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Precursor-product discrimination by La protein during tRNA metabolism

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SUMMARY

La proteins bind pre-tRNAs at their UUU-3'OH ends, facilitating their maturation. While the mechanism by which La binds pre-tRNA 3' trailers is known, the function of the RNA-binding β-sheet surface of RRM1 is unknown. How La dissociates from UUU-3'OH-containing trailers after 3' processing is also unknown. La preferentially binds pre-tRNAs over processed tRNAs or 3' trailer products through coupled use of two sites: one on the La motif and another on the RRM1 β surface that binds elsewhere on tRNA. Two sites provide stable pre-tRNA binding while processed tRNA and 3' trailer are released from their single sites relatively fast. RRM1 loop-3 mutations decrease affinity for pre-tRNA and tRNA but not UUU-3'OH trailer, and impair tRNA maturation *in vivo*. We propose that RRM1 functions in activities that are more complex than UUU-3'OH binding. Accordingly, the RRM1 mutations also impair a RNA chaperone activity of La. The results suggest how La distinguishes precursor from product RNAs, allowing it to recycle onto a new pre-tRNA.

INTRODUCTION

La is the first protein to interact with nascent pre-tRNAs in eukaryotes and remains bound during tRNA processing and modification1,2. In its best characterized activity, the La domain, comprised of a La motif (LM) and RNA recognition motif (RRM1) in fixed arrangement, protect UUU-3'OH-containing RNAs from 3'-exonucleolytic digestion1–4.

Because the LM and RRM1 are required for UUU-3'OH binding5, it was expected that this binding would involve the β-sheet surface of La RRM16–8. Surprisingly, a human La (hLa) crystal structure shows that while the LM and RRM1 form a UUU-3'OH binding cleft, most RNA contacts are to the LM, leaving the β-sheet surface of RRM1 unoccupied4. This documented sequence-specific recognition by a RRM that does not involve its β-sheet surface4. Solving of four more structures of hLa with different RNAs confirmed the LM

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AUTHOR CONTRIBUTIONS

M.A.B. performed all experiments. R.J.M. and M.A.B. designed the study and analyzed the data. R.J.M wrote the paper with editing by M.A.B.

mode of recognition with the RRM1 β-sheet surface unoccupied9. Thus the LM-RRM1 mediates induced fit UUU-3'OH binding9 while leaving the RRM1 β- sheet surface unoccupied and its role in RNA binding unclear3,4,9,10.

Some La-related proteins (LARPs) that contain a La domain are telomerase subunits11,12. LARP7 (a.k.a. PIP7S) is a tumor suppressor that binds 7SK snRNA to regulate P-TEFb13,14. Ciliate LARPs recognize UUU-3'OH-containing telomerase RNA, and LARP7 recognizes UUU-3'OH of 7SK snRNA, while neither are associated with nascent pre-tRNAs13,15. Since LARPs have conserved the LM-RRM arrangement they likely use RNA binding modes similar to genuine La. Yet, how the LM and RRM1 β-sheet binding surface work together is not known for any protein.

Some activities of La proteins are more complex than UUU-3'OH binding. Yeast La is required for the maturation of structurally impaired pre-tRNAs16–18, can promote tRNA folding19, and acts redundantly with tRNA modification enzymes, consistent with a role in RNA structural integrity20. Ciliate LARP p65 induces structural rearrangement of telomerase RNA12,21, and hLa exhibits RNA chaperone activity18,22,23. While these studies document complex functions related to RNA structure/folding, the mechanisms that distinguish these activities from simple UUU-3'OH binding are unknown. To understand this it will be necessary to know how the LM and RRM interact with RNA dynamically, during simple and complex activities, and in specific pathways of RNA metabolism.

Modified nucleotides found on La-associated pre-tRNAs indicate that La is bound to pre-tRNAs during modification24,25. La remains associated with pre-tRNA until RNase Z-mediated endonucleolytic cleavage separates the tRNA body from the UUU-3'OH-containing 3' trailer, the latter of which varies for different pre-tRNAs16,17,26–29. While pre-tRNA processing occurs in minutes, the half life of La is hours24,30, and although abundant, La can be limiting for tRNA maturation31. To participate in multiple rounds of tRNA maturation, La must dissociate from the UUU-3'OH-containing trailer products of processing and recycle onto newly synthesized pre-tRNAs. Since cleaved 3' trailers contain UUU-3'OH, the highest affinity sequence-specific ligand known for La, it was unclear how efficient dissociation would occur.

We hypothesized that La might distinguish pre-tRNA from processed products using two binding sites which together provide higher affinity than either alone. After processing, the tRNA and 3' trailer would readily dissociate from their single binding sites. We show that the RRM1 β-sheet surface forms a RNA binding site distinct from the UUU-3’OH binding site that promotes tRNA maturation *in vivo*. We also show that this RRM1 binding site contributes to the RNA chaperone activity of La.

RESULTS

La binds tRNA using non-UUU-OH mediated contacts

To test for a tRNA binding site on hLa distinct from the UUU-3'OH binding site, we used the electrophoretic mobility shift assay (EMSA) on a 73 nt $tRNA^{Arg}ACG$ transcript (which ends with UCG-3'OH), and a 12 nt 3’ trailer that ends with UUU-3'OH, as these represent

cleavage products of RNAse Z26 (indicated as tRNA and UUU-OH 3' trailer in Fig 1). hLa exhibits several-fold higher affinity for this trailer than for the same trailer ending in AAA-3'OH, consistent with prior results18 and confirming UUU-3'OH-specific recognition in our EMSA (not shown). A standard EMSA that contains Mg^{2+} revealed that hLa bound avidly to both the 12 nt UUU-3'OH trailer and the tRNA (Fig. 1A, B).

Although the tRNA does not end with UUU-3'OH, it may still interact with the UUU-3'OH binding site, which can accommodate, albeit with lower affinity, bases other than U4. Therefore, we examined binding to hLa-Q20A/Y23A/D33R (hLa-QYD in Fig. 1C, D) which is mutated in the LM UUU-3'OH binding site: Q20 makes uracil-specific contact; Y23 stacks with U; and D33 makes bidentite contacts to 2'OH and 3'OH of the last nucleotide4,9. hLa-QYD was debilitated for UUU-3'OH trailer binding but supported more binding to the tRNA (Fig. 1C, D). This suggested a UUU-3'OH-independent mode of tRNA binding that is mediated by a site on La other than the RNA 3'OH-end binding site in the LM4,9.

Differential sensitivity to Mg^{2+} reflect distinct binding modes

To further examine for differential binding we compared dissociation constants (K_d) for three relevant RNA species, pre-$tRNA^{Arg}$ACG, $tRNA^{Arg}$ACG and the free 12 nt UUU-3'OH trailer, in two extremes of [Mg^{2+}], 0 and 10 mM, as well as 1 mM (Fig. 2A–C). Of note is the absence of divalent cations in the crystal of hLa bound to UUU-3'OH4, and the recognized association of divalent cations with tRNA32. The 85 nt pre-$tRNA^{Arg}$ACG used in Fig. 2 lacks a 5' leader but contains the 12 nt UUU-3'OH-containing trailer covalently linked to $tRNA^{Arg}$ACG (i.e., as a contiguous T7 transcript), as this is a substrate for RNase Z26, while the 73 nt $tRNA^{Arg}$ACG and the 12 nt UUU-3'OH trailer represent cleavage products. We used two EMSA methods, each with reactions containing 0 or 10 mM Mg^{2+}. In Method 1, varying amounts of hLa were added to trace amount of ^{32}P-RNA. This showed that 12 nt trailer binding was no different in 0 and 10 mM Mg^{2+}, while tRNA and pre-tRNA binding were dramatically compromised in 10 mM versus 0 Mg^{2+} (Supplementary Fig 1, 0 and 10 mM Mg^{2+}). By method 1, K_d is roughly estimated as the La concentration at which 50% of the RNA is shifted. K_d for the pre-tRNA and tRNA were each estimated at 5–10 nM in 0 mM Mg^{2+}, and ~100 nM in 10 mM Mg^{2+}, whereas K_d for the 12 nt trailer was ~20 nM, with little if any difference in 0 versus 10 mM Mg^{2+}. The relatively large effect of Mg^{2+} on pre-tRNA and tRNA but not on 12 nt trailer was confirmed by EMSA method 2 as described below (Fig 2A–C).

For Method 2, analyses focus on RNA concentrations around the K_d obtained by method 1. For Method 2, varying amounts of unlabeled RNA were added to constant amounts of ^{32}P-RNA and La33. Bound and free ^{32}P-RNA fractions were quantified and K_d determined by Scatchard analysis. EMSAs were performed in triplicate. Method 2 supports a greater amount of data, is independent of the fraction of active protein, and analysis includes quality assurance parameters33. Results for 0 and 10 mM Mg^{2+} are shown in Fig. 2A and B, respectively and summarized in Fig. 2G (also see Supplementary Fig. 2 & Fig 3). The K_d of 13.6 nM that we obtained for the 12 nt UUU-3'OH trailer in 10 mM Mg^{2+} is similar to 7.1

nM and 25 nM obtained by others in the presence of Mg^{2+} for similar ligands4,34. Our K_d of 2.4 nM for pre-$tRNA^{Arg}$ is similar to 7.3 nM for pre-$tRNA^{Val}$, in the absence of Mg^{2+}35.

We asked if the K_ds were reflective of different dissociation rates (k_{off})33. The data indicated that hLa binds more stably to pre-tRNA than to the UUU-3'OH trailer or tRNA (Fig. 2D–F, representative EMSAs shown in Supplementary Fig 4). The $t_{1/2}$ for pre-tRNA, tRNA and UUU-3'OH trailer were 6.2, 2.2, and 3.3 min., respectively (Fig. 2G).

La binding to pre-tRNA and tRNA was inhibited by 10 mM Mg^{2+} whereas binding to the UUU-3'OH trailer was slightly enhanced (Fig. 2G, columns 0 Mg^{2+}, 10 Mg^{2+} and 0 Mg/10 Mg). The major conclusion we draw from this analysis is that the binding modes used for tRNA and UUU-3'OH ligands are biochemically distinct.

Although differential sensitivity to Mg^{2+} provides biochemical evidence of distinct binding modes, neither 0 nor 10 mM Mg^{2+} represent physiological conditions. As summarized in Fig. 2G (column 1 mM Mg), pre-tRNA had the highest affinity for La followed by 3' trailer and tRNA.

A most striking difference in binding avidities was revealed by competition. In parallel reactions containing 1 mM Mg^{2+}, we examined ^{32}P-pre-tRNA and ^{32}P-trailer for hLa binding in the presence of 100 nM La and increasing amounts of unlabelled competitor RNAs (Fig. 3A & B). Unlabelled 3' trailer was largely ineffective for competition with ^{32}P-pre-tRNA even when present at higher concentration than La, indicating that La exhibits strong preference for pre-tRNA over the UUU-3'OH-containing trailer (Fig. 3A & C). The same unlabelled 3' trailer readily competed with ^{32}P-trailer (Fig. 3B, D), indicating its activity as a competitor. As expected, unlabelled pre-tRNA competed effectively with ^{32}P-pre-tRNA and ^{32}P-trailer. The tRNA, which lacks UUU-3'OH, competed with ^{32}P-pre-tRNA but significantly less so with the ^{32}P-UUU-3'OH-trailer, consistent with La accommodating both the tRNA and trailer substrates using distinct binding sites. We conclude that La exhibits strong preference for pre-tRNA over the 12 nt UUU-3'OH trailer, consistent with the idea that pre-tRNA uses two binding sites while the 12 nt UUU-3'OH-containing trailer uses only one.

As shown below, a La RRM1 loop-3 mutant exhibits decreased affinity for tRNA, but not the UUU-3'OH-trailer binding *in vitro*, and decreased tRNA maturation *in vivo*, providing support for the physiologic relevance of the second binding site on RRM1.

La RRM1 functions in pre-tRNA recognition

The Mg^{2+}-sensitive UUU-3’OH-independent binding activity was mapped to the hLa domain (not shown). To further localize this activity we examined surface charge distribution (see Supplementary Fig S5) and mutated candidate basic surface patches to evaluate their effect on RNA binding. Several different mutated proteins were examined for tRNA binding, and in some cases other non-UUU-3'OH-containing RNAs (not shown, indicated as "other" in Table 1*a*. RRM1 loop-3 (R143A/R144A/K148A/K151A, referred to as “hLa-loop” mutant below), which connects RRM1 β2 and β3 strands, reduced binding to pre-tRNA and tRNA 4–5 fold relative to hLa, without much decrease in binding to the

UUU-3'OH trailer (Table 1*b*, also see Supplementary Figs S6 and S7). Moreover, the residual tRNA binding observed for hLa-loop was insensitive to Mg^{2+} (not shown). Decreased tRNA binding observed for the hLa-loop protein is specific since many other mutated proteins did not decrease tRNA binding (Table 1*a*). The fact that hLa-loop mutated protein exhibited normal UUU-3'OH-mediated binding, which requires LM-RRM1 inter-motif contacts,4,9 indicates that the La domain is not grossly misfolded. We conclude that loop-3 of RRM1 is an important determinant of a UUU-3'OH-independent RNA binding site on La. The basic residues mutated in La-loop-3 are highly conserved in La proteins but not other RRM proteins such as U1A and PABP (Fig. 4A). Loop-3 forms a basic wall on one side of the RRM β-sheet surface (Fig. 4B).

La RRM1 loop-3 functions in tRNA maturation *in vivo*

We next examined the hLa-loop mutant for tRNA maturation in *S. pombe* as monitored by tRNA-mediated suppression (TMS) of a premature stop codon in *ade6-704*, which alleviates the accumulation of red pigment. By this assay, hLa can replace the function of *S. pombe* La protein (Sla1p) *in vivo*17, 18. We previously characterized suppressor tRNA alleles that vary in dependence on La, Rrp6 (3' exonuclease component of the exosome), and the pol III termination subunit Rpc11p (a pol III-associated 3'-5' exonuclease), for efficient maturation18,31. Mutation of the conserved aromatic residues, Y114 and F155, on the RNP-1 and -2 motifs of the RRM1 β-surface compromised maturation of the structurally-defective suppressor pre-tRNA in the strain, ySK5, but had less effect in ySH9, which contains a wild-type suppressor tRNA18. For the present study, analysis was limited to the wild-type suppressor tRNA in ySH9 (Fig. 5A–B). Consistent with prior data, hLa was more active for TMS than the negative controls pRep vector and hLa26-408 which lacks residues involved in UUU-3'OH recognition17,18,36 (Fig 5A, sectors 1 & 2). hLa-Y114A/F155A also served as a control (sector 3). hLaloop-3 was less active than hLa (Fig. 5A, compare sectors 3 & 5), indicating that one or more of the basic residues in loop-3 that mediate UUU-OH-independent pre-tRNA binding contribute to functional tRNA maturation *in vivo*. We also combined the RRM1 loop-3 and the β-sheet mutations. These mutants, hLaY114A-loop and hLaY114A/F155A-loop were increasingly compromised for TMS (Fig. 5A, sectors 6 & 7). As shown below, these RRM1 mutants maintain pre-tRNA UUU-OH 3' end protection activity.

Analysis of endogenous pre-$tRNA^{Lys}_{CUU}$ by northern blot is a standard by which the 3' end protection function of La protein is monitored17,18,36–38. An intron probe detects three pre-$tRNA^{Lys}_{CUU}$ species, the upper and middle bands, which contain the 3' trailer and require La for accumulation, and the lower band which accumulates independently of La17,18,36–38. The upper band represents a nascent pol III transcript, while the middle species has had its 5' leader removed, and the lower band reflects the 5' and 3' end matured species (as indicated to the right of Fig. 5B upper panel). U5 snRNA was probed on the same blot to serve as a control for quantitation (Fig. 5B, lower panel). In cells lacking La or in La26-408, the upper and middle bands run as a smear (lanes 1–2) due to 3' exonucleolytic nibbling17,37. The upper band is stabilized by hLa17, 37, but progressively less so in hLa-Y114A, hLa-F155A, and hLa-Y114A/F155A mutants18.

The hLa-loop mutant accumulated less nascent pre-tRNA$^{Lys}_{CUU}$ (upper band) than did hLa (Fig. 5B, compare lanes 3 & 5), consistent with less stable interaction of the pre-tRNA with the hLa-loop protein relative to hLa due to decreased RRM1-mediated binding.

The compound RRM1 mutant hLaY114A/F155A-loop, with mutations to the β-sheet surface and loop-3, was most compromised for nascent pre-tRNA accumulation (Fig. 5B, lane 7), consitent with its low TMS activity (Fig. 5A). Note that for this and other RRM1 mutants, the upper and middle pre-tRNA species appear as distinct bands, a hallmark of 3'-end protection (Fig. 5B, compare lanes 1 & 2 with lanes 4–7). Thus, two different regions of the RNA-binding surface of RRM1 indicate that RRM1 contributes to tRNA maturation *in vivo*. Moreover, the RRM1 effect is distinct from pre-tRNA 3' end protection. The cumulative results provide evidence that La RRM1 contributes to tRNA maturation by mediating UUU-3'OH-independent binding to pre-tRNA.

RRM1 contributes to the RNA chaperone activity of La

The hLa protein exhibits RNA chaperone activity in a *cis*-splicing assay that uses a self-splicing intron23. This RNA can misfold *in vitro* and become trapped in an inactive conformation that can be resolved by La and other RNA chaperone proteins such as StpA, NCp7 and Hfq22. We noted previously that hLa and a mutated derivative, hLa-Y114A/F155A, were comparably active in this assay, consistent with their indistinguishable RNA binding activities18. We compared hLa and hLa-loop protein in the *cis*-splicing assay, by monitoring the disappearance of full length intron-containing RNA and the appearance of spliced product (Fig. 6, ln *U/Uo*, Y-axis). In agreement with previous reports, hLa activity was reflected by a steep slope in the initial fast phase of the assay23 (Fig. 5C). While the no protein and BSA controls showed relatively little activity as expected, hLa was most active, and the hLa-loop protein was intermediate. We note that the RNA used in this assay does not end in UUU-3'OH. A decrease in RNA chaperone activity for hLa-loop correlates with a decrease in its non-UUU-3'OH-mediated RNA binding activity whereas hLa-Y114A/F155A exhibits no decrease in RNA chaperone or RNA binding activities as noted previously18.

DISCUSSION

We report here biochemical and mutational analyses of La RRM1-mediated binding to pre-tRNA. The results indicate that the LM and RRM1 β-sheet surface comprise two binding sites that bind UUU-3'OH and non-UUU-3'OH RNAs respectively. Concurrent use of both sites as occurs for nascent pre-tRNA provides high affinity stable binding, whereas either site alone provides lower affinity, with less stable binding of processed tRNA and cleaved 3' trailers.

Two binding sites support directionality in tRNA maturation

Consistent with its presence in a human pol III holoenzyme and localization at pol III transcribed genes in yeast and human cells,39–41 La is poised to be the first protein to bind newly synthesized pre-tRNAs. How La would dissociate from UUU-3'OH after separation of the trailer and recycle onto new pre-tRNAs had been unknown. This is an issue because the large amount of 3' trailers carrying the sequence-specific ligand, UUU-3'OH, that are

produced during tRNA processing, might consume La if there was no mechanism for dissociation. The ability to withstand challenge by an excess of 12 nt UUU-3'OH RNA revealed a strong preference of La for pre-tRNA (Fig. 3). This coupled with the relatively fast dissociation of La from UUU-3'OH trailers illustrates a mechanism by which the potential consumption of La by UUU-3'OH trailers can be avoided. The ability to dissociate after 3' trailer cleavage supports directionality of the tRNA pathway (Fig. 6). After dissociation, the end-matured tRNAs become substrates of 3' end-modifying proteins such as CCA-adding enzyme and tRNA synthetases, and are bound by export factors Los1/Xpo-t and exported from the nucleus27. La can then recycle onto a new pre-tRNA.

Stable pre-tRNA binding contributes to tRNA structural integrity

Although nascent pre-tRNAs likely have minimal structure, data suggest they acquire structure while associated with La. While evidence indicates that La promotes correct folding of pre-tRNA$^{\mathbf{Arg}}$ *in vivo*19, the misfolded pre-tRNA$^{\mathbf{Arg}}$ was not converted to correct folded pre-tRNA$^{\mathbf{Arg}}$ by La *in vitro*, suggesting involvement of other factors *in vivo*. Genetic evidence points to tRNA modifying enzymes as such factors.20,42–44 As a result of high affinity, stable binding, pre-tRNAs would have enough time to acquire structure-stabilizing modifications, some of which occur while associated with La24,25,45,46 (Fig. 6).

For certain functions, it may be beneficial for a RNA-binding protein to preferentially associate with RNA precursors and dissociate from products. Our data indicate this for La. We are unaware of another non-catalytic RNA binding protein for which this has been demonstrated.

RRM1 loop-3 is adjacent to and distinct from the LM binding site

It was thought that the LM and RRM1 β-sheet surface form a single site that recognizes UUU-3'OH6–8. With the appearance of multiple co-crystal structures of hLa bound to UUU-3'OH RNAs4,9, this idea has given rise to a two-site model3,4,9,10.

We provided *in vitro* and *in vivo* evidence for RRM1 function in the metabolism of a normal pre-tRNA. We used three RNA substrates, differential Mg^{2+} sensitivity and mutagenesis, which led to identification of RRM1 loop-3, adjacent to the β-sheet surface, as important for La-pre-tRNA binding, but not UUU-3'OH binding, *in vitro* and pre-tRNA maturation *in vivo*.

For other RRM proteins, loop-3 contacts RNA, in some cases via a basic side chain47 (Fig. 4C). Loop-3 of La forms a basic wall on one side of the RRM β-sheet surface that projects in the same plane as the conserved aromatic side chains (Fig. 4D). Involvement of La loop-3 in UUU-3'OH-independent binding is in agreement with prior results. None of the mutated residues in RRM1 loop-3 were observed to contact any other parts of La or UUU-3'OH-containing RNA in the structures reported4,6, suggesting availability to bind RNA. NMR revealed that one of the La-loop mutated residues, K151, exhibited a large chemical shift variation in the presence of RNA6. That loop-3 residues contribute to a RNA-binding platform is supported by the compound mutant, LaY114A,F155A,-loop, which is more severely defective for TMS and *in vivo* pre-tRNA accumulation than either of the hLa-

loop-3 or hLa-Y114A/F155A mutants is alone (Fig. 5A & B). The data support the idea that stable RNA-binding by RRM1 is mediated by multiple contacts to the β-sheet surface and loop-3, and possibly other residues.

Although the LM and RRM1 form independent structures in the absence of RNA,6,7 their orientations are fixed by contacts to bound RNA4,9. In the structure reported by Teplova et al., double-stranded RNA4 raised concern about the exit path of the RNA10. Additional co-crystal structures with all single-stranded RNAs have resolved this concern and added new insight9. In addition to closer LM-RRM1 packing, induced-fit, and plasticity in the UUU-3'OH binding cleft, the new structures show a RNA exit path that indeed differs from the Teplova structure, as appreciated by comparing both (Supplementary Fig 8A and B). These and other considerations9 favor two disparate sites on La9, that could bind distant regions of an RNA separated by intervening RNA structure4 as in Supplementary Fig 8C.

As a separate binding platform, RRM1 contributes to versatility

The basic nature of RRM1 loop-3 suggests RNA backbone contacts that could support sequence-independent binding to a variety of RNAs that can derive additional affinity from the UUU-3'OH binding site. This possibility, and plasticity of the UUU-3'OH site9 provide insight into how La can exhibit versatility for different RNAs.

We note that the affinity gained by two-site binding may be more complex than a simple addition of the affinity of each site individually. We also note that the percent La protein that was active for high affinity pre-tRNA binding was lower than the percent active for tRNA and 3' trailer binding, in all Mg conditions tested, and using the same batch of La protein for the different RNAs. We believe that this should not be unexpected since pre-tRNA binding requires both RNA binding sites be active whereas 3' trailer and tRNA binding each require only one site be active. Thus, the chances that either of the two sites required for pre-tRNA binding will be inactive would seem relatively high. We suspect that the orientations of the LM and RRM1 become more fixed relative to each other than when either is bound singly to its isolated RNA. Moreover, the manner in which the LM and RRM1 are fixed relative to each other may vary for different RNAs.

La RRM1 functions in a complex activity

La proteins also function in the metabolism of non-UUU-3’OH containing RNAs. We showed that RRM1 is important for a complex, previously characterized activity of La, RNA chaperone activity. Holding different parts of an RNA by two disparate binding sites connected by intervening RNA may promote RNA folding or other rearrangements by La domain proteins, as has been suggested for other RNA chaperones48,49. Conserved residues on RRM1 β-sheet specifically, of hLa and Sla1p, have been shown to promote the maturation of structurally-impaired pre-tRNA18. We suspect that the results reported here will be useful toward understanding complex activities of La in its interactions with mRNA and other RNAs, and to LARPs and their RNAs. Coupled use of distinct binding modes may be an important mechanism for RNP dynamics more generally.

METHODS

Mutagenesis

We carried out mutagenesis by QuikChange XL (Stratagene) using pREP4-hLa17 and pET28a-La5 as templates, and verified all constructs by sequencing.

Protein purification

Plasmids encoding hLa (pET28a-La) and mutated derivatives were expressed in *Escherichia coli* BL21 Star(DE3)pLysS (Invitrogen). Induced proteins were purified by nickel chromatography, concentrated and desalted into 25 mM Tris (pH 8.0), 1 mM Mg^{2+}, 100 mM KCl, 0.5% (v/v) NP-40, 10% (v/v) glycerol and 1mM DTT. Total protein yield was quantified using BCA Protein Assay (Pierce) and further assessed by SDS-PAGE/ Coomassie staining to ensure equal quantities of the various proteins.

RNA binding assays

The sequences of the 12 nt trailer, pre-tRNA and mature tRNA substrates were based on that for the human $tRNA^{Arg}_{ICG}26$, with the pre-tRNA sequence identical to that for the "R-11TUUU" plus one extra terminal uracil. Radiolabeled RNAs were prepared and purified as described18. Briefly, 12 nt trailer (5'GUGUAAGCUUUU, IDT Technologies) was end-labeled using (^{32}P-γ)ATP and T4 polynucleotide kinase. Radiolabeled pre-tRNA and mature tRNA ligands were synthesized by T7 RNA polymerase (Ambion Megascript) using DNA templates generated by PCR of the human $tRNA^{Arg}_{ICG}$ gene cloned into plasmid pUC19/R-11TUUU (gift from M. Nashimoto). Labeled RNAs were then PAGE purified. For Method 1, approximately 15,000 cpm of 5' 32P end-labeled 12 nt trailer or 3,000 cpm of pre-tRNA or mature tRNA ligand, (~0.1 nM each) were incubated with various amounts of hLa proteins in 20 ul containing 20 mM Tris pH 8.0, varying concentrations of Mg^{2+}, 100 mM KCl and 5 mM β-mercaptoethanol. Complexes were incubated at 37°C for 30 minutes, cooled on ice for 20 minutes, and then resolved on 8% (w/v) polyacrylamide nondenaturing gels at 4°C and 150V. Dissociation constants (K_d) were approximated as the concentration of protein at which half of the RNA substrate was bound33.

Method 2 was performed as described33. Briefly, after K_ds were determined by Method 1, an appropriate concentration of protein was incubated with varying RNA concentrations estimated to give between 20% and 80% protein occupancy, calculated using scatplan.xls50. The concentration of protein active for each ligand under different conditions can be derived from Scatchard analysis of Method 250. The active La concentration (and the mass concentration, in nM, used for each titration in Fig. 2A–C is listed here in parentheses) determined for pre-$tRNA^{Arg}$ACG, $tRNA^{Arg}$ACG and 12 nt UUU-3'OH trailer, to be 5.5 (10), 21 (20) and 51.5 (45) nM, respectively in 0 Mg^{2+}, 84 (250), 94 (250), and 46 (60) nM, respectively in 10 mM Mg^{2+}, and 40 (80), 86 (80) and 129 (160) nM, respectively, in 1 mM Mg^{2+}. Complexes were formed using constant RNA concentrations of ^{32}P-RNA (~0.1 nM) supplemented with cold RNA to yield the final RNA concentrations noted in the figures. These RNA-protein complexes were formed and resolved as for Method 1. Bound and free fractions were quantitated using a Fuji-FLA 3000 phosphorimager; in most cases bound included cpm with mobility above the free band. Using the bound and free quantities and the

known total concentrations of RNA added, K_ds were determined using a non-linear curve fitting algorithm (the scatchd2.xls spreadsheet found at the Setzer lab page50) that fit the data to a standard Scatchard analysis. Our Scatchard plots of Method 2 yield straight lines indicative of 1:1 stoichiometry. We note that ours and others analysis of La RNA binding by Method 1 do indicate 1:2 stoichiometry, this occurs only at high concentrations of La (e.g., see Supplementary Fig. 1, hLa concentration ≥40 nM, reflected by a second RNP complex higher in the gel). Consistent with a 1:1 stoichiometry, all of our Method 2 analyses were performed with relatively low La concentrations for which only one RNP is seen.

Dissociation rate constant assays were performed in the absence of Mg^{2+}, essentially as described, in triplicate33 using the ratern0.xls spreadsheet50. Typical EMSA gels used for dissociation rate are shown in Supplementary Fig. S4. Briefly, La-RNA complexes were formed in EMSA buffer using a concentration of La (200 nM) determined to give approximately 95% 32P-RNA binding. Concentrations of unlabeled competitor RNA were then determined that when mixed with the 32P-RNA prior to La addition, yielded 10–20% La bound ^{32}P-RNA (2000 nM; Supplementary Fig. S4 lanes 1). Dissociation rate constants were determined by first forming complexes using only ^{32}P-RNA (as in lane 2), then at time = 0 adding the pre-determined amount of competitor cold RNA (as per lane 1), and loading aliquots of the dissociation reaction at the noted time points. By measuring the dissociation of the ^{32}P-RNA from the La-bound form to the unbound form over time, the dissociation rates of La for different RNA substrates were calculated.

tRNA mediated suppression 18 and northern blots17 were performed as described. The probe for U5 RNA was 5’CTGGTAAAAGGCAAGAAACAGATACG.

RNA Chaperone activity was monitored as described23, except that RNA was radiolabeled with ^{32}P instead of ^{35}S, and that proteins assayed were added simultaneously with GTP. Intron self-splicing was measured by quantitation of the exponential decay of the unspliced precursor (*Unspliced/Spliced = U*) from the starting value of unspliced precursor (*Uo/So = Uo*), expressed as ln U/Uo over time. Unspliced and spliced products were measured using phosphorimager analysis. The DNA template for the precursor RNA was made by PCR amplification of a minimal T4 phage self-splicing intron (gift from R. Schroeder, Vienna, using an oligonucleotide containing a T7 promoter), followed by T7 transcription and PAGE purification.

Supplementary Material

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Abbreviations

LM	La motif
LD	La domain
hLa	human La
RRM	RNA recognition motif
TMS	tRNA-mediated suppression
pol III	RNA polymerase III

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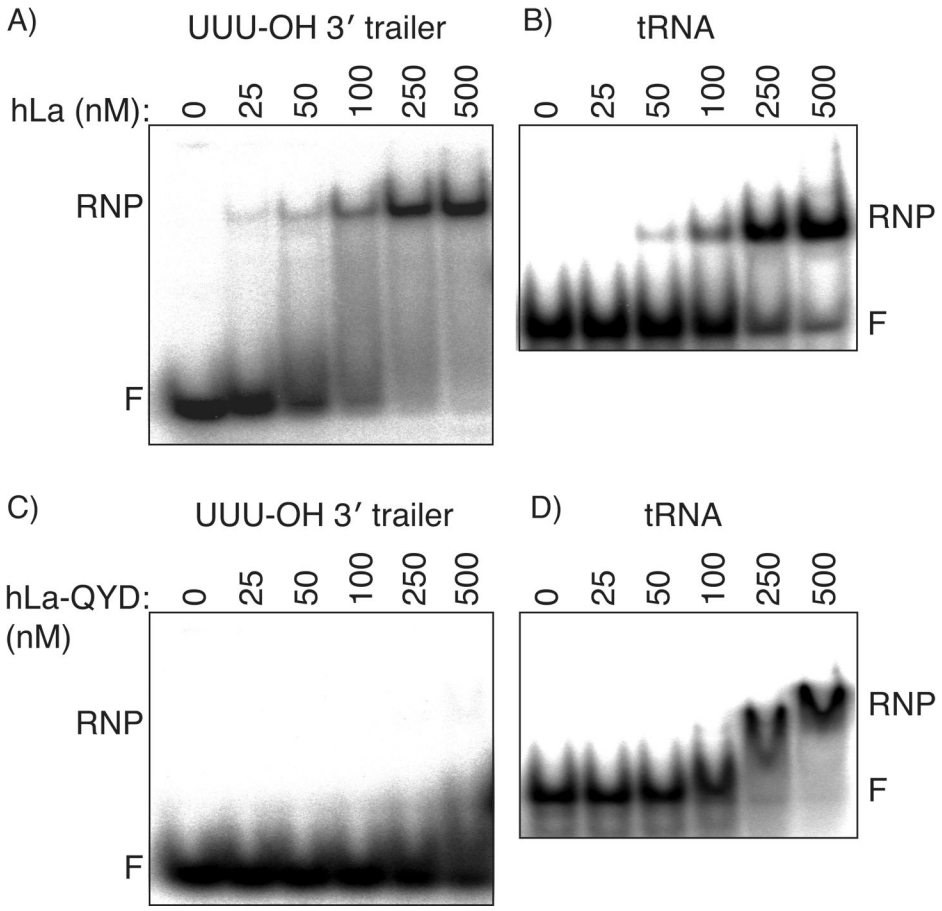
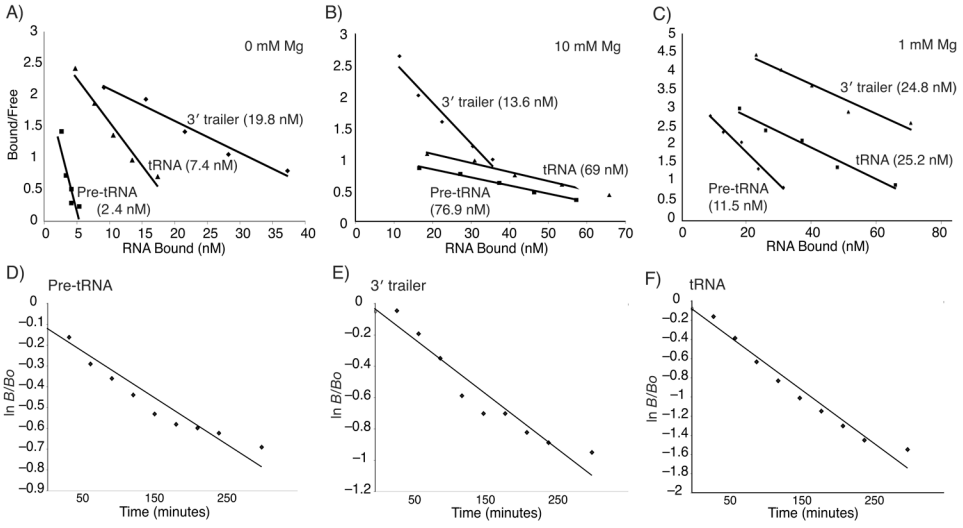


Figure 1.

La can bind non-UUU-3OH-containing RNA via contacts that are not mediated by the previously characterized RNA 3'OH binding site in the La motif. (a) EMSAs reveal human La protein (hLa) binding to the 12 nt UUU-3'OH-containing trailer and (b) a $tRNA^{Arg}ACG$ transcript which lacks UUU-3'OH. (c & d) show binding by the mutated protein hLa-Q20A/Y23A/D33R (abbreviated hLa-QYD) that contains three substitutions in the LM that are known to be critical for UUU-OH-specific binding4 (see text). For each, a constant trace amount of ^{32}P-RNA (~0.1 nM) was incubated with varying concentrations of La protein as indicated above the lanes in nM.



G)

	K_d (nM)				k_{off} (min^{-1})	$t_{1/2}$ (min)
	0 Mg	10 mM Mg	1 mM Mg	0 Mg/10 Mg		
pre-tRNA	2.4	76.9	11.5	0.031	0.113	6.2
tRNA	7.2	69.0	25.2	0.105	0.321	2.2
3' trailer	19.8	13.6	24.8	1.45	0.208	3.3

Figure 2.

Two distinct RNA binding sites on La together enhance stable binding to pre-tRNA. (a–c) Scatchard analyses of Method 2 EMSAs performed on pre-$tRNA^{Arg}ACG$, $tRNA^{Arg}ACG$ and the 12 nt UUU-3'OH trailer at 0, 10 and 1 mM Mg^{2+}. Each titration was done at a constant concentration of La with concentrations of RNA varying, although the La concentration differed for each individual Scatchard plot; K_ds are provided in each panel next to each ligand and in panel G. (d–f) Analysis of dissociation of hLa from pre-$tRNA^{Arg}ACG$, $tRNA^{Arg}ACG$ and the 12 nt UUU-3'OH trailer from data derived from EMSAs, was performed and analyzed as described33. Time scales are 0 to 350 seconds. (g) K_ds derived from A–C above and the numerical fraction of the K_ds at 0 and 10 mM Mg^{2+} (0 Mg/10 Mg) as indicated. Standard errors derived from triplicate determinations for pre-$tRNA^{Arg}ACG$, $tRNA^{Arg}ACG$ and the 12 nt UUU-3'OH trailer were 14.7%, 11.7% and 10.9%, respectively in 0 Mg^{2+}, 5.5%, 7.3% and 8.9%, respectively in 10 mM Mg^{2+}, and 7.7%, 5.9% and 13.9% in 1 mM Mg^{2+}. k_{off}s were derived from D–F above using standard calculations33. The $t_{1/2}$s were then derived from k_{off}. Standard errors for dissociation of pre-$tRNA^{Arg}ACG$, $tRNA^{Arg}ACG$ and the 12 nt UUU-3'OH trailer were 8.5%, 7.1% and 10.8%, respectively. The units for k_{off} and $t_{1/2}$ are min^{-1} and min, respectively.

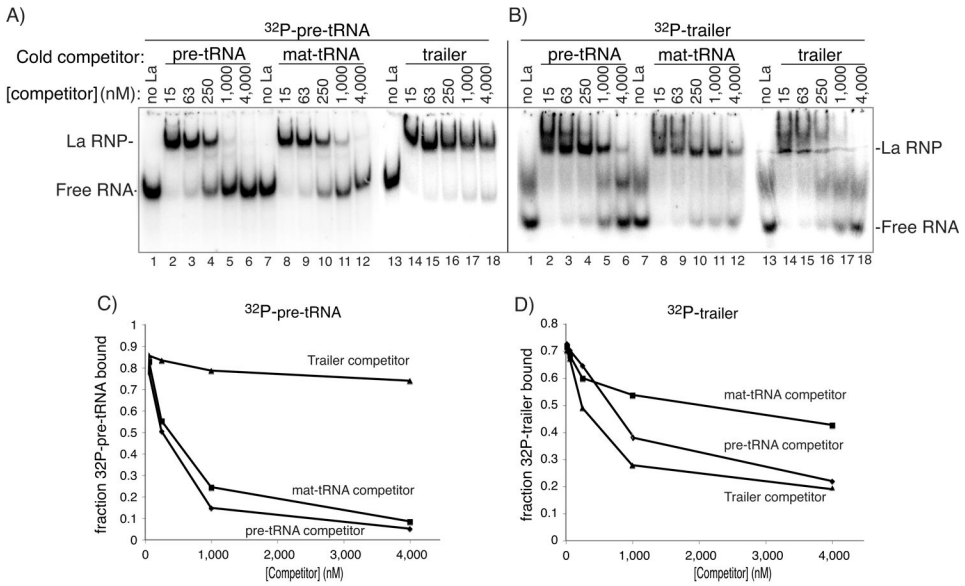


Figure 3.

hLa exhibits strong preference for pre-tRNA over a UUU-3'OH trailer. (a & b) Binding reactions contained constant amounts of hLa protein (100 nM) and a trace amount of ^{32}P-RNA (~0.1 nM, pre-tRNA in A, or UUU-3'OH trailer in (a), and varying amounts of unlabelled (cold) competitor RNAs as indicated above the lanes and described in the text, in 1 mM Mg^{2+}. The data in A and B were quantified and analyzed in (c) and (d) respectively.

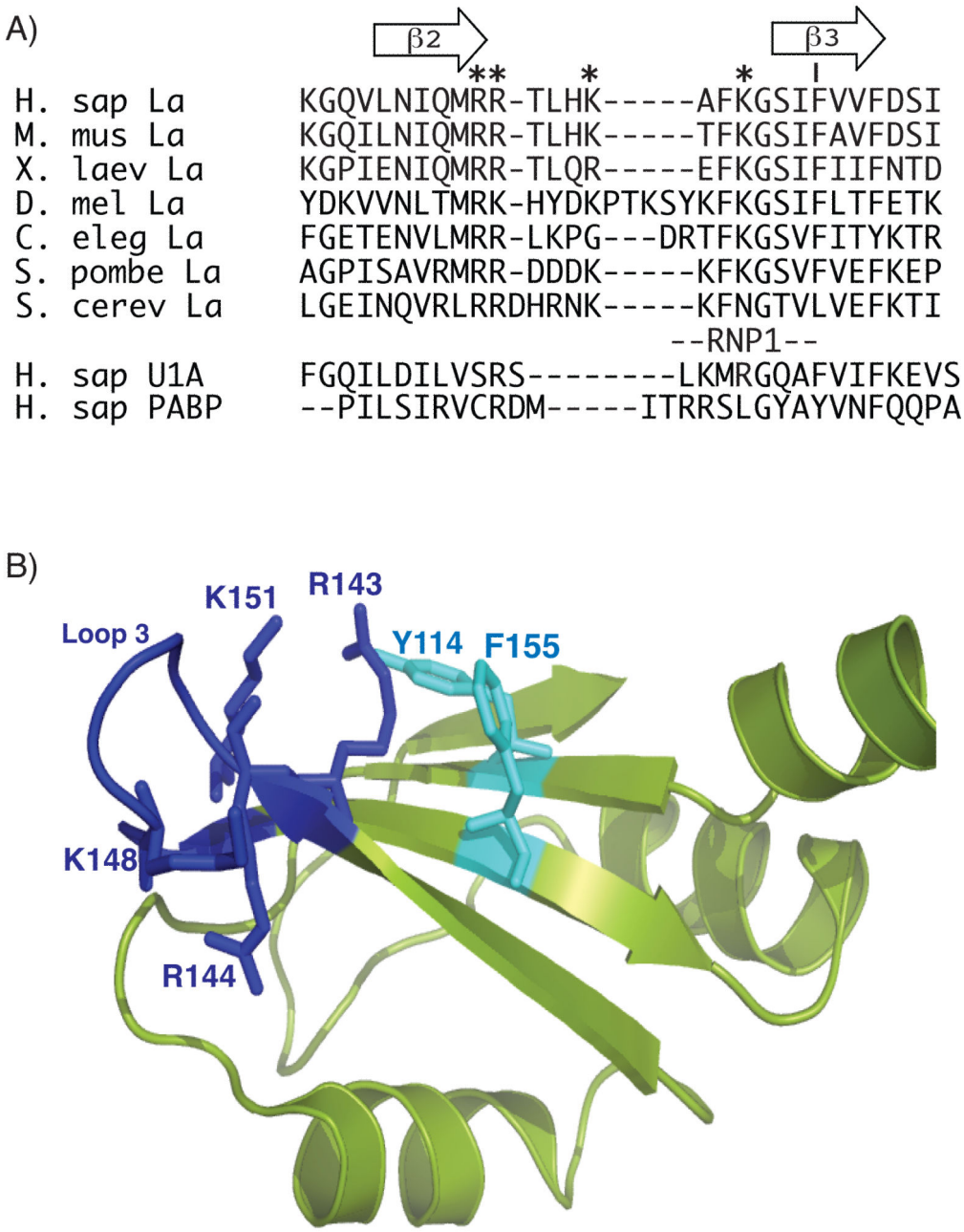


Figure 4.

La RRM1 loop-3 mediates UUU-3'OH-independent tRNA binding. (a) Sequence alignment of the β2-loop 3-β3 regions of the RRM1s of the La proteins from seven organisms followed by the homologous regions of U1A and PABP. Asterisks indicate the basic residues conserved in loop 3 of La proteins. RNP1 is indicated47; in hLa this contains F155, indicated by vertical line above the sequence. (b) Structure of the loop-3 side chains relative to the RRM1 β-sheet surface (adopted from Kotik-Kogan et al., PDB accession # 2VON). The loop-3 basic residues mutated for this study are shown in blue. RRM1 is shown in

green, with the conserved aromatic residue side chains (Y114 and F155) on β-strands 1 and 3, in cyan.

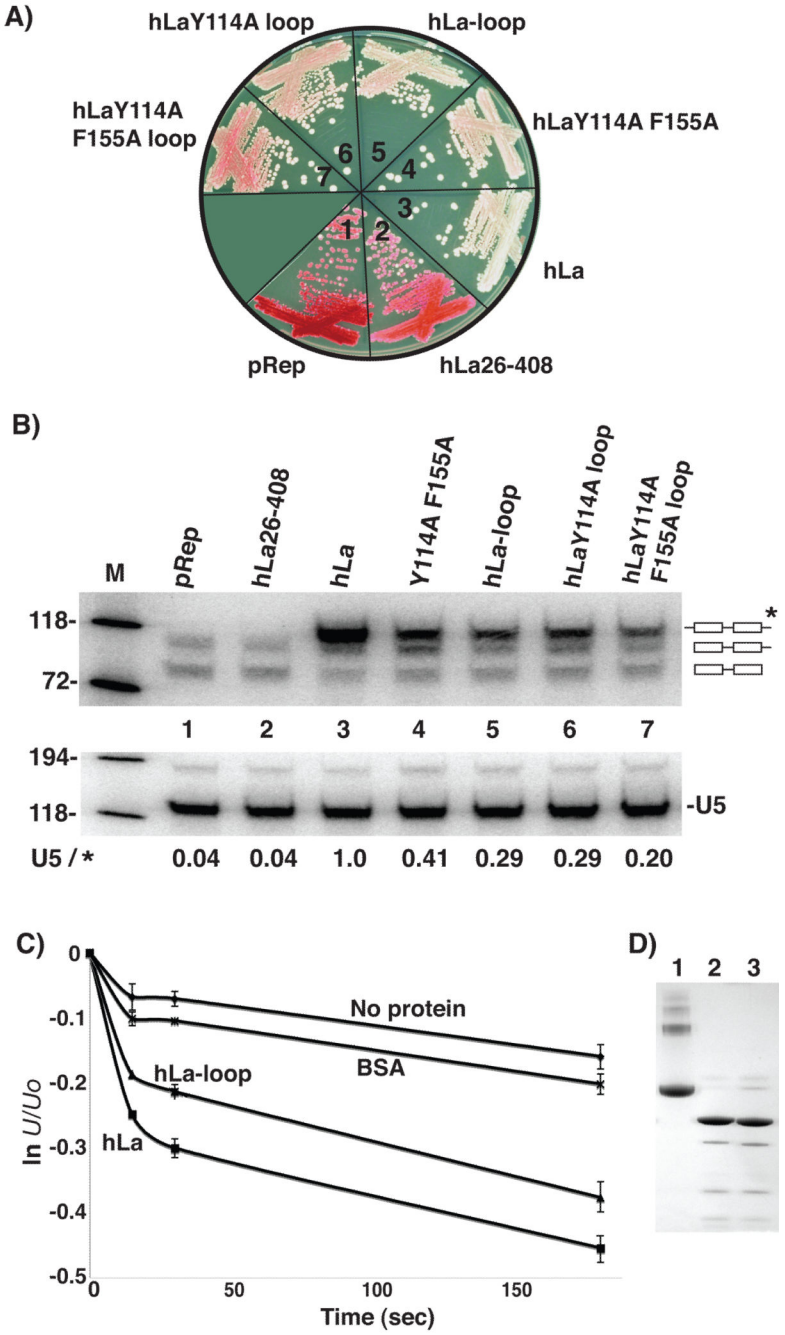


Figure 5.

The hLa loop mutant is defective in tRNA maturation in vivo. (a) tRNA-mediated suppression (TMS) activity was assayed in *S. pombe*; pRep is the empty vector; all other hLa constructs indicated were cloned in pRep17,18,36,38. (b) Northern blot analysis of RNAs extracted from cells in A above. Upper panel shows blot probed for intron-containing pre-tRNA^{Lys}CUU species, which migrate as upper, middle and lower bands (see text). Lower panel shows the same blot reprobed for U5 snRNA, which served as a loading control for quantitation, normalized to hLa = 1.0, lane 3. (c) RNA chaperone assays were

performed *in vitro* using the cis-splicing intron RNA23. Activity is reflected by a decrease in unspliced RNA (ln U/Uo, Experimental Procedures) for each of the proteins indicated. Error bars reflect triplicate samples for each point. (d) Coomassie blue stained gel of the BSA (lane 1), hLa (lane 2) and hLa-loop (lane 3) proteins used in the assay.

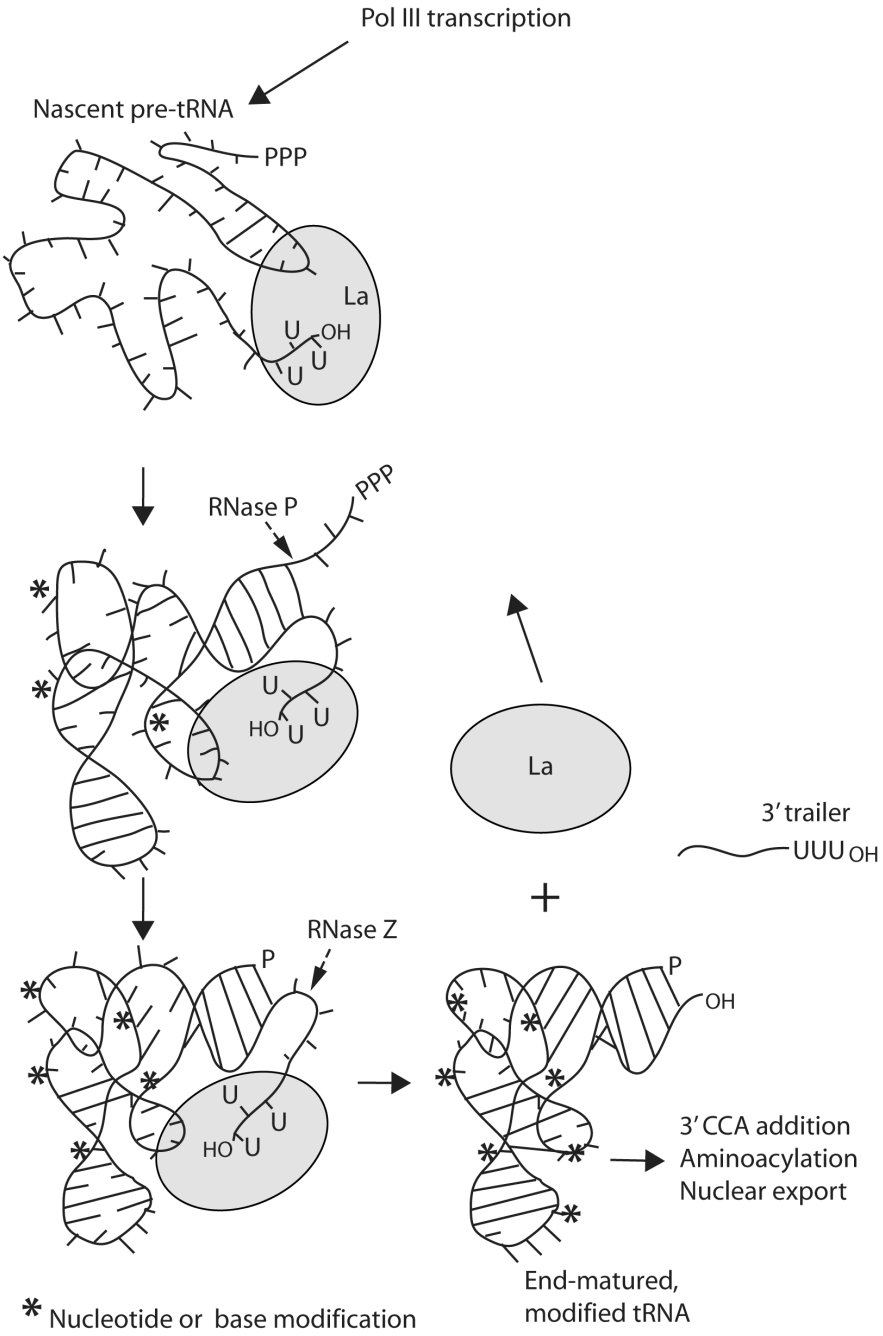


Figure 6.

Model of involvement of La protein in a tRNA maturation pathway. La appears to be the first protein that binds nascent pol III-transcripts including pre-tRNAs (see text), and presumably does so via its UUU-3'OH binding cleft and/or RRM1. Different regions of the RNA may become juxtaposed to RRM1 as the pre-tRNA folds, acquires nucleotide modifications (indicated by asterisks), and becomes a substrate for 5' processing by RNase P. After separation of the tRNA and 3' trailer by cleavage by the endonuclease, RNase Z, La is no longer tethered to two sites on a single RNA and readily dissociates, free to associate

with new nascent pre-tRNA. The released, end-matured, modified tRNA becomes the substrate of 3' end-modifying proteins such as the CCA-adding enzyme and tRNA synthetases, and is exported from the nucleus.

Table 1

a, Effects on RNA binding of different hLa mutated proteins. ***b***, Dissociation constants (K_d) of hLa-loop protein and the relationship of the K_ds for hLa-loop and hLa (expressed as a fraction: K_d hLa/K_d hLa-loop) for three RNA ligands.

(*a*) hLa mutant	La region	tRNA binding	UUU-OH 3' trailer
Q20A Y23A D33R	LM	Slight reduced	Much reduced
R32A K34A K37A	LM	Unaffected	ND
K105A K109A	RRM1-α0	Unaffected	ND
Y114A F155A	RRM1-β2 β3	Unaffected	Unaffected
R143A R144A	RRM1-loop 3	3–4X reduced	Unaffected
K148A K151A			
K185A K191A	RRM1-α3	Unaffected	ND
K192A			
R57A R60A	LM	NA (other)	Unaffected
K16A H19A	LM	NA (other)	Unaffected
R143A K151A	RRM1-loop 3	Unaffected	ND
R144A K148A	RRM1-loop 3	Unaffected	ND

(*b*) Ligand	K_d hLa-loop	K_d hLa/hLa-loop
pre-tRNA	12.9 nM	0.19
tRNA	25.8 nM	0.28
UUU-OH 3' trailer	20.5 nM	0.97