PRECURSORS OF DISTINCT SIZE FOR CHICKEN α^A, α^D and β GLOBIN mRNAs

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1. Introduction

Globin pre-mRNA molecules extracted from avian erythroblast nuclei have been characterized by fractionation on DMSO–sucrose gradients [1]. Recently, high M_r globin pre-mRNA including upto 15 kilobases, from the same source, were found after fractionation of labelled nuclear RNA, by molecular sieving on a Biogel A50 column followed by electrophoresis on polyacrylamide gels in pure formamide at high temperature (45–50°C, see [2]). RNA fractions of chosen M_r range were titrated for globin-specific sequence content by RNA excess hybridization in liquid phase using labelled cDNA as probe. This very sensitive technique allowed detection of as few as 1 globin mRNA molecule/cell. The labelled probe, however, contained copies of both α- and β-globin mRNAs and hence did not distinguish between the different globin pre-mRNAs.

High M_r nuclear RNAs ($2–5 \times 10^6$) were shown containing, in covalent linkage, a globin coding sequence [2]. Furthermore, such analysis revealed that the distribution of globin pre-mRNA was heterodisperse encompassing the entire size spectrum of nuclear RNA. It was maximal between $2–6 \times 10^5$ M_r and represented molecules the size of mature globin mRNA as well as the '15 S' globin pre-mRNA. The latter species is the best described globin precursor in the erythroid systems studied [3–9]. At steady state it accounts for ~2000 molecules/cell. However, discrete populations of globin pre-mRNA of ~28 S have also been detected [10–13].

We have investigated by Southern transfer and hybridization the steady state size distribution of precursors of α^A, α^D and β globin mRNAs using clone purified globin cDNA as probes.

2. Materials and methods

2.1. *Preparation of nuclear RNA*

Immature red blood cells from acetylphenylhydrazine-induced anemic chicks were labelled with [^{3}H]uridine (100 μCi/ml packed cells) for 45 min. The labelled cells were then subjected to 'isotonic lysis' and the nuclei were isolated as in [14]. RNA was extracted from the free nuclei as in [2].

2.2. *Fractionation of nuclear RNA by gel filtration chromatography*

Total nuclear RNA was fractionated on a 2.6 × 100 cm column (~500 ml vol.) of Biogel A50m (100–200 mesh) in 50 mM lithium acetate, 10 mM acetic acid and 1 mM EDTA, adjusted to pH 6.0, at 4°C. Nuclear RNA (12 mg) was taken up in 5 ml buffer composed of 10 mM triethanolamine (pH 7.4) and 10 mM NaCl, heat denatured at 65°C, cooled, and applied to the column. Fractionation was performed at a flow-rate of 12 ml/h and fractions were collected every 20 min. Absorbance was monitored at 254 nm by an ISCO UA5 absorbance unit. Aliquots (10 μl) were taken from each fraction for radioactivity measurement. Fractions from the exclusion volume, coinciding with the peak of the radioactivity profile (fractions 34–39), were pooled, ethanol precipitated, and the RNA recovered after centrifugation.

2.3. *Preparation of chick globin chain-specific cDNA probes*

Recombinant plasmid DNA containing either 250 μg α^A, α^D or β duck globin DNA were each linked covalently to individual strips of chemically activated diazobenzyloxymethyl (DBM) paper. All the DBM strips were pooled, and the linked DNA hybridized in a common reannealing reaction to 45 μg highly puri-

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fied chick globin mRNA (9 S) for 36 h. The mRNAs specific for α^A, α^D and β globin chains were separately eluted from the DNA–RNA hybrids.

The mRNAs were transcribed into complementary DNAs using RNA-dependent DNA polymerase of AMV with all 4 deoxynucleotides labelled with ^{32}P [1].

2.4. *Agarose gel electrophoresis*

Preparation of the gel, glyoxalation of the RNA, and the in situ enzymatic cleavage of the fractionated RNA was as in [15]. In brief, 30 μg nuclear RNA fractionated by gel filtration on a Biogel A50m column, was denatured by treatment with glyoxal and separated by electrophoresis on a 1% agarose gel. The gel was then exposed to the action of micrococcal nuclease and the in situ cleaved RNA transferred on nitrocellulose sheets as in [16]. The RNA blots were hybridized with 5×10^6 cpm/cm^2 of ^{32}P-labelled globin chain-specific DNA probes of spec. act. $3–4 \times 10^9$ cpm/μg. All subsequent steps of washing and autoradiography were as in [15,16].

3. Results

Immature red blood cells from anemic chicks were labelled with [^{3}H]uridine (21 Ci/mmol) at 100 μCi/ml of packed cell volume. The controlled lysis of cells eliminated most, if not all, of the cytoplasmic RNAs without disruption of lysosomes and yielded free nuclei. Extensive treatment of the detergent disrupted nuclei with DNase (RNase free) and hot phenol extraction at low pH, eliminated most of the DNA from the RNA preparation. A second cycle of DNase treatment followed by phenol extraction further purified the nuclear RNA without affecting their size. The RNA obtained had spec. act. 4.4×10^3 cpm/μg.

Gel filtration chromatography using the molecular sieving properties of Biogel A50m at low pH and low ionic conditions eliminated small RNA molecules and DNA fragments generated due to the action of DNase added during the nuclear RNA preparation. About 5% of the RNA was recovered from the exclusion volume which contained over 50% of the radioactivity. Fig.1 shows the elution profile of such a column, indicating the fractions, corresponding to the peak of the radioactivity profile, pooled for further analysis.

The probes employed for the detection of globin pre-mRNA sequences were ^{32}P-labelled chick globin chain-specific cDNAs. Although we had at our disposal the various chick globin cDNA clones, we preferred here the use of in vitro synthesized cDNA and not nick-translated cloned DNA, since the former gave spec. act. $3–4 \times 10^9$ cpm/μg, which represented a 5–10-fold increase in radioactivity/μg DNA and hence more sensitivity for our hybridization procedures.

Recombinant plasmid DNA containing α^A, α^D or β globin DNA of duck, bound to the DBM paper, were employed for the isolation of chick α^A, α^D and β globin mRNAs. The rationale of this approach in which duck globin DNA containing plasmids were used for isolating chick globin mRNAs was based on the following observations:

(i) Duck and chick globin mRNAs share ~75–85% sequence homology;
(ii) Both α and β globin RNAs share sufficient homology so that probes of one cross-hybridizes with the other;

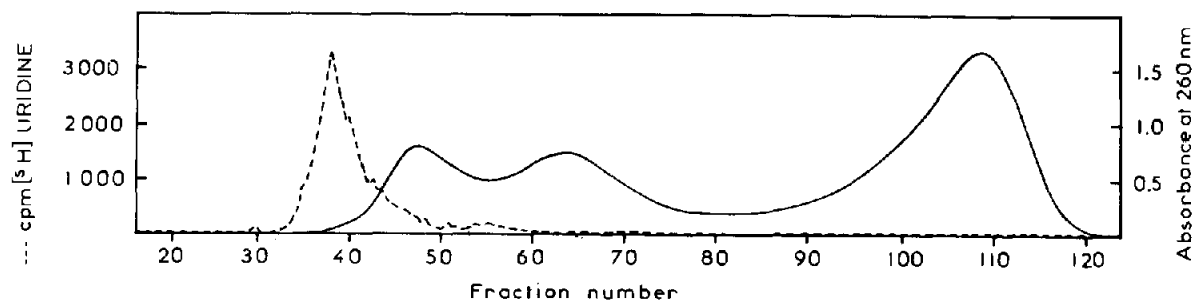

Fig.1. Chick erythroblast RNA (12 mg) was denatured by heating at 65°C, cooled and applied over a 2.6 × 100 cm long Biogel A50 column. Fractionation was performed at 12 ml/h flow rate and fractions were collected every 20 min; (——) shows the absorbance profile measured at 254 nm; 10 μl aliquots from each fraction were precipitated with trichloroacetic acid and radioactivity profile (· · · ·) of the elute determined.

(iii) This is true for the globin mRNAs of either ducks or between those of chicks;
(iv) However, the α of ducks do not share extensive sequences capable of forming stable hybrids with β of chicken and vice versa.

Our procedure for hybridization selection of chain-specific globin mRNAs took advantage of the extensive homology between like-chains and the absence of cross hybridization between unlike chains of one globin species with that of another.

The relative scarcity of large globin pre-mRNA transcripts (~10 molecules/cell; see [2]) prompted us to employ the most sensitive method possible during the current type of experimental approach. This involved synthesis of probes with increased specific activity on one hand, and on the other, devising means of quantitative transfer of RNA from concentrated gels to nitrocellulose sheets prior to hybridization [15]. Fig.2 shows the autoradiogram of the nuclear RNA from chick erythroblasts after hybridization of the blots with ^{32}P-labelled globin chain specific probes. The bands obtained on the autoradiographs are compared to marker RNAs of known M_r that were electrophoresed simultaneously in parallel slots and visualized after ethidium bromide staining and UV illumination. Each probe revealed the existence of two bands corresponding to two M_r classes of globin sequence containing RNA. With all 3 globin-specific probes we observed the existence of a band corresponding to ~600 bases (the size of globin mRNA, 9 S), while the second band in each corresponded to a distinct size precursor. They were 1.0, 1.3 and 1.8 kilobases for the α^A, α^D and β globins, respectively.

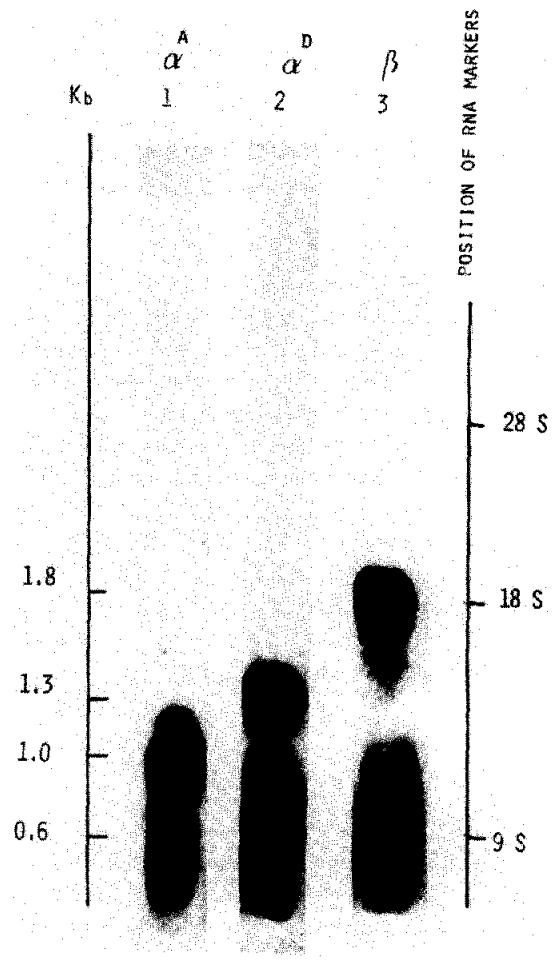

Fig.2. Total nuclear RNA was fractionated on a Biogel A50 column under non-denaturing conditions. RNA from the exclusion volume (30 μg/slot) was denatured by glyoxal treatment and further fractionated on 1% agarose slab gel after which it was cleaved in situ by micrococcal nuclease and transferred to nitrocellulose sheets. The RNA blots were hybridized with 5 × 10^6 cpm/cm^2 of ^{32}P-labelled globin chain-specific probes of spec. act. 3–4 × 10^9 cpm/μg and the hybrids visualized after autoradiography. Globin 9 S mRNA and ribosomal 18 S and 28 S RNA served as M_r markers during electrophoresis.

4. Discussion

The autoradiograms of the hybrids formed between the nuclear RNA and the chain-specific globin cDNA each showed 2 distinct bands. All 3 DNA probes hybridized to their respective RNA homologues at positions corresponding to ~600 nucleotides and hence the size of mature globin mRNAs. The presence of this prominent band indicates that gel filtration chromatography at low pH and low ionic strength on Biogel A50, but in aqueous buffer, had not fully eliminated molecules with the size of globin mRNA from the exclusion volume. The second band in the autoradiogram corresponds to globin mRNA precursors. The sizes of the mRNA precursors observed for each of the 3 globin chains were distinctly different. The α^A globin mRNA showed a precursor of 1.0 kilobases while the α^D and β globins showed precursors of 1.3 and 1.8 kilobases, respectively. The relative size of the different globin mRNAs coding for the 3 different types of chains being about the same, the difference in the size of their precursors must then be attributed to the difference in the size of their intervening sequences.

The size of the chick β-globin gene has been estimated to be ~1.6 kilobases long and shown to contain 2 intervening sequences totalling 950 nucleotides [17]. The size of the β-globin mRNA precursor observed by us is ~1.8 kilobases. The difference of 200 nucleotides between the pre-mRNA size and the length of the β-globin gene from 'Cap-to-poly-(A)' site remains unexplained. A very similar situation was observed for mouse β globin pre-mRNA of 1.8 kilobases [9,11,13] for which the 'gene' size was reported to be 1.6 kilobases [18].

The primary structure of the 'stress' α and 2 embryonic α-like [19] globin coding messengers are now known and the chromosomal organization of α and α-like globin genes have been established [20–22]. However, the fine structure of the DNA regions containing the α-globin genes has not yet been elucidated. In the mammalian system, the size of the intervening sequences in the α-globin genes are smaller than those in the β-genes [18,23,24]. If an analogous situation exists for the avian globin genes, then the difference in precursor size reflects also the difference in their intervening sequences.

The 3 abundant and stable precursor mRNAs of 1.0, 1.3 and 1.8 kilobases for the α^A, α^D and β globins, respectively, observed by us may also represent the major globin pre-mRNA populations observed in the avian erythroid system [1,5,10,25].

Analysis of nuclear RNA from reticulocytes of β^+ thalassemia patients showed the presence of several intermediate-sized precursors, in the range of 1.8 and 0.6 kilobases, for the β globin mRNA [26]. No such intermediates have been observed by us in the chicken system.

Electrophoretic separation and liquid phase hybridization analysis with labelled globin cDNA probes have enabled the determination of globin sequence containing nuclear RNA within the size range of 6–15 kilobases [1,2,10]. Pulse-chase experiments [11] and electronmicrographs of 'R-loops' formed between globin gene containing DNA and large M_r pre-mRNA have added supportive evidence for the existence in the nuclear RNA, of large globin sequence containing transcripts. Using the blot-hybridization technique, we have been unable to detect any of these large M_r globin pre-mRNAs. Admittedly the technique of blot-hybridization employed here, is not adequate for discerning a continuous spectrum of fugitive molecules of globin pre-mRNA. The main obstacle to their visualization is their rarity and the rapidity with which they are processed, forcing the weak signals, if any, in the autoradiogram to merge into each other and become indistinguishable from the inherent background noise. Given the limitations of the technique and the nature of the globin gene transcripts, these results are confined to the demonstration of globin mRNA precursors that are stable, abundant and perhaps representing processing intermediates.

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