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RNA Chaperone Activity of Human La Protein Is Mediated by Variant RNA Recognition Motif*[S]

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La proteins are conserved factors in eukaryotes that bind and protect the 3′ trailers of pre-tRNAs from exonuclease digestion via sequence-specific recognition of UUU-3′OH. La has also been hypothesized to assist pre-tRNAs in attaining their native fold through RNA chaperone activity. In addition to binding polymerase III transcripts, human La has also been shown to enhance the translation of several internal ribosome entry sites and upstream ORF-containing mRNA targets, also potentially through RNA chaperone activity. Using *in vitro* FRET-based assays, we show that human and *Schizosaccharomyces pombe* La proteins harbor RNA chaperone activity by enhancing RNA strand annealing and strand dissociation. We use various RNA substrates and La mutants to show that UUU-3′OH-dependent La-RNA binding is not required for this function, and we map RNA chaperone activity to its RRM1 motif including a noncanonical α3-helix. We validate the importance of this α3-helix by appending it to the RRM of the unrelated U1A protein and show that this fusion protein acquires significant strand annealing activity. Finally, we show that residues required for La-mediated RNA chaperone activity *in vitro* are required for La-dependent rescue of tRNA-mediated suppression via a mutated suppressor tRNA *in vivo*. This work delineates the structural elements required for La-mediated RNA chaperone activity and provides a basis for understanding how La can enhance the folding of its various RNA targets.

La proteins are conserved throughout nearly all eukaryotes and are highly expressed RNA binding factors with important functions in the processing and metabolism of a variety of RNA targets (1). Their best characterized function is to engage the UUU-3′OH trailers of polymerase III transcripts, such as pre-tRNAs, and protect them from exonuclease digestion during their processing. Although La is essential in higher eukaryotes, including *Drosophila* and mice (2, 3), La can be deleted in budding yeast and fission yeast (4, 5), and La study in yeast has shown that La binding to pre-tRNAs can also influence the order of tRNA processing events (6). Yeast La has also been shown to bind certain noncoding polymerase II transcripts via a terminal UUU-3′OH motif that they obtain transiently during their processing (7, 8).

La proteins contain a conserved N-terminal domain called a La domain or La module, which is responsible for specific UUU-3′OH binding (1). The La domain is made up of two motifs: an N-terminal La motif, similar in structure to a winged helix fold, and an RNA recognition motif (RRM),[3] separated by a short linker (9, 10). Crystallographic studies on co-crystals containing the La domain and a UUU-3′OH containing short RNA, as well as accompanying biochemical work, have revealed conserved residues in the La motif that are important for UUU-3′OH-dependent RNA binding, and mutations to these cause enhanced degradation of pre-tRNAs by 3′ exonucleases *in vivo* (11–13). In addition to UUU-3′OH-dependent contacts, regions important for UUU-3′OH independent binding of pre-tRNAs have been mapped to other regions of La proteins, including the loop 3 region of the conserved RRM1 motif and the less well conserved C-terminal domain, but the structural requirements of RNA targets for these binding modes are less well understood (14, 15).

In addition to protecting the 3′ ends of pre-tRNAs, La proteins have also been hypothesized to function as RNA chaperones for these. Although the simple capacity of La proteins to enhance the propensity of pre-tRNAs to be correctly processed through their transient binding and associated 3′ end protection would be sufficient to characterize them as molecular chaperones (7), other data point to a function for La proteins in RNA folding more directly. For example, La deletion in budding yeast is synthetically lethal with tRNA mutations predicted to result in their misfolding or with mutations to tRNA modification enzymes thought to stabilize tRNA structure (6, 16–19). Furthermore, yeast La has been shown to stabilize the native structure of mutated, misfolded tRNA anti-codon stems *in vitro*, and can rescue the respective mutant tRNAs *in vivo* (16). Finally, mutations to the RRM1 domain of human La (hLa) and *Schizosaccharomyces pombe* La (Sla1p) have been associated with defects in the rescue of mutated pre-tRNAs despite normal 3′ end binding and protection activity (13). Consistent with this hypothesis, human La is active in an *in vitro* assay in which a misfolded, self-splicing intron relies on an RNA chaperone to acquire the native fold required for catalysis (14, 20).

In addition to binding UUU-3′OH containing RNA targets, La binds to a significant number of viral and cellular coding

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[3] The abbreviations used are: RRM, RNA recognition motif; hLa, human La; IRES, internal ribosome entry site; LARP, La-related protein.

mRNA transcripts and has been implicated in the translation of these. A common feature of these mRNAs is that they typically harbor atypical translation initiation motifs, including internal ribosome entry sites (IRESs), upstream ORFs or 5′ terminal oligopyrimidine sites (21–28). La can either enhance or repress the translation of these, but for the IRES and upstream ORF mRNA targets, La is generally thought to act as a translational enhancer. Notably, the very first IRES *trans*-acting factor, characterized as a protein important for the enhancement of translation from the poliovirus IRES, was identified to be human La (21). One mechanism that has been hypothesized for La function for such mRNAs is that it can assist in the correct folding of RNA structures required for optimal translation by acting as an RNA chaperone (22, 29–31). Thus, the capacity for La to act as an RNA chaperone has been suggested to represent a unifying feature for La function between its pre-tRNA and mRNA targets (32, 33). Despite the importance that such a role may play in La function, a rigorous analysis of La-dependent RNA chaperone activity is currently lacking.

Recent FRET-based assays have been developed that are capable of measuring strand annealing and strand dissociation capabilities of RNA chaperones (34, 35). In this work, we use such an assay to demonstrate that human La harbors both of these RNA chaperone activities and that this is a conserved feature between *S. pombe* and human La proteins. Consistent with a role in the folding of mRNA transcripts, we show that La-dependent RNA chaperone activity does not require UUU-3′OH-mediated RNA binding. Using deletion and point mutants of hLa, we show that the N-terminal La domain harbors both strand annealing and strand dissociation activity; specifically, the RRM1 motif is necessary and sufficient for these. Notably, an α-helix present at the C terminus of RRM1 that is not part of the typical RRM fold is important for this activity. We further demonstrate the relevance of this α-helix in RNA chaperone activity by appending it as a fusion onto the C terminus of an unrelated RRM, that from the human U1A protein, and show that we can enhance the capacity of this motif to act as an RNA chaperone. Finally, we show that hLa mutants defective in RNA chaperone activity are also compromised in their capacity to rescue a mutated pre-tRNA *in vivo*. These data confirm that RNA chaperone activity is a conserved feature of La proteins and identify the motifs of La required for this function, as well as provide insight on the RNA binding modes used by La for this process. It is expected that this work will inform future research on the mechanisms by which La proteins contribute to the metabolism of both their noncoding and coding RNA targets.

EXPERIMENTAL PROCEDURES

Cloning—The gene for the bacterial StpA protein was amplified by *Escherichia coli* colony PCR and cloned into the NcoI and HindIII restriction sites of pET30a. Deletion mutants of hLa, except 105–202 and 105–229, are described in Ref. 36. hLa 1–235 α-3 was constructed by QuikChange using hLa pET28a hLa 1–235 as a template (36). hLa 1–235 Y114A/F155A and hLa 1–235 loop3 were cloned into the pREP4 and pET28a vectors using the SalI and BamHI or NcoI and HindIII restriction sites, respectively, using their full-length hLa equivalents (pET28a) as templates (14). Full-length U1A was cloned as a cDNA from total RNA (HeLa cells) into the HindIII and NdeI sites of pET28a, which was subsequently used as a template for the other U1A variants. All of the clones were confirmed through sequencing analysis.

Protein Purification—His-tagged proteins were expressed in BL21 Star (DE3) pLysS or RosettaBlue (DE3) pLysS. Protein production was induced with 0.1–1 mM isopropyl β-D-thiogalactopyranoside for 3 h to overnight and purified using cobalt affinity chromatography (His-TRAP; ThermoFisher). Proteins were then concentrated and desalted into RNA chaperone buffer (50 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, and 1 mM DTT) and quantified using SDS-PAGE and Coomassie staining.

FRET Assays—RNA chaperone assays were performed as described (37), with the following modifications: RNA substrates (see text and supplemental Table S1 for sequences) were obtained from IDT and Dharmacon and labeled with Cy5 (upper strand) and Cy3 or Dy547 (lower strand). FRET reactions took place in a total volume of 400 μl in a heated cuvette at 37 °C. For hLa and mutants, the concentration of each labeled RNA was 25 nM, and the concentration of protein was 0.1 μM. For the positive control StpA, the concentrations of each labeled RNA substrate was 5 nM, and the concentration of protein was 0.5 μM. At time = 180 s, excess, nonlabeled competitor RNA, with sequence 5′-ACUGCUAGAGAUUUUCCACAU-3′, was injected at 10-fold excess compared with the protein. All of the fluorescence readings were obtained by a Cary Eclipse fluorimeter. FRET indexes and rate constants were obtained as described (37): briefly, a FRET index (emission at 680 nm *versus* emission at 590 nm) was calculated over time (s) in half-second time points and normalized between 0 and 1 using Graphpad Prism 5.0. To obtain rate constants, the phase I for strand annealing was least square-fitted with the exponential function for signal increase, and phase II was least square-fitted with an exponential function for signal decay or signal increase depending on the presence or absence of strand displacement activity, respectively. Histograms and tables with rate constants show the results of a minimum of three independent experiments.

tRNA-mediated Suppression—tRNA-mediated suppression was performed as described (14). Briefly, plasmids encoding hLa and mutants cloned into the SalI and BamHI sites of pREP4 were transformed into ySH18 (13), and representative single colonies were selected and streaked out on selective media containing 5 μg/ml adenine.

RESULTS

To investigate strand annealing and strand dissociation activities of La proteins, we performed FRET-based assays that rely on the annealing of 21-nucleotide complementary RNA strands labeled at their 5′ ends with the fluorophores Cy5 (top strand) or Cy3 (bottom strand) (35, 37) (Fig. 1*A*). Briefly, the RNA substrates were incubated together in a heated fluorimeter cuvette held at a constant temperature of 37 °C, and the rate of strand annealing (k_{ann1}) in the presence or absence of an RNA chaperone was measured as an increase in FRET index between the fluorophores over time (phase I; Fig. 1*B*). In phase II (at t = 180 s), the capacity of candidate RNA chaperones to dissociate annealed duplexes formed in phase I was determined

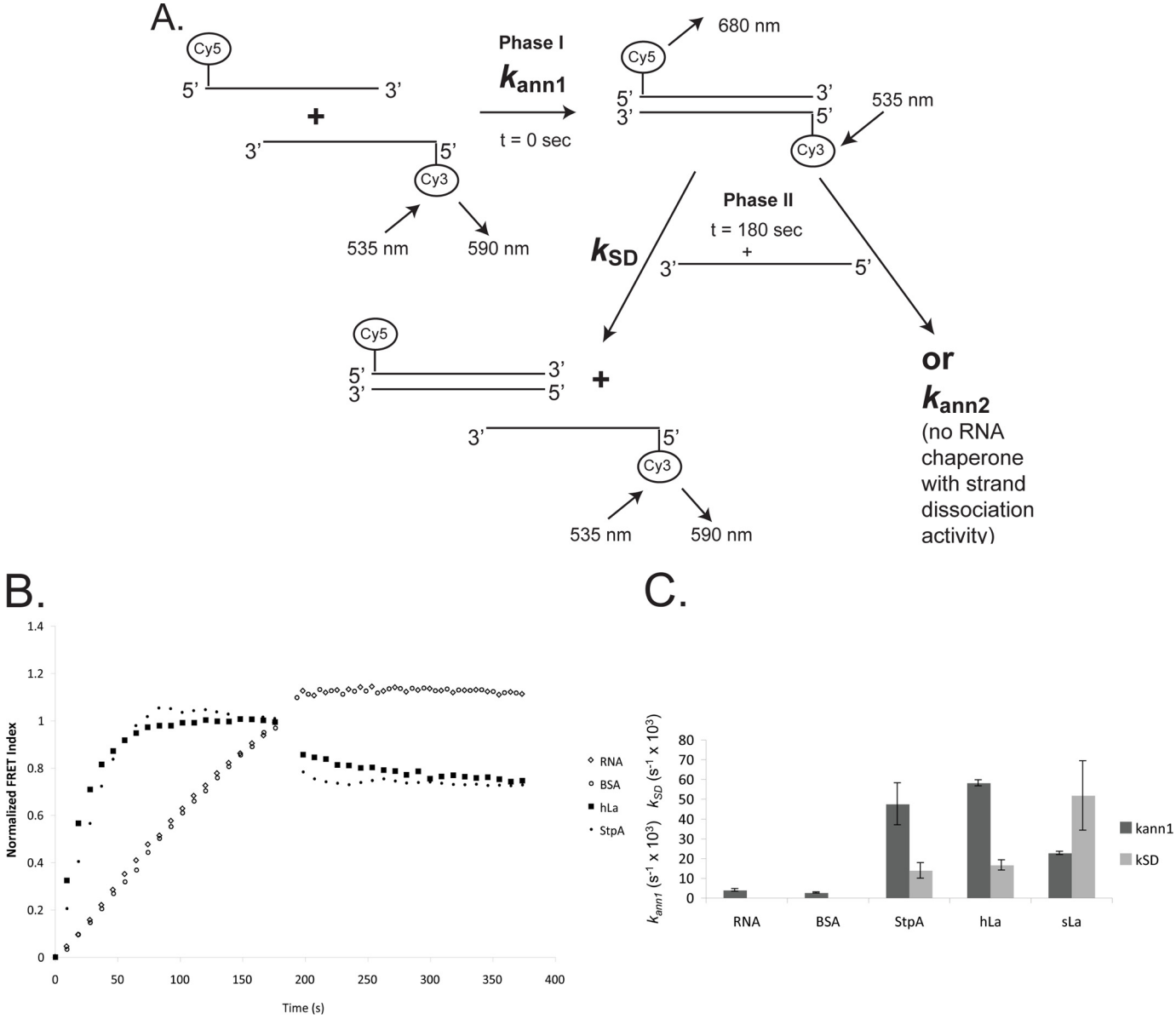

FIGURE 1. **hLa and Sla1p display RNA strand association and strand dissociation activity.** *A*, assay for strand annealing and strand dissociation. *B*, RNA strand annealing (phase I) and strand dissociation (phase II) of hLa compared with StpA was measured as a change in FRET index between complementary Cy5- and Cy3-labeled RNA strands over time. *C*, FRET index data over time were used to calculate strand annealing rates (k_{ann1}, phase I), strand dissociation rates ($k_{S.D.}$, phase II) and strand annealing in phase II in the absence of strand dissociation (k_{ann2}, phase II). The *error bars* show the standard deviation of at least three separate experiments.

by injection of an excess of unlabeled bottom strand. In the absence of an RNA chaperone with strand dissociation activity, this results in an increase in measured strand annealing (k_{ann2}) because the concentration of one of the partners in the annealing reaction has now increased (37). However, in the presence of an RNA chaperone with strand dissociation activity, the excess unlabeled bottom strands can trap dissociated upper strands resulting in a decrease in FRET index (Fig. 1*B*; measured as the rate of strand dissociation, $k_{S.D.}$). As negative controls, we measured the rates of strand annealing and strand dissociation in the absence of added protein or in the presence of BSA. As a positive control, we performed assays using the *E. coli* StpA protein, which has been previously characterized as harboring both strand annealing and dissociation activity (37).

Conserved RNA Chaperone Activities of La Proteins—To assay the strand annealing and dissociation activities of purified hLa, we first used a modified, 33-nucleotide top strand containing the 21-nucleotide region of complimentarity followed by a 12-nucleotide trailer ending with UUUU-3′OH (21F Cy5 Trailer UUUU; supplemental Table S1 and Fig. 2*A*), because we had previously shown this trailer sequence binds hLa with high affinity using the UUU-3′OH-dependent binding mode (14). Incubation of the positive control StpA with the 21F Cy5 Trailer UUUU top strand and the bottom strand (21R Cy3) resulted in an approximate 10-fold increase in the rate of strand annealing (k_{ann1}) compared with RNA alone or BSA, confirming that this modification of the top strand sequence was not prohibitive to the assay (FRET indices shown in Fig. 1*B*; derived

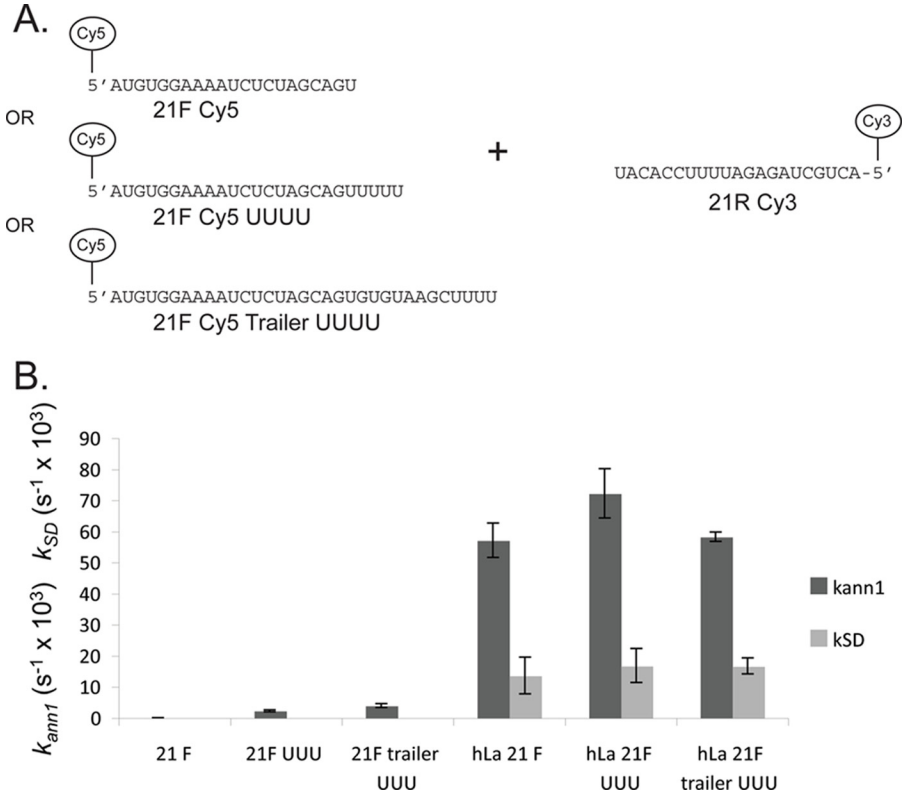

FIGURE 2. **Enhancement of RNA strand annealing and dissociation by hLa does not require UUU-3′OH-mediated RNA binding.** *A*, Cy5-labeled top strands containing varying UUU-3′OH contexts at their 3′ ends were assayed in the FRET-based assay to determine whether this motif was required for hLa-dependent RNA chaperone activity. *B*, hLa has similar RNA strand annealing and dissociation activity for RNAs lacking UUU-3′OH or containing this sequence with or without an intervening trailer.

rate constants from these shown in Fig. 1*C*; all specific rate constants and associated standard deviations given in supplemental Table S2). Likewise, the addition of excess unlabeled bottom strand in phase II resulted in a decrease in the amount of FRET in the presence of StpA ($k_{S.D.} = 0.014\ s^{-1}$), but not with RNA alone or BSA ($k_{S.D.} = 0$), which instead showed continued annealing, k_{ann2} (k_{ann2} rates provided in supplemental Tables S2, S3, and S4). Substitution of hLa in the strand annealing phase also resulted in a greater than 10-fold increase in the rate of FRET compared with RNA alone or BSA (Fig. 1, *B* and *C*), and addition of unlabeled bottom strand in phase II also resulted in comparable strand dissociation ($k_{S.D.} = 0.017\ s^{-1}$) to StpA compared with zero loss of FRET for the negative controls. Titration of hLa into the assay showed a concentration-dependent increase in RNA chaperone activity that effectively became saturated at a concentration of 100 nM (supplemental Fig. S1), compared with 500 nM for StpA (data not shown). Notably, this saturating concentration for hLa was similar to estimates of hLa concentration in human cell extracts (50 nM) (38). As a control for the validity of the assay, we also varied the length of the annealing phase in both the presence and absence of RNA chaperone (hLa *versus* RNA alone) to ensure that this had no effect on our assignments of strand annealing or dissociation activity (supplemental Fig. S2). We found that increasing phase I from 180 to 600 s had negligible effects on k_{ann1} or $k_{S.D.}$ rates in either the presence or absence of an RNA chaperone. From these data we conclude that like StpA, hLa contains both RNA strand annealing and dissociation activities. Because yeast La has been hypothesized to function as an RNA chaperone during the rescue of misfolded pre-tRNAs (6, 13, 16), we also tested the *S. pombe* La homolog Sla1p for RNA chaperone activity and found that it also enhanced strand annealing and dissociation compared with negative controls (Fig. 1*C*). We conclude that RNA chaperone activity is conserved between *S. pombe* and human La.

hLa RNA Chaperone Activity Functions Independently of UUU-3′OH-dependent Binding—The best characterized binding mode for La proteins involves the specific recognition of UUU-3′OH, largely by amino acids on the conserved La motif. For pre-tRNA maturation, binding of the UUU-3′OH trailer is hypothesized to be distinct from the binding and enhancement of folding of the main tRNA body via RRM1, and consequently La-mediated RNA chaperone activity may depend more strongly on UUU-3′OH-independent RNA binding (13, 14). Consistent with this, our previous work revealed that point mutation of the RRM1 β2-β3 loop-3 of hLa causes both a decrease in UUU-3′OH-independent RNA binding, as well as the ability of hLa to resolve a misfolded self-splicing intron (14). To test the importance of the La UUU-3′OH binding mode in RNA chaperone activity more directly, we compared strand annealing and strand dissociation rates using three different top strand substrates with the same bottom 21R Cy3 substrate (Fig. 2*A*): (i) a 21-nucleotide substrate complementary to the 21-nucleotide bottom strand and lacking UUUU-3′OH (21F Cy5, ending in CAGU-3′OH), (ii) a 25-nucleotide substrate containing the same 21-nucleotide sequence but with an added

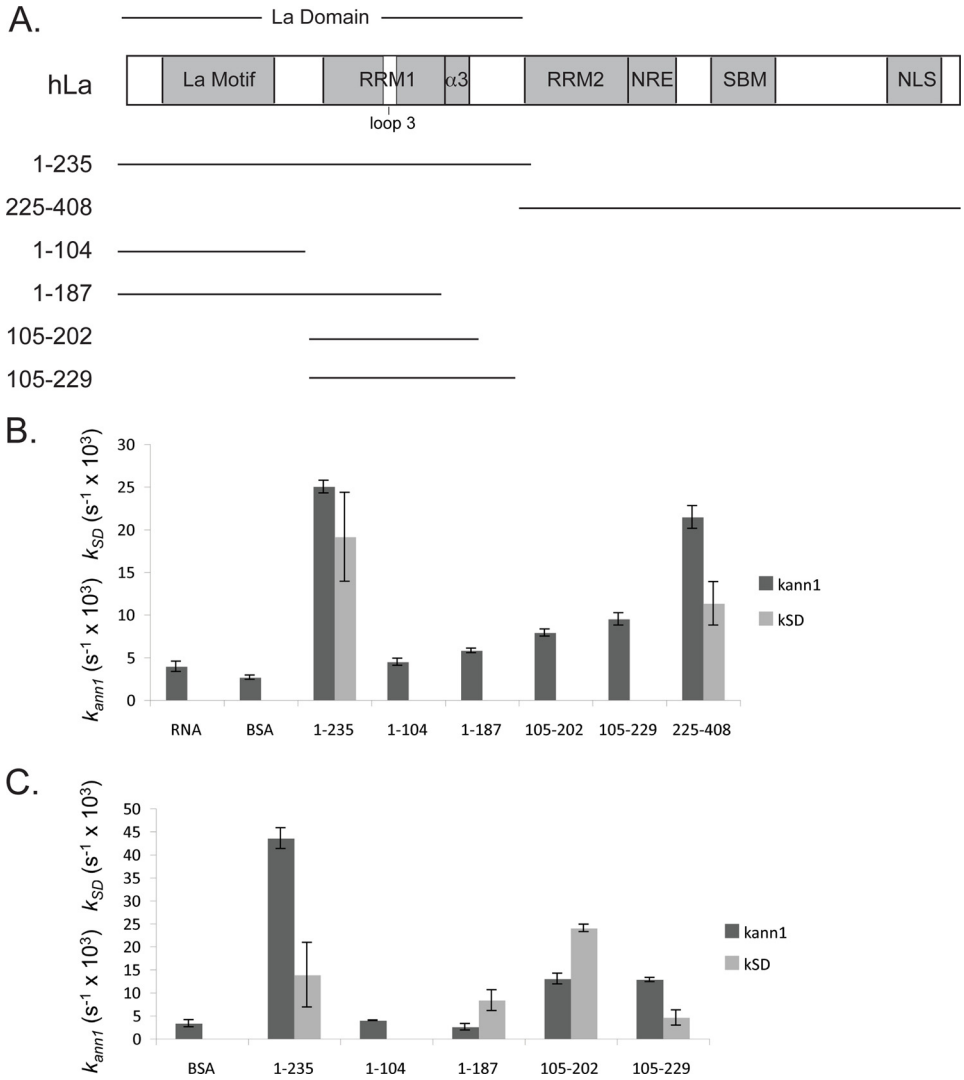

FIGURE 3. **Domain mapping of the RNA chaperone activity of human La.** *A*, architecture of the human La protein and the deletion mutants used in this study. *NRE*, nuclear retention element; *SBM*, short basic motif; *NLS*, nuclear localization signal. *B*, k_{ann1}, $k_{S.D.}$, and k_{ann2} rates for indicated mutants of the La domain at 100 nM (saturating concentration for wild-type hLa). *C*, k_{ann1}, $k_{S.D.}$ and k_{ann2} rates for indicated mutants of the La domain at 5× concentration (500 nM).

UUUU-3′OH overhang (21F Cy5 UUUU), and iii) the 21F Cy5 Trailer UUUU substrate. In the absence of any added proteins, minimal strand annealing and strand dissociation rates were observed for all three top strand substrates (Fig. 2*B*). The addition of hLa to these various substrates resulted in similar increases in strand annealing and strand dissociation rates, consistent with the UUU-3′OH-dependent RNA binding not contributing significantly to La-dependent RNA chaperone activity.

Mapping of hLa Structural Determinants Required for Strand Annealing and Dissociation—We performed strand annealing and dissociation assays using various point and deletion mutants of hLa to identify which elements were required for each activity (Fig. 3 and 4). Surprisingly, we found that both the N- and C-terminal halves of hLa (hLa 1–235 and hLa 225–408) harbored both strand annealing and strand dissociation activity, although neither had strand annealing activity at the same level as full-length hLa (hLa 1–235 $k_{ann1} = 0.025\ s^{-1}$ and hLa 225–408 $= 0.021\ s^{-1}$ *versus* hLa $k_{ann1} = 0.058\ s^{-1}$; Fig. 3*B*). Because the C-terminal half of human La is not conserved and is largely absent in La proteins from fission and budding yeast (see "Discussion"), we focused on studying the N-terminal La domain shared between all La homologs to gain insight into how La-dependent RNA chaperone activity should function generally across eukaryotes. This N-terminal region includes the winged helix fold containing La motif followed by RRM1, which in addition to having the expected $\beta1\alpha1\beta2\beta3\alpha2\beta4$ RRM fold (39) contains an extra C-terminal α-helix ($\alpha3$) not typically found in unrelated RRMs and previously hypothesized to be in an orientation particular to La proteins (Fig. 4*A*) (9). Notably, this helix includes three basic lysine residues, two of which point toward the canonical RNA binding RRM1 β-sheet surface, as well as a universally conserved aromatic residue (hLa: Tyr-188) that stacks upon a conserved aromatic (hLa: Tyr-114) also on this β-sheet (39). We found that the La motif in isolation (amino acids 1–104) had insignificant strand annealing or dissociation activity compared with controls (Fig. 3*B*). Surprisingly, we found that including both the La motif and the canonical $\beta1\alpha1\beta2\beta3\alpha2\beta4$ RRM fold (hLa 1–187) also showed very little activity, suggesting that the region between hLa 187 and

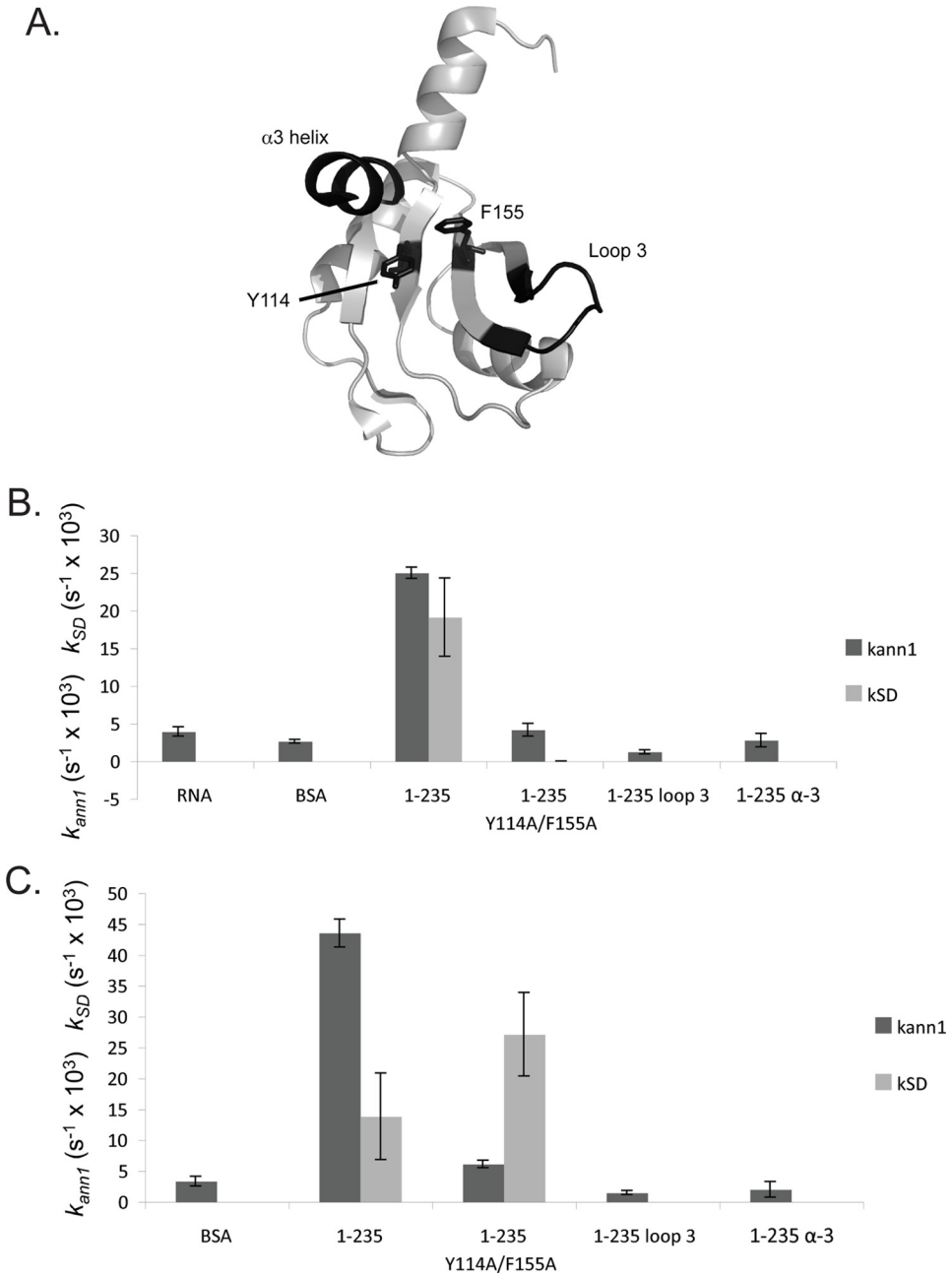

FIGURE 4. **The canonical RRM1 RNA binding surface and the α3-helix are required for RNA chaperone activity by the La domain.** *A*, regions of the hLa RRM1 containing point mutations and tested for RNA chaperone activity are colored in *black*. The α3 helix mutations were: hLa 1–235 K185A/K191A/K192A; the loop-3 mutations were: hLa 1–235 R142A/R143A/K148A/K151A. *B*, k_{ann1}, $k_{S.D.}$ and k_{ann2} rates for indicated point mutations of the La domain at 100 nM (saturating concentration for wild-type hLa). *C*, k_{ann1}, $k_{S.D.}$, and k_{ann2} rates for indicated point mutations of the La domain at 5× concentration (500 nM).

235 is important for RNA chaperone activity. This region includes the noncanonical α3-helix as well as a predicted unstructured linker region between RRM1 and RRM2.

To examine the importance of the canonical RRM, the RRM1 α3-helix, and linker region further, we tested RRM1 without the La motif but including successively greater sections of the region between amino acids 187 and 235 (hLa 105–202 and hLa 105–229; Fig. 3, *A* and *B*) in the assays. We found that these RRM1 mutants acquired modest strand annealing activity with successively greater C-terminal extensions (hLa 1–187 k_{ann1} = 0.0058 s^{-1}, *versus* hLa 105–202 k_{ann1} = 0.0079 s^{-1}, *versus* hLa 105–229 k_{ann1} = 0.0095 s^{-1}), consistent with the RRM, α3-helix, and adjacent linker representing the necessary elements for strand association activity. Interestingly, we noted that the hLa 105–202 and 105–229 mutants, but not the hLa 1–104 or 1–187 mutants, showed significantly higher k_{ann2} rates (supplemental Table S2; hLa 105–202 and hLa 105–229 k_{ann2} = 0.449 and 0.198 s^{-1}, respectively, *versus* hLa 1–104 and hLa 1–187 k_{ann2} = 0.024 and 0.067 s^{-1}, respectively). This led us to consider the possibility that the hLa 105–202 and 105–229 mutants only had decreased affinity for substrate in strand annealing and that the higher levels of substrate available in phase II upon competitor addition were sufficient to rescue some strand annealing activity. To test this possibility, we performed the

assays again but increased the concentration of protein in the assay by 5-fold (Fig. 3*C* and supplemental Table S3). We found that this indeed increased the strand annealing activity of hLa 105–202 ($k_{ann1} = 0.013\ s^{-1}$ at 5× *versus* $0.0079\ s^{-1}$ at 1×) and hLa 105–229 ($k_{ann1} = 0.013\ s^{-1}$ at 5× *versus* $0.0095\ s^{-1}$ at 1×) and rescued strand dissociation activity for hLa 105–202 ($k_{S.D.} = 0.024\ s^{-1}$ at 5× *versus* 0 at 1×) and 105–229 ($k_{S.D.} = 0.0046\ s^{-1}$ at 5X *versus* 0 at 1×), but not for the isolated La motif (hLa 1–104 $k_{ann1} = 0.0039$ at 5×, *versus* $0.0046\ s^{-1}$ at 1×; $k_{S.D.} = 0$ at 5× and 1×). No rescue in strand annealing was observed at 5× protein concentration for hLa 1–187, but strand dissociation was rescued. These data are consistent with a model in which the RRM1 (including its α3-helix) and adjacent linker regions form the basis for strand annealing activity, whereas the canonical RRM1 domain is sufficient for strand displacement activity. The decrease in strand annealing activity between 1–235 and 105–229 is also consistent with the La motif providing an accessory domain that enhances affinity of hLa for RNA substrates even in the absence of the UUU-3′OH motif (Fig. 2; see "Discussion").

Point Mutations of RRM1 That Inhibit RNA Chaperone Activity—Previous work has shown that mutation of conserved aromatics in the RRM1 RNP motifs of both human (hLa Y114A and F155A) and fission yeast La results in defective La-dependent rescue of tRNA-mediated suppression without a loss in their 3′ end protection. Furthermore, point mutation of basic residues in the β2-β3 loop-3 region of RRM1 of hLa decreased tRNA binding and the ability of hLa to resolve a misfolded, self-splicing intron, but not UUU-3′OH-dependent binding, suggesting that the canonical RRM1 RNA binding surface may play important functions in the binding and folding of substrates lacking UUU-3′OH. We therefore decided to test point mutants in the canonical RRM1 RNA binding surface of the La domain (Fig. 4*A*) in our assay. We found that mutation of the conserved RNP aromatic residues (hLa 1–235 Y114A/F155A; $k_{ann1} = 0.0041\ s^{-1}$) or of the basic residues of RRM1 β2-β3 loop3 (hLa 1–235 loop 3 = hLa 1–235 R142A/R143A/K148A/K151A; $k_{ann1} = 0.0012\ s^{-1}$) resulted in a loss of strand annealing activity compared with the 1–235 control ($k_{ann1} = 0.025\ s^{-1}$; Fig. 4*B*). Both mutants also lacked strand displacement activity. Notably, our previous work has shown that these point mutations have negligible effects on the binding of UUU-3′OH-containing RNAs, suggesting that neither set of mutations cause significant misfolding of the RRM (13, 14). Because our work here also suggested that the RRM1 α3-helix and subsequent linker participates in RNA chaperone activity, we also mutated basic residues in the α3-helix (1–235 α3 = hLa 1–235 K185A/K191A/K192A) and tested this in our assay. Like the point mutations to the basic residues of RRM1 β2-β3 loop3, this mutant was also defective in both strand annealing ($k_{ann1} = 0.0028\ s^{-1}$) and dissociation activity. Like the Y114A/F155A and β2-β3 loop 3 point mutants, this mutant was also active in RNA binding as determined by gel shift (data not shown). Taken together, these data suggest that the canonical RNA binding surface of RRM1, including the α3-helix particular to La proteins, forms the minimal functional requirement for La domain-associated RNA chaperone activity.

To test whether the point mutations in RRM1 and the α3-helix were defective only in the binding of RNA substrates, as we had hypothesized for deletion of the La motif, we also tested these point mutants at 5× concentration. Contrary to the hLa 105–202 and 105–229 mutants, however, each of these point mutants were incapable of strand annealing at 5× concentration (Fig. 4*C*; see rates, supplemental Table S3), suggesting that an intact RRM1 motif/α3-helix forms the minimal requirement for this activity. Notably, the strand dissociation of hLa 1–235 Y114A/F155A ($k_{S.D.} = 0.027\ s^{-1}$), but not of hLa 1–235 loop3 or hLa 1–235 α3, was rescued at 5× concentration, suggesting that the basic amino acids of the RRM1 β2-β3 loop3 and the α3-helix form the critical element for hLa strand dissociation.

hLa RRM1 α3-Helix Can Enhance RNA Chaperone Activity of an Unrelated RRM—Our results suggest that the α3-helix at the end of RRM1 of hLa is important for the strand annealing activity of this motif. Structural analysis of the hLa N-terminal domain indicates that this helix is relatively short (hLa amino acids 185–192) before becoming disordered, and both the α3 helix and unstructured region have a high number of basic residues (see "Discussion") (12). Notably, most of the basic residues in the helix (Lys-185 and Lys-192) project toward the canonical RNA binding surface of RRM1, as does a universally conserved aromatic residue (hLa Tyr-188) that stacks on the aromatic residue projecting from the RRM RNP2 motif (hLa Tyr-114) typically associated with canonical RRM-mediated RNA binding (39). Because the RNA recognition motif is a highly ubiquitous motif, we decided to test the hypothesis that this particular α3-helix might be capable of conferring strand annealing activity to an unrelated RRM by appending it to its C terminus. To test this, we chose the highly studied N-terminal RRM (U1A 1–102) from the snRNP-associated human U1A protein. Notably, the U1A protein also contains an α3-helix C-terminal to its RRM (starting around U1A amino acid 91), but this helix is shorter, has fewer basic amino acids, is in a different orientation with respect to the RRM, and begins after a longer linker sequence (5 amino acids *versus* 1) compared with the equivalent α3 helix in hLa (Fig. 5*A*) (40).

We first tested the U1A 1–102 fragment, consisting of the wild-type U1A RRM and C-terminal α3-helix (Fig. 5*B* and supplemental Table S4) in the RNA chaperone assay and found that it harbored a low level of strand annealing activity compared with RNA alone (U1A 1–102 $k_{ann1} = 0.0072\ s^{-1}$ *versus* RNA alone $k_{ann1} = 0.0039\ s^{-1}$). Based on our work on human La, we hypothesized that this low level of U1A RRM activity might be dependent on its own α3-helix, and so also we tested U1A 1–88, in which we deleted this helix and some of the intervening linker, but this mutant had approximately the same low level of strand annealing activity as U1A 1–102 (U1A 1–88 $k_{ann1} = 0.0083\ s^{-1}$). To test the importance of the α3-helix of hLa in RNA chaperone activity, we appended the hLa α3 helix and the first 10 unstructured residues (hLa 185–202) onto the U1A RRM (U1A 1–88 hLa α3) and tested this in the assay. Consistent with our hypothesis, we found that U1A 1–88 hLa α3 showed enhanced RNA strand annealing activity compared with U1A 1–88 and U1A 1–102 (U1A 1–88 hLa α3 $k_{ann1} = 0.0209\ s^{-1}$; Fig. 5*B*). No U1A variants or the U1A-hLa fusion showed strand displacement activity. From these data, we con-

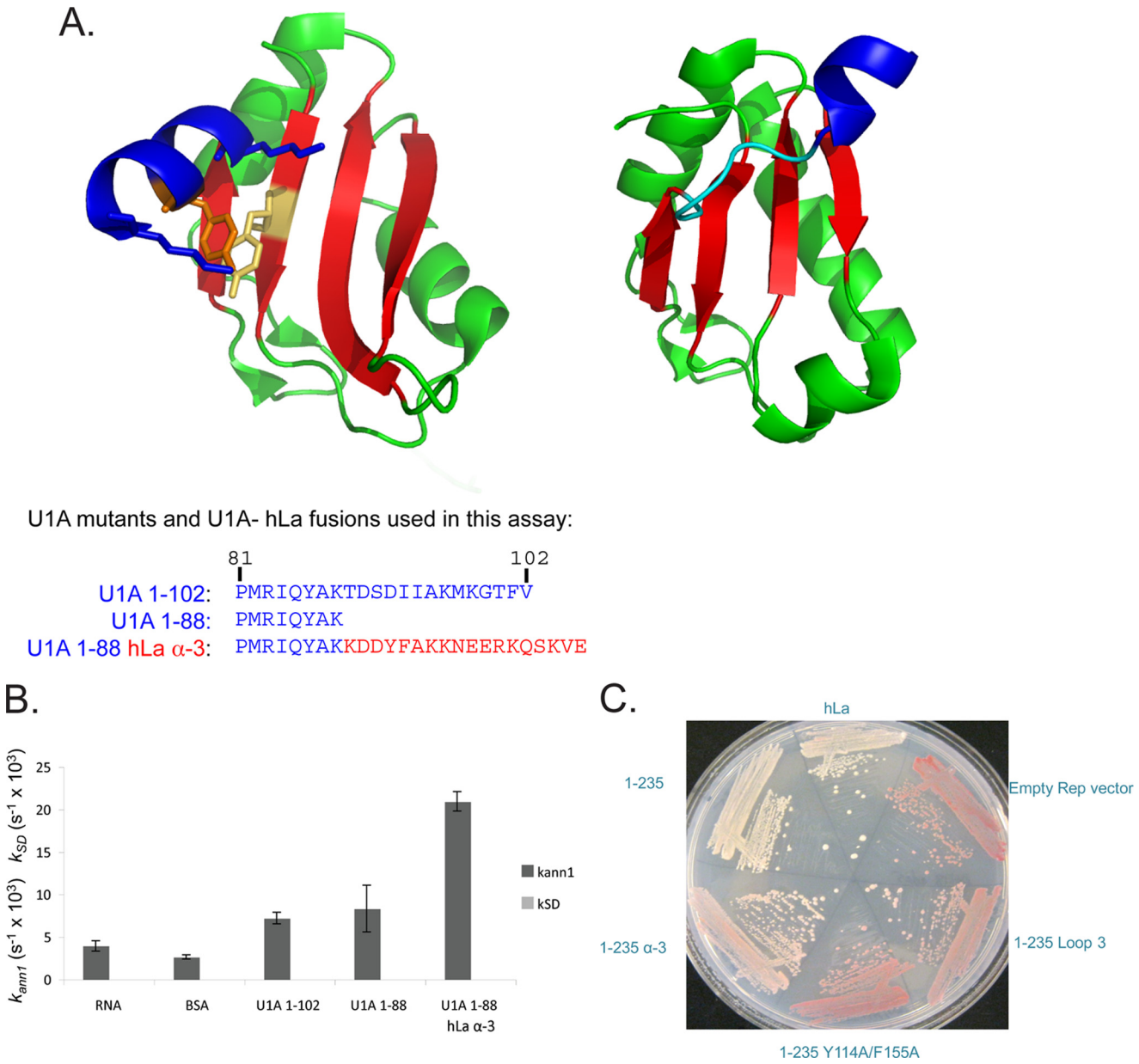

FIGURE 5. **The α3-helix of hLa can enhance RNA chaperone activity of the U1A protein *in vitro* and is required for tRNA mediated suppression via a mutated pre-tRNA *in vivo*.** *A*, comparison of the hLa RRM1(*left panel*) and human U1A N-terminal (*right panel*) RRMs. β-Sheet strands are shown in *red*, α3-helices are in *blue*, and the spacer between β4 and α3 (0 amino acids in hLa; 5 amino acids in U1A) are in *cyan*. The stacking interaction between the α3-helix and β-sheet surface of hLa is also shown, with Tyr-187 and Tyr-114 in *orange* and *gold*, respectively. *Inset*, amino acid identities for U1A and U1A/hLa proteins tested. *B*, k_{ann1}, $k_{S.D.}$, and k_{ann2} rates for U1A 1–88, U1A 1–102 (wild-type), and the U1A/hLa fusion are given. *C*, point mutation of the canonical hLa RRM1 RNA binding surface (1–235 Y114A/F155A or 1–235 Loop-3) or the α3-helix (1–235 α3) causes defects in La-dependent rescue of tRNA-mediated suppression in *S. pombe*, compared with wild-type hLa or the wild-type hLa La domain (1–235).

clude that the α3-helix found at the end of the hLa RRM1 enhances the strand annealing activity of this RRM and that this α-helix can enhance the strand annealing of an unrelated RRM when appended to its C terminus.

Mutants Defective in RNA Chaperone Activity in Vitro Are Defective in Rescue of Mutated pre-tRNA in Vivo—La function in tRNA processing can be assessed *in vivo* using a red-white suppressor tRNA assay in *S. pombe* in which La-dependent rescue of a mutated suppressor tRNA results in suppression of red pigment accumulation via readthrough of an in-frame stop codon in the *ade6-704* allele (41). We transformed plasmids encoding either wild-type hLa, hLa 1–235, or our RRM1 point mutants shown to be defective in *in vitro* RNA chaperone activity into a La null *S. pombe* strain (*sla1*$^-$) containing an integrated suppressor tRNA allele previously characterized to require La for maturation and suppression of red pigment accumulation. Mutation of the RRM1 RNP aromatic residues (hLa 1–235 Y114A/F155A) or the basic amino acids of β2-β3 loop3 (hLa 1–235 loop3) showed defects in rescue of suppression of red pigment accumulation, compared with wild-type hLa and the N-terminal hLa 1–235, which showed comparable activity (Fig. 5*C*). Notably, mutation of basic residues in the α3-helix of RRM1 (hLa 1–235 K185A/K191A/K192A) also showed defects in suppression of red pigment accumulation *in vivo*. These data

are consistent with a model in which mutations that cause defects in RNA strand annealing and dissociation *in vitro* also cause defects in La-dependent rescue of mutated pre-tRNAs *in vivo*, suggesting that La may act directly as an RNA chaperone *in vivo* for misfolded pre-tRNAs.

DISCUSSION

In addition to a hypothesized function in the folding of misfolded pre-tRNAs ending in UUU-3′OH, La proteins have also been hypothesized to function as an RNA chaperone for mRNAs lacking this motif by enhancing the correct folding of complex 5′-UTR structures required for optimal translation. In this work, we show that both human La and *S. pombe* La harbor RNA strand annealing and dissociation activity and as such can be considered genuine RNA chaperones. Focusing on the conserved N-terminal La motif and RNA recognition motif from human La, we mapped strand annealing to the RRM1 and adjacent α3-helix, with the predicted disordered region between RRM1 and RRM2 of human La enhancing this activity. Point mutation to the canonical RRM RNA binding surface (RNP mutations Y114A/F155A), a basic loop shown to be important in tRNA binding (β2-β3 loop 3), or to basic residues in the α3-helix all inhibited strand annealing activity at both 1× protein concentration (saturating concentration for wild-type hLa) and 5× concentration *in vitro* and La-dependent rescue of a mutated pre-tRNA *in vivo*. For strand dissociation, mutation of conserved basic residues of RRM1 (β2-β3 loop 3 or the α3 helix) resulted in a loss of activity at both 1× and 5×, whereas the Y114A/F155A mutant was still active in strand displacement at 5× protein concentration, suggesting that the basic residues of the RRM are more highly associated with this function. Our data are consistent with the conserved RRM1 and adjacent α3-helix and linker representing the minimum element required for La domain associated RNA chaperone activity.

Although strict structural requirements for RNA chaperone activity in other proteins have not been rigorously delineated, it has been noted that RNA chaperones frequently contain regions predicted to be disordered, and these regions have been hypothesized to function in RNA chaperone activity through entropy transfer via RNA binding-dependent folding (37). Notably, we have found that the unstructured region between the RRM1 α3-helix and RRM2 of hLa contributes to strand annealing associated with the conserved La domain. Furthermore, the RRM1 α3 helix and subsequent unstructured region between RRM1 and RRM2 have a high propensity of basic amino acids (10 of 24 or 42% of residues 185–209 K or R; pK_a = 9.78), a feature also commonly found in other RNA chaperones (42). Although the well ordered La motif, RRM1 and RRM2 from human La have been well studied both structurally and biochemically (9–12, 43), the function of unstructured regions of La proteins is less well understood. Recent work indicates that in addition to the modular La domain, the disordered region C-terminal to RRM1 in the *S. cerevisiae* La homolog Lhp1p is required for the correct folding of pre-tRNA anticodon stems *in vivo* (15) and in human La in binding to the hepatitis C IRES (31).

La proteins in higher eukaryotes (including humans) often contain a second RRM (RRM2 or RRMc), which like RRM1 is also followed by a C-terminal α-helix (previously characterized in human and *S. pombe* La to harbor a nuclear retention element (44, 45)), although this is in a different orientation to the α3 helix found after RRM1 (43). In human La, the α-helix after RRM2 is also followed by a predicted disordered region, previously described as the short basic motif, which has been shown to interact with the 5′ end of the pre-tRNA, with access of this region to the pre-tRNA 5′ leader controlled by the phosphorylation of hLa at serine 366 (38). We found that both the N- and C-terminal halves of human La were capable of strand annealing and strand dissociation, although with rates of strand annealing that were lower than that of the full-length protein (Fig. 3*B* and supplemental Table S2). Although the C-terminal half of human La is less conserved and is largely absent in La proteins of lower eukaryotes, it would be interesting to test whether RRM2 and its associated C-terminal α-helix and subsequent disordered region may function equivalently to the La domain counterparts found in the La proteins of all species that contain them.

We used three different Cy5-containing RNA substrates containing varying 3′ ends to test the importance of the UUU-3′OH motif in La-dependent RNA chaperone activity. All three substrates showed similar RNA association and dissociation rates, consistent with the UUU-3′OH-dependent RNA binding not playing a critical role in these processes. These data are consistent with models in which La-dependent RNA chaperone activity plays an important function in the translation of mRNAs lacking the UUU-3′OH motif, as well as previous data indicating that the UUU-3′OH-independent RNA binding mode by which La engages the main body of pre-tRNAs (*i.e.* through RRM1 β2/β3 loop 3) is also important for RNA chaperone activity (14). Crystallographic and biochemical data have shown that the La motif is primarily responsible for UUU-3′OH binding and associated 3′ pre-tRNA trailer protection from exonucleases (11, 12), but this motif in isolation was found to be incapable of supporting RNA chaperone activity (Fig. 3 and supplemental Table S2). However, significant drops in strand annealing and dissociation rates were nonetheless observed upon deletion of the La motif from the greater context of the La domain (compare 1–235 with 105–229; Fig. 3*B*). Incubation of the RNA substrates with a 5-fold increase in 105–229 protein was able to rescue significant strand annealing and dissociation, suggesting that with respect to RNA chaperone activity, the La motif may function as an accessory domain that enhances the binding of La to RNA targets, even in the absence of the UUU-3′OH motif. Consistent with this possibility, various nucleotide substitutions in the UUU-3′OH motif of short RNAs decrease hLa affinity for these targets by at most 10-fold (11), and the deletion of the UUU-3′OH-containing trailer of a pre-tRNA reduced affinity for the respective tRNA sequence by only 3-fold (14). It is therefore possible that the La motif may play an important role in the folding of RNA targets irrespective of these ending in UUU-3′OH by enhancing the affinity of La for them.

Recently the study of La motif-containing proteins has expanded into the investigation of La-related proteins (LARPs),

which share a conserved La motif (and often an RRM1 or RRM1-like domain) with genuine La proteins but have evolved independent functions (1, 46). RNA chaperone activity has not yet been formally investigated in the LARPs, but it is interesting to note that the *Tetrahymena thermophila* telomerase associated protein p65, a La motif containing member of the LARP7 family, is active in the remodeling the telomerase RNA and required for the assembly of the telomerase RNP (47). Based on secondary structure prediction (48), there is a weak prediction that the regions immediately following the RRM1 or RRM1-like motifs from the human LARPs HsLARP1, HsLARP4A, HsLARP6 and HsLARP7 also form α-helices (data not shown), but further structural and biochemical investigation of the LARPs will have to be performed before it can be concluded that any of these may harbor RNA chaperone activity or may function *in vivo* as RNA chaperones.

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