Efficient synthesis, termination and release of RNA polymerase III transcripts in Xenopus extracts depleted of La protein

Nathalie Lin-Marq and Stuart G.Clarkson

Department of Genetics and Microbiology, University Medical Centre (C.M.U.), 9 avenue de Champel, 1211 Geneva 4, Switzerland

La proteins are conserved, abundant and predominantly nuclear phosphoproteins which bind to the 3'-termini of newly synthesized RNA polymerase III transcripts. The human La protein has been implicated in the synthesis, termination and release of such transcripts. Here we examine the potential transcriptional properties of La in Xenopus laevis, using a homologous tRNA gene as template. Immunodepletion of La from cell-free extracts leads to the formation of tRNA precursors lacking 3'-U residues. This shortening can be uncoupled from RNA polymerase III transcription, indicating that it results from nuclease degradation rather than incomplete synthesis. Extracts containing <1% of the normal La protein content synthesize tRNA precursors just as well as complete extracts, with no change in termination efficiency, and the vast majority of these full-length transcripts are not associated with the template or with residual La protein. Hence, Xenopus La seems not to function as an initiation, termination or release factor for RNA polymerase III. Consistent with the recently discovered role of La in yeast tRNA maturation in vivo, recombinant Xenopus La prevents 3'-exonucleolytic degradation of tRNA precursors in vitro. A conserved RNA chaperone function may best explain the abundance of La in eukaryotic nuclei.

Keywords: nuclease/RNA chaperone/transcription/tRNA gene/tRNA processing

Introduction

The existence of eukaryotic La proteins was first realized because the 47 kDa human version is a frequent autoantigen in systemic lupus erythematosus and Sjögren’s syndrome patients (Mattioli and Reichlin, 1974; Alspaugh and Tan, 1975). Since the cloning of human La (Chambers et al., 1988), homologues have been found in other mammals (Chan et al., 1989; Töpfer et al., 1993), frogs (Schery et al., 1993), insects and yeasts (Yoo and Wolin, 1994; Lin-Marq and Clarkson, 1995; Van Horn et al., 1997). It is now clear that La proteins are highly conserved, mostly nuclear phosphoproteins. They are also abundant, being present at ~2×10^7 copies per mammalian cell; they are particularly abundant in Xenopus laevis oocytes, in which they accumulate to ~8×10^11 copies. The extent of this stockpiling during oogenesis strongly suggests that La has a crucial function during early embryogenesis.

Despite these indications of their importance, La proteins are not needed for yeast viability (Yoo and Wolin, 1994; Lin-Marq and Clarkson, 1995; van Horn et al., 1997).

La proteins are RNA-binding proteins, showing a preference for RNAs with 3'-terminal U residues (Stefano, 1984). This is a characteristic feature of most nascent transcripts produced by RNA polymerase III (pol III), suggesting that La may be involved in pol III transcription termination. Indeed, evidence has been presented over the years that human La may function as a pol III termination, release and even initiation factor (Gottlieb and Steitz, 1989a,b; Marais et al., 1994; Marais, 1996; Fan et al., 1997; Goodier et al., 1997). Other reports suggest, however, that accurate transcription termination and transcript release can occur with purified vertebrate pol III without the need for additional proteins (Cozzarelli et al., 1983; Watson et al., 1984; Campbell and Setzer, 1992), and there is no evidence to suggest that yeast La proteins have functions in pol III transcription (Yoo and Wolin, 1997).

Every pol III transcription unit requires TFIIIB, most require TFIIIC, and 5S RNA genes also need a third factor, TFIIIA, for accurate and efficient initiation (reviewed in White, 1994). For efficient termination, an accessory factor might be needed by those pol III transcription units with an intrinsically weak terminator. For X. laevis 5S RNA genes, an efficient termination site comprises a stretch of four or more T residues embedded in G–C-rich DNA in the non-coding DNA strand (Bogenhagen and Brown, 1981). These two features are not found at the end of every pol III gene, however, even in X. laevis. For example, termination efficiency is increased rather than decreased by a mutation that makes the sequences surrounding the T cluster behind a X. laevis tRNA^5S gene more A–T-rich (Mazabraud et al., 1987). A second exception is provided by a X. laevis tRNA^phe gene. Its natural terminator is a T4 stretch with three G–C base pairs (bp) on each side, yet, as shown below, significant read-through occurs from this site in vitro.

We have used this tRNA^phe gene as a paradigm of poorly terminating pol III transcription units to determine whether X. laevis La protein increases the efficiency of termination and/or transcript release at its T4 site in homologous cell-free extracts. Our results provide no evidence for such activities, or for a pol III transcription initiation function, but they are consistent with X. laevis La having a chaperone function in tRNA processing comparable with that recently described for the yeast La homologues (van Horn et al., 1997; Yoo and Wolin, 1997).

Results

Reagents

The cloned tRNA^phe gene used here is normally found together with seven other tRNA genes on a tandemly
repeated 3.18 kb fragment of *X. laevis* DNA (Müller et al., 1987). In homologous S-100 extracts (Weil et al., 1979), pol III initiates transcription from this gene with a pppA located seven nucleotides (nt) upstream of the mature tRNA^Phe^ sequence. Most of the primary transcripts terminate with three or four U residues within a downstream T4 tract (P2; Figures 1 and 2B). Read-through products stop within a T15 tract, the natural terminator of a *X. laevis* tRNA^Phe^ gene (Gouilloud and Clarkson, 1986), which was placed further downstream to act as a 'fail-safe' terminator (P1; Figures 1 and 2B). The 5' leaders of these primary transcripts are removed by RNase P to yield processing intermediates (P1a and P2a); these in turn are cleaved by a 3' endonuclease to yield 3' trailers (P4 and P5) and, after 3'-CCA addition, the mature-length tRNA^Phe^ (P3).

All experiments made use of S-100 extracts from which the La protein had been progressively removed by immunodepletion. For this approach, rabbit polyclonal antibodies were raised against recombinant *X. laevis* La protein that had been expressed in *Escherichia coli* and partially purified by heparin–Sepharose chromatography. The anti-La antibodies were mixed with protein A–Sepharose beads; the beads were washed with transcription buffer, incubated with extract, and the immobilized antigen–antibody complexes were removed by centrifugation. The partially depleted extracts were routinely subjected to at least two further rounds of immunodepletion.

As revealed by immunoblotting, most of the La protein is removed from S-100 extracts by two rounds of anti-La immunodepletion, whereas two rounds of treatment with pre-immune serum have little effect (Figure 2A, compare α-La1, 2, PI2 and NT). A third round of immunodepletion (α-La3) reduces La levels even further. From immunoblots of diluted extracts (for example Figure 5A), we estimate that 1–3% of the original La protein remains after three rounds of immunodepletion. Additional rounds of immunodepletion remove some, but not all, of the remainder (data not shown).

**tRNA^Phe^ precursors are more heterogeneous in length in La-depleted extracts**

These immunodepleted and mock-depleted extracts are able to transcribe the tRNA^Phe^ gene template but both quantitative and qualitative differences are evident when compared with untreated S-100 extracts. First, there is an inverse relationship between the number of pol III transcripts and the number of rounds of depletion.

Although most noticeable with La-depleted extracts, this effect also occurs with mock-depleted extracts (Figure 2B, compare lanes c, g and k) and is due to the dilution of the extract that occurs at each round of depletion, which in turn reduces transcriptional ability. This effect can be overcome by concentrating the depleted extracts (see below).

Addition of an excess of recombinant La protein to the extracts stabilizes the transcripts and/or slows down RNA processing, in particular 3' processing. This is also a general effect, as shown by the increased levels of the 5' processed precursor P1a and reduced levels of mature tRNA^Phe^ P3, in untreated as well as depleted extracts (Figure 2B, compare lanes a and b, c and d, e and f). In apparent contradiction with this, the 3' trailer RNAs P4 are also more prominent in La-supplemented extracts. These 3' trailers are fully immunoprecipitable by anti-La antibodies (data not shown). Together, these results suggest that bound La protein inhibits the degradation of these processing products.

The major effect specific to La immunodepletion is that the tRNA^Phe^ precursors are more heterogeneous in length.

---

**Fig. 1.** Schematic diagram of the *X. laevis* tRNA^Phe^ gene and its pol III transcripts. Transcription initiation (arrowed) occurs 7 nt upstream of the mature tRNA coding region (boxed) and terminates within T4 or T15 tracts. The lengths of the primary transcripts P1 and P2 and their processing products are shown to the right.

---

**Fig. 2.** Transcription of the tRNA^Phe^ gene in S-100 extracts progressively immunodepleted of La protein. (A) Western blot analysis of the extracts probed with a polyclonal anti-La antibody. S-100 extracts were not treated (NT) or subjected to one, two or three rounds of depletion with immobilized anti-La antibody (α-La1, 2, 3) or pre-immune serum (PI1, 2, 3). (B) Transcription and processing products of the tRNA^Phe^ gene during a 2 h incubation at 20°C with the indicated extracts. Reactions contained [α-32P]GTP and either no additional La protein (-) or 500 ng of recombinant *X. laevis* La (+). Transcripts were purified and resolved on a 12% denaturing polyacrylamide gel. Arrows indicate the tRNA precursors and mature tRNA shown schematically in Figure 1. The bracket indicates the 3' trailer P4 containing three or four 3'–U residues. The star indicates Px, the pol III transcript found in La-depleted extracts without supplementary La protein.
than normal. Some are longer and some are normal length, while others are shorter. Although some transcripts longer than P1 are also evident in untreated and mock-depleted extracts, those found in La-depleted extracts are more heterogeneous in length and represent a more substantial fraction of the precursors (Figure 2B, compare lanes a, c and e); their formation is prevented by addition of recombinant La protein to the extracts (Figure 2B, lanes b, d and f). Transcripts longer and shorter than P2 are seen most clearly after two rounds of immunodepletion; they are not found in the corresponding La-supplemented extract, which instead generates the normal P1 and P2 tRNA\(^{\text{Phe}}\) precursors and 5’ processing intermediates (Figure 2B, compare lanes i and j).

The P1 precursors made in untreated extracts terminate within a T15 tract; most of them possess UUUU\(^{\text{OH}}\) at their 3’-terminus, the remainder having three or five U residues (data not shown). Thus, in principle, the RNAs longer than P1 could result from pol III transcription extending further into the T15 tract. The P2 precursors terminate within a T4 tract and have UUUU\(^{\text{OH}}\) or UUUU\(^{\text{OH}}\) at their 3’-terminus (see below). Any extension of these RNAs by pol III would imply transcription beyond the T4 tract into G–C-rich DNA. We consider it more likely that the transcripts longer than P2 represent pol III primary transcripts that are extended by another polymerase. Such elongated, labelled RNAs are found when unlabelled P2 precursor is incubated with La-depleted extract in the presence of [\(\alpha^{\text{32P}}}\)UTP but not [\(\alpha^{\text{32P}}}\)GTP (data not shown). This may indicate addition of U residues to the 3’-terminal (U)UUU\(^{\text{OH}}\) by a poly U polymerase (Andrews and Baltimore, 1986). These longer transcripts have not been investigated further.

A truncated tRNA\(^{\text{Phe}}\) precursor is made in La-depleted extracts

The most prominent shorter transcript found in La-depleted extracts is a novel RNA with an electrophoretic mobility intermediate between that of the P2 primary transcript and its 5’ processed product P2a (Figure 2B, Px, lane i). To examine this RNA in more detail, we incubated the tRNA\(^{\text{Phe}}\) gene template in a La-depleted extract in the presence of [\(\alpha^{\text{32P}}}\)UTP but not [\(\alpha^{\text{32P}}}\)GTP (data not shown). This may indicate addition of U residues to the 3’-terminal (U)UUU\(^{\text{OH}}\) by a poly U polymerase (Andrews and Baltimore, 1986). These longer transcripts have not been investigated further.

Formation of the truncated tRNA\(^{\text{Phe}}\) precursor is separable from pol III transcription

To try to distinguish between these possibilities, we inserted the \(X.\text{laevis}\) tRNA\(^{\text{Phe}}\) gene into a plasmid down-stream of a promoter for T7 RNA polymerase (T7 pol) and inserted an A4 tract after the T4 termination signal for the pol III-generated P2 primary transcripts. After digestion with the restriction enzyme \(D\text{ral}\) (cut site \(\text{T}\text{TTTT}\text{AAA}\)), T7 pol synthesizes a transcript that mimics the P2 tRNA\(^{\text{Phe}}\) precursor in that the 5’ leader of 14 nt starts with a triphosphorylated purine and the 3’ trailer contains the potential hairpin followed by a stretch of U residues. We then followed the fate of this T7P2 RNA, labelled with [\(\alpha^{\text{32P}}}\)GTP, after incubation in S-100 extracts that had been through three rounds of anti-La immunodepletion or mock-depletion.

In both kinds of extracts, as with genuine pol III transcripts, processing of T7P2 RNA by RNase P precedes 3’ processing. Thus, the 5’ processed precursor P2a is the only processing intermediate to appear (Figure 4). Again, as with pol III transcription reactions, addition of recombinant La protein stabilizes the transcripts and/or inhibits their 3’ processing. Most importantly, the T7P2 RNAs migrate more rapidly after incubation in La-depleted extracts. Initially these RNAs are quite heterogeneous in length, but with longer incubation they are converted to a discrete T7Px transcript or processed to the mature tRNA\(^{\text{Phe}}\) P3. Length heterogeneity and T7Px formation are completely suppressed by addition of recombinant La to the La-depleted extracts (Figure 4B).

These results demonstrate that Px RNA can be formed...
La-depleted extracts perform multiple rounds of pol III transcription

To determine if *X. laevis* La has a transcript release function, we examined the efficiency of pol III transcription in La-depleted extracts in the presence and absence of heparin to distinguish single from multiple rounds of transcription (Kassavetis et al., 1989). Several factors were varied in preliminary experiments to optimize this approach. In the final protocol, the S-100 extracts were stabilized by addition of bovine serum albumin (BSA) to 0.1 mg/ml; endogenous NTPs were severely depleted by centrifugation through Sephadex G-50 spin columns and extracts were subjected to five rounds of anti-La immunodepletion or mock-depletion and then concentrated ~2.5-fold by centrifugation through ultrafiltration units to regain the original protein concentration. This protocol removes ~99% of the La protein originally present in the S-100 extract (Figure 5A).

In other preliminary experiments, the concentration of the DNA template was varied to find the point at which pol III transcription reached a plateau; this was achieved at ~4 nM. Keeping the total DNA concentration constant at this level, decreasing amounts of the template were then mixed with pBR327 plasmid DNA in order to engage
the maximum number of template molecules in productive pol III transcription. A combination of 10 ng template (3.34 fmol) and 200 ng pBR327 (94 fmol) generated the same number of tRNA* coding transcripts as 300 ng template (100 fmol) in 25 μl reactions (data not shown).

The DNA template was also modified by inverting the T–A base pair located 1 bp upstream of the mature tRNA\(^{\text{he}}\) coding sequence. With this template, the first UMP to be incorporated into the primary transcript is at position 15. Thus, in principle, pol III reactions done in the presence or absence of 300 μM heparin (see below).

In the experiment shown in Figure 5, preinitiation complexes were assembled for 3 h in the presence of 0.2 mM ATP (Bieker et al., 1985), CTP, UTP, [α\(^{32}\)P]GTP and additional ATP were then added in the presence or absence of 300 μg/ml heparin and in the presence or absence of recombinant La protein. The RNAs made in 0.5, 2 and 5 min were recovered for gel electrophoresis and phosphorimager analysis. Aliquots of the 5 min reactions were also challenged with anti-La antibodies before RNA extraction. In the presence of heparin, comparable numbers of P1 and P2 RNAs are synthesized in La-depleted and mock-depleted extracts (1.13 and 1.07 fmol, respectively). Most of this synthesis occurs within 0.5 min and is complete within 2 min (Figure 5, lanes a and b). These results indicate that transcription is limited to a single round and that 33–35% of the DNA template is committed. In contrast with the RNAs made in the mock-depleted extract, none of the precursors made in the La-depleted extract is immunoprecipitable by anti-La antibodies (Figure 5, lanes c and d). The presence of recombinant La protein has no effect on transcript levels but precursors are now immunoprecipitable from both extracts (lanes e–f).

In the absence of heparin, transcripts accumulate in a time-dependent manner in both kinds of extract (Figure 5, lanes g–i). After 5 min, the La-depleted extract contains 29 fmol of P1 and P2 RNA; the corresponding value for the mock-depleted extract is 30 fmol. Since heparin inhibits reinitiation, these transcript levels imply that each committed DNA template undergoes ~25–28 rounds of transcription in 5 min. Many of the RNAs made in the mock-depleted extract are immunoprecipitable by anti-La antibodies, whereas only a very minor fraction of the precursors made in the La-depleted extract appears to be immunoprecipitable, and even this may be a gel artefact (Figure 5B, lanes i and j). As with the single-round conditions, transcription efficiency is unaffected by the presence of recombinant La, but most newly synthesized RNAs are now immunoprecipitable when challenged with anti-La antibodies (Figure 5B, lanes i–l).

These results demonstrate that X.laevis cell-free extracts containing <1% of the normal La protein content are capable of efficient pol III transcription under both single-round and multiple-round conditions. Few, if any, of the transcripts made in depleted extracts under multiple-round conditions are associated with La. This strongly suggests that the residual La protein in such extracts is not functioning to strip nascent transcripts from their template.

**Naevus pol III transcripts are released from template DNA in La-depleted extracts**

To test the latter suggestion more directly, we incubated La-depleted and mock-depleted extracts with a DNA template immobilized on magnetic streptavidin beads and then asked if the newly synthesized pol III transcripts are associated with the template. For the experiment shown in Figure 6, extracts were again subjected to five rounds of anti-La immunodepletion or mock-depletion, concentrated, and incubated with the immobilized template and 0.2 mM ATP for 3 h. The preinitiation complexes were then either rinsed or not with transcription buffer (which contains 70 mM KCl). After 1 h of transcription in the presence of [α\(^{32}\)P]GTP, newly synthesized RNAs contained in the total reactions, supernatants and attached to the beads were analysed on a denaturing gel. Aliquots of the supernatant fractions were also challenged with anti-La antibodies before RNA extraction.

In both mock-depleted and La-depleted extracts containing <1% of the normal La protein content, the vast majority of the transcripts are found in the supernatant fraction, i.e. released from the template, irrespective of whether or not the preinitiation complexes are rinsed (Figure 6, compare lanes a, b and d; e, f and h; i, j and l; m, n and p). Most of the precursors made from unrinsed preinitiation complexes in mock-depleted extracts are immunoprecipitable with anti-La antibodies (Figure 6, lanes b and c) whereas this is true of only a minor fraction of precursors made in the comparable La-depleted extract (Figure 6, lanes j and k). Rinsing the preinitiation complexes in La-depleted extracts abolishes this minor fraction without affecting the efficiency of transcript release (Figure 6, lanes m, n and o). It is interesting to note that some mature tRNA is found under these conditions but not after rinsing the preinitiation complexes from mock-depleted extracts (Figure 6, compare lanes m and n with lanes e and f). This is consistent with the earlier observation (Figures 2 and 4) that processing is more rapid in the absence of La. More importantly, these results provide no evidence that X.laevis La functions as a pol III transcript release factor.

**Discussion**

**La and pol III transcription**

The preferential binding of human La to 3'–U residues (Stefano, 1984), the hallmark of most nascent pol III transcripts, was an early indicator that this protein may be involved in pol III transcription termination. Subsequent work with human cell-free extracts has lent support to that idea and has resulted in some specific proposals, namely that La not only binds to the 3'–U residues of nascent pol III transcripts but is also required for their synthesis (Gottlieb and Steitz, 1989a) and for the subsequent release of the transcripts from the DNA template (Gottlieb and Steitz, 1989b; Maraia et al., 1994). Most recently, human La has been invoked as a pol III initiation
factor (Maraja, 1996) whose activity depends on a particular residue, Ser366, being dephosphorylated (Fan et al., 1997) and on the integrity of a nearby basic region of the protein (Goodier et al., 1997).

Our interest in the X. laevis La protein also stemmed from the hope that it would function as a pol III termination factor, but the experiments reported here provide no support for that view. Progressive immunodepletion of La from X. laevis S-100 extracts reduces pol III transcriptional activity (Figure 2). This effect is not due to the loss of La, however, but rather to the inevitable dilution of the extracts that occurs at each round of immunodepletion, which can be overcome by concentrating the extracts. A pol III transcript lacking 3'-U residues is found in La-depleted extracts (Figures 2 and 3), but the 3'-U residues of a presynthesized T7pol transcript are also missing after incubation in La-depleted extracts (Figure 4). This strongly suggests that the 3' truncated pol III transcript results from nucleolytic degradation of the primary transcript rather than incomplete synthesis on a stalled transcription complex. Extracts containing <1% of the normal content of La protein sustain very efficient levels of pol III transcription, under both single- and multiple-round conditions (Figure 5), with the vast majority of these full-length tRNA precursors being released from the template without being associated with La (Figure 6). Similarly, HeLa extracts depleted of La are competent for transcription of the adenovirus VAI RNA gene and the resulting transcripts are not precipitable with anti-La antibodies (Francoeur and Mathews, 1982). In addition, both X. laevis and calf thymus pol III seem capable of accurate and efficient termination, polymerase recycling and transcript release in the absence of other proteins (Cozzarelli et al., 1983; Watson et al., 1984; Campbell and Setzer, 1992). Together, these results provide no evidence for La functioning as a pol III initiation, termination or release factor in X. laevis, and they question such proposed roles for La in other vertebrates.

In vitro studies also provide no evidence for La functioning as a pol III transcription factor in Saccharomyces cerevisiae (Kassavetis et al., 1995; Dieci and Sentenac, 1996). In particular, in the presence of TFIIIB and TFIIIC, limiting quantities of pol III preferentially reinitiate on tRNA gene templates that have already undergone transcription (Dieci and Sentenac, 1996). This facilitated recycling pathway also generates multiple-round transcripts of the yeast U6 gene; the template in this case requires only recombinant TFIIIB and highly purified pol III. If an accessory factor were needed for each transcript made, and not just for each template used, it should be present in substantial amounts in the polymerase fraction, yet highly purified yeast pol III preparations show little sign of even sub-stoichiometric amounts of additional polypeptides (Huet et al., 1996). More specific to La, there is no difference in pol III activity between extracts of S. cerevisiae strains carrying wild-type or chromosomally disrupted La genes (Yoo and Wolin, 1997). As noted earlier, La is not needed for viability in either S. cerevisiae or Schizosaccharomyces pombe, so no essential pol III gene can depend on La for its expression in either yeast species. In S. cerevisiae, efficient pol III termination requires longer T tracts than are found in vertebrates (Allison and Hall, 1985), perhaps eliminating the need for an accessory termination factor.

The yeast La homologues are significantly shorter than human La; the three proteins are most related at their N-termini and the yeast proteins may be missing some C-terminal residues implicated in transcription in the human protein (aligned in Van Horn et al., 1997). The frog and human La sequences are much more related. In fact there are two distinct forms of La in X. laevis, the result of an ancient whole genome duplication and

Fig. 6. Transcription of the tRNA<sup>Phe</sup> gene immobilized on magnetic streptavidin beads in mock-depleted and La-depleted extracts. S-100 extracts were subjected to five rounds of depletion with immobilized pre-immune serum (PI) or anti-La antibody (α-La) and then were concentrated by ultrafiltration. The La-depleted extract contained <1% of the normal content of La protein (as Figure 5A, data not shown). Preinitiation complexes were either rinsed or not with transcription buffer (+R, –R) and pol III transcription was allowed to proceed for 1 h at 20°C in the presence of [α-<sup>32</sup>P]GTP. Transcripts found in the total reaction (T), supernatant (S) and pellet (P) were recovered. A fourth aliquot was immunoprecipitated with fresh anti-La antibody (Im). Transcripts were purified and resolved on a 12% denaturing polyacrylamide gel.
subsequent sequence drift (Bisbee et al., 1977). The two frog proteins are 90% identical (95% similar) and more than 61% identical (≥74% similar) to human La (Scherly et al., 1993). Both *X.laevis* La proteins contain a serine at position 366 (and also one at 367) corresponding to the phosphorylated residue in the human protein (Fan et al., 1997). Given this high degree of relatedness, it is difficult to reconcile the disparate pol III transcription results obtained with the frog and human La proteins. Contributing factors could include the different DNA templates, extracts and experimental protocols used. We emphasize that the tRNA<sub>3′</sub> gene used here does not possess an intrinsically strong termination site, that perhaps has no need of a factor required at less efficient sites. On the contrary, this gene was chosen because significant read-through occurs beyond its T4 termination site (Figure 2). Read-through at this site is neither increased by La depletion nor decreased by addition of recombinant, presumably unphosphorylated La protein (Figures 2, 5 and 6).

Hence, despite their sequence similarities, frog and human La proteins seem to have distinctly different properties with respect to pol III transcription. Alternatively, they may have similar properties and the residual <1% La protein remaining in La-depleted *X.laevis* extracts may be sufficient for a transcriptional function(s). We cannot exclude the latter possibility but the primary pol III transcripts made in La-depleted *X.laevis* extracts are released from the DNA template without being associated with La (Figures 5 and 6). This strongly suggests that the residual La in these extracts does not have a transcript release function. Moreover, if <1% of the normal La content is sufficient for a transcriptional function(s), the remainder is presumably needed for another, even more important purpose.

**La and other functions**

Other functions have indeed been ascribed to the La protein. For *X.laevis* La, these include retaining certain pol III transcripts in the nucleus (Boelens et al., 1995; Simons et al., 1996) and mediating RNA import from the cytoplasm to the nucleus (Grimm et al., 1997). Human La has been implicated in regulating the activation of interferon-inducible protein kinase PKR (Xiao et al., 1994), the stabilization of histone mRNAs (McLaren et al., 1997) and in internal translation initiation of poliovirus (reviewed in Belsham and Sonenberg, 1996) and hepatitis C virus (Ali and Siddiqui, 1997). Evidence is accumulating that human and frog La proteins may have similar roles in the translation of some cellular mRNAs (McBratney and Sarnow, 1996; Peek et al., 1996; Pellizzoni et al., 1996).

Few of these functions readily account for the high concentration of La in eukaryotic nuclei. Recently, however, La has been found to be involved in yeast tRNA maturation *in vivo*. In both *S.cerevisiae* and *S.pombe*, binding of La to many tRNA precursors is needed for the correct endonucleolytic removal of their 3′ trailer sequences (Van Horn et al., 1997; Yoo and Wolin, 1997).

In the absence of La, these precursors are degraded by a 3′-exonuclease(s) (Yoo and Wolin, 1997) whereas the normal processing pattern can be re-established in *S.pombe* cells genetically depleted of La by introducing the human or yeast La cDNAs (Van Horn et al., 1997). The experiments reported here are consistent with these results. Bona fide and synthetic tRNA precursors are subject to 3′-exonuclease attack in La-depleted *X.laevis* extracts (Figures 2–4). Addition of recombinant La prevents this exonucleolytic degradation of the precursors while stabilizing the endonucleolytically cut 3′ trailers (Figure 2). Over-expression of La in *S.pombe* appears to stabilize pol III transcripts and/or slow the rate of their processing (Van Horn et al., 1997). The same effects are seen *in vitro* with an excess of recombinant *X.laevis* La, independent of pol III transcription (Figures 2 and 4).

In conclusion, the one function that appears to be shared between yeast, frog and human La proteins is an involvement in tRNA processing. Since La binding is not restricted to tRNA precursors, it may well be involved in the protection or maturation of other RNA species. A conserved RNA chaperone function may best explain the high nuclear abundance of these enigmatic proteins.

**Materials and methods**

**Purification of recombinant LaB1**

Full-length *X.laevis* LaB1 cDNA (Scherly et al., 1993) was cloned behind the *λ* promoter in the heat-inducible vector CBA (gift of B. Allet) and transfected into *E.coli* HB101 cells. 100 ml L-broth containing 5 lg/ml tetracycline was inoculated with a single colony and was shaken overnight at 28°C, then mixed with 300 ml L-broth and shaken for 3 h at 42°C. Cells were harvested and sonicated on ice several times in phosphate-buffered saline (PBS), then in PBS containing 6 M urea. The extract was progressively diluted with 25 mM Tris–HCl pH 8, 3 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.5 mM DTT (B buffer) containing 0.1 M KCl, until the urea concentration was <0.1 M. It was then loaded on a 1.5 ml CL4B heparin–Sepharose column (Pharmacia). The column was rinsed with 0.1 M KCl in B buffer and then step-eluted with increasing concentrations of KCl in B buffer. Full-length La protein (49 KDa) was eluted at 0.4 M KCl, together with two proteolytic fragments of ~42 and ~39 KDa (Figure 5A).

**Immunodepletion**

Polyclonal rabbit antibodies were raised against recombinant La protein in the heparin–Sepharose 0.4 M KCl fraction and purified on protein A–Sepharose columns (Bio-Rad). S-100 extracts (Weil et al., 1979) were prepared from a *X.laevis* kidney cell line as described (Koski et al., 1980). 0.1 ml of packed protein A–agarose beads (Boehringer Mannheim) was suspended in 0.4 ml of 30 mM HEPES pH 7.9, 3 mM MgCl<sub>2</sub>, 140 mM KCl (S buffer), and the suspension was mixed gently with 0.1 ml pre-immune serum or anti-La IgG by end-over-end rotation for at least 2 h at 4°C. The beads were centrifuged for 3–4 s at 14 000 g and then washed three times with 1 ml S buffer. 0.4 ml S-100 extracts containing 0.1 mg/ml BSA was gently mixed with 0.1 ml antibody-coupled beads for 20 min at 4°C as above. This step was repeated two to four times and the extracts were then transferred to UFMC ultraltrifiltration units (Millipore) and centrifuged at 2000 g for 1–1.5 h at 4°C to achieve an ~2.5-fold concentration.

**Immunoprecipitation**

Antibodies were coupled to protein A–agarose as described above in 50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% NP-40 (NET150 buffer). The beads were mixed with transcription reactions by end-over-end rotation for 1 h at 4°C, and then washed three times with NET150 buffer. Bead-associated RNAs were purified by phenol extraction and resolved on 12% denaturing polyacrylamide gels.

**Immunoblotting**

Proteins were resolved on 12.5% polyacrylamide–SDS gels and then transferred to nylon membranes (Boehringer Mannheim) in 25 mM Tris, 192 mM glycine, 20% methanol in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) for 16 h at 30 V and 4°C. Membranes were soaked for 1.5 h in 2% blocking reagent (Boehringer Mannheim) in 0.1 M Tris–HCl pH 7.5, 0.15 M NaCl (TBS), then incubated for 1 h with anti-La serum diluted to 1/250 with TBS containing 0.1% Tween 20. Blots were washed, then incubated for 0.5 h with an alkaline phosphatase-conjugated anti-rabbit antibody (Promega) diluted to 1/100.
000 in the same buffer. The nylon sheets were incubated for 1 min with AMPPD (Boehringer Mannheim) diluted to 1/100 in 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂, dried briefly then incubated 15 min at 37°C before autoradiography.

**In vitro transcription**

Clone pJ52 contains the *X.laevis* tRNA<sup>Phe</sup> gene in pBR322 with the natural T15 terminator of the T7rDNA (Goulioud and Clarkson, 1986) placed behind the weaker T4 termination site of the tRNA<sup>Phe</sup> gene. Clone pH4 Era contains the *X.laevis* tRNA<sup>Phe</sup> gene behind the T7 promoter of the pGEM4 vector and a series of A residues downstream of the T4 tract.

For T7 polymerase transcription, clone pH4 Era was linearized after the T4 tract with DpnI and then incubated at 37°C with T7 polymerase in 40 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 10 mM DTT, 1 mM each of ATP, CTP and UTP, 0.1 mM GTP, 10 μCi [α-<sup>32</sup>P]GTP. Pol III transcription was performed with S-100 somatic extracts in 20 mM Hepes–KOH pH 7.9, 70 mM KCl, 5 mM MgCl₂ (T buffer) containing 0.5 mM DTT, 10 mM creatine phosphate, one nucleoside α-<sup>32</sup>P triphosphate at 25 μM and the remaining three NTPs at 200 μM, as described previously (Mazabraud et al., 1987). Reactions were incubated at 20°C for the stated times. In some experiments, the template was incubated with extract previously passed through a G-50 spin column to remove endogenous NTPs for 3 h at 20°C in the presence of MgCl₂ and ATP to allow assembly of preinitiation transcription complexes (Bieker et al., 1985). For single-round transcription conditions, reactions contained 300 μg/ml heparin to prevent reinitiation (Kassavetis et al., 1989). RNAs were purified by phenol extraction and resolved on 12% denaturing polyacrylamide gels.

**In vitro transcription with immobilized templates**

Clone pH4 Era was linearized with EcoRI, and the site filled in with the Klenow polymerase in the presence of Biotin-7dATP (Gibco-BRL) as described previously (Arias and Dynan, 1989). Non-incorporated NTPs were removed on G-50 Sephadex columns and, after digestion with ScaI, the 2079 bp fragment containing the tRNA<sup>Phe</sup> gene was incubated overnight at 4°C with magnetic streptavidin beads (Dynal) in 10 mM Tris–HCl pH 7.6, 1 M NaCl, 1 mM EDTA. The distance between the bead attachment site and the transcription start site is ~90 bp. After coupling, beads were washed three times with the above buffer, then three times with T buffer containing 10% glycerol and 250 μg/ml BSA. To form preinitiation complexes, 1.5×10<sup>5</sup> DNA coupled beads were incubated with 10 μl of S-100 extract for 2 h at 37°C in T buffer containing 0.5 mM DTT, 0.2 mM ATP and 250 μg/ml BSA in a total volume of 20 μl. When indicated, preinitiation transcription complexes were washed three times with 200 μl of T buffer, and subsequently supplemented described above. Transcription was initiated with 25 μl of T buffer containing 0.5 mM DTT, 10 mM creatine phosphate, 200 μM ATP, 100 μM each of CTP and UTP, 25 μM GTP and 10 μCi [α-<sup>32</sup>P]GTP. Reactions were performed for 1 h at 20°C. RNAs from the released and retained fractions were phenol extracted, precipitated and resolved on 12% denaturing polyacrylamide gels.

**RNA sequence analyses**

RNA from gel slices was digested with RNase T1, then fingerprinted by ionophoresis on cellulose acetate strips at pH 3.5 followed by homochromatography on polyethyleneimine–cellulose thin layers. These analyses and secondary analyses of the T1 oligonucleotides were performed as described previously (Koski and Clarkson, 1982).

**Acknowledgements**

We thank J. Corlet for expert technical assistance and D. Scherly and F. Stutz for discussions and helpful advice. This work was supported by the Swiss National Science Foundation.

**References**


pol III transcription in La-depleted extracts


White, R.J. (1994) RNA Polymerase III Transcription. R.G. Landes, Austin, Texas, USA.


Received December 22, 1997; revised January 23, 1998; accepted January 26, 1998.