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Identification of the proteins in direct contact with duck globin mRNA

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Proteins in direct contact with translationally active and repressed duck globin mRNA were determined by irradiating blood or lysates with ultraviolet light. Cross-linked proteins from polyribosomes and free mRNP particles were ^{14}C-labeled by reductive methylation and identified on SDS-polyacrylamide gels upon autoradiography. Results indicate that ten cross-linked proteins are common to both polysomal and free mRNP, however, a 44 kDa protein appears to be specific for repressed mRNP particles. Furthermore, the notable lack of cross-linked proteins in the 20–30 kDa range in free mRNP supports the view that the characteristic low molecular mass 'prosomal' proteins, previously found associated with translationally repressed duck globin free mRNP [(1984) EMBO J. 3, 29–34], do not interact directly with the mRNA molecule.

Reticulocyte; Messenger ribonucleoprotein; Ultraviolet cross-linking; Prosome; (Duck)

1. INTRODUCTION

Selective activation and repression of mRNA molecules is an important regulatory mechanism in eukaryotic cells [1–3]. Such control is thought to be mediated either by proteins which can bind to certain regions of an mRNA and increase their translational efficiency [2,3], or by proteins and/or RNA molecules which can mask specific molecular determinants required for translation [1,4–10]. Numerous studies suggest that the globin 20 S free mRNP particle present in nucleated duck reticulocytes is translationally repressed [6,7,11]. Such repression appears to be genuine and has been shown to resist treatment with high salt [6]. Recently, a novel type of RNP particle termed 'prosome' has been found to be associated with translationally repressed duck globin mRNA [12,13]. This particle contains a characteristic set of some 25 proteins (20–30 kDa range) and a number of low molecular mass RNA molecules [12,13]. It has been suggested that these particles may be involved in the cytoplasmic repression of duck globin mRNA [12,13].

Though numerous proteins have been found to be associated with the duck globin 20 S free mRNP particle isolated by conventional techniques [6,7], proteins in direct contact with repressed mRNA, however, have not yet been identified. Photo-induced cross-linking is a 'zero-length' probe, which can monitor intimate contact between a protein and nucleic acid [14–16]. Here, we have identified the proteins in direct contact with both translationally active and repressed duck globin mRNA by ultraviolet light-induced cross-linking. Our results indicate that ten cross-linked proteins are common to both polysomal and free mRNP, however, a 44 kDa and likely a 125 kDa protein

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are found specifically enriched in repressed free mRNP particles. Furthermore, the notable scarcity of cross-linkable proteins within the 20–30 kDa size range in repressed mRNA suggests that the characteristic low molecular mass 'prosomal' proteins may not be in direct contact with the duck globin mRNA.

2. MATERIALS AND METHODS

2.1. *Ultraviolet light-induced cross-linking*

Induction of reticulocytes in Pekin ducks has been described [17]. 50 ml freshly drawn blood from anemic ducks was divided into two 25-ml sterile petri dishes and, while stirring gently at 4°C, irradiated with ultraviolet light (λ = 254 nm) at a dose of 4–7 × 10^5 erg/mm^2 using four 15-W germicidal lamps (no.34 UVP Inc.). Irradiation of reticulocyte lysates was carried out similarly. Irradiation doses were measured with a model J-225 Blak-Ray UV meter (Fisher Scientific).

2.2. *Purification of cross-linked globin mRNP*

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) buffer, 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) buffer and 2 μg/ml of PLA (pepstatin, leupeptin, aprotinin). 4.5 ml lysate diluted to 9.0 ml with gradient buffer was layered per gradient and centrifuged at 4°C for 40–48 h in an SW28 rotor at 95000 × g. Pooled gradient fractions containing either polyribosomes or free mRNP were brought to a final concentration of 1% *N*-lauroylsarcosine (Sarkosyl), 10 mM β-mercaptoethanol, 10 mM EDTA, 500 mM NaCl, and 1 mM PMSF. Cross-linked mRNP was purified by oligo(dT)-cellulose column chromatography [18]. Poly(A)-containing fractions were pooled and brought to a final concentration of 15 mM EDTA in 1% SDS, heated for 2 min at 90°C, and further purified by centrifugation through linear 15–30% (w/w) sucrose gradients containing 0.5% SDS [19]. The yield of purified free mRNP was estimated to be 6–8 μg per duck.

2.3. *Radiolabeling and analysis of cross-linked proteins*

Purified cross-linked mRNP samples were labeled by reductive methylation with [^{14}C]formaldehyde (New England Nuclear, 52 mCi/mmol) and further purified as in [18]. ^{14}C-labeled mRNP samples were dissolved in 20 μl T_2 digestion buffer (0.5% *N*-lauroylsarcosine, 1% β-mercaptoethanol, 1 mM EDTA, 20 mM NH_4OAc, pH 4.5), and incubated for 2 h at 37°C with 0.3 U/μl of T_2 ribonuclease (Sankyo). Digestion mixtures were lyophilized to dryness and proteins analyzed by electrophoresis on 10% SDS-polyacrylamide gels [20]. 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.

2.4. *Analysis of the globin free mRNP particle*

Duck globin free mRNP particles isolated by sucrose density centrifugation were ethanol precipitated, deproteinized with phenol [19], purified by oligo(dT)-cellulose column chromatography, and the resulting mRNA labeled with [^{32}P]Cp using T_4 RNA ligase [21]. The (3′-^{32}P)-end-labeled mRNAs were electrophoresed on denaturing 3.5% polyacrylamide gels [22], and the individual α- and β-globin mRNAs excised upon autoradiography and their radioactivity quantitated.

3. RESULTS

Fig.1 shows the isolation and analysis of free globin mRNP particles from duck reticulocytes. Free mRNP that is prepared by direct centrifugation of lysate without pelleting and resuspension of post-ribosomal supernatants [6] resolves into a major 20 S particle and a minor 35 S species. The 20 S particle contains duck globin mRNA which is comprised of ~66% duck α and ~34% duck β mRNA. The α and β mRNA designations were confirmed by nucleotide sequence analysis of the 5′-terminal regions (not shown) [23]. The 35 S free mRNP consists of a heterogeneous mRNA population and is devoid of globin mRNA [7]. The 20 S free mRNP particle was found to be translationally inactive in both mRNA-dependent rabbit reticulocyte and wheat germ cell-free systems, even

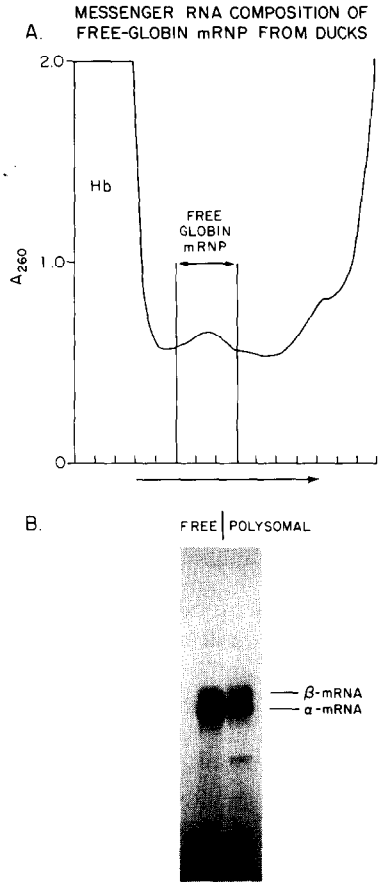
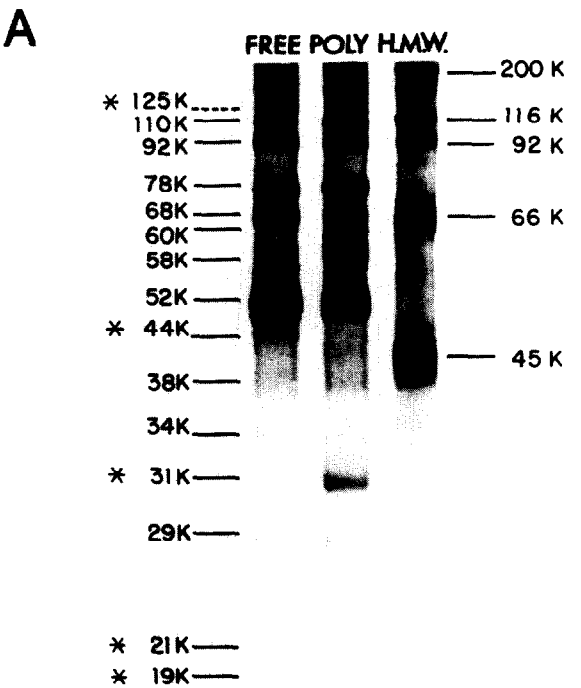


Fig.1. (A) Isolation of duck reticulocyte globin free mRNP by sucrose density gradient centrifugation. Fractions designated by the double-headed arrow were pooled and their polyadenylated mRNA purified. (B) Autoradiogram of in vitro (3′-^{32}P)-end-labeled mRNA purified from both the 20 S free mRNP particle and duck polyribosomes, and electrophoresed on a 3.5% polyacrylamide gel in 7 M urea. Gel dimensions were 20 cm long × 20 cm wide × 1.5 mm thick.

though the purified mRNP-mRNA was active (not shown).

Photo-induced cross-linking of RNA to protein in intact cells rather than after purification or in vitro reconstitution overcomes the many difficulties encountered in identifying proteins naturally associated with mRNA. Proteins in direct contact with duck globin mRNA were determined by irradiating either blood or lysates from anemic ducks with ultraviolet light (λ = 254 nm) as detailed in section 2. Fig.2A shows an SDS-



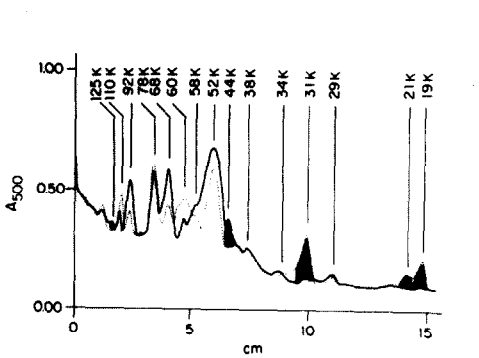


Fig.2. (A) Autoradiogram of a 10% polyacrylamide gel electrophoretic analysis of the ^{14}C-labeled proteins cross-linked to polysomal (POLY) or free mRNP (FREE) isolated from ultraviolet-irradiated blood of anemic ducks. HMW, ^{14}C-labeled high molecular mass marker prteins. (*) Designates proteins specifically enriched in either polysomal or free mRNP. 30000 cpm were loaded per lane. (B) Microdensitometric scans of both the free mRNP (——) and polysomal mRNP (···) lanes superimposed. Shaded areas indicate major differences in absorbance within specific peaks.

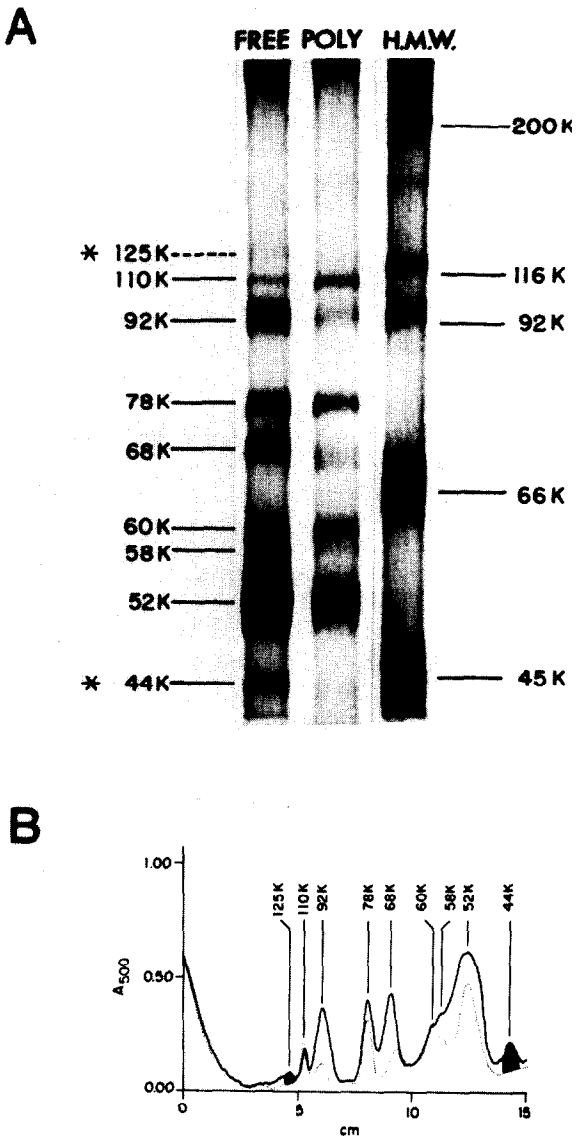


Fig.3. (A) Autoradiogram of a 10% polyacrylamide gel electrophoretic analysis of the ^{14}C-labeled proteins analyzed in fig.2A, but run for a longer time. HMW, ^{14}C-labeled high molecular mass marker proteins. (*) Designates proteins specifically enriched in free mRNP. 30000 cpm were loaded per lane. (B) Microdensitometric scans of both the free mRNP (——) and the polysomal mRNP (···) lanes superimposed. Shaded areas indicate major differences in absorbance within specific peaks.

polyacrylamide gel electrophoretic analysis of ^{14}C-labeled proteins cross-linked to both free and polysomal mRNA. Fig.2B shows microdensitometric scans of both the polysomal and free mRNP lanes superimposed. About ten proteins (110, 92, 78, 68, 60, 58, 52, 38, 34, 29 kDa) were found common to both polysomal and free mRNA though many appeared to differ in relative amounts. Some of these proteins (92, 78, 68, 60, 52, 34 kDa) are similar in mobility to those recently determined to be cross-linked to rabbit globin mRNA [18]. Three proteins (31, 21, 19 kDa) appeared to be specifically enriched in translationally active mRNP. Surprisingly, very few proteins were present in this region of the gel with free mRNP. A 44 kDa and possibly a 125 kDa protein, however, were found to be specifically enriched in repressed mRNP. For better resolution and a more accurate size determination of the higher molecular mass proteins, ^{14}C-labeled samples were electrophoresed for a much longer time on similar gels as shown in fig.3A. Microdensitometric scans of both lanes shown in fig.3B clearly indicate that the 44 kDa protein is predominant to free mRNP and is labeled to a similar extent as are the 21 and 19 kDa proteins predominant to polysomal mRNP. Both the 44 and 125 kDa proteins appear to be specific to avian globin mRNA and were not found cross-linked to rabbit globin mRNA [18].

4. DISCUSSION

Previous studies on proteins associated with duck globin free mRNP particles purified by conventional isolation techniques indicate the presence of possibly 35–50 proteins [7]. Since it is likely that not all of these proteins are in direct contact with the mRNA, we have set out to identify those proteins which are close enough to be cross-linked with ultraviolet light. As shown in fig.2, at least 12 proteins can be cross-linked to the repressed mRNA. Because of the residual RNA oligomer still covalently linked to the protein, it was not possible to determine clearly protein composition by two-dimensional gel electrophoresis. Many of the observed proteins (92, 78, 68, 60, 52, 34 kDa) have similar mobilities to those previously found cross-linkable to other mRNA molecules [18,24,25]. The 44 kDa and possibly the 125 kDa protein, however, appear to be specific for the free mRNP

whether blood or lysates are irradiated. It is noteworthy that a marked reduction of cross-linkable proteins was observed if pooled sucrose gradient fractions containing 20 S mRNP particles were irradiated directly, underscoring the necessity to avoid fractionation prior to irradiation. Both the 44 and 125 kDa proteins appear to be specific to avian globin mRNA and are not found cross-linked to mammalian globin mRNA [18]. Previous analysis of proteins associated with sucrose gradient-purified free mRNP by Vincent et al. [7] indicated the presence of many proteins including a predominant ~120 kDa protein and a phosphorylatable ~48 kDa species. These two proteins were specific for the 20 S mRNP and were not found in the heterogeneous 35 S particle. Whether our cross-linked ~44 and ~125 kD a species are the same proteins and whether they are involved either directly or indirectly in translational repression are currently under investigation.

The existence of the three proteins (31, 21, 19 kDa) specifically enriched in polysomal mRNP suggests that they may be important for the translation of duck globin mRNA. Most striking is the notable lack of free mRNP-specific proteins in this region. Such data support the notion that the characteristic ~25 low molecular mass prosomal proteins within the 20–30 kDa size range are not in direct contact with the duck globin mRNA molecule. Whether the small cytoplasmic RNA molecules (scRNAs) associated with these particles actually interact with the mRNA is currently under study.

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