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A *Xenopus laevis* Homologue of the La Autoantigen Binds the Pyrimidine Tract of the 5' UTR of Ribosomal Protein mRNAs *in Vitro*: Implication of a Protein Factor in Complex Formation

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In *Xenopus* and other vertebrates, ribosomal protein mRNAs share a common sequence in the 5' untranslated region (5' UTR), in particular a pyrimidine tract at the 5' end, which has been demonstrated to be involved in the translational regulation of this class of mRNAs. In previous studies, carried out in the *Xenopus* system, we demonstrated the specific binding of two proteins (57 kDa and 47 kDa) to the pyrimidine tract of the mRNAs for three different ribosomal proteins. Here, we show that the two binding proteins are in fact one; one being the cleavage product of the other. By immunoprecipitation and protein purification, this binding protein has been identified as the *Xenopus* homologue of the human La autoantigen, an RNA-binding protein previously reported to be implicated in RNA polymerase III transcription termination and in translation initiation of poliovirus and immunodeficiency virus type 1 RNAs. We show that the specific interaction of La with the 5' pyrimidine tract of ribosomal protein mRNA is mediated by a protease-sensitive factor, which, after assisting La-RNA binding, dissociates from the complex and becomes again available to promote further binding. We show that mutations in the 5' UTR pyrimidine tract, known to disrupt the translational control of ribosomal protein mRNA, severely impair La binding. Although a direct relationship between ribosomal protein mRNA translation and La binding is not yet available, the properties of the interaction suggest that La protein, possibly together with other components, might be involved in translational regulation.

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Keywords: La protein; ribosomal protein mRNA; RNA/protein interaction; translational control; *Xenopus*

Introduction

The coordinated synthesis of the numerous ribosomal proteins (r-proteins) is achieved in vertebrates by regulation at post-transcriptional levels. In particular, a translational regulation mechanism controls the efficiency of utilization of the mRNAs specific for ribosomal proteins (rp-mRNAs) according to the cellular need for ribosomes, as observed during development and in cultured cells under different growth conditions (Geyer *et al*., 1982); Pierandrei-Amaldi *et al*., 1982, 1985; Agrawal & Bowman, 1987; Loreni & Amaldi, 1992). In *Xenopus*, as in other systems, the mRNAs for different r-proteins share similarities in the 5' untranslated region (5' UTR), which has been shown to have a role in their regulated translation (Mariottini & Amaldi, 1989; Levy *et al*., 1991; Pierandrei-Amaldi & Amaldi, 1994). In particular, a pyrimidine tract located at the 5' end of these mRNAs is necessary, though not sufficient, to exert the translational control in *Xenopus* and in mammals (Levy *et al*., 1991; Kaspar *et al*., 1992; Avni *et al*., 1994). It has been found that this particular 5' UTR is present in the rp-mRNA and in the mRNA

Abbreviations used: PMSF, phenylmethylsulfonylfluoride; UTR, untranslated region; r-protein, ribosomal protein; rp-mRNA, ribosomal protein mRNA.

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for other proteins related to the synthesis and the function of the translation apparatus, such as EF-1α and EF-2, which are consequently translationally coregulated with r-proteins (Loreni *et al*., 1993; Jefferies *et al*., 1994; Terada *et al*., 1994). This class of mRNAs, called TOP-mRNAs from the Terminal Oligo-Pyrimidine tract (Meyuhas *et al*., 1996), represents about 15 to 20% of the mRNA of the animal cell.

In our previous studies (Cardinali *et al*., 1993) a number of proteins were identified to bind specifically to the 5' UTR of *Xenopus* L4 mRNA (formerly L1†). Two of them, named A and B (57 kDa and 47 kDa), were found to interact with the pyrimidine tract, while the others, C and D (31 kDa and 24 kDa), were shown to bind the downstream region of the 5' UTR. Similarly, a protein of 57 kDa has been described to bind the pyrimidine tract of mouse rp-L32 mRNA (Kaspar *et al*., 1992; Severson *et al*., 1995). Here, we report the purification and characterization of the *Xenopus* 57 kDa protein that binds the L4-mRNA pyrimidine tract, and its identification as La, the *Xenopus* homologue (Scherly *et al*., 1993) of human La autoantigen (reviewed by Pruijn *et al*., 1990). Moreover, we show that the interaction of La with the pyrimidine sequence is dependent on the presence of a protein factor. Interestingly, a stretch of pyrimidines is invariably present also in the internal ribosome entry segment (IRES) of all picornavirus RNAs, a region that interacts with cellular proteins as La autoantigen in the case of poliovirus, and PTB (pyrimidine tract binding protein) in the case of encephalomyocarditis virus (EMCV). These proteins have been suggested to be implicated in the internal translation initiation process of picornavirus RNAs (for a review, see Jackson & Kaminski, 1995).

Results

Characterization of the proteins that bind the pyrimidine tract of L4 mRNA

In our previous study we established that the 12 nt RNA fragment F5, corresponding to the 5' pyrimidine tract of rp-L4 mRNA, was the minimal element still able to bind proteins A and B. The binding assay utilized in that study, as in the present one, consists of the incubation of *in vitro* transcribed radiolabeled RNA with whole or fractionated cytoplasmic extracts; after UV cross-linking and RNase digestion, the resulting in-directly labeled proteins are analyzed by SDS-PAGE (Cardinali *et al*., 1993). We observed that binding activity was maintained with RNA fragments with 3' progressive deletion downstream of the pyrimidine tract, but was lost when this tract was deleted. The specificity of binding was proved by competition experiments with related and unrelated RNAs (Cardinali *et al*., 1993). This binding activity was found and studied only in *Xenopus* embryo extracts. Now, using the same RNA fragment F5, we have analyzed extracts from other sources, namely *Xenopus* oocytes, and liver

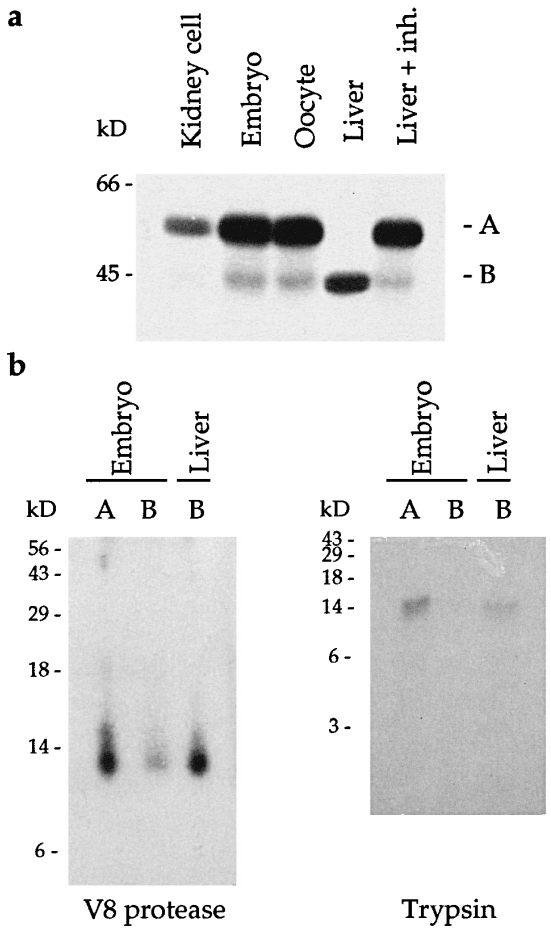

Figure 1. Characterization of the L4 pyrimidine tract binding proteins. a, Binding analysis of different *Xenopus laevis* extracts. *In vitro* transcribed radioactive F5 RNA (50,000 cpm) was incubated under standard conditions with S100 extracts from different sources in a volume of 15 μl: B 3.2 kidney cultured cells (30 μg), stage 15 embryos (40 μg), stage VI oocytes (40 μg), adult liver (100 μg), and adult liver (100 μg) prepared with the addition of the protease inhibitor leupeptin (10 μg/ml). After treatment with heparin, UV-crosslinking and RNase digestion the reactions were analyzed on a 12% polyacrylamide/SDS gel and autoradiographed. b, Protease mapping. Bands corresponding to indirectly labeled protein A and B from S100 embryo extract and protein B from S100 liver extract were excised from the gel, subjected to V8 protease (left) or trypsin (right) digestion, analyzed on a 17% poly-acrylamide/SDS gel and autoradiographed. Molecular mass markers are shown. A, Protein A; B, protein B; inh, inhibitor.

† In previous papers the numbering of *Xenopus* ribosomal proteins followed the system introduced in our first study (Pierandrei-Amaldi & Beccari 1980). The large amount of sequencing data now accumulated in many species allows to adopt, for a unified nomenclature, the rat system (Wool *et al*., 1990). Thus the *Xenopus* r-protein that we previously designed L1 is here identified as L4.

and kidney-derived cultured cells. Figure 1a shows that extracts from oocytes and cultured cells have a binding pattern very similar to that of embryo extracts, with protein A much more abundant than B. In contrast, in liver extract only binding of protein B is observed. However, when the liver extract was prepared in the presence of leupeptin, the pattern appeared identical to the other sources, with protein A more abundant than B, suggesting that B is a cleavage product of A. Support for this conclusion came from experiments of peptide mapping with V8 protease and trypsin (Figure 1b). The bands corresponding to indirectly ^{32}P-labeled proteins A and B from embryo extract and B from liver extract, prepared in the absence of leupeptin, were excised from the gel, loaded onto a 17% polyacrylamide/SDS gel and digested with V8 protease as described in Materials and Methods. The labeled peptides originating from bands A and B comigrate in the gel at a position corresponding to 12 kDa (Figure 1b), suggesting an identity of the two proteins. Digestion with trypsin of the same bands also resulted in the production of an identical labeled peptide, of 14 kDa (Figure 1b). We conclude that proteins A and B, previously described to bind the pyrimidine tract of L4 mRNA, are in fact the same protein, in that B is a cleavage product of A.

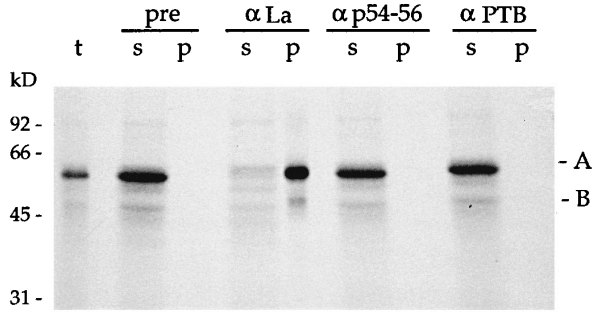

Figure 2. Immunoprecipitation analysis of the complex. Binding reactions with radioactive F5 RNA and a liver extract prepared in the presence of inhibitors were performed according to the standard protocol. UV-cross-linked samples were immunoprecipitated with a preimmune serum, with anti La (αLa), anti p54–56 (αp54–56) or PTB (αPTB) antisera, respectively. The binding reactions before immunoprecipitation (t), the supernatants (s) and the precipitates (p) were separated by SDS-12% PAGE and indirectly labeled proteins were revealed by autoradiography. A, Protein A; B, protein B.

Comparison of protein A with other RNA-binding proteins

As discussed previously (Cardinali *et al*., 1993), several RNA-binding proteins have been described that, for size and features, resemble in some way our protein A. Among these the *Xenopus* p56 (Murray *et al*., 1991), the pyrimidine tract binding protein PTB (Garcia-Blanco *et al*., 1989) and the autoantigen La (Lerner & Steitz, 1979). To find out if protein A was one of those proteins, immunoprecipitation experiments of the protein A/RNA complex were carried out. The binding reaction was performed with radiolabeled RNA fragment F5 and liver extracts prepared in the presence of the protease inhibitor leupeptin. After UV cross-linking and RNase treatment, the indirectly labeled proteins were immunoprecipitated with p56, PTB and La antibodies. Figure 2 shows that La antibodies immunoprecipitate the complex but p56 and PTB antibodies do not. The same results were obtained with the whole L4 mRNA, or using oocyte extracts (not shown). This result indicates the presence of La in the F5 binding complex.

Purification of the binding protein

To verify the identity of protein A and La, chromatographic procedures previously used to purify La were carried out (Stefano, 1984; Bachman *et al*., 1990; Meerovitch *et al*., 1993) and the capacity of the various fractions to bind F5 RNA was tested. A first attempt to purify the 5' pyrimidine tract binding protein from oocytes was abandoned as these are known to contain an abundant amount of the RNA-binding protein p56 (Murray *et al*., 1991), which may interfere with our pyrimidine tract binding assay, and a large amount of proteins that copurify with our activity. Of the other sources of material tested (see above), liver extract proved to be the most convenient. In fact, although it has an F5 binding activity three to four times lower than oocytes (see the legend to Figure 1), it contains with respect to oocytes, a much smaller amount of proteins that coelute with the binding activity through purification. Protease inhibitors were omitted during extract preparation, since they were inefficient in preventing cleavage during the purification steps of large-scale preparations. Nevertheless, protein B, although shorter than A, maintains the same RNA-binding capacity necessary to follow the protein during purification.

Thus liver S100 extracts were sequentially fractionated by chromatography on heparin-Sepharose and poly(U)-Sepharose, and the presence of protein B was monitored by the F5 binding assay of all fractions. The S100 extracts were loaded onto a heparin-Sepharose matrix and eluted as described in Materials and Methods (Figure 3a). The binding assay shows that protein B is eluted to 0.4 M KCl (Figure 3b). This fraction, after dialysis, was loaded onto a poly(U)-Sepharose column and a linear salt gradient was applied for elution (Figure 3a'). Protein B activity eluted at 1 to 1.5 M KCl (Figure 3b'), corresponding to a fairly homogeneous protein in the stained gel (Figure 3a'). Binding activity progressively decreased throughout purification, with a 70% drop during the heparin-Sepharose step and a further loss during the poly(U)-Sepharose fractionation (Table 1).

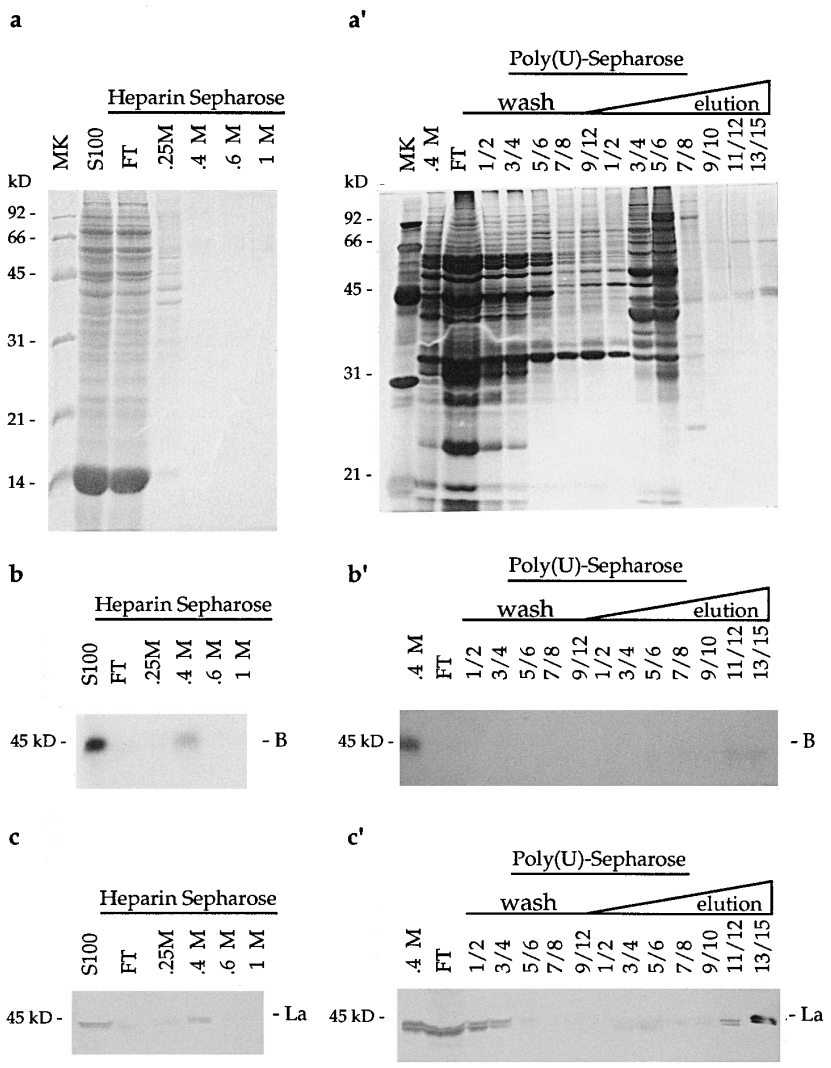

Figure 3. Purification of the RNA binding protein B from liver. a, Protein elution pattern from the heparin-Sepharose column loaded with the S100 liver extract. Fractions were collected and 0.003% of the volume of each was run on a 12% polyacrylamide/SDS gel and stained with Coomassie blue. a', Elution pattern (0.1 M to 1.5 M KCl gradient) from a poly(U)-Sepharose column loaded with the 0.4 M KCl heparin-Sepharose fraction: 0.5% of this fraction and 2.5% of each poly(U)-Sepharose fraction were analyzed by electrophoresis on a 12% polyacrylamide/SDS gel followed by silver staining. Molecular mass markers (MK) are indicated. b, The same amount of protein of the S100 and of heparin-Sepharose fractions used in a was tested for ability to bind *in vitro* transcribed radioactive F5 RNA; b', 0.6% of the fractions used in a' were tested in binding assay with F5 RNA. c and c', The same amount of protein shown in a and a', respectively, were fractionated on 12% polyacrylamide/SDS gels, blotted onto a nitrocellulose filter and incubated with rabbit polyclonal anti-La antiserum (diluted 1:200). Bound antibodies were revealed by the alkaline phosphatase method. B, Protein B; La, La autoantigen; FT, flow-through.

La antibodies were used in Western blot experiments to confirm the presence of La during protein purification. F5 binding activity and La protein coelute in the 0.4 M KCl fraction from the heparin-Sepharose column (Figure 3b and c) and in the 1 to 1.5 M KCl fractions from the poly(U)-Sepharose column (Figure 3b' and c') and coincide with the homogeneous protein in the stained gel (Figure 3a'). Notice that only a relatively small amount of La binds to the poly(U)-Sepharose column, a large part of it being found in the flow-through and in the wash fractions. If these fractions are loaded onto a new poly(U)-Sepharose column they are again excluded, implying that their exclusion is not due to limiting amounts of beads (not shown). It can be observed (Figure 3b' and c') that in the flow through and wash fractions, in spite of the large amount of La present, there is no F5 binding activity. This could be attributed to different isoforms of the protein or to some modification of it as already reported (Bachman *et al*., 1990). However, the progressively lower recovery of the F5 binding activity throughout purification (see above) suggests the possibility of a loss during purification of some factor involved in binding.

Table 1. Purification of protein B (p45)

Fraction	Volume (ml)	Protein Conc.(mg/ml)	Protein Total (mg)	Yield (%)[a]	Fold purification
Liver extract	90	36	3240	100	1
Heparin-Sepharose	100	0.17	17	35	66.5
Poly(U)-Sepharose	20	0.001	0.02	5	8100

[a] Yield was calculated by quantitation of the amount of label transferred to proteins (values normalized to that of S100).

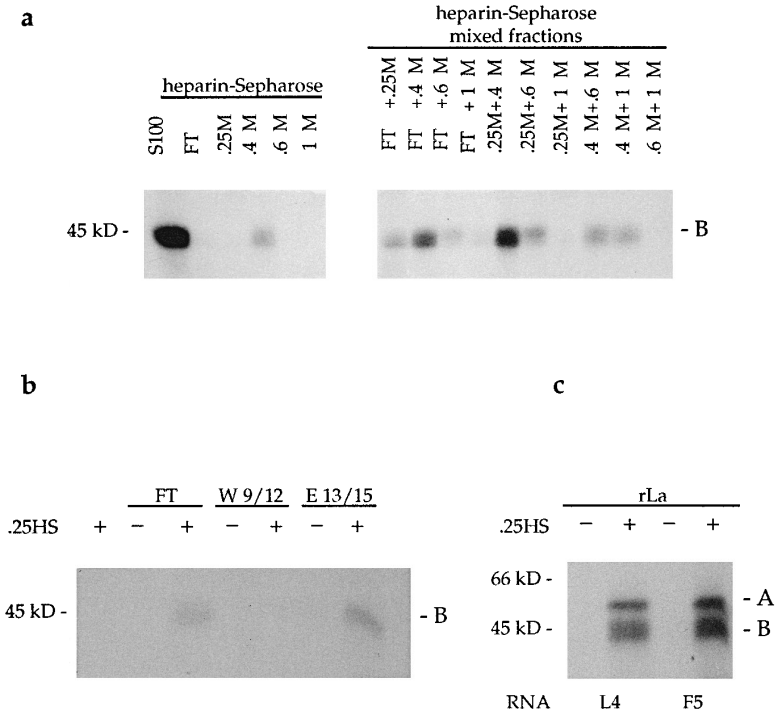

Figure 4. Binding reconstitution experiments. a, Binding assay of radioactive F5 RNA with liver S100 extract and heparin-Sepharose eluted fractions alone (left panel) or mixed (right panel). b, Poly(U)-Sepharose flow-through (FT), wash 9/12 and elution 13/15 fractions were tested for binding, alone or mixed with the 0.25 M KCl fraction of heparin-Sepharose (.25HS). The amount of protein fractions utilized in a and b was identical to that indicated in the legend to Figure 3b and b', respectively. c, Purified (100 ng) bacterial-expressed *X. laevis* recombinant LaB1 protein (Scherly *et al*., 1993) was utilized alone or mixed with the .25HS in binding experiments with the full length L4 RNA or the F5 RNA. The binding reactions were separated on a 12% polyacrylamide/SDS gel and autoradiographed. B, Protein B; rLa, recombinant La.

The binding activity is determined by components that elute separately during purification

In order to investigate this last hypothesis, reconstitution experiments were carried out mixing different fractions of the various purification steps. Equivalent amounts of different fractions were mixed and used for F5 RNA binding assay. The left panel of Figure 4a shows the F5 binding activity of heparin-Sepharose individual fractions and the right panel shows the activity after mixing the fractions in all combinations. Figure 4 shows that some agent is present in the flow-through and in the 0.25 M KCl fraction (hence named .25HS fraction), that is able to potentiate the F5 binding activity of the 0.4 M KCl fraction to levels comparable to that of the S100 extract, and to rescue some activity in flanking fractions. This activity is not present in the 0.6 M or 1 M KCl fractions.

We have tested the .25HS potentiating activity on some relevant poly(U)-Sepharose fractions. In Figure 4b it appears that, upon addition of the .25HS fraction, some F5 binding activity is detected in the poly(U)-Sepharose flow-through, *per se* inactive. Enhancement of F5 binding is observed on the already active 1 to 1.5 M KCl fractions, while wash fractions are not affected. By comparing the results of Figure 4b with those of Figure 3c', it can be observed that binding activity can be rescued by .25HS only in fractions containing La, suggesting a connection between La and the rescuing agent.

To further investigate this relationship, so far observed in tissue extracts, experiments were carried out with purified *Xenopus* recombinant La protein (Scherly *et al*., 1993). Figure 4c shows that recombinant La alone does not bind F5 RNA nor full-length L4 mRNA, while in the presence of the .25HS fraction it binds both RNAs strongly. This finding supports the notion that La is the L4 pyrimidine tract binding protein and that the factor plays an essential role in binding.

To gain information about the nature of the rescuing factor, the .25HS fraction was treated with protease or nuclease before binding. Aliquots of this fraction were incubated with proteinase K, immobilized Pronase-CB, immobilized-TPCK trypsin, or nuclease S_7. After inactivation or elimination of the enzymes (as described in Materials and Methods), the .25HS-treated samples were added to the heparin-Sepharose 0.4 M KCl fraction to test their ability to enhance the F5 binding activity. Figure 5 shows that treatment with all three proteases abolished the .25HS enhancing activity, leaving the basal binding level of the 0.4 M KCl fraction. Treatment with nuclease S_7 had no effect. In control experiments it was checked that no residual protease or nuclease activity was present after inactivation (not shown), as shown by the unaffected basal binding of the 0.4 M fraction (Figure 5). These results indicate that at least a protein is responsible for the .25HS activity.

Modality of interaction of the components in complex formation

In order to investigate the interaction between La protein and the factor in relation to the binding to the pyrimidine tract, we developed an experimental approach based on the affinity selection of the protein/biotinylated F5 RNA complex by streptavidin magnetic beads. In planning this exper-

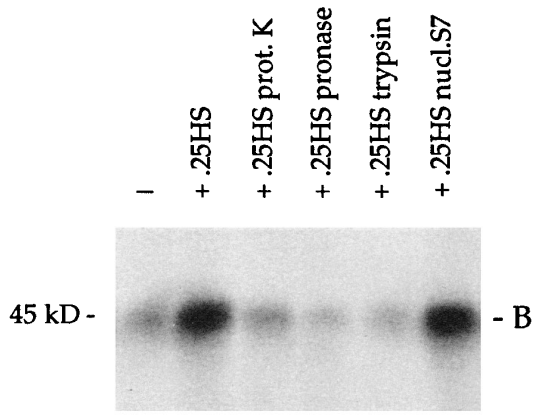

Figure 5. Protease sensitivity of the .25HS fraction activity. Different aliquots of the .25HS fraction were respectively treated with proteinase K, pronase or trypsin immobilized on agarose beads and nuclease S_7 as described in Material and Methods. Binding assays were carried out by incubating radioactive F5 RNA with the 0.4 M KCl heparin-Sepharose fraction alone or mixed with the untreated, or the protease and nuclease-treated .25HS fraction, respectively. The amount of the protein fractions utilized was as for Figure 3b. The reactions were analyzed by SDS/PAGE and autoradiography. B, Protein B.

iment we kept in mind that in our first attempts to purify protein B by affinity selection we could never recover any binding activity with the proteins eluted from the selected complex. A saturating amount of radiolabeled F5 RNA, biotinylated at its 3' end, was incubated with liver S100 extract followed by UV crosslinking (Figure 6a and a'). An aliquot was kept before selection (total) and the bulk was affinity-selected with an excess amount of streptavidin magnetic beads to capture all biotinylated RNA. Equivalent amounts of the total (t) and of the affinity-selected pellet (p) and supernatant(s) fractions were analyzed by SDS-PAGE and autoradiography to show the indirectly labeled protein (Figure 6a), or by Western blot to show the La distribution (Figure 6a'). Figure 6a shows that the protein associated in the complex is mostly found in the pellet, while the F5-bound La represents only a small part of the total La present in the extract (Figure 6a'), thus indicating that most La is unable to bind RNA. Since the RNA was in excess, this experiment indicates that the complex present in the pellet corresponds to all the La protein that can bind. A parallel experiment was carried out using amounts of RNA, extract, binding conditions and affinity selection identical to the one just described, but omitting UV crosslinking (Figure 6b and b'). In this way a functional recovery of the selected fractions and their use for further binding analysis was possible. After affinity selection the proteins associated in the complex were eluted with 2 M KCl and dialyzed. Equivalent amounts of the extract before binding (t), of the supernatant fraction (s) and of the pellet eluate (p) were tested for binding activity by addition of radiolabeled F5 RNA (Figure 6b) or analyzed by Western blot (Figure 6b'). In Figure 6b it can be seen that the eluted protein (p) has no binding activity, in contrast the affinity supernatant fraction (s)

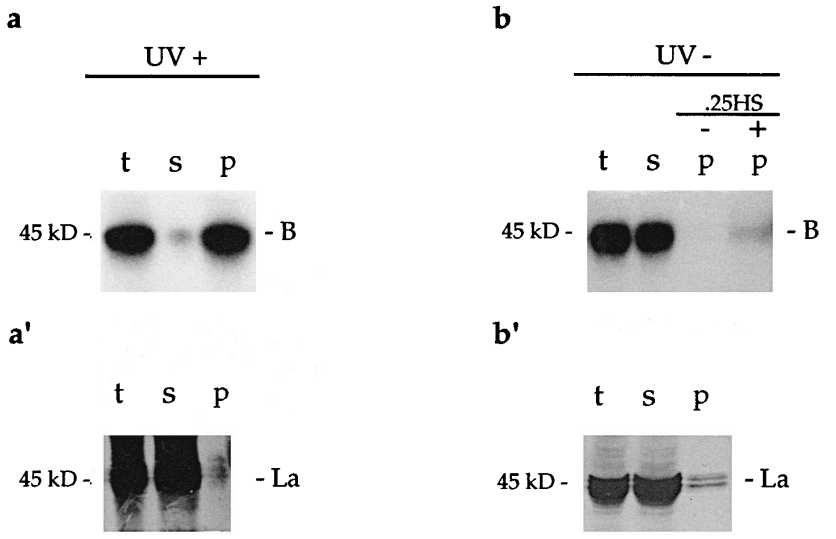

Figure 6. Affinity-selection analysis of the *in vitro* L4 pyrimidine tract RNA/protein complex. a and a', Affinity selection of the complex. The liver S100 extract was incubated under standard binding conditions with a saturating amount of 5' end-labeled, 3' end-biotinylated F5 RNA. The complex was UV-cross-linked and captured with an excess amount of streptavidin beads. The unbound fraction (super) and the bound fraction (pellet), were analyzed and compared to the binding mixture before capture (total). Equivalent amounts of total (t), pellet (p) and supernatant (s) were loaded onto two identical 12% polyacrylamide/SDS gels. One (a) was dried and autoradiographed showing the indirectly labeled proteins, the other (a') was Western blotted and incubated with anti-La antiserum. b and b', Recovery of binding activity and of La, respectively, after affinity selection. An experiment identical to that described above was done, with the exception that the UV-crosslinking was omitted before streptavidin capture of the complex, thus making possible binding analysis after affinity. The unbound fraction (super) and the bound fraction eluted from the beads with a 2 M KCl buffer (pellet) were analyzed for recovered binding activity and compared to the S100 liver extract before binding (total). Equivalent amounts of the total (t), supernatant (s) and the pellet (p), this last alone or mixed with the .25HS fraction, were analyzed (b) by binding assay or (b') by Western blot analysis performed with anti-La antibodies. B, Protein B; La, La protein.

Table 2. rLa relative binding to wild-type (wt) and mutant F5 (L4 Py tract)

F5	L4 RNA	Length (nt)	% rLa binding
wt	CCUUUUCUCUUC	12	100
del 1	CCUUUUC	7	100
mut 1	GCCUUUUCUCUUC	13	100
mut 2	GGCCUUUUCUCUUC	14	100
mut 3	ACCUUUUCUCUUC	13	20
mut 4	ACUUUUCUCUUC	12	15
mut 5	UUUUCUCUUC	10	30

contains an activity comparable to that of the extract before affinity selection (t). The Western blot in Figure 6b' shows that the amount of La in the pellet, as in the other fractions, is similar to that in Figure 6a', indicating that there is no protein loss due to the omission of UV crosslinking or to recovery procedures. In an attempt to find out if binding activity in the pellet eluate could be rescued by addition of the enhancing factor, the .25HS fraction was added to the pellet eluate binding assay. Figure 6b shows that under these conditions activity is restored. This suggests that La is still able to bind after being complexed, but possibly, once in the complex, loses a factor necessary for binding, as suggested by the complete recovery of F5 binding in the supernatant. These findings explain our previous unsuccessful efforts to recover binding activity after affinity selection when attempting to purify the protein.

Binding analysis of mutated La RNA pyrimidine tracts

After identifying the L4-mRNA pyrimidine tract binding protein as La, and showing that binding is determined by the presence of another factor, we wanted to investigate how mutations in the pyrimidine tract could interfere with that interaction. The F5 RNA fragment and some mutagenized forms were incubated with recombinant La (rLa) plus the .25HS fraction to test their binding capacity. Some of these mutants (mut 1 and mut 2) have been tested for binding with extracts (Cardinali *et al*., 1993). The T7 *in vitro* transcripts of the correct size were gel purified and used in the reactions at the same molar amounts. Table 2 shows that the addition of one or two G bases (mut 1 and mut 2) in front of the pyrimidines does not affect the binding, nor does the deletion of the last five nucleotides (del 1). In contrast, the addition of one A at the 5' end (mut 3) or the substitution of the first C with A (mut 4) decreases the binding to 20% and 15%, respectively, while the deletion of the first two C bases (mut 5) brings the binding to 30%. Similar results were obtained by using the S100 extract or the 0.4 M heparin-Sepharose fraction (not shown). In all cases the binding of La to the different RNAs requires the binding factor.

Discussion

We present here the identification and characterization of the protein that we have previously shown to interact specifically with the pyrimidine tract of the 5' UTR of the mRNAs for some r-proteins. We described in *Xenopus* embryo extracts two proteins, named A (57 kDa) and B (47 kDa), that bind the pyrimidine tract of L4-mRNA (Cardinali *et al*., 1993). Now we have demonstrated their presence in other *Xenopus* cell types (oocytes, liver and kidney cultured cells), and that protein B is a cleavage product of A. Moreover, we have identified this binding protein as the *Xenopus* homologue of the human La autoantigen (Lerner & Steitz, 1979). This identification is based on recognition by La antibodies, similar chromatographic behavior and binding properties identical to recombinant La protein.

Xenopus La, recently cloned and characterized by Scherly *et al*. (1993), is 60% homologous to human and bovine La. The major difference is present in the C terminus, where the frog protein contains an insertion of 20 amino acid residues proximal to the protease-susceptible PEST site. Conserved RNA binding motifs are located more proximally to the N terminus. Thus a cleavage in the susceptible region is compatible with the sizes of proteins A and B, and with the preservation of their binding ability.

The low ability of La to bind poly(U)-Sepharose has been reported; Bachman *et al*. (1990) observed that only part of mammalian La binds to poly(U)-Sepharose and they attributed the binding failure of most La to a post-translational modification of the protein. In our case, the limited poly(U) binding capacity of La could be interpreted in the same way. On the other hand, the limited F5 binding activity of La appears to be due to a different reason; namely, the loss throughout purification of a factor that elutes in a heparin-Sepharose fraction different from that containing La. This factor is able to potentiate or rescue F5 RNA binding activity when added to an La-containing fraction. As one might have expected, the recombinant La is absolutely devoid of any F5 binding activity if the factor is not added, supporting the idea that it is necessary for F5 RNA binding. We have shown that the factor activity is abolished by treatment with protease but not nuclease, indicating the involvement of protein and not of RNA, although the presence of a protected RNA cannot be excluded. The nature of the interaction between La and the factor is not clear, but it appears to be independent of the post-translational modification of La that, as mentioned above, influences binding to poly(U) (Bachman *et al*., 1990). In fact, the factor rescues F5 binding activity of La, whether poly(U)-Sepharose excluded or retained. This is in agreement with our previous observation that poly(U) competes poorly with the F5 fragment or with L4 mRNA in binding assays with embryo extracts (Cardinali *et al*., 1993).

The affinity-selection experiments of the RNA/protein complex provided information about some features of complex formation. First, they showed that only a small percentage of the available La accounts for all the binding capacity of the extract, possibly due to limiting amounts of the factor. Second, they indicate that, after mediating complex formation, the factor becomes available again to recruit new La molecules until an equilibrium is reached among the components. This is in agreement with the fact that addition of the factor-containing fraction to the La eluted from the pellet restores the binding, but to a lesser extent than the corresponding supernatant, due to the different amounts of La present. The possibility that a covalent modification of La is responsible for its binding seems unlikely. It is difficult to suppose that, once modified and captured by affinity selection of the complex, La can revert by itself to the unmodified state, being no longer able to bind. It is more likely that the factor promotes a conformational change of La that increases its affinity for F5 RNA, possibly through protein-protein interaction.

The role attributed so far to La is as an RNA polymerase III transcription termination factor, a notion supported by the finding that La protein binds to nascent RNA polymerase III transcripts carrying 3' oligo(U) residues and that La depletion impairs transcription and RNA processing (Stefano, 1984; Gottlieb & Steitz, 1989). La has been found associated with a few polymerase II transcripts, which however also possess a short tract of U residues that seems to be the common feature principally responsible for binding (Chambers *et al*., 1983; Mathews & Francouer, 1984; Madore *et al*., 1984; Kurilla & Keene, 1983). The results of immunofluorescence studies are consistent with a prevalent nuclear localization of La; although it has been shown that part of La is present in the cytoplasm and shuttles with the nucleus in the form of RNP (Hendrick *et al*., 1982; Habets *et al*., 1983; Bachman *et al*., 1989; O'Brien *et al*., 1993; Peek *et al*., 1993; Simons *et al*., 1994).

Interestingly, the protein p52, which binds to the poliovirus RNA 5' UTR (Sonenberg & Meerovitch, 1990) has been recently identified as La (Meerovitch *et al*., 1993; Svitkin *et al*., 1994). This host cell protein interacts with a region of the viral RNA that includes the highly conserved and functionally important pyrimidine motif involved in internal initiation of translation of the poliovirus RNA. In particular, La is able to specifically enhance translation and to correct aberrant initiation of poliovirus RNA in rabbit reticulocyte lysates, presumably through the interaction with the mRNA 5' UTR (Meerovitch *et al*., 1993). Moreover, in the same study it was reported that, following virus infection, La is redistributed in the cell from the nucleus to the cytoplasm, suggesting for La a function as a cytoplasmic factor involved in cap-independent translation of poliovirus RNA. A role for La has been described also in cap-dependent translation of the human immunodeficiency virus type 1 (HIV-1) RNA. By interacting strongly and specifically with the *trans*-activation response element (TAR), La relieves the *cis*-inhibitory effect on translation of this sequence (Chang *et al*., 1994; Svitkin *et al*., 1994). Now the question arises of whether La is involved in translation only upon viral infection or whether it normally exerts an activity in translation of some cellular mRNAs that is adopted by the virus during infection. It is attractive to speculate that these cellular mRNAs are those coding for r-proteins, which in fact contain an La binding 5' UTR pyrimidine tract. It is worth noting that the involvement of a factor promoting La activity had been postulated by Meerovitch *et al*. (1993) to explain the tenfold lower effect of recombinant La as compared to HeLa extracts on *in vitro* translation of poliovirus RNA.

It was demonstrated that the integrity of the pyrimidine tract is necessary for regulation of r-protein mRNA translation. In fact, mutations in the pyrimidine tract of mammalian S16 mRNA, where the first C was substituted or preceded by an A, were found to abolish the translation control (Avni *et al*., 1994). We have shown here that the same mutations in the L4 pyrimidine tract, which is identical to that of S16, severely affect La binding. Although a direct relationship between binding of La to the 5' UTR of r-protein mRNAs and a functional role in translational regulation is not yet available, the two properties appear to be somehow connected.

The mutations described result in an increased translation of the mRNA, suggesting a negative control by a translation repressor (Avni *et al*., 1994). On the other hand, La has been shown to be a positive effector in the inefficient translation of poliovirus and HIV-1 RNAs in reticulocyte lysate. Translation of rp-mRNAs also is inefficient in reticulocyte lysate and these mRNAs are poor competitors for cap-binding proteins (Hammond *et al*., 1991). Moreover, rp-mRNA translation repression is not relieved by overexpression of eIF4E (Meyuhas *et al*., 1996). These observations would suggest a positive role of La in translation of rp-mRNA, in contrast with the idea of the repressor derived by the pyrimidine tract mutation experiments. Understanding of the role of the factor could provide a way to reconcile this apparent contradiction. Although it is premature to propose a model, one could speculate that La interacts with the rp-mRNA both in translationally active and repressed states. The association of the factor with the complex might determine the repressed state by preventing a positive effect of La. Conversely, the dissociation of the factor from the complex would leave the possibility of La exerting its positive effect.

The mechanism of control of the r-protein mRNAs might not depend simply on the action of La on the pyrimidine tract of the mRNA. It appears that other proteins, which have been shown to interact with the 5' UTR of r-protein mRNA

downstream of the pyrimidine tract (Cardinali *et al*., 1993), might be involved in the regulation. It has been found that this downstream region, together with the pyrimidine tract, is relevant in translational regulation in mammalian cells (Avni *et al*., 1994) and that mutations in this sequence alter protein binding (Severson *et al*., 1995). However, other elements that may be implicated in a more complex mechanism have to be kept in mind. An important one is the phosphorylation of ribosomal protein S6, induced by mitogens, that has been found to lead to a selective translation increase of pyrimidine tract-containing mRNAs (Jefferies *et al*., 1994; Terada *et al*., 1994). The proteins that recognize the 5' UTR of this class of mRNA could represent mediators between specific and general cell signals and r-protein mRNA translation.

Materials and Methods

In vitro transcription and RNA/protein binding analysis

F5 transcript (CCUUUUCUCUUC) and its mutated forms were generated by phage T7 polymerase *in vitro* transcription of a DNA oligonucleotide template carrying the T7 promoter in the presence of 50 μCi of [α-^{32}P]UTP (800 Ci/mmol) according to Milligan *et al*. (1987) and with the modality previously described (Cardinali *et al*., 1993). Transcripts corresponding to the whole L40 RNA (1300 nt) were obtained as described (Cardinali *et al*., 1993); the procedures for RNA/protein interaction, heparin treatment, UV crosslinking, RNase digestion and complex analysis were as described (Cardinali *et al*. 1993). For crosslinking, a Spectrolinker XL4000 (Spectronics Corp.) at 1×10^6 μJ/cm^2 energy was used. In the reconstitution binding experiments, the indicated amounts of different fractions were mixed just before the addition of the labeled RNA. Quantification of radioactive bands was performed with a Molecular Dynamics Series 400 Phosphorimager and ImageQant version 3.2 software.

Extract preparation and protein purification

S100 extracts from oocytes, embryos, liver and cultured cells (B3.2) were prepared as described (Cardinali *et al*., 1993). For protein purification, 60 g of liver from *X. laevis* adult frogs (Nasco, Wisconsin) was homogenized and an S100 cytoplasmic extract was prepared. Fractionation on heparin-Sepharose (Pharmacia) was carried out essentially as described (Neupert *et al*., 1990). The S100 extract (90 ml) was diluted to reach the final KCl concentration of buffer A (10 mM Hepes (pH 7.5), 40 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, 5% (v/v) glycerol) and incubated for one hour at room temperature, under slow shaking, with 40 ml of preswollen packed beads. The unbound fraction was collected and the beads were washed with six volumes of buffer A, then packed into a column. Bound proteins were step-eluted with 100 ml of buffer A containing KCl at 0.25 M, 0.4 M, 0.6 M and 1 M, respectively, at a flow-rate of 2 ml per minute. The RNA-binding fraction, 0.4 M KCl, was dialyzed against the poly(U) binding buffer (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol) and passed through a column containing 30 ml of preswollen poly(U)-Sepharose beads (Pharmacia) equilibrated in binding buffer, at room temperature and at a flow-rate of 1 ml per minute. The column was washed with 120 ml of the same buffer, collecting 10-ml fractions. Elution was performed with a 160-ml linear gradient of 0.1 M to 1.5 M KCl in binding buffer at the same flow-rate, collecting fractions of 10 ml.

All extracts and purification fractions utilized for the RNA binding analysis were dialyzed against buffer D (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol) according to Dignam *et al*. (1983).

Protein analysis and immunoprecipitation

Analysis of proteins was carried out on 12% polyacrylamide gels and visualized by Coomassie blue or silver staining. Protein quantitation was performed with the Protein Assay kit (BIO-RAD). Western blots were performed according to Scherly *et al*. (1993) and were developed with the alkaline phosphatase assay kit (BIO-RAD) following the manufacturer's instructions.

In immunoprecipitation experiments, antibodies were incubated with protein A-Sepharose CL-4B (Pharmacia) in 0.25 ml of Ipp_{150} buffer (10 mM Tris HCl (pH 7.6), 150 mM KCl, 0.1% (v/v) Nonidet P-40) at 4°C for two hours with rotation. Following three 0.5 ml washes with Ipp_{150} buffer, 60 μl of UV-crosslinked and RNAse-treated binding reactions between F5 radioactive transcripts and S100 liver extracts were added to the beads and brought to 0.25 ml with IPP_{150} buffer. After incubation for two hours at 4°C with rotation, the beads were washed three times with 0.5 ml of ice-cold Ipp_{500} (0.5 M KCl) buffer and once with Ipp_{150} buffer. Supernatants and immunoprecipitated proteins, released from the beads by heating at 100°C in the presence of SDS-gel loading buffer, were analyzed by SDS-PAGE. Indirectly labeled proteins were revealed by autoradiography. The antibodies used in these experiments were: anti La αLa79 (Scherly *et al*., 1993), anti p54-56 (Murray *et al*., 1991) and anti PTB (Patton *et al*., 1991) sera.

Peptide mapping

Binding reactions were UV-crosslinked and run on a 12% polyacrylamide/SDS gel. After exposure, the bands corresponding to the indirectly labeled proteins A and B were excised from the dried gel, equilibrated for 15 minutes and placed into the wells of a 17% polyacrylamide/SDS gel for digestion with *Staphylococcus aureus* V8 protease (Cleveland *et al*., 1977). At the end of the run the gel was dried and autoradiographed. For digestion with trypsin, the excised bands were equilibrated for ten minutes in a 10% (v/v) isopropanol, 10% (v/v) acetic acid solution, then in 50% (v/v) methanol and dried for one hour under vacuum. The bands were added to 1 ml of 50 mM freshly prepared $(NH_4)_2CO_3$ containing 75 μg of trypsin and incubated overnight at 37°C with end-over-end rotation. The solutions were recovered, lyophilized, resuspended in SDS sample buffer and separated on a 17% polyacrylamide gel that was dried and exposed at –70°C for autoradiography.

Nuclease and protease treatments

The 250 mM heparin-Sepharose (.25HS) fraction was subjected to different enzymatic digestions. In the treatment with micrococcal nuclease, 50 μl of the fraction was incubated for one hour at 37°C in the presence of

2 mM $CaCl_2$ and 45 units of nuclease S_7 (Boehringer). The reaction was stopped by addition of EGTA (pH 8) at a final concentration of 2 mM. In the treatment with protease, 100 µl of the same fraction was incubated for four hours at 37°C in the presence of proteinase K (20 µg/ml), or with 200 µl (3 units) of immobilized TPCK-trypsin swollen beaded agarose (Pierce), or with 200 µl (0.5 unit) of pronase-CB swollen beaded agarose (Pierce), both previously washed five times with 1 ml of buffer D. The proteinase K reaction was stopped by adding PMSF at a final concentration of 1 mM. Trypsin and pronase-treated supernatants were recovered by centrifugation of the beads. The treated extracts were directly utilized in mixed binding experiments.

Affinity selection

In affinity-selection experiments we used an RNA 12-mer, identical to F5 except for being biotinylated at its 3' end (F5 biot), synthesized by National Biosciences, Inc. Two identical binding reactions were prepared: 5 mg of S100 liver extract (30 mg/ml) was incubated in standard binding conditions in a final volume of 1 ml in ice for 60 minutes with 45 pmol of non-radioactive F5 biot and 5 pmol of the same oligo, labeled at the 5' end with phage T4 polynucleotide kinase (Biolabs) in the presence of [γ-^{32}P]ATP. The specific activity of the radioactive oligo was 3×10^8 cpm/µg. At the end of the incubation, heparin was added at a final concentration of 7.5 mg/ml, and the reaction mixture was incubated for an additional 15 minutes. One sample was UV-crosslinked, and the other was not. For affinity selection, 100 µl (1 mg) of Dynabeads M-280 streptavidin (Dynal), previously prepared according to the manufacturer's protocol, were equilibrated with five washes each of 500 µl of washing buffer (same as binding buffer with addition of 0.1% (w/v) bovine serum albumin and 7.5 mg/ml heparin), divided into two parts and concentrated with the Dynal MPC (Magnetic Particle Concentrator). Then each binding reaction was mixed with one aliquot of beads and incubated for 60 minutes at 4°C under constant mild agitation. The beads were concentrated from each sample with Dynal MPC. The supernatants were collected and the beads were washed four times with 500 µl of the washing buffer containing 0.05% NP40 and once with the washing buffer alone.

RNA/protein complexes of the UV crosslinking experiment were eluted by boiling the beads 10 minutes in SDS sample buffer. Fractions before and after affinity selection were analyzed by SDS/PAGE gels followed by autoradiography or Western blot. Proteins of the experiment without UV crosslinking were eluted from the beads with 500 µl of 2 M KCl in binding buffer and the eluate was dialyzed against buffer D. Fractions before and after affinity were either analyzed by SDS/PAGE followed by Western blotting or analyzed for binding assay with radioactive F5 RNA according to the standard procedure.

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