Efficient translation and polyribosome binding of ^{125}I-labelled rabbit globin messenger ribonucleoprotein

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Rabbit polyribosomal globin messenger ribonucleoprotein (mRNP) was labelled under mild conditions, using ^{125}I and Iodogen, in the protein moiety so that the fate of mRNA-associated proteins could be followed during translation. ^{125}I-mRNP was shown to retain functional activity in the nuclease-treated reticulocyte lysate translation system under optimal labelling conditions. Polyribsome binding of ^{125}I-mRNP and its sensitivity to cycloheximide indicated a functional- and translation-dependent binding of mRNP proteins. The results constitute a successful and direct approach to the study of mRNA-associated proteins in translational control.

Radioiodination *Messenger ribonucleoprotein* *Translation* *Polyribosome binding*

1. INTRODUCTION

Eukaryotic messenger RNA (mRNA) normally exists in association with a specific complement of proteins as messenger ribonucleoprotein particles (mRNP) [1,2]. The mRNP proteins have been implicated in the processing [3], transport [4], storage [5–9] and translational control [10–12] of mRNA (review [13]). There is, however, no direct evidence that mRNP proteins participate in the translation of mRNA since no difference in the translation in vitro of mRNA and mRNP has been observed [14], although mRNP has been shown to bind to ribosomes under conditions where mRNA will not [10,15]. Recently it has been proposed that mRNP proteins freely exchange with a cytoplasmic pool of proteins and recycle on and off the mRNA [11]. Whether this exchange has a function in translation has not yet been demonstrated and awaits an experimental system in which these proteins can be directly studied. One approach would be to monitor the binding and release of radiolabelled mRNP proteins during the ribosome cycle in a system similar to that used to study initiation factors in the assembly of initiation complexes under conditions of active protein synthesis [16,17].

Accordingly and as a prerequisite for the direct investigation of the role of mRNP proteins in translation, radioactive and functionally active mRNP has now been prepared. Here, we describe and characterise the labelling in the protein moiety of rabbit reticulocyte polyribosomal globin mRNP under mild conditions using Na^{125}I and Iodogen to give a highly labelled product which retains messenger activity, whereas other radioiodination techniques have given labelled mRNP of variable quality [M.S. Fenster, unpublished work; 13]. We also show a translation-dependent binding of the full complement of mRNP proteins to 80 S ribosomes and polyribosomes. The results demonstrate that mRNP in which the proteins are labelled by this procedure may be successfully used for the direct study of mRNP proteins in the control of translation.

2. METHODS

Polyribosomal globin mRNP was prepared from rabbit reticulocytes by EDTA dissociation of

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0.5 M KCl washed polyribosomes and purified by sucrose density gradient centrifugation [14]. The protein complement of mRNP was characterised by electrophoresis on 10% polyacrylamide–SDS gels [18] and visualised with Coomassie blue or by autoradiography in the case of ^{125}I-labelled mRNP.

Radioiodination was performed essentially as in [19,20] at 20°C in a 0.5 ml plastic conical microcentrifuge tube coated with 5 μg Iodogen (Pierce and Warriner, England), evaporated from 50 μl dichloromethane. The reaction mixture contained 0.1–0.2 A_{260} units of mRNP, 1–5 μCi Na^{125}I (Amersham International; 15.1 mCi/μg I) in a final volume of 20 μl of 10 mM Tris–HCl (pH 7.6). The amount of Na^{125}I used varied according to the experimental requirement (see table 1); to achieve low specific activity labelling with high iodine substitution levels, cold carrier KI was also added. The reaction was terminated by removal of the reaction mixture from the Iodogen coated tube and made 10 mM in dithiothreitol (DTT). Incorporation of ^{125}I into mRNP was measured by precipitation from 1 ml cold 20% trichloroacetic acid in the presence of carrier KI (100 mM), tRNA (20 μg) and ovalbumin (20 μg). The precipitate was washed onto Whatman GF/C filters with excess 5% trichloroacetic acid containing 10 mM KI and counted at 35% efficiency in a gamma counter (Berthold, LB MAG 310). The number of labelled molecules of mRNP in a given preparation was calculated on the assumption of a M_r of 220000 for 9 S globin mRNA, a 64% protein content based on a buoyant density in Cs_2Cl of 1.42–1.44 g/ml [21] and an $A_{260/280}$ of 1.7–1.9.

Since removal of free ^{125}I from the labelling reaction mixture could not be accomplished without considerable inactivation of mRNP, control and ^{125}I-labelled mRNP were translated immediately in a nuclease-treated reticulocyte lysate translation system [22] at 30°C and at concentrations equivalent to 30–45 μg RNA/ml lysate. The incorporation of [^{35}S]methionine (Amersham International, 1225 Ci/mmol) into protein over 30 min was measured [22] in the presence of carrier KI and counted on GF/C filters at 80% efficiency for [^{35}S]methionine with a ^{125}I spill over of 64%.

Polyribosome binding was performed by incubation of ^{125}I-mRNP at 1–3 μg RNA/ml final concentration in 250 μl of a reticulocyte lysate [22] without nuclease treatment. Lysates were preincubated at 30°C for 10 min prior to addition of ^{125}I-mRNP. Translation was stopped after a further 20 min by adding an equal volume of ice-cold TKM-1 buffer (25 mM Tris–HCl, pH 7.6, 75 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) containing 500 μg

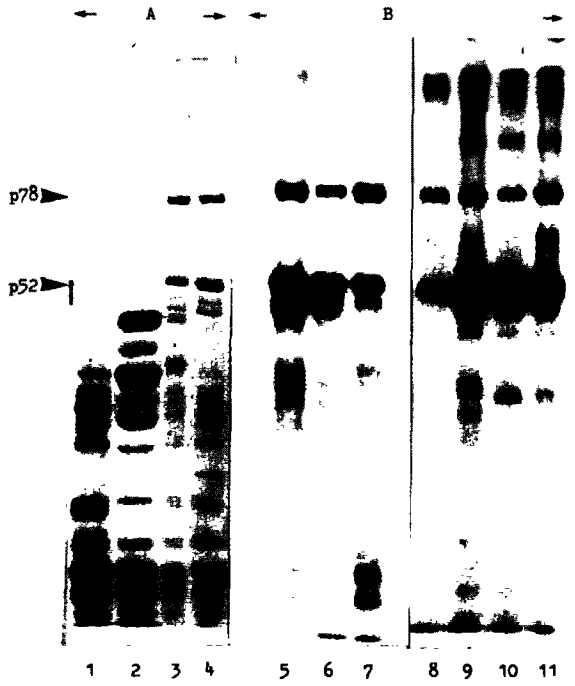

Fig.1. Electrophoretic analysis on 10% polyacrylamide gels of the protein components of rabbit globin mRNP. (A) 14 S mRNP (lanes 3 and 4) was released from salt washed polyribosomes by treatment with EDTA and was separated from the 30 S small ribosomal subunit (lane 1) and the 8 S ribosomal RNP subparticle (lane 2) by sucrose density gradient centrifugation: 0.5 A_{260} units/lane; Coomassie blue stained. (B) Autoradiographic detection of mRNP proteins directly after labelling with ^{125}I (conditions as table 1a) of several separate mRNP preparations (lanes 5–7; 3 × 10^4 cpm/lane) and after translation of ^{125}I-mRNP so labelled (4.54 × 10^8 dpm/A_{260} unit) in a reticulocyte lysate polyribosomal binding assay system, recovered as free mRNP (lane 8) or as mRNP bound to the 40–60 S region (lane 9), the 80 S region (lane 10) and the polyribosome region (lane 11) of a parallel gradient profile to that shown in fig.4. About 0.1–0.3 A_{260} units of gradient fraction material and 3 × 10^4 cpm were loaded in lanes 8–11. Bands were visualised by exposure to Kodak X-Omat film. The two main mRNP proteins are indicated: p52 and p78.

cycloheximide/ml. The lysates were then layered over 12 ml exponential sucrose gradients (15–28.1% (w/v) in TKM-1 buffer) and centrifuged at 196000 × *g* for 1.5 h. Fractions (300 μl) were collected with continuous UV monitoring and trichloroacetic acid-precipitable ^{125}I counts measured as above. Electrophoretic analysis of the protein components of ^{125}I-mRNP bound to the polyribosome, 80 S and 40–60 S regions of the gradient was performed on the pellets of pooled fractions which had been centrifuged at 100000 × *g* for 4 h at 4°C. Unbound ^{125}I-mRNP was loaded directly onto gels.

3. RESULTS

3.1. *Radioiodination of polyribosomal mRNP*

Purified polyribosomal globin mRNP was characterised by electrophoresis on 10% polyacrylamide–SDS gels and was shown to have a complement of proteins consisting of the two major proteins, M_r 52000 (p52) and 78000 (p78), that have been commonly observed in polyribosomal mRNP from various sources [2]. Additionally a few minor proteins together with some contaminating lower-M_r ribosomal proteins were also present (fig.1A, lanes 3 and 4). After radioiodination, mRNP was electrophoresed on a parallel gel and autoradiography revealed labelling of all the protein components (fig.1B, lanes 5–7).

Optimal labelling conditions were selected on the basis of a minimal loss in the translational activity of ^{125}I-mRNP, expressed as a percentage of the [^{35}S]methionine incorporated after 30 min when unlabelled mRNP was used as a control. Maximum incorporation of ^{125}I was attained after

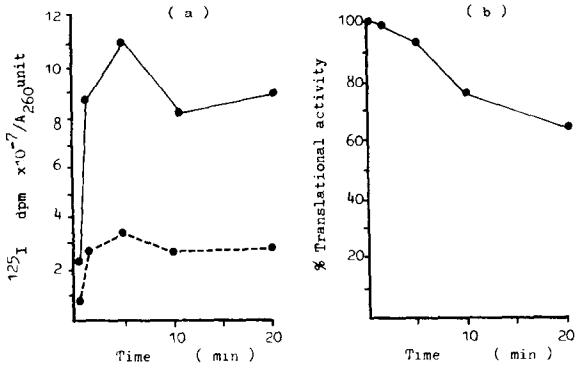

Fig.2. (a) Kinetics of the incorporation of ^{125}I into mRNP: 0.1 A_{260} unit of mRNP, labelled with 5 μg Iodogen and 2 μCi (●---●) or 20 μCi (●——●) of carrier-free Na^{125}I, labelling conditions as table 1a. The incorporation of ^{125}I was measured over 20 min by trichloroacetic acid precipitation of 2 μl aliquots of the reaction mixture in the presence of carrier KI, tRNA and ovalbumin. (b) The effect of exposure to Iodogen on the translational activity of mRNP: 5 μl aliquots of the labelling reaction were removed at time intervals, adjusted to 10 mM DTT and immediately translated in a nuclease-treated lysate system at 30–45 μg RNA/ml final concentration. Translational activity of ^{125}I-mRNP was measured as the [^{35}S]methionine incorporation into trichloroacetic acid-insoluble products after 30 min and expressed as a % of the activity of control unlabelled mRNP not exposed to Iodogen.

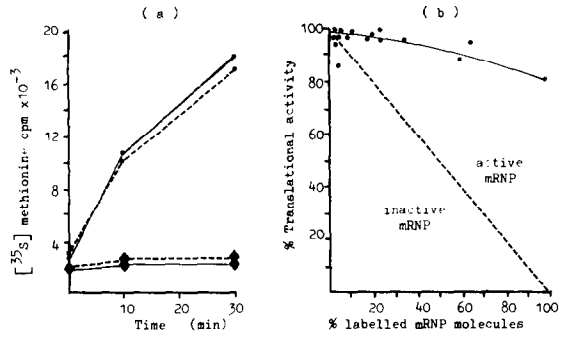

Fig.3. (a) Translation of ^{125}I-mRNP: Unlabelled control mRNP (●——●) and ^{125}I-mRNP (4.05 × 10^6 dpm/A_{260} unit; labelled as in table 1a) (●---●) were introduced into nuclease-treated lysates at 45 μg/ml, incubated at 30°C and the [^{35}S]methionine (final conc. 200 μCi/ml lysate) incorporation into trichloroacetic acid-insoluble material was measured over 30 min. Lysates were also incubated at 0°C or after 10 min preincubation in the presence of 250 μg cycloheximide/ml (◆=-=◆). (b) Evidence for the functional integrity of ^{125}I-mRNP. Low specific activity ^{125}I-mRNP preparations were prepared with varying levels of iodine substitution (0.005–0.98 iodine molecules/molecule of mRNP: 0.5–98.0% of mRNP molecules labelled) by labelling with 1 μCi Na^{125}I and increasing amounts of carrier KI (1–500 pmol; conditions as in table 1b). The translational activity of these ^{125}I-mRNP preparations was measured, expressed as a % of control mRNP and plotted as a function of the % number of molecules of mRNP labelled: (– – –) correlation expected if mRNP molecules were inactivated by iodine substitution; (●——●) correlation obtained.

5 min (fig.2a) and further exposure to Iodogen produced a reduction in messenger activity (fig.2b). Unlabelled control mRNP similarly treated in the absence of Iodogen showed little loss of activity. Thus the reduction in translational efficiency caused by exposure to the mild oxidative effect of Iodogen may also account for the much greater losses of mRNP functional activity observed in earlier experiments in this laboratory using more oxidative iodination techniques [13].

3.2. *Translation of ^{125}I-labelled mRNP*

The incorporation of [^{35}S]methionine into acid-precipitable products during translation of ^{125}I-mRNP (4.05×10^6 dpm/A_{260} unit) and unlabelled control mRNP at equivalent concentrations (45 μg RNA/ml lysate) is shown in fig.3a. Translation was abolished at 0°C or by preincubating the lysate with 250 μg cycloheximide/ml. Evidence that ^{125}I-mRNP retained functional integrity and that [^{35}S]methionine incorporation in the translation assay was not simply due to the translation of unlabelled mRNP species was obtained from a series of experiments which correlated translational efficiency with the level of iodine substitution in ^{125}I-mRNP preparations. A high level of substitution could be obtained by increasing the amount of ^{125}I used (table 1a). However, at high levels of ^{125}I, spillover into the ^{35}S channel is significant and small differences in [^{35}S]methionine incorporation cannot be determined accurately. This problem was overcome by isotope dilution with cold carrier KI and a range of ^{125}I-mRNP preparations with low specific radioactivities was prepared with iodine substitutions of between 0.005 and 0.98 iodine molecules/molecule of mRNP (0.5–98% of molecules labelled) as shown in table 1b. The translational activity of a range of these iodine-substituted mRNP preparations was then measured and the results from several separate experiments are combined in fig.3b. A small loss in translational activity of these mRNP preparations compared with control mRNP (no Iodogen) was noticed and is due largely to the reduction in translational activity caused by the 5 min exposure to Iodogen during the labelling reaction (fig.2b). That increasing the number of labelled mRNP molecules did not cause a parallel decrease in the number of active mRNP molecules indicates that most ^{125}I-mRNP molecules prepared in this way are functionally intact and translationally active and may therefore be legitimately used to monitor the fate of mRNP proteins during the translation of mRNP.

Table 1

Incorporation of ^{125}I into mRNP

(a) The effect of increasing [Na^{125}I]

(b) The effect of carrier KI

^{125}I (μCi)	Carrier KI (pmol)	Spec. act. ^{125}I (dpm/nmol)	μCi ^{125}I/ A_{260} unit	Spec. act. mRNP		% mRNP molecules labelled
				dpm/A_{260} unit	dpm/nmol	
(a)						
2	–	4.15×10^9	15	4.2×10^6	2.03×10^7	0.5
20	–	4.15×10^9	150	4.3×10^7	2.1×10^8	5.1
200	–	4.15×10^9	1500	1.2×10^9	5.6×10^9	134.0
(b)						
1	–	4.15×10^9	20	9.8×10^6	4.7×10^7	1.1
1	4	4.93×10^8	20	5.5×10^6	2.7×10^7	5.4
1	40	5.47×10^7	20	1.8×10^6	9.1×10^6	16.6
1	400	5.47×10^6	20	1.1×10^6	5.4×10^6	98.7

Reaction conditions: 0.1–0.2 A_{260} units mRNP, 5 μg Iodogen in 20 μl 10 mM Tris–HCl (pH 7.6). Counting efficiency = 35%. % number of mRNP molecules labelled = {[spec. act. mRNP (dpm/nmol)]/[spec. act. Na^{125}I (dpm/nmol)]} × 100

3.3. *Polyribosomal binding of ^{125}I-labelled mRNP*

In non-nuclease treated reticulocyte lysates, ^{125}I-mRNP proteins became associated with 80 S ribosomes and higher order polyribosomes under conditions of active protein synthesis (fig.4). In the presence of 250 μg cycloheximide/ml, the binding of ^{125}I-mRNP to polyribosomes was significantly reduced with an increased binding to the 80 S ribosome as a result of the accumulation of initiation complexes following inhibition of elongation. Control lysate incubations of the ^{125}I labelling reaction mixture without added mRNP were performed and loaded onto gradients (fig.4). The profile obtained indicates that non-specific association of free ^{125}I with lysate components does not occur. Together, the reagent blank and cycloheximide controls establish a translation dependent binding of ^{125}I-mRNP proteins. The fact that ^{125}I counts did not follow the A_{260} polyribosome profile also indicates the absence of significant non-specific association of ^{125}I-labelled proteins with ribosomes. Moreover, electrophoresis of the 80 S and polyribosome-bound ^{125}I-mRNP proteins revealed the presence of the p52 and p78 proteins (fig.1, lanes 8–11), thus showing that most of the radioactivity bound to ribosomes was due to the major mRNP proteins and not ribosomal protein contaminants.

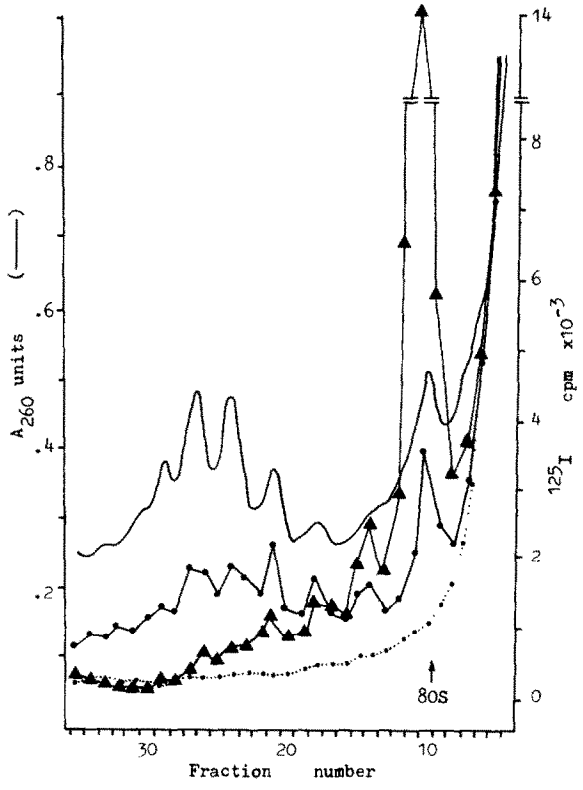

Fig.4. Polyribosome binding of ^{125}I-mRNP during protein synthesis: ^{125}I-labelled mRNP (4.54×10^8 dpm/A_{260} unit) was added to a reticulocyte lysate (2.5 μg RNA/ml lysate) which had been preincubated for 10 min at 30°C in the presence (▲——▲) and absence (●——●) of 250 μg/ml cycloheximide. After a further 20 min, the lysate was diluted with ice-cold buffer, fractionated on sucrose gradients and analysed for trichloroacetic acid-precipitable ^{125}I counts. Fractions from parallel gradients representing the polyribosomes (13–35), the 80 S ribosome (9–12), the 40–60 S region (5–8) and free mRNP (2–4) were pooled, pelleted and the ^{125}I-labelled protein components analysed by SDS–polyacrylamide gel electrophoresis as shown in fig.1, lanes 8–11. Control lysate incubations of the ^{125}I-labelling reaction mixture without mRNP were performed (•·····•); insufficient counts in the gradient compartments precluded gel analysis of non-specifically labelled proteins.

The appearance of an M_r 105000 band in ribosome-bound fractions (fig.1, lanes 9–11) compared with free mRNP (lane 8) may represent a functional accumulation of a minor mRNP protein of that M_r-value [2,13] or, alternatively, may result from an overloading of lanes 9–11 relative to lane 8 due to counting error in the presence of a massive excess of free ^{125}I at the top of the gradient (fig.4, fractions 1–5) with a resultant enhancement of minor bands. Other changes in minor proteins within the ribosomal protein M_r-range were also observed (fig.4, lanes 8–11), although these changes have not been characterised.

4. DISCUSSION

Polyribosome binding of translationally active ^{125}I-mRNP and its sensitivity to cycloheximide indicates a functional and translation-dependent binding of mRNP proteins to ribosomes during protein synthesis. Our experiments confirm the presence of a p78 protein on polyribosomal mRNP [23,24] and show that this 3′-poly(A)-binding protein [2,13] is not released during translation, unlike the reported deadenylation and loss of a poly(A)–protein complex associated with translation of free cytoplasmic mRNP in *Physarum*

polycephalum [25], thus arguing against a possible role for poly(A) turnover in translation control [26].

The location of mRNP proteins within polyribosomes [23] and initiation complexes [10] other than at the 3′-poly(A)-termini had faced serious problems with the unambiguous detection of mRNP proteins which are present only in low amounts relative to ribosomal proteins and which, although structurally distinct from initiation factors [27] co-electrophorese with them [28]. The use of purified, characterised and functionally active mRNP labelled in the protein moiety with ^{125}I avoids these disadvantages and allows the role of mRNP proteins and their relocation during translation to be evaluated. The results provide direct evidence that the full complement of mRNP proteins remain closely associated with the mRNA throughout the ribosome cycle, with translation of an intact particle as suggested [13], although the possibility of a quantitatively minor exchange with a pool of cytoplasmic proteins has not been rigorously excluded.

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