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Interaction of proteins with the mRNA for ribosomal protein L1 in *Xenopus*: structural characterization of *in vivo* complexes and identification of proteins that bind *in vitro* to its 5'UTR

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ABSTRACT

***Xenopus* r-protein mRNAs are known to be coordinately regulated at the translational level. To find out if RNA/protein interactions are involved in this control mechanism, we have characterized the particles containing the translationally repressed rp-mRNA and we have investigated the proteins that specifically bind to this type of mRNA. By sedimentation analysis and isopycnic centrifugation we have found that the repressed rp-mRNAs are assembled in slow sedimenting complexes where the RNA is prevalent over the protein mass (2.3 to 1). This composition is maintained also after *in vitro* reconstitution of the particle. We carried out also a detailed analysis of *in vitro* RNA/protein complex formation by focusing our attention on the 5'UTR, very similar in different rp-mRNAs and important in the translational regulation. We describe specific interactions of L1 mRNA with four proteins. The binding site of two of them, 57 kD and 47 kD, is in the typical pyrimidine sequence at the 5' end and is position dependent. Proteins of the same size interact also with the analogous region of r-protein S1 and L14 mRNA, not with unrelated RNAs. Binding of two other proteins, 31 kD and 24 kD, in the downstream region of the 5'UTR was also observed. The most evident 57 kD protein has been partially purified. Although the binding of these proteins to the r-protein mRNA 5'UTR is specific, their involvement in the translation regulation remains to be proved.**

INTRODUCTION

In the frog *Xenopus laevis* the coordinate synthesis of the over 80 ribosomal proteins (r-proteins) is attained by regulation at various levels (1). In particular a translational regulation controls the efficiency of utilization of the mRNAs specific for ribosomal proteins (rp-mRNA) according to cellular demand for ribosomes. This translational regulation is clearly observed during *Xenopus* development, when the production and accumulation of rp-mRNA are uncoupled from its utilization. In fact newly synthesized rp-mRNA is mostly kept unused as free mRNPs for several hours and it is mobilized onto polysomes when the maternal store of ribosomes is exhausted (2, 3, 4, 5). The partition of the mRNA between the polysomal and subpolysomal (mRNP) compartments, analyzed by sucrose gradient sedimentation, is typical of the developmental stage and it is specific for this class of mRNA (2, 4, 6). Other mRNA show a different behaviour, for example histone RNA is fully loaded on polysomes for the great part of development (7), as does putative troponine C mRNA (8) whereas EF-1α (9) and other mRNA (10) show different patterns; this specificity was considered indicative of a regulation. Similar translational controls have been shown to be involved in the regulation of ribosomal protein synthesis of other eukaryotic systems such as cultured mammalian cells (11, 12), *Drosophila melanogaster* (13, 14) and *Dictyostelium discoideum* (15), as an immediate response to changes in requirement for ribosomes in the cell.

The observation that this translational behavior is specific and common to the mRNAs for the numerous r-proteins, resulting in their coordinate synthesis, suggested a search for particular sequences and structures that could differentiate this class of mRNA from others and confer on them their typical translational properties. Sequence comparison revealed the presence of some common features in the 5' untranslated regions (5'UTR) of the mRNAs for the *Xenopus* r-proteins analyzed. Most typically they are always short and start with a oligopyrimidine tract at the 5' terminus (for a review see reference 16). It seemed possible that these characteristics, shared also with mammalian rp-mRNAs (17), might have a role in the translational regulation. Support for this hypothesis comes from experiments of microinjection into *Xenopus* fertilized eggs of a fused gene which produces a mRNA containing the 5'UTR of *Xenopus* r-protein S19 joined to the coding portion of a reporter gene. The translation pattern of the chimeric mRNA in the developing embryo mimicked that of the r-protein mRNA, with the typical translational delay (7). Analogous results have been obtained in the mammalian system, where it was also shown that the 5' terminal pyrimidine tract

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plays a critical role in the translational control mechanism (18, 19).

Translational regulation, in analogy to transcriptional controls, can depend on specific interactions between *cis* regulatory elements in the mRNA and *trans*-acting factors. Thus we decided to investigate the rp-mRNA/protein complexes (rp-mRNP) present *in vivo* and to search for possible factors that specifically interact *in vitro* with the 5'UTR typical of these mRNAs. We describe here binding experiments between rp-mRNA fragments and cytoplasmic extracts from *Xenopus* embryos and oocytes and the identification of cytoplasmic proteins forming specific complexes with definite tracts of the 5' UTR of the mRNA for r-protein L1. Some physical characteristics of the rp-mRNA particles *in vivo* have also been established. Recently a protein, possibly homologous to one of ours, was found to be related to the pyrimidine tract of a murine rp-mRNA (18).

MATERIAL AND METHODS

Materials

Xenopus laevis were purchased from Horst Kahler, Hamburg, Germany. Embryo staging is according to Nieuwkoop and Faber (20) and manipulation of oocytes and embryo was performed as previously described (4).

Sucrose gradients, density gradients and RNA analysis

Cytoplasmic extract preparation and RNA analysis by Northern blot hybridization were performed essentially as previously described (3). For density gradient fractionation 200 embryos of stage 25 were homogenized in1 ml of HNM (30 mM Hepes pH 7.2, 100 mM Na Cl, 3 mM $MgCl_2$) containing 0.005% Triton, 1mM DTT and 400 U/ml of RNase inhibitor (Boehringer). Nuclei were removed at 2000 rpm, the supernatant was treated with 1% final deoxycholate for 10 minutes, cleared at 10.000 rpm for 10 minutes and centrifuged at 100.000×g for 1 hour at 4°C. The S100 supernatant was fixed in 7% formaldehyde for 2 hours, then added to a solution of CsCl prepared in HNM to bring the volume to 5 ml; the final density of the solution was 1.7 g/ml and the concentration of formaldehyde in the gradient was about 2%. As a control the RNA equivalent of 50 embryos was dissolved in 1ml of HNM, fixed and added to CsCl as above. In reconstitution experiments binding was performed as described in the following sections; three standard binding reactions were pooled, fixed and treated as the other samples. Gradients were centrifuged in the SW65 rotor at 30.000 rpm for 70 hours at 20°C. The collected 0.3 ml fractions were diluted with three volumes of HNM and 6 μg of carrier RNA, ethanol precipitated at 4°C and carefully washed with 80% ethanol. Precipitates were collected and the RNA was extracted and analyzed by Northern hybridization. The RNA recovered from gradient fractions of the reconstitution experiment was analyzed on a 4% polyacrylamide gel/7% urea, dried and autoradiographed.

Synthesis of transcripts

In preliminary experiments L1 transcripts obtained with the available full length L1 cDNA (21), which contained a stretch of polyT at the 5' end, gave partly unreliable results due to protein binding to the poly U sequence. For this reason we have deleted the extra polyT tract and part of the Bluescript vector linker from the cDNA clone by oligonucleotide mediated mutagenesis (22). Long L1 transcripts were obtained by T3 polymerase transcription of this mutated L1 cDNA (see Figure 2): for L1 RNA 1300 nt (whole L1 mRNA) the plasmid was linearized by digestion at the 3' end in the *BamHI* site of the linker; to obtain the L1 RNA 700 nt and the L1 RNA 160 nt, the plasmid was truncated by *AvaII* and *HindII* digestion respectively. Control transcripts for α1 cardiac actin were obtained by SP6 polymerase transcription of plasmid pα164, which contains the *PstI-PvuII* fragment from the cDNA coding for the α1 cardiac actin (23) inserted in pSP64, digested with *FokI* to obtain run off transcripts to base 143. Similarly the plasmid pSP65 was digested with *PvuII* to obtain a 240 nucleotide transcript. Transcription by T3 and SP6 polymerases were carried out according to Melton et al. (24). RNA was synthesized in a total volume of 20 μl containing the appropriate buffer, 500 μM ATP, GTP and CTP, 50 μM UTP, 16 μCi [α-^{32}P] UTP (800 Ci/mmol), 1.5 μg of DNA, 1 unit/μl of RNase inhibitor and 20 units of polymerase (Promega). Short L1 transcripts (L1 52, F1, F2, F3, F4, F5, F5a, F5b, F5c; see Figure 2) and those for L14 and S1 RNAs, were synthesized by T7 polymerase using as templates DNA oligonucleotides containing the sequence of T7 promoter and different regions of the 5'UTR of L1 mRNA and following the protocol described by Milligan et al. (25). Since it has been shown that the efficiency of T7 transcription from oligonucleotides strongly depends on the presence of G at position +1 and +2, one or two G were inserted at these positions when the natural template did not have them. Before doing that, we have checked by UV crosslinking experiments that the presence of one or two G at the start site did not change the binding properties of the RNA when compared to the same RNA starting with C, whose *in vitro* synthesis was very inefficient (as an example see figure 6A lane F1 and figure 6B lane F1'). Moreover the possibility that, in the absence of a G, the polymerase skips the stretch of pyrimidine and starts downstream with a purine, was ruled out by checking transcript size on acrylamide gel: this was particularly evident for short transcripts. When not specified the radioactive transcripts were obtained by incorporating [α-^{32}P]UTP. Occasionally, according to the particular sequence to be analyzed, labeling was carried out also with other nucleotides. [α-^{32}P]ATP was not used for labeling polyadenylated RNA so that poly(A) binding activity was not detectable. Large quantities of competitor unlabeled RNAs for L1, actin and pSP65 were synthesized by adding to the unlabeled nucleotides trace amounts of [α-^{32}P]UTP to quantify the yield of RNA (24). Transcripts were separated on denaturing polyacrylamide gel and eluted with 0.2M Na Acetate pH 5.5, 1 mM EDTA pH 7, followed by phenol extraction and ethanol precipitation (24). Generally all transcripts used were uncapped. When required, capping of the RNA was performed by addition of mG(5')ppp(5')G to the transcription mixture as described (26).

Extracts preparation and protein purification

S100 cytoplasmic extracts were prepared according to Dignam et al. (27). Typically 300 embryos were washed and then homogenized in one volume of homogenization buffer. Extract aliquots were stored at −70°C. Nuclear extracts from *Xenopus* stage V−VI oocytes were prepared from manually collected nuclei following the same procedure as for cytoplasmic extracts. Fractionation on heparin sepharose (Pharmacia) was carried out from isolated oocytes essentially as described by Neupert et al. (28). The S100 extract obtained as above was made 50 mM KCl and incubated for 3 hours at room temperature, under constant shaking, with 2.5 ml of preswollen beads in buffer A (10 mM Hepes pH7.5, 50 mM KCl, 3 mM $MgCl_2$, 5% glycerol). After

washing, the beads were packed in a column and proteins were eluted subsequently with 0.2 M and 0.4 M KCl in the presence of 0.5 mM DTT. A small aliquot of each fraction was dialyzed according to Dignam et al. (27) and then tested for binding. The 0.4 M KCl fraction, containing the binding activity, was concentrated in a Centricon 30 (Amicon) and the sample was loaded on a HPLC TSK 3000 (LKB) gel filtration column in 100 mM KCl. Fractions of 0.5 ml were collected and aliquots dialyzed to be tested for binding activity and also to be used for protein quantitative determination and for analysis by polyacrylamide gel electrophoresis and silver staining.

RNA/protein interaction and analysis of the complexes

The S100 cytoplasmic extracts (30 μg), corresponding to about six oocytes or embryos, were mixed with 30.000 cpm of [^{32}P]RNA in the binding conditions described by Leibold and Munro (29) in a final volume of 15 μl, with salt concentration in the mixture 40 mM KCl and 3 mM $MgCl_2$. Incubation was carried out at 15°C for 30 min, then continued for 10 more minutes in the presence of 5 mg/ml of heparin. Competitions were carried out by preincubating the extract with a large molar excess of specific or unspecific RNA competitor, at 15°C for 10 minutes, before addition of labeled RNA. The RNA-protein complexes were then analyzed by band shift or by UV crosslinking. In band shift experiments a 6% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 30:1) in 45 mM Tris-borate (pH 8.3) and 0.45 mM EDTA was prerun for 10 minutes and run at 8 V/cm for 3 hours, then dried and autoradiographed. In UV crosslinking experiments the heparin treated binding reaction products were irradiated 3 cm under a 15W UV lamp (Philips 254 nm) for 4 min. The samples were then incubated with 1 U of RNase T1 (BRL) and 60 μg/ml of RNase A (Boerhinger) for 15 min at the binding temperature. The results were not affected by higher concentrations of RNase. The samples were boiled for 5 min in 1× Laemmli buffer and run on SDS/12% polyacrylamide gel (30). The gels were then stained with Comassie blue to make visible the molecular mass markers (BioRad), destained and autoradiographed. In some experiments the samples were incubated with Proteinase K (500 μg/ml) at room temperature for 30 minutes before RNase treatment.

RESULTS

Characterization of mRNPs which contain rp-mRNA

In *Xenopus* oocyte and embryo the rp-mRNA is completely or partly excluded from polysomes since it is associated in mRNPs (2, 4, 6). We have analyzed some physical properties of the r-protein mRNPs that contain the repressed rp-mRNA. In sucrose gradient sedimentation (not shown), it was found that this mRNA is not bound to the 40s ribosomal subunits, excluding its association in blocked initiation complexes, but it is contained in small particles, somewhat heavier than naked mRNA, whereas histone mRNA is found associated with polysomes, as already described for this (7) and other mRNAs (9, 10).

In order to have more information on the structure of the rp-mRNPs we used isopycnic centrifugation in cesium chloride, a technique that allows the separation of free proteins, RNA/protein complexes and free RNA according to density. Moreover the buoyant density of RNA/protein complexes is a function of the protein/RNA ratio thus giving information on the relative quantitative contribution of the two components. A postribosomal extract (S100) from embryos at stage 15−20, which according to previous experiments were expected to contain rp-mRNA but not histone mRNA or other non regulated mRNAs, was centrifuged in cesium chloride gradients as described in Materials and Methods. RNA was extracted from each fraction and analyzed by Northern blot hybridization. In preliminary experiments we used methods that are known to maintain structurally intact authentic snRNPs without fixation (31); however in these conditions the hybridization was found scattered in different fractions of the gradient and the size of the RNA was indicative of a severe degradation. For this reason we then preferred to fix the particles with formaldehyde before centrifugation. In Figure 1 the distribution along a CsCl gradient of naked L1 mRNA and that of L1 mRNA/complexes are compared. The naked mRNA (Figure 1A) pellets at the bottom of the gradient, whereas the complex (Figure 1B) bands at a density of 1.72 gr/ml that corresponds (32) to a RNA/protein mass ratio of 2.3/1; a minor amount of the mRNA behaves as free RNA. Hybridization with a probe for r-protein L14 mRNA appears in the same region (not shown).

A first criterion of reconstitution is the formation of particles with the correct buoyant density. Radioactive full length L1 RNA carrying 20 residue poly(A) tail, obtained by *in vitro* transcription, was incubated with a cytoplasmic extract as described below; the mixture was then fixed and centrifuged in CsCl gradients. Figure 1C shows that L1 mRNA is assembled into particles of the same density as the endogenous counterparts. Attempts to identify the bound proteins failed due to formaldehyde fixation of the sample.

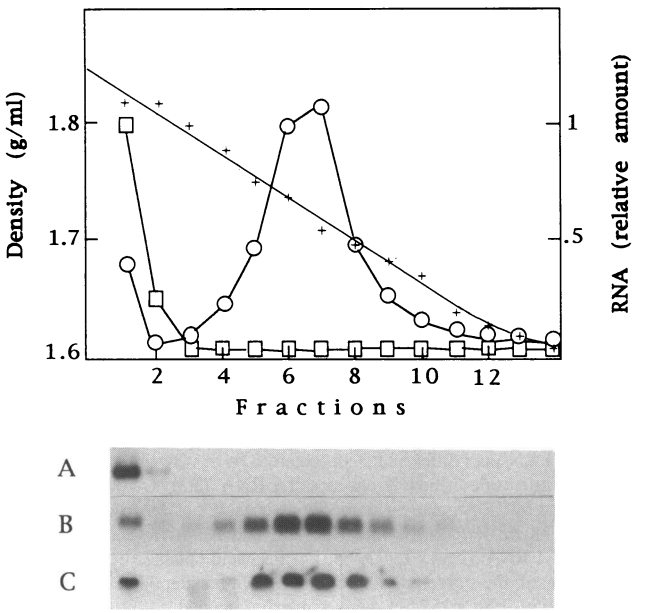

Figure 1. Isopycnic CsCl gradient centrifugation of rp-mRNPs. RNA extracted from CsCl gradient fractions (left, bottom; right, top) of an embryo cytoplasmic extract **(B)** and of the purified RNA as a control **(A)**, was analyzed by Northern blot hybridization with the probe for r-protein L1. The reconstituted particle, obtained by *in vitro* binding of radioactive L1 RNA and extract, was subjected to CsCl gradient as above; the radioactive RNA was extracted and then analyzed on a polyacrylamide gel **(C)**. A lane has been cut out in the photo between lanes 7 and 8 due to an empty defective well. The graph shows the extent of hybridization (values expressed relative to the highest point) estimated by densitometric scanning of autoradiograms B (○) and A (□); buoyant density is given (+).

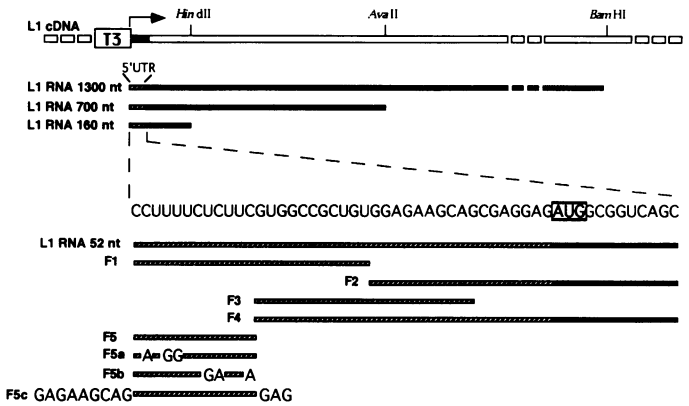

Figure 2. L1 RNAs of different length, obtained by *in vitro* transcription, utilized in the experiments described. Transcripts L1 RNA 1300 nt, 700 nt and 160 nt, were obtained by T3 polymerase transcription of the L1 cDNA containing plasmid truncated with *BamHI* (in the linker), *AvaII* and *HindII* respectively. Smaller fragments (L1 RNA 52 nt, F1, F2, F3, F4, F5) were obtained by T7 polymerase transcription of the corresponding synthetic oligonucleotides. Mutated RNA fragments (F5a, F5b and F5c) were similarly obtained.

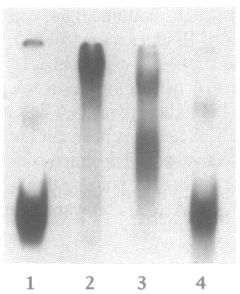

Figure 3. Gel retardation analysis of specific complexes between L1 RNA and S100 embryo cytoplasmic extract. [^{32}P] L1 RNA 160 nt was incubated at 15°C for 30 min with or without the extract. The product was analyzed by electrophoresis on a non denaturing polyacrylamide gel: RNA alone (lane 1); RNA incubated with the extract (lane 2), and the same treated with heparin after incubation (lane 3); competition with a 200 fold excess of the same non radioactive RNA (lane 4).

These results suggest that the *in vitro* reconstituted L1 mRNA particles are structurally similar to the authentic ones, and prompted us to analyze in detail the *in vitro* binding proteins.

Complex formation between L1 rp-mRNA and embryo extracts

In order to determine whether proteins present in embryo cytoplasmic extracts could recognize specific sequences in the 5′ region of rp-mRNAs, *in vitro* synthesized uncapped L1 RNAs were incubated with extracts, and the complexes formed were analyzed by the gel retardation technique. [^{32}P] L1 RNA, 700 and 160 nucleotide long and containing the 5′UTR, were obtained by *in vitro* transcription of L1 cDNA (Figure 2). They were incubated at 15°C with S100 cytolpasmic extracts prepared from embryos at stage 15–20 when rp-mRNA translation is repressed. The product of the reaction was analyzed on a non denaturing polyacrylamide gel (29). The experiment described in Figure 3, relative to the RNA of 160 nucleotides, shows that the addition of the S100 extract retards the RNA mobility (lane 1 and 2). Treatment with heparin, that eliminates aspecific protein binding,

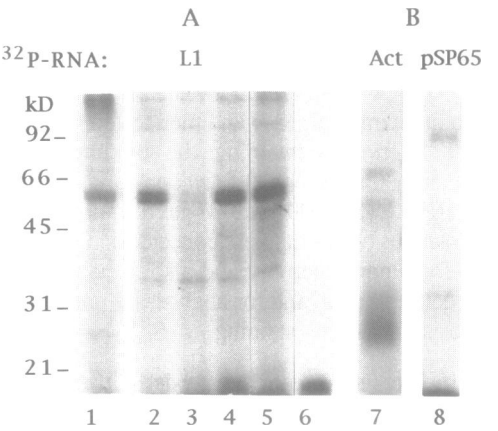

Figure 4. Gel electrophoresis analysis of indirectly labeled proteins that bind to L1 RNAs. [^{32}P] L1 RNAs were incubated with the extract and treated with heparin; after UV irradiation they were digested with RNase and run on a SDS/polyacrylamide gel (see Materials and Methods). **(A)** Binding to L1 RNA 1300 nt (lane1) and to the L1 RNA 160 nt (lane 2). Binding of the L1 RNA 160 nt was competed with the same non radioactive RNA (lane 3) or with the non-specific competitors 5′ region of α1 actin mRNA (lane 4) and pSP65 RNA (lane 5). Binding reaction with L1 RNA 160 nt was treated with Proteinase K (lane 6). **(B)** [^{32}P] α1 actin RNA and [^{32}P] pSP65 RNA were incubated with the extract and analyzed as above (lanes 7 and 8). The position of molecular mass markers is indicated.

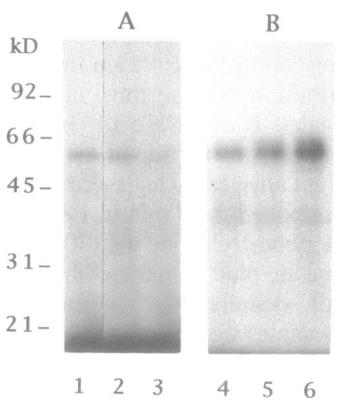

Figure 5. Effect of temperature and salt concentration on binding of the extract to L1 RNA 160 nt. **(A)** Indirect labeling was carried out as described in Figure 4 with extract preincubated for 5 min at 37°C and 60°C (lanes 2 and 3) and control (lane 1). **(B)** The binding reaction was carried out in standard salt conditions, 0.04 M KCl, or in the presence of 0.2 or 0.4 M KCl (lanes 4–6). The position of molecular mass markers is indicated.

maintains retarded complexes (lane 3) which are completely competed by a 200 molar excess of non radioactive L1 RNA (lane 4). Similar results were obtained with the 700 nucleotide L1 RNA (not shown).

These first observation suggested that there is a specific interaction between some component(s) present in the extract and a portion of L1 mRNA that contains the 5′ untranslated leader and a small part of the coding region.

Detection of proteins interacting with L1 mRNA

To identify the nature of the components that bind to the conserved 5′UTR of the rp-mRNAs, the binding reaction between

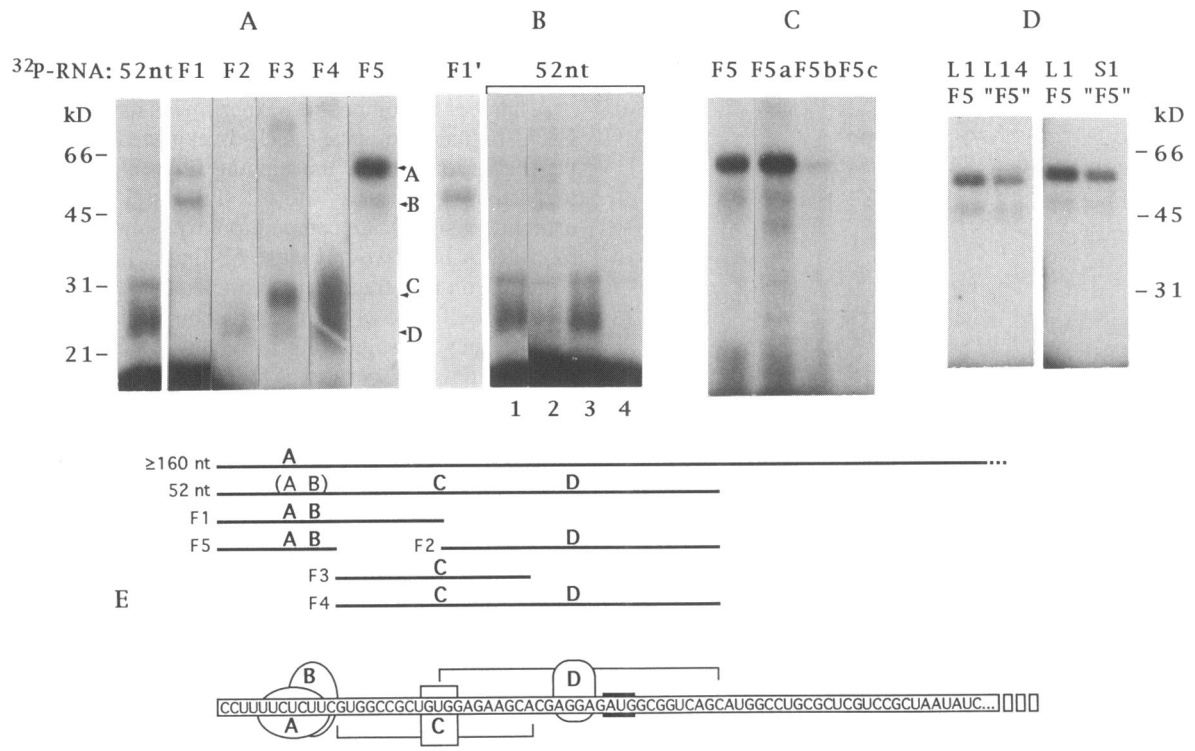

Figure 6. Localization of the binding sites within the L1 RNA 5'UTR region. **(A)** [^{32}P] L1 RNA 52 nt and [^{32}P] RNA subfragments described in figure 2 (F1−F5) were incubated with the extract and the indirectly labeled proteins analyzed as in Figure 4. **(B)** Binding activity of F1 RNA starting with C (F1') to be compared with F1 starting with G in panel A; binding of L1 RNA 52nt, uncompeted (lane 1) or competed with the same non radioactive RNA (lane 2) or with fragments F5 and F4 (lane 3 and 4). **(C)** Binding activity of mutated F5 transcripts (F5a, F5b and F5c, see Figure 2) compared to that of the normal sequence (F5). **(D)** Binding activity of the pyrimidine tract (F5) of L1 mRNA was compared to that of the homologous tracts of rp-L14 and S1 mRNAs (L14'F5' and S1'F5'). The sequence of these tracts are: CCUUUCCUCCCC for L14 and CCUUUCCU for S1. The four binding proteins (A−D) are indicated. The position of molecular mass markers is shown. **(E)** Schematic representation of protein binding activity of the various fragments and localization of proteins along the 5' UTR.

radioactive L1 RNA 160 nt and cytoplasmic extract was treated with heparin, UV crosslinked and then digested with RNAse. The indirectly labeled complexes were then analyzed on SDS-polyacrylamide gels (Figure 4A). In lane 2 of this figure a band of 57 kD is observed that sometimes appears as a doublet and that is sensitive to proteinase K treatment (lane 6). Lane 3 shows that this band is strongly competed by a 200 fold molar excess of non radioactive L1 RNA (the two minor bands of about 100 and 35 kD are not competed and thus not to be considered specific). On the contrary non specific competitors, such as a 5'UTR containing 143 nucleotide region of α1 actin mRNA (lane 4) and a pSP65 transcript (lane 5) are totally ineffective in competition at 200, and even at 500, molar excess as also are yeast total RNA, poly(U) and poly (IC) (not shown). Binding experiments with radioactive α1 actin RNA and pSP65 RNA give completely different patterns (Figure 4B). It can be observed that the binding pattern of the whole L1 mRNA is essentially similar to the one observed with L1 RNA 160 nt (Figure 4A, lane 1).

Incubation of the extract at different temperatures prior to binding, showed that the binding protein is stable at 37°C, but inactivated at 60°C (Figure 5A). Complex formation dependence on temperature has been also examined, and it was found that binding occurs at 0°C and 20°C, but not at 37°C (not shown); this is in agreement with the physiological temperature of *Amphibia*. For this reason the temperature routinely used in our binding experiments was 15°C.

Figure 5B shows the effect of different salt concentrations on complex stability. It appears that the binding improves with KCl concentration. To test if salt stabilization was due to the involvement of an RNA-RNA interaction, the extract was pretreated with micrococcal nuclease; this however did not change the binding property of the extract (not shown).

In order to find out if the specific binding proteins presented some qualitative or quantitative difference during development, extracts from the same number of oocytes (stages II and VI) and embryos (stages 8, 20, 40) were incubated with the L1 RNA 160 nt. No relevant differences were found (not shown). The binding capability of nuclear and cytoplasmic extracts was also tested by preparing extracts from manually isolated nuclei of oocytes; it was found that the activity is mostly cytoplasmic (not shown).

Localization of the binding sites within the L1 5'UTR

In order to localize more precisely the site of protein/RNA interaction, a 52 nt RNA that covers the 5'UTR and the first four codons of L1 RNA (Figure 2) was incubated with the extract. Using this part of the RNA the binding pattern changes with respect to the one produced by the longer RNAs: two bands of 31 kD and 24 kD appears, whereas the 57 kD band becomes quite faint (Figure 6A). They are all competed by specific competitors. We will refer the proteins as A (57 kD), C (31kD) and D (24 kD).

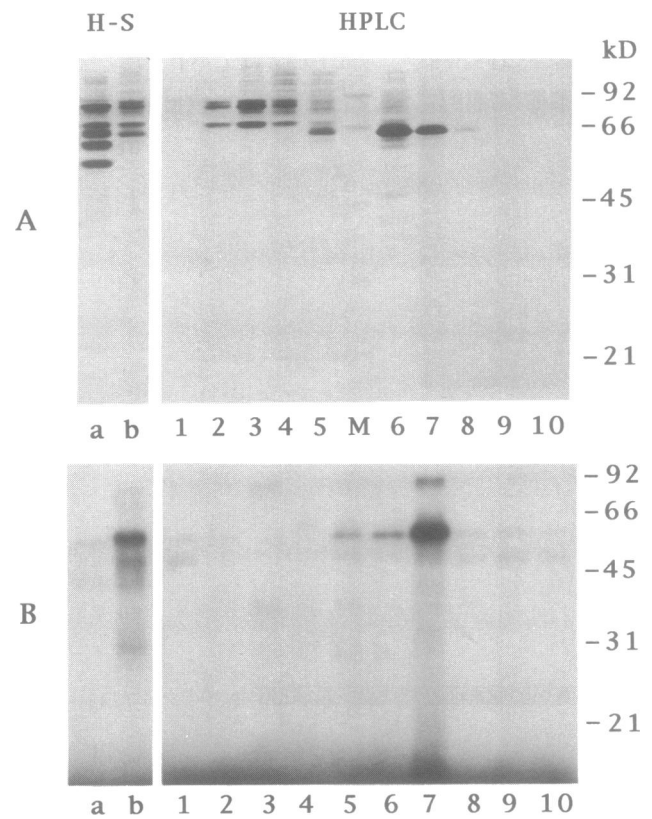

Figure 7. Partial purification of protein A. Fractionation of oocyte S100 extracts was carried out by heparin-sepharose chromatography followed by HPLC gel filtration. Aliquots of the eluted fractions were prepared for binding assay as described in Materials and Methods and **(A)** analyzed on SDS-polyacrylamide gels and silver stained, or **(B)** utilized for detection of binding activity to [^{32}P] L1 RNA 160 nt by indirect labeling as in Figure 4. Lanes a and b refer to the fractions eluted from heparin-sepharose with 0.2 M and 0.4 M KCl respectively. Lanes 1 to 10 refer to subsequent fractionation of the 0.4 M heparin-sepharose eluate by HPLC gel filtration. Lane M in panel A was loaded with the molecular mass markers indicated at right.

To map the position of the various binding proteins within these first 52 nucleotides of L1 mRNA, we further dissected that region into fragments by *in vitro* transcription of synthetic oligonucleotides with T7 polymerase, (see Figure 2). When the two halves of these region were incubated with the extract, the 5′ half (F1) was found to interact with protein A and a 47 kD protein, referred to as B (Figure 6A). The same figure shows that the extreme reduction of the 5′ region to the pyrimidine tract (F5), also resulted in binding to bands A and B, although with quite different relative intensities. In contrast, the 3′ half (F2) was found to be absolutely negative for binding to these proteins, but formed a complex with protein D. Fragment F3, partially overlapping with F1 and F2, binds protein C, while fragment F4, which spans over F2 and F3, binds both protein C and D. The involvement of the pyrimidine tract in binding A and B was further examined by substituting some of the pyrimidines with purines (F5a and F5b, see Figure 2); a modification of the extreme 5′ end at positions 2, 4 and 5 had no effect, whereas a modification at positions 8, 9 and 12 abolished binding of both A and B (Figure 6C). The same figure shows that A and B binding is abolished also when the pyrimidine tract was moved from the extreme 5′ end to a more downstream position (F5c). The possibility that in this experiment a binding at the 5′ end could occur but was undetectable due to lack of U, the RNA was also labeled with [α-^{32}P]ATP; no binding was observed.

A number of competition experiments have been carried out. Each fragment competes itself as expected. F1 and F5 RNAs were able to compete the binding of the 160 nucleotide RNA to protein A, whereas the F2, F3 and F4 were not. As an example, a significant competition experiment on the binding of 52 nt RNA is shown in Figure 6B (lane 1). Binding of proteins C and D on this fragment is competed by an excess of the 52 nt RNA itself (lane 2), not competed by F5 (lane 3), but competed by F4 (lane 4), suggesting a binding specificity for C and D proteins in the F4 portion of the 5′ UTR. Considering the high content of pyrimidines in F5, competition with large amounts of poly(U) was also carried out: it was found that binding to proteins A and B was not affected.

To see if other mRNAs of the ribosomal protein class interact with the same proteins, binding experiments with the pyrimidine tracts present at the 5′ end of L14 and S1 rp-mRNAs were performed (Figure 6D); the results obtained indicate an interaction with proteins of the same size as A and B. All these results and the localization of binding proteins along the 5′ UTR are schematically represented in Figure 6E.

Considering that the binding occurs at the 5′ end we have checked if protein A was related with RNA capping; no differences in binding was observed with capped or uncapped L1 RNA; moreover other RNAs such as plasmid RNA, fragments of actin mRNA or internal portions of L1 RNA itself, capped or uncapped, did not react with protein A (not shown).

Enrichment of binding protein A

A preliminary attempt to purify protein A was carried out for convenience from oocytes. The S100 extract (100 mg of protein) was fractionated on heparin-sepharose; the fractions eluted with 0.2M and 0.4M KCl were analyzed by gel electrophoresis and for binding activity to L1 RNA 160 nt (Figure 7A and 7B, lanes a and b). It appears that the 57 kD protein A is selectively eluted by 0.4 M salt. Silver staining of SDS gel shows in this same fraction four major bands, all of larger size (Figure 7A, lane b). The calculated enrichment of protein A in this fraction is of about 30 fold. The 0.4 M fraction (3 mg of protein) was then loaded on a HPLC gel filtration column and the eluted fractions analyzed both by gel electrophoresis and for binding activity (Figure 7A and 7B, lanes 1 to 10). Also here it is evident that protein A does not correspond to any of the four major proteins; moreover it appears also that protein A does not correspond, both for elution position and gel migration, to any of the faint bands visible in the silver stained gel. The enrichment of protein A with respect to the extract can be calculated to be approximately of 300 fold in fraction 7, which still contains part of one of the heparin-sepharose eluted major proteins.

DISCUSSION

We have presented here a characterization of the mRNP containing the translationally repressed mRNA for r-protein L1, and of the proteins interacting with the 5′ region of this mRNA. The basic motivation behind our interest is that this mRNA, like the mRNAs for all other r-proteins, is regulated at the translational level and that its 5′UTR is somehow involved in this control (7, 18, 19).

The free rp-mRNPs sediment slower than the 40S ribosomal subunit, indicating that the repressed rp-mRNA is not engaged in blocked initiation complexes, but somewhat faster than the naked mRNA implying the presence of some other components. By isopycnic centrifugation experiments we have determined that the rp-mRNA particles (rp-mRNP) have a density of 1.72 g/ml, indicating that the RNA mass is prevalent over the protein with a ratio of 2.3 to 1. This composition, found also after *in vitro* reconstitution of the particle from L1 mRNA and cytoplasmic soluble protein, suggests that the L1 mRNA, 1300 nt plus poly-A tail, is associated with a small number of proteins for a total of approximately 200 kD. Richter and Smith (33) have described in *Xenopus* oocytes RNP complexes containing poly(A)$^+$RNA with a density of 1.4 g/ml, and concluded that in these particles, that contain the masked maternal mRNA, the protein mass is prevalent. As the rp-mRNAs do not belong to the category of masked maternal mRNAs, on the contrary their translation is continuously and finely regulated, it is reasonable that they are assembled in a different type of mRNPs, possibly associated with proteins in a more dynamic physical structure than masked mRNAs.

Searching in cytoplasmic extracts for factors forming complexes with RNA, *in vitro* binding experiments were carried out by gel shift assay and UV crosslinking; binding with the RNA fragments was preferred to RNase mapping as it could give direct information on the binding proteins. (For simplicity the poly(A) binding activity has been eliminated by using mostly non polyadenylated RNA fragments, or by labeling the RNA with nucleotides different from A). Using the entire mRNA sequence or several different fragments in its 5′ region, we have identified some RNA binding proteins whose specificity for L1 RNA was determined by competition. A protein of 57 kD (protein A) binds to the whole L1 RNA or to a fragment of 160 nucleotides that contains its 5′UTR. The binding strength of this protein becomes weaker when the RNA is reduced to the 5′UTR containing fragment of 52 nucleotides. In this case three other proteins appear with different intensity, called B, C and D, of 47, 31 and 24 kD respectively, whose binding positions in the 5′ UTR have been mapped. Further shortening of the RNA to half of the 5′UTR, or even better to only the characteristic pyrimidine tract, results in the re-establishment of a strong binding of protein A while binding of B is maintained and binding of C and D are lost. This behaviour might suggest that the binding of these proteins is somehow affected by alternative conformations assumed by the 5′ end of the mRNA. Accordingly a computer analysis of L1 sequences of different length, from 52 to 600 nt all starting at the 5′ end, has revealed for the 5′UTR two possible configurations, both with the same stem loop but differing from each other in the situation of the pyrimidine tract. One could speculate that, according to the translational state of the mRNA, alternative conformations might expose different sites of the 5′UTR to protein recognition. Although suggestive, this view remains speculative. At this point we have proved only the specific binding of these proteins to sites in the 5′ UTR region of L1 mRNA and of other rp-mRNA.

Of the four binding proteins observed, we have considered with most attention protein A; this gives the strongest signal, and binds very specifically to the three different rp-mRNA tested on their 5′ pyrimidine tract, a sequence which is very typical of all rp-mRNA of Vertebrates and that has been shown to be involved in the translational control of this class of mRNAs (7, 19). We have also shown that the binding of protein A is dependent on the position of the pyrimidine sequence, being lost when this region is moved from the very 5′ extremity to several nucleotides downstream. This result could be correlated with the observation that the internalization of the pyrimidine containing region of the 5′UTR in the mRNA for mammal r-protein S16 results in the loss of the translational control (34). A protein of 56 kD, probably homologous to our protein A, has been recently described to bind the pyrimidine tract of murine L32 rp-mRNA; this protein did not bind to RNAs lacking the polypyrimidine sequence (18). Binding activity was similar in extracts made from resting and activated cells.

Avidly mRNA-binding polypeptides of 54 kD and 56 kD have been described in *Xenopus* oocytes as an mRNP constituent (35, 36, 37). The p56 protein has been shown also to have DNA binding properties (38). Our protein A shares some properties with these proteins raising the possibility that we may be dealing with one of them: the size is rather close, the binding is stabilized at higher ionic strength and it is insensitive to micrococcal nuclease. However there are also some remarkable differences: first of all, fractionation of extracts by heparin-sepharose and HPLC has shown that our binding activity A is not associated with any major band of the electrophoretic pattern, while the above mentioned proteins have been described as major components in the oocyte cytoplasmic extract. Second, while these have a general binding ability for any RNA (36, 39), our protein A is very specific for rp-mRNA pyrimidine tracts; moreover its binding activity does not substantially change in oogenesis and embryogenesis, at variance with those proteins whose binding was primarily found in oocytes (36). These different properties indicate that we are dealing with different proteins or at least, and even more interestingly, with a particular member of the same family of RNA binding proteins postulated for p54 and p56 (40).

An identity of protein A with the PTB protein and its isoformes that interact with the polypyrimidine tract of introns (41, 42) is very unlikely as they differ in many properties, including their respectively cytoplamic and nuclear localizations and their different sequence specificity, which in the case of the PTB protein is for a particularly U rich sequence different from the rp-mRNA pyrimidine tract (41).

Although we might have missed some protein either *in vivo* and *in vitro*, the data obtained in the cesium cloride and in the RNA/protein binding experiments suggest that the rp-RNP particle cannot contain much more than the proteins described above even in the possible case of alternative binding.

The fact that the described proteins are mostly bound to the 5′ untranslated region could suggest a role in translational regulation of this class of mRNA. In fact some other mRNAs have shown a similar situation, the best studied example being represented by the ferritin mRNA whose translation is repressed by a protein factor which binds to an iron responsive element (IRE) located in the 5′UTR (43 and references therein). This element, found in ferritin, transferrin and δ-aminolevulinic acid synthase mRNAs, and the corresponding binding protein represent the common elements for the coordinate regulation of the synthesis of the proteins involved in iron utilization and metabolism (44). One can imagine a similar situation for the class of r-protein mRNAs. In fact the 5′UTR of the rp-mRNAs has been demonstrated to represent the *cis* acting element of this type of regulation in *Xenopus* (7) and in mammals (18, 19, 34), and now we have reported that this element can bind specific proteins. However there is no proof that the described proteins themselves

act as translational repressors; in fact one can alternatively envisage these proteins always associated with the 5'UTR of rp-mRNA to form a peculiar 5' end structure sensitive to translational control by some other factor(s). In this case the described proteins could be always bound to rp-mRNA, also when this is being translated on polysomes, as some preliminary results not shown here seem to suggest. A complex situation of this kind is suggested by our observation that the binding activity is equally present at different developmental stages when the repression of rp-mRNA translation declines. To establish the role of the proteins described here in the translational regulation of rp-mRNA they have to be purified and then tested in functional assays.

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