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Characterization of mouse reticulocyte free globin mRNP

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Globin messenger ribonucleoprotein particles (free and polysomal) from mouse reticulocyte lysates were characterized for their mRNA composition, translational activity as well as the proteins in direct contact with them. In contrast to the homogeneous single-peak distribution of rabbit and duck reticulocyte free mRNPs, mouse free mRNP particles were heterogeneously dispersed on the sucrose density gradient into two major domains called region I and region II. Region I appeared enriched with α-globin mRNP and region II with β-globin mRNP. mRNP from both regions was translationally active. Examination of lysates prepared from β-thalassemic mice revealed a reduction of translatable β^{minor} mRNP within region I, supporting the hypothesis of a compensatory recruitment of β^{minor} free mRNP into polysomes in β-thalassemic mice.

Messenger ribonucleoprotein; Ultraviolet crosslinking; Thalassemia; (Mouse, Reticulocyte)

1. INTRODUCTION

Recruitment and repression of mRNA molecules are important mechanisms in the translational control of gene expression in eukaryotic cells. Within mammalian reticulocytes, approximately 5–10% globin mRNA molecules exist as free messenger ribonucleoprotein particles (mRNPs) [1]. Free globin mRNP in mouse spleen erythroblasts infected with Friend leukemia virus is reported to be translationally repressed [2]. Various models for human thalassemias result from deletions in either α- or $\beta^{Maj.}$-globin gene locus. Interestingly, erythroid cells from these thalassemic mice can partially compensate for the missing gene at the level of translation [4,5]. The partial normalization of α- and β-globin chain ratio in mutant mice could involve sequestration of either α- or β-globin mRNA as a translationally repressed mRNP particle. Mouse free globin mRNP particles are stable and provide a unique opportunity to determine the native structure of α- and β-globin mRNAs in the cells which may lead to confirmation of the secondary structure model proposed for mouse $\beta^{Maj.}$-globin mRNA [6]. To understand the regulatory mechanism of translational control in mouse reticulocytes, we have characterized the free globin mRNP particles in mouse reticulocyte lysates. Here we report their mRNA composition, translational activity, relative α- and β-globin chain synthesis, as well as analysis of the proteins intimately associated with them. Furthermore analysis of free globin mRNP particles from homozygous (Hbb^{thal}/Hbb^{thal}) β-thalassemic mouse lysates has been carried out.

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2. MATERIALS AND METHODS

2.1. *Analysis of free and polysomal globin mRNP particles*

Induction of reticulocytes in Swiss mice and preparation of lysates have been described [7]. Polyribosomes were isolated from lysates by centrifugation through linear 15–30% (w/w) sucrose gradients containing 85 mM KCl, 5 mM $MgCl_2$, 25 mM triethanolamine-HCl (pH 7.5) and 0.5 mM PMSF (phenylmethylsulfonyl fluoride). Free mRNP particles were isolated by centrifugation through 28 ml linear 20–40% (w/w) sucrose gradients containing 20 mM KCl, 1.5 mM $MgCl_2$, 25 mM triethanolamine-HCl (pH 7.5) and 2 μg/ml PLA (pepstatin, leupeptin, aprotinin). 4.0 ml of lysate diluted to

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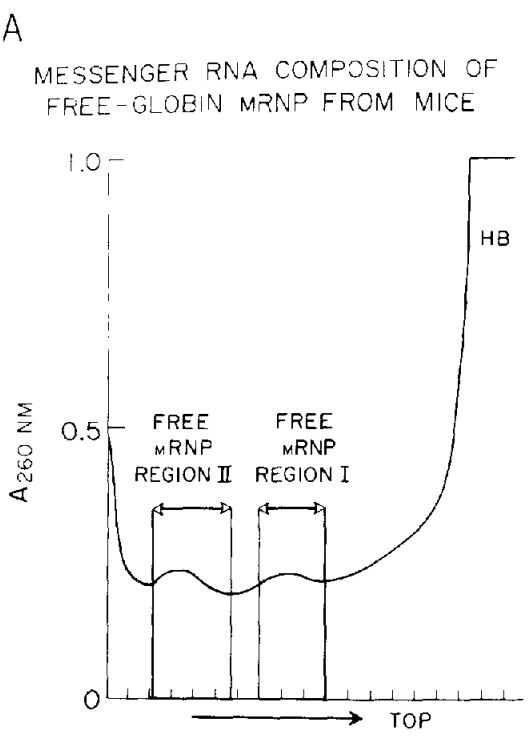

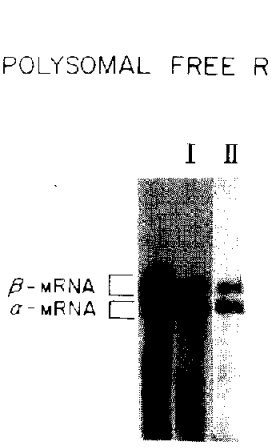

Fig.1. (A) Isolation of mouse (normal) reticulocyte free globin mRNP by sucrose density gradient centrifugation. Fractions shown by the double-headed arrow were pooled and their polyadenylated mRNA purified. (B) Autoradiogram of in vitro 3′-^{32}P-end-labeled mRNA purified from both the free mRNP particles and polyribosomes, electrophoresed on a 3.5% polyacrylamide gel in 7 M urea.

9.0 ml with gradient buffer were layered per gradient and centrifuged at 4°C for 44 h in SW28 rotor at 95000 × *g*. Fractions in region I and region II (fig.1A) were pooled, ethanol precipitated, deproteinized with phenol [8], and purified by oligo(dT)-cellulose column chromatography. The resulting mRNA was labeled with [^{32}P]Cp using T_4 RNA ligase [9]. The 3′-^{32}P-end-labeled mRNAs were electrophoresed on denaturing 3.5% polyacrylamide gel [10]. The individual α- and β-globin mRNAs were excised upon autoradiography and their radioactivity quantitated.

2.2. *In vitro translation of mRNA and mRNP*

The translational activity of mouse free mRNP particles as well as mRNA obtained either from polyribosomes or individual free mRNP was assayed in mRNA-dependent in vitro translation systems prepared from both wheat germ and rabbit reticulocyte lysates (Bethesda Research Laboratories) as detailed in [11] in the presence of L-[^{35}S]methionine (NEG-009T, New England Nuclear). Radiolabeled globin chains synthesized in the rabbit reticulocyte lysates were fractionated by carboxymethyl-cellulose chromatography on a Whatman CM52 column [12]. The relative synthesis of α- and β-globin chains was determined by comparing the distribution of L-[^{35}S]methionine in α- and β-globin peaks.

2.3. *Ultraviolet light-induced cross-linking*

Mouse reticulocyte lysate or freshly drawn blood from anemic animals was irradiated with ultraviolet light (λ =

Table 1

Distribution of relative quantities of α- and β-globin mRNAs and ratio of globin chains synthesized by mRNAs in polysomal and post-polysomal (free) region of sucrose density gradient (normal mouse)

Sample	% mRNA		α:β mRNA	α:β globin chain
	α	β		
Polysomal mRNA	61.08 ± 2.0	38.92 ± 2.0	1.57	0.42
Region I mRNA	75.24 ± 2.0	24.76 ± 2.0	3.04	1.48
Region II mRNA	58.12 ± 2.0	41.88 ± 1.0	1.39	0.69

The data represent the average of four independent experiments

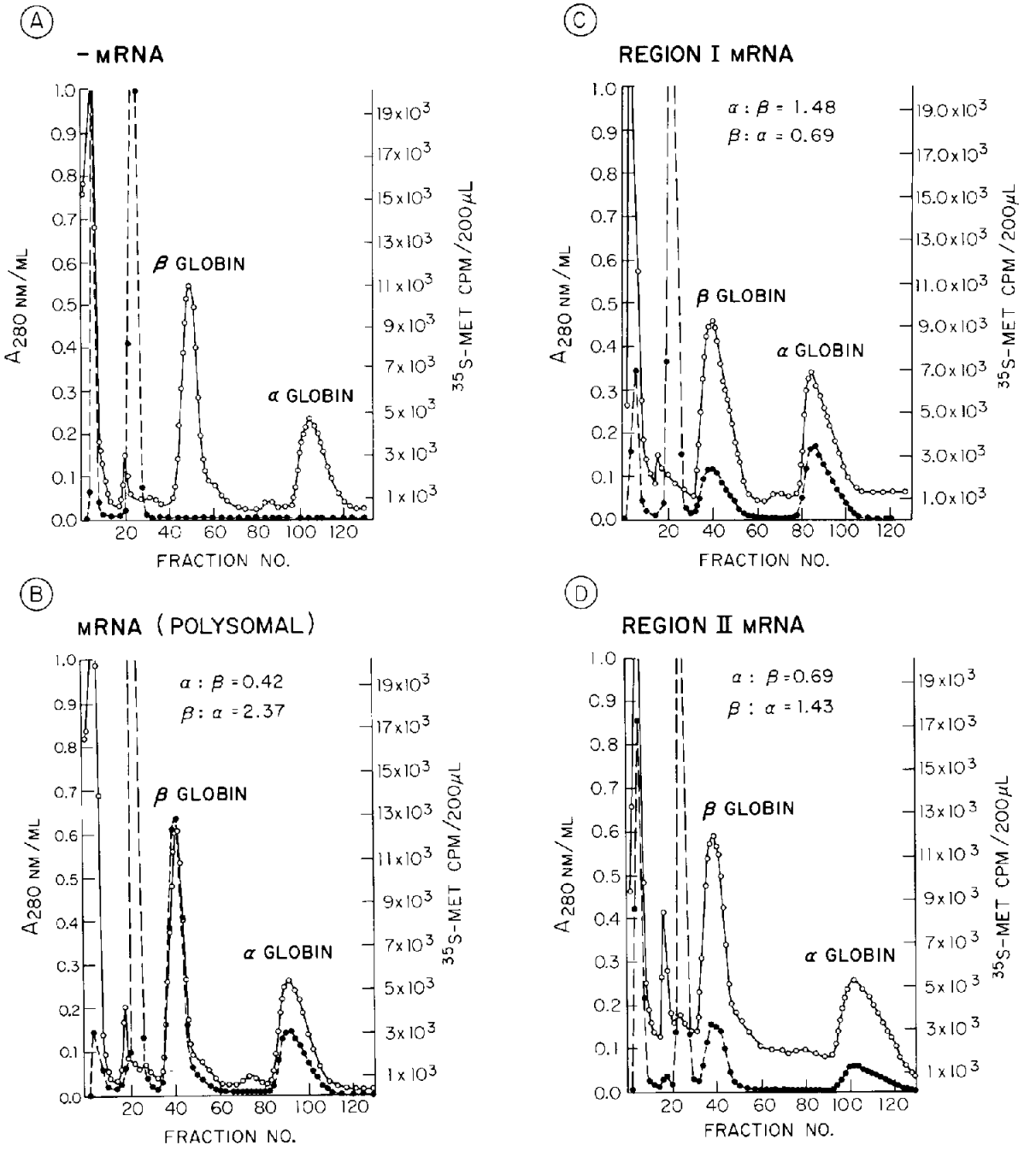

Fig.2. Urea-carboxymethyl-cellulose column chromatography of globin synthesized in the presence of L-[^{35}S]methionine in a rabbit cell free translation system programmed with mouse hemoglobin mRNA at near saturation concentration. After incubation for 60 min at 30°C, the cell free system was mixed with 45 mg of mouse carrier hemoglobin. Globin was prepared by a modification of the method detailed in [12]. Globin dissolved in 0.01 M Na_2HPO_4-H_3PO_4 buffer, pH 6.9, containing 8 M urea and 0.05 M β-mercaptoethanol, was applied to 1.5 cm × 15 cm Whatman CM-52 carboxymethyl-cellulose column. The column was eluted with a linear gradient of 0.01 M Na_2HPO_4/0.022 M H_3PO_4 buffer, pH 6.9, containing 8 M urea and 0.05 M β-mercaptoethanol at 60 ml/h. 5 ml fractions were collected, their absorbance measured at 280 nm (○—○) and the radioactivity (^{35}S) determined (●---●) in a liquid scintillation counter. Globin synthesis: (A) in the absence of external mRNA; (B) in the presence of polysomal mRNA; (C and D) in the presence of mRNA recovered from region I mRNP and region II mRNP, respectively.

254 nm) at a dose of $5-7 \times 10^5$ ergs/mm^2 using four 15-W germicidal lamps (no.34 UVP Inc.) as detailed in [13].

2.4. *Purification and radiolabeling of cross-linked proteins*

UV irradiated lysates were fractionated on sucrose density gradients as described in section 2.1. Gradient fractions containing cross-linked either polysomal or free mRNP were pooled and purified as described in [8,13,14]. The yield of purified total free mRNP (region I and II) was estimated to be ~5 μg per 100 mice. Purified cross-linked mRNPs were radiolabeled by reductive methylation with [^{14}C]formaldehyde (New England Nuclear, 52 mCi/mmol) as described in [13,14]. Labeled proteins were analyzed by electrophoresis on 10% SDS-polyacrylamide gels [15]. After electrophoresis gels were impregnated with Amplify (Amersham), dried and fluorographed with Kodak XAR-5 film, autoradiograms were scanned using a Shimadzu dual wavelength chromato-scanner, model CS-930.

3. RESULTS

Fig.1 shows the isolation and analysis of free mRNP particles from normal mouse reticulocytes. Unlike the single peak of free mRNP in rabbit [16] or duck [13], the mouse free mRNP reproducibly fractionates into two distinct translationally active regions on sucrose density gradients (fig.1A, region I and region II). This is the first report of such subfractionation of free globin mRNP in any organism. A polyacrylamide gel electrophoretic analysis of 3'-^{32}P-end-labeled polyadenylated RNA (fig.1B) from each region as shown in table 1 indicated the relative enrichment for α-mRNA in region I and for β-mRNA in region II. The individual free mRNP from region I and region II were comparably active showing translational activity similar to their corresponding protein-free mRNAs at subsaturating concentrations (2.5 μg/ml) in both in vitro translation systems.

In addition to analysing the mRNAs from these fractions, the translational product of mRNAs from region I and region II was also investigated. Since the mouse globin chains do not resolve in 12.5% phosphate buffered SDS-polyacrylamide gels, the globin synthesized in a rabbit cell-free system programmed with mouse mRNA/mRNP was fractionated by urea-carboxymethyl-cellulose column chromatography. As seen in fig.2 and table 1, there is relatively greater synthesis of α-globin chains in region I mRNA and β-globin chains in region II mRNA. Examination of the reticulocyte lysate prepared from β-thalassemic mice using the approaches described above reveal-

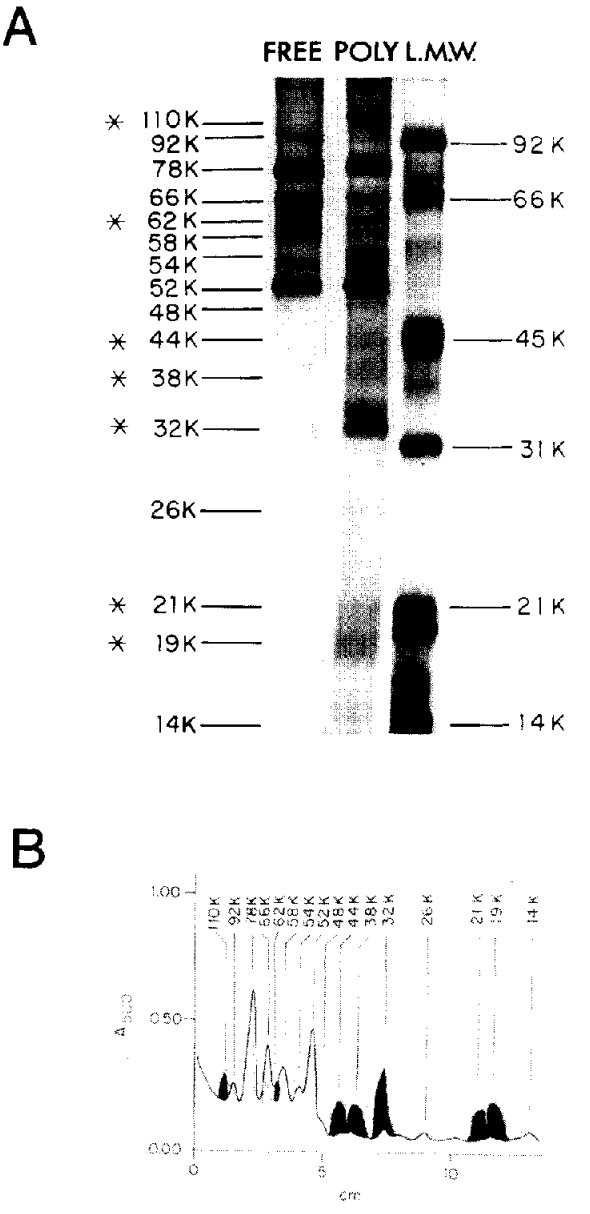

Fig.3. (A) An autoradiogram of a 10% polyacrylamide gel analysis of the ^{14}C-labeled proteins cross-linked to polysomal (POLY) and free mRNP (FREE) isolated from UV-irradiated blood of anemic mice. LMW, ^{14}C-labeled low-molecular-mass marker proteins. (*) Designates proteins specifically enriched in either polysomal or free mRNP. 30000 cmp were loaded per lane. (B) Densitometric scans of the free (——) and the polysomal mRNP (···) lanes superimposed. Shaded areas indicate major differences in absorbance within specific peaks.

ed a reduction of translatable β^{minor} mRNP within region I (not shown).

To avoid difficulties in identifying mRNP proteins using conventional isolation techniques or in vitro reconstitution [14,17,18], we used an alternative approach [13] in which intact cells were irradiated with ultraviolet light to induce photo-cross-linking of RNA to proteins. Proteins in direct contact with mouse (normal) globin mRNA (free and polysomal) were determined as detailed in section 2. Fig.3A shows an SDS-polyacrylamide gel electrophoretic analysis of ^{14}C-labeled proteins cross-linked to free (region I and II combined) and polysomal mRNA. Fig.3B shows densitometric scans of polysomal and free mRNP (lanes superimposed). The 92-, 78-, 66-, 58-, 54-, 52-, 44-, 38-, 32-, 26-, and 14-kDa proteins were cross-linked to both polysomal and free mRNP with significant enrichment of 32-, 38-, and 44-kDa proteins in polysomal mRNP particles. Some of these proteins (92-, 78-, 58-, 52-, and 38-kDa) are similar in mobility to those determined to be cross-linked to rabbit globin mRNA [14] and duck polysomal mRNA [13]. As seen in fig.3A proteins 19-, 21-, and 110-kDa are specifically associated with polysomal mRNA, while the other visible bands are common to both, 'Free' and 'Poly' lanes. Because of the limited quantity of material present in free mRNP region I and region II it was not possible to examine proteins cross-linked within these individual mRNPs.

4. DISCUSSION

The data presented above constitute the first report on characterization of free mRNP particles from mouse reticulocytes which encode globin. The data also clearly show that in the mouse reticulocyte system, β-globin mRNA is a better initiator than α-globin mRNA. As opposed to the translationally repressed free mRNP in mouse spleen erythroblasts infected with Friend leukemia virus [2], mouse reticulocyte free mRNP particles are translationally active. The dissimilarity in translational activity between spleen and reticulocytes particles could be a reflection of either the different stages in erythrocyte differentiation or the Friend leukemia viral infection; or both. Free mRNPs in β-thalassemic mouse lysates are also translationally active. Although further experimentation is necessary, the relative reduction of translatable β^{minor} mRNP in region I supports the suggestion of a compensatory recruitment of β^{minor} free mRNP into polyribosomes as a mechanism of translational regulation in β-thalassemic mice.

Ultraviolet light-induced cross-linking of proteins 110, 21-, and 19-kDa exclusively in polysomal mRNP suggests that these proteins are specifically required for active translation of mRNA. Many of the proteins photo-cross-linked in mouse globin polysomal mRNP are quite similar in molecular mass to those previously found cross-linked to polysomal mRNA from HeLa cells [14,18], L cells [14], Ehrlich ascites tumor cells [19], chicken muscle cells [20] as well as from adenovirus [21]. The striking similarity in proteins cross-linked to reticulocyte and other cellular mRNAs [14,18,19] suggests that these mRNA-bound proteins have been conserved throughout evolution and likely have a defined function within a cell. We are currently exploring the possibility of identifying some of these cross-linked proteins as initiation factors, elongation factors, ribosomal or cytoskeletal proteins.

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