RNA actually contained either α- or β-globin sequences, the RNA retained by the globin cDNA cellulose column was annealed to DNA from plasmids containing the

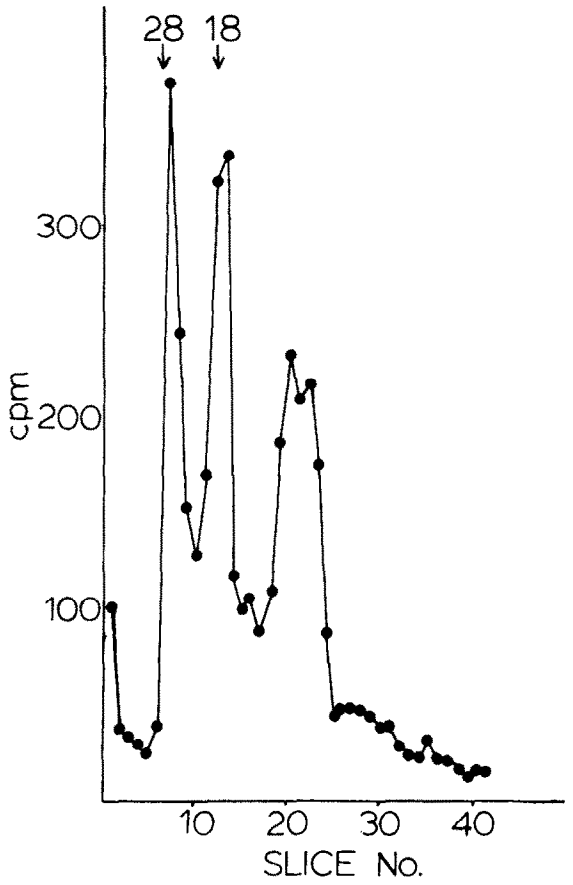

Fig.1. Polyacrylamide gel analysis of globin cDNA cellulose-bound RNA isolated from pulse-labeled Friend erythroleukemia cells. The 3 peaks represent the 9 S globin mRNA, the 16 S β-globin mRNA precursor and the 27 S putative globin mRNA precursor.

α-globin (pCR1αGM10) and β-globin (pCR1βGM9) DNA. The recombinant plasmids were cleaved with the restriction endonuclease *Hin*dIII, denatured with alkali, and bound to nitrocellulose filters prior to the hybridizations.

Filter hybridizations were carried out with 2–3 fold excess of DNA. Following the hybridizations, the filter-bound material was eluted with 90% formamide and both the bound and unbound fractions precipitated with ethanol and analyzed on formamide gels (fig.2). Of the input radioactivity, 20% and 16%, respectively, hybridized to the α- or β-cDNA-containing filters. Mature globin mRNA annealed to both

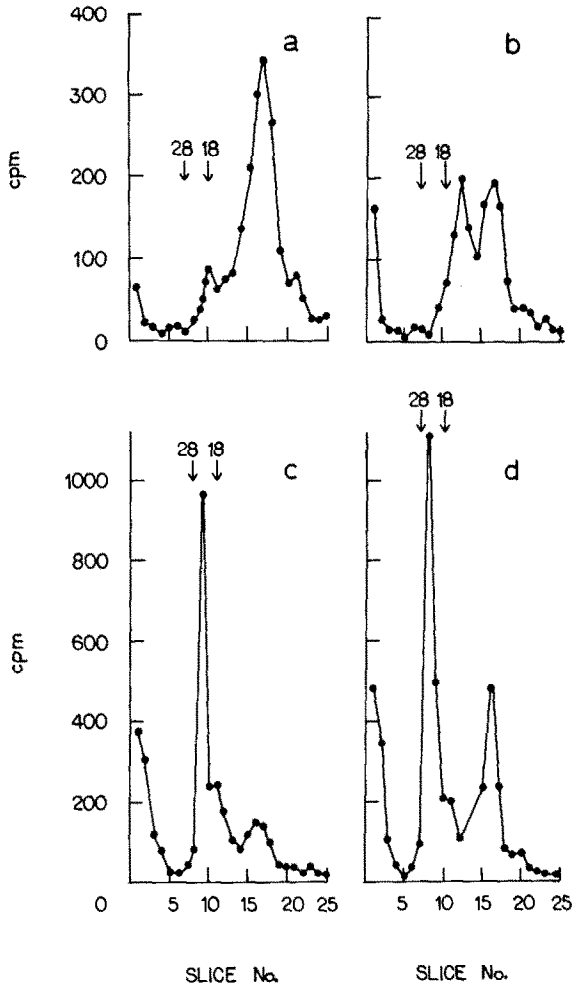

Fig.2. Polyacrylamide gel analysis of bound and unbound RNA after hybridization to α- and β-globin cDNA-containing filters. Globin cDNA cellulose bound RNA from a pulse-labeling experiment was applied to the α- and β-globin cDNA-containing filters under conditions of normal hybridization stringency as in section 2. (a) RNA bound to the α-globin cDNA-containing filter. (b) RNA bound to the β-globin cDNA-containing filter. (c) Unbound RNA from the α-filter hybridization reaction. (d) Unbound RNA from the β-filter hybridization reaction.

filters whereas the 16 S globin mRNA precursor hybridized to only the β-filter. This is in agreement with [4]. The 27 S RNA hybridized to neither filter and was found exclusively in the unhybridized fractions.

It should be noted that some radioactivity was found in the first one or two slices of the gels. This phenomenon was variable and probably represents RNA trapped at the gel origin. Material at the top of the gel is sometimes observed when gradient purified globin mRNA alone is electrophoresed.

It is possible that the 27 S RNA is transcribed from a minor β-globin gene [13], and would not cross-hybridize with plasmid DNA containing sequences from the major β-gene. The globin cDNA cellulose used in these studies was synthesized from total globin mRNA isolated from mice synthesizing α, β-major, and β-minor globins. Thus, the existence of minor β-globin sequences in the 27 S RNA would allow it to be retained by the cDNA cellulose column but possibily not permit it to hybridize to the plasmid cDNAs which contained only α- and β-major sequences. Friend cells express both the major and minor β-globin sequences [13].

The possibility that the 27 S RNA contained β-minor globin sequences was ruled out by hybridizing globin cDNA-bound RNA to α- and β-filters under conditions where the hybridization stringency was reduced significantly below the point required for cross-hybridization to occur between major and minor β-globin mRNAs [14]. Bound and unbound RNA from the low stringency filter hybridizations were analyzed on polyacrylamide gels in the presence of 99% formamide (fig.3). The results clearly show the absence of globin mRNA sequences in the 27 S RNA by its failure to hybridize to the α- or β-filters under conditions that have been shown to allow extensive cross-hybridization between major and minor β-globin sequences [14]. This observation together with the lack of any other large RNA hybridizing to globin cDNA indicates that the 16 S β-globin RNA may be the largest globin transcript present.

4. Discussion

The failure of either the α- or β-globin cDNA-containing plasmids to hybridize to the 27 S RNA clearly demonstrates the absence of globin mRNA sequences in this RNA. Even under conditions where the hybridization stringency is reduced, the 27 S RNA does not anneal, indicating that it is not a precursor

to a minor globin mRNA. The hybridization studies also show that no other RNA larger than 16 S is present in the globin cDNA-bound RNA from erythroleukemia cells. These findings are observed regardless of the stage of induction or the length of labeling (data not shown).

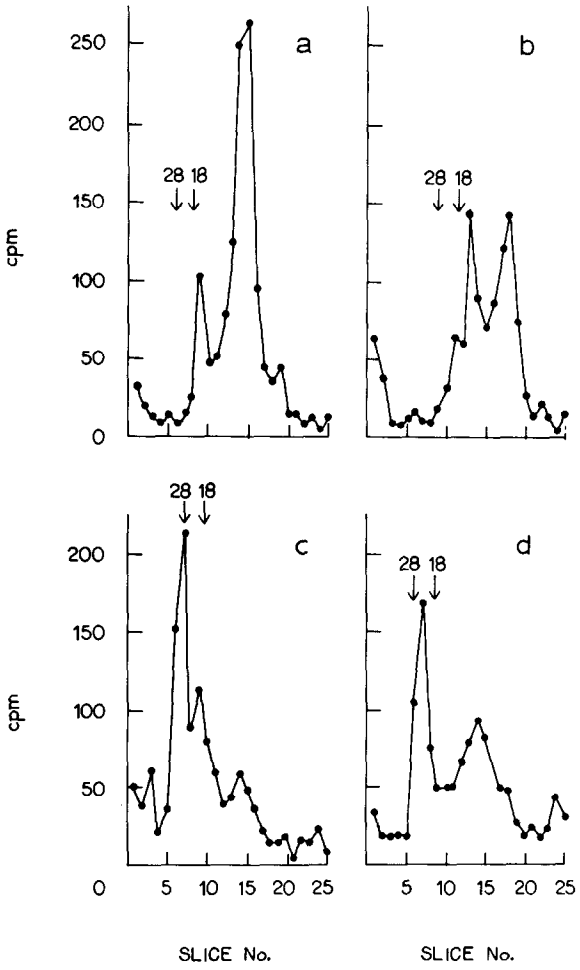

Fig.3. Polyacrylamide gel analysis of bound and unbound RNA after hybridization to α- and β-globin cDNA-containing filters. Globin cDNA cellulose bound RNA from a pulse labeling experiment was applied to the α- and β-globin cDNA-containing filters under conditions of reduced hybridization stringency as in section 2. (a) RNA bound to the α-globin cDNA-containing filter. (b) RNA bound to the β-globin cDNA-containing filter. (c) Unbound RNA from the α-filter hybridization reaction. (d) Unbound RNA from the β-filter hybridization reaction.

Our data is in contradiction with competition hybridization experiments [5]. Mature globin mRNA, 16 S RNA, and the 27 S RNA were all observed to compete with the hybridization of ^{125}I-labeled globin mRNA to globin cDNA. Increasing amounts of these competitors eventually achieved greater than 90% inhibition of ^{125}I-labeled globin mRNA hybridization to globin cDNA. Since the 16 S RNA contains only β-globin sequences, it should be expected to compete against no more than 50% of the labeled globin mRNA hybridization. These competition results could be explained by the presence of contaminating 9 S globin mRNAs in the 16 S and 27 S RNA preparations.

The reason that the 27 S RNA is retained by the globin cDNA cellulose is unknown but it probably can be explained by a contaminating RNA in the globin mRNA used to synthesize cDNA cellulose. Only a small amount of contamination in the globin cDNA would be required to bind a non-globin RNA with high specific activity such as the 27 S RNA. This is particularly so as the column is used under conditions of DNA excess.

It should be noted that 9 S globin mRNA from a short exposure to [^{3}H]uridine contains more α-globin sequences than β-globin sequences (fig.2,3). This suggests that the initial transcript of the α-globin gene may be identical to or close to the size of the mature mRNA. Thus, 9 S α-globin RNA would appear earlier since its accumulation would require little or no processing.

Acknowledgements

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