The La Motif and the RNA Recognition Motifs of Human La Autoantigen Contribute Individually to RNA Recognition and Subcellular Localization*

The human La autoantigen (hLa) protein is a predominantly nuclear phosphoprotein that contains three potential RNA binding domains referred to as the La motif and the RNA recognition motifs RRMs 1 and 2. With this report, we differentiated the contribution of its three RNA binding domains to RNA binding by combining in vitro and in vivo assays. Also, surface plasmon resonance technology was used to generate a model for the sequential contribution of the RNA binding domains to RNA binding. The results indicated that the La motif may contribute to specificity rather than affinity, whereas RRM1 is indispensable for association with pre-tRNA and hY1 RNA. Furthermore, RRM2 was not crucial for the interaction with various RNAs in vivo, although needed for full-affinity binding in vitro. Moreover, earlier studies suggest that RNA binding by hLa may direct its subcellular localization. As shown previously for RRM1, deletion of RNP2 sequence in RRM2 alters nucleolar distribution of hLa, not observed after deletion of the La motif. Here we discuss a model for precursor RNA binding based on a sequential association process mediated by RRM1 and the La motif.

The La protein is a phosphoprotein expressed in a variety of different organisms (for review, see Refs. 1 and 2) and was first discovered as an autoantigen in rheumatic diseases (3, 4). La is involved in a number of RNA metabolic pathways and interacts with all classes of transcripts. La is probably the first factor that interacts with RNA polymerase (pol)1 III precursor transcripts such as pre-tRNAs to guide these molecules through their initial steps of maturation (1, 2, 5, 6). Interaction of La with pol III transcripts occurs by the specific recognition of a 3’- (UUU)-OH terminus unique to these RNAs (7–9). Recently, it was shown that the yeast La homologue Lhp1p acts as an RNA chaperone (10). It is assumed that Lhp1p keeps attached RNA in a specific shape and protects it from unspecific or premature cleavage and degradation (11). La also interacts with RNA pol II products such as sn-/snoRNA. Here, too, it is involved in the stabilization of precursors and in snRNP formation (12–14). In the case of yeast U3 snoRNA, Lhp1p binds the processing intermediates after partial maturation, which produces a terminal poly(U) sequence (12, 50). Besides its interaction with cellular mRNAs, such as histone mRNA (15) and telomerase mRNA (16) or TOP-mRNAs (17, 18), La binds to a variety of viral RNAs, such as those of human immunodeficiency virus (19), polio virus (20), and hepatitis B and C virus (21–23).

The N terminus of La is well conserved from yeast to humans, whereas the human La protein gained an additional C-terminal domain absent in the yeast Lhp1p (2, 24). Recent structural data revealed that the N-terminal La motif does not adopt an RRM structure but folds into a winged helix motif, whereas the central and C-terminal RRMs (RRMs 1 and 2, respectively) resemble RNA recognition motifs (25–27). We have shown that the RNP2 signature of the RRM1 is of general importance for RNA binding (28, 29). Others reveal that the La motif is supposed to reconcile the recognition of UUUOH and specificity of binding (2, 27). The involvement of this particular domain may be determined by the RNA substrate, because it is involved in pre-tRNA recognition (2, 24) but seems dispensable for binding of some viral RNAs, such as hepatitis B and C virus RNAs (28, 30).

In addition, human La autoantigen (hLa) also contains a putative Walker A motif and a dimerization domain as well as a conserved basic region, nuclear retention elements, and a nuclear localization signal and nucleolar localization signal for nuclear and nucleolar targeting, respectively (2, 24, 29). Several studies have described the subcellular localization of hLa by immunofluorescence as largely confined to the nucleus (Ref. 2 and references therein), except for certain situations, such as apoptosis (31, 32) or certain viral infections (33–35). In contrast to earlier studies (36, 37), others have shown that subcellular and subnuclear distribution may well be mediated by phosphorylation (38, 39). In addition, we have recently shown that the nucleolar localization signal, together with the RRM1 motif, are involved in nuclear trafficking of hLa (29).

In the present study, we compared the involvement and dynamics of the La motif and RRMs 1 and 2 of hLa in pre-tRNA recognition by analyzing different internal deletion mutants. For this purpose, we used gel shift analysis, UV cross-links, and co-immunoprecipitations as equilibrium systems. Further-
more, real-time determination of dynamic binding parameters was performed using surface plasmon resonance (SPR) technology. In all assays, RR1M is indispensable for pol III RNA binding, both in vitro and in vivo. Interestingly, the La motif is not essential for high affinity pre-tRNA binding in vitro but appears to mediate specificity and/or protection of associated RNAs in vivo. Moreover, RR2 contributes to overall affinity and is also involved in pre-tRNA binding in vivo. Additionally, we used GFP fusion proteins to show that specific mutations of RRMs 1 and 2 (but not of the La motif) lead to partial nuclear accumulation of La. Particularly, the acquirement is restricted to yield a mean difference of 1000–1300 RU, where 1000 a negative control. Protein attachment to the chip surface was manually and is also involved in pre-tRNA binding in vivo. All four flow cells without La protein were used as a reference. Data were evaluated with the Biacore 3000 software (BIAcore), and the obtained sets of sensorgrams were fitted into Langmuir’s model ratio of 1:1 binding. Calculated $\chi^2$ values were always below 0.8, indicating a reliable representation of experimental data by the chosen binding model. $R_{max}$ values were in the expected ranges, and residuals had a mean deviation from fitted graphs of generally $<1.0$ (not shown).

Estimated $k_d$ of a given protein complex was calculated as $t_{1/2} = \ln(0.5) / k_d$. Note, we included a complete regeneration step between every single measurement to exclude the possibility that still-associated RNA could interfere with subsequent experiments. This was included in all experiments to ensure standardized and comparable results.

**Experimental Procedures**

**Recombinant Proteins, Gel Shift Analysis, UV Cross-links, Co-immunoprecipitation, GFP Fusions, and Immunofluorescence—**Previous publications (28, 29) give details for expression vectors as well as proteins and relevant methods. Electrophoretic mobility shift assays (EMSAs) and UV cross-links contained 50 nM protein. GFP fusion proteins were produced using the strategy described previously (29). For confocal microscopy of living cells, 7 x $10^5$ HeLa cells were plated in Lab-Tek double chamber systems (Nunc) and transfected on the next day with 1 $\mu$g of respective GFP-hLa plasmid using FuGENE 6 (Roche Applied Science). 12–16 h post-transfection, DrsQ5 (Alexis Biochemicals) was added to the cells, and confocal microscopy was performed with a LCM501 device (Leica). For co-immunoprecipitations, 293T cells were transfected and prepared as described previously (29). The transfection efficiency of the 293T cells with calcium phosphate was much higher than that of HeLa cells with FuGENE 6 (not shown). Note, independent of the transfected GFP-hLa construct, mean numbers as well as overall shapes of nuclei were not changed in transfected cells (data not shown).

**In Vitro Transcription and Purification of RNA—**Plasmids, DNA templates, and protocols used for in vitro transcription of (pre-)tRNA were described previously (29). Additional primers for the generation of mature tRNA were: 5'-CAA TCG ATT AAT ACC ACT CAC TAT AGT TTC CCG AGT GTA GTG TTC ATG ACA G-3' (TT promoter sequence underlined) and 5'-TGG TGG TGT TCG CCG GGT TGG TAC A-3'. Where unlabeled RNAs were synthesized, the samples were suspended in 2 volumes of sample buffer, heated (95 °C, 10 min), and separated on a prerun (1 h) denaturing 7% urea, 15% polyacrylamide gel with 600 V for 3 h. Elution of RNAs by UV shadowing was performed according to our group (28, 29) give details for expression vectors as well as overall shapes of nucleoli were not changed in transfected cells (data not shown).

To understand the interaction of hLa with pre-tRNA, it is crucial to know how the different RNA binding surfaces contribute to the binding. Recently, we have shown that RR1M is essential for interaction with different RNAs (28, 29), revealing that this motif is of general importance for RNA binding both in vitro and in vivo. In this report, we extended this analysis to a comparison of all known RNA binding motifs of hLa.

As outlined in Fig. 1A, internal deletion mutants of recombinant hLa were constructed to either lack the La motif completely (hLa-Δ1) or the RR2C or -1 consensus sequences of RR1M (hLa-Δ2, hLa-Δ3) or RR2M (hLa-Δ4, hLa-Δ5). His-tagged, recombinant hLa was expressed in Escherichia coli and purified by nickel-nitrilotriacetic acid chromatography. Proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 1B) and Western blotting (not shown) to verify usage of equal amounts of recombinant hLa protein. Proteins were analyzed for the binding of in vitro transcribed radioactively labeled pre-tRNA in multiple assays. First, we established EMSAs with stringent conditions and verified that hLa interacted specifically with pre-tRNA whereas mature tRNA was barely bound (Fig. 2A, lanes 2–5 and 7–10, respectively).

**Results**

The La Motif and RRMs 1 and 2 of Human La Contribute Differently to Pre-tRNA Binding in Vitro—To understand the interaction of hLa with pre-tRNA, it is crucial to know how the different RNA binding surfaces contribute to the binding. Recently, we have shown that RR1M is essential for interaction with different RNAs (28, 29), revealing that this motif is of general importance for RNA binding both in vitro and in vivo. In this report, we extended this analysis to a comparison of all known RNA binding motifs of hLa. As outlined in Fig. 1A, internal deletion mutants of recombinant hLa were constructed to either lack the La motif completely (hLa-Δ1) or the RR2C or -1 consensus sequences of RR1M (hLa-Δ2, hLa-Δ3) or RR2M (hLa-Δ4, hLa-Δ5). His-tagged, recombinant hLa was expressed in Escherichia coli and purified by nickel-nitrilotriacetic acid chromatography. Proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 1B) and Western blotting (not shown) to verify usage of equal amounts of recombinant hLa protein. Proteins were analyzed for the binding of in vitro transcribed radioactively labeled pre-tRNA in multiple assays. First, we established EMSAs with stringent conditions and verified that hLa interacted specifically with pre-tRNA whereas mature tRNA was barely bound (Fig. 2A, lanes 2–5 and 7–10, respectively). Without these conditions, we subsequently analyzed the interaction of hLa mutants with pre-tRNA in vitro. Unexpectedly, deletion of the La motif (hLa-Δ1) (Fig. 2A, lane 3) did not impair binding of pre-tRNA, which was tested with multiple protein preparations and is validated by other methods (see next page).
For unexplained reasons, the smeary appearance of hLa-Δ1/pre-tRNAVal RNPs was always observed, independent of the hLa-Δ1 protein preparation used. One explanation might be that the overall structural heterogeneity was increased after deleting amino acids 11–99. We calculated some of the theoretical biophysical parameters using the ExPASy server and noticed that some parameters were changed. The pI of hLa-Δ1 was more acidic (pI 6.68) than the pI of hLa-WT (pI 7.0), the aliphatic index was lowered when the La motif was deleted (aliphatic index: hLa-Δ1 = 63.08; hLa-WT = 70.32). We do not know whether these changes account for the smeary appearance of the hLa-Δ1-pre-tRNA complex or whether it might depend on the RNA substrate bound. This possibility should be considered, because we did not observe a smeary appearance for the hLa-Δ1-hepatitis B virus RNA complexes (28). Deletion of either one of the RNP consensus sequences in RRM1 abolished any RNA binding activity of hLa-Δ1 and hLa-Δ3 proteins (Fig. 2B, lanes 4 and 5), respectively. These results unveil that both RNP signatures in this domain are essential for in vitro binding of pre-tRNAVal. Mutations in RRM2 had diverse effects, as deletion of RNP2 (hLa-Δ4) abolished the interaction with pre-tRNAVal, although complex formation after deletion of RNP1 (hLa-Δ5) was weakly affected (Fig. 2B, compare lanes 6 and 7). These data imply that RRM1 and 2 are required for binding, whereas the La motif seems to be of minor importance.

Next we performed similar studies with UV cross-links, which may be more suited to detecting short-lived interactions and estimating the ability of hLa to protect bound RNAs against RNase treatment. As expected, hLa-WT bound pre-tRNAVal in a dose-dependent manner (Fig. 2C, panel 1). Again, deletion of the La motif (hLa-Δ1) did not abolish binding of pre-tRNAVal but caused a reduction in signal intensities when compared with hLa-WT (Fig. 2C, compare panels 1 and 2). It should be noted that, in contrast to EMSAs, signal intensities in UV cross-linking assays are sensitive to RNase A applied in the experiment. The signal intensity of hLa-pre-tRNA complexes depends on the number of covalently bound radiolabeled UTPs and the accessibility of the bound RNA molecule to the degradation by RNase A added in the experiment. Therefore, it is important to note that the α-helical part of the wing helix motif formed by the La motif is in close proximity to the RRM1 and may be required to protect RNAs bound via the RRM1 (26, 27). The combination of both the EMSA and the UV cross-linking experiments suggests that the La motif is not essential for binding of pre-tRNAVal in vitro but rather is of importance for the protection of bound pre-tRNAs associated with the hLa protein. Mutations of either RNP2 or -1 in RRM1 abolished any RNA binding activity (hLa-Δ2, hLa-Δ3, respectively) (Fig. 2C, panels 3 and 4), confirming the data presented above (Fig. 2B) and previously (29). Weak signals were detectable with hLa-Δ4 (ARNP2 in RRM2), potentially indicative of a short-lived interaction, because this was not observed in EMSAs (compare Fig. 2C, panel 5, with Fig. 2B, lane 6). Deletion of RNP1 in RRM2 strongly reduced the ability of hLa-Δ5 to form stable RNPs (Fig. 2C, panel 6). Deviation in signal intensities with hLa-Δ5 in EMSAs and UV cross-links (Fig. 2B, lane 7 and 2C, panel 6) also imply that RNP1 is not essential for pre-tRNA binding per se and that associated RNAs are less protected against RNases in the absence of functional RRM2. In summary, the combination of two independent methods reveal distinct contributions of the RNA binding surfaces of hLa to binding and/or protection of pre-tRNAVal in vitro.

Real-time Determination of Binding Parameters—To determine the dynamics of complex formation of hLa with pre-tRNA, we performed BIAcore SPR analysis. In these assays, we focused on hLa-Δ1, hLa-Δ2, and hLa-Δ4, as these mutations affect the individual RNA binding motifs.

We used the same hLa proteins mentioned previously (Figs. 1 and 2) together with in vitro transcribed, unlabelled and gel-purified pre- and mature tRNAVal. First, His-tagged hLa was captured onto a nickel-coated nitrioltriatic acid sensor chip surface, generating a stable association and only rarely a drifting baseline. Afterward, pre- or mature tRNAVal was injected in several different concentrations, and the kinetics of association and dissociation were monitored. Oriented capturing of recombinant hLa via the N-terminal His tag generated an active and specific surface. Pre-tRNAVal injected at different concentrations gave a concentration-dependent response (Fig. 3A). BIAcore data were corrected for noise artifacts and fitted to a model ratio of 1:1 interaction of hLa and pre-tRNA. The calculated difference between experimental data and fit ($\chi^2 < 0.8$) indicates that the 1:1 ratio interaction model is indeed adequate to describe the binding situation. More complex models taking into account heterogeneity, two-step reactions, and mass transport phenomena did not improve the fit and did not indicate rate-limiting structural changes of hLa in the course of pre-tRNA association (data not shown).

Complex formation of hLa-WT and pre-tRNAVal occurred with a $k_{\text{off}}$ of $6.9 \times 10^{-5} \text{M}^{-1} \text{s}^{-1}$ and a $k_{\text{on}}$ of $4.9 \times 10^{-9} \text{s}^{-1}$, yielding a $K_D$ of $7.3 \times 10^{-9} \text{M}$ (Fig. 3, A and E). The calculated half-life of this complex is 141 s. The high affinity of hLa for pre-tRNAVal is similar to other reported $K_D$ values in the nanomolar range, e.g. for pre-tRNAAsp, or hY-1 and hY-4 RNAs (19, 41, 42). Furthermore, captured hLa-WT retained its specificity in SPR experiments, because the affinity for mature tRNAVal ($K_D = 1.4$}
Different studies show individual contributions of the La motif and RRMs 1 and 2 of hLa to pre-tRNA<sub>Val</sub> binding in vitro. Specificity of EMSAs is shown in A, as mature tRNA<sub>Val</sub> was hardly bound when compared with pre-tRNA<sub>Val</sub> (as indicated under “Experimental Procedures”). 100 ng of recombinant hLa-WT were incubated with labeled pre-tRNA<sub>Val</sub> and with increasing amounts (2.5, 5, 7.5, 10 μg, respectively) of poly(C); reaction without hLa on lanes 1 and 6. B, 100 ng of each of the proteins outlined in Fig. 1A were incubated with labeled pre-tRNA<sub>Val</sub> (plus 2 μg poly(C)) and electrophoretically separated; reaction without hLa on lane 1. C shows binding analysis of the different hLa mutants with pre-tRNA<sub>Val</sub> in UV cross-linking experiments with the same conditions. Increasing amounts (25, 50, 100, 150 ng, respectively) of proteins were incubated with labeled pre-tRNA<sub>Val</sub>; UV cross-linked, and separated by 12.5% SDS-PAGE. All gels were simultaneously exposed for the same time.

As expected from previous data (29), deletion of RNP2 in RRM1 (GFP-hLa-D1) abolishes any association with pre-tRNA<sub>Val</sub> at all concentrations tested (Fig. 3A). Interestingly, no other domain showed compensatory effects that could counterweigh deletion of RNP2 in RRM1. Thus, pre-tRNA binding by hLa is first established by RRM1 before any other motif contributes to the interaction.

In accordance with EMSAs and UV cross-links (see above), deletion of RNP2 in RRM2 (GFP-hLa-D4) caused a decreased affinity (K<sub>d</sub> = 2.1 × 10<sup>-8</sup> μM) as a result of a 2-fold lowered association rate constant and a slightly faster dissociation (Fig. 3). A and E). In addition, a shortened half-life of the RNP complex was caused by this mutation, indicating that RNP2 in RRM2 is needed for hLa-WT to bind pre-tRNA<sub>Val</sub> with full affinity and comparable binding characteristics. Taken together, the diverse in vitro binding studies indicate that RRM1 and 2 are required for high affinity binding and suggest that both La motif and RRM2 participate in protection of pre-tRNAs.

Contributions of the La Motif, RRMs 1, and RRMs 2 to RNA Binding in Vivo—The effect of mutations in hLa on its RNA binding activity in vivo was studied on 293T cells transfected with plasmids encoding for GFP fusions of the constructs described above. Co-immunoprecipitations (co-IPs) from cell lysates were performed to detect GFP-hLa-associated pre-tRNA<sub>Tyr</sub> and pre-tRNA<sub>Val</sub> (and pre-tRNA<sub>Aeu</sub> and pre-tRNA<sub>Aan</sub>, data not shown) and hY1 RNA. Fig. 4A shows efficient and specific precipitation of GFP-hLa proteins. Note that only faint amounts of GFP-hLa-WT were precipitated in the control experiment and that no endogenous La was detectable in any IP pellet. Furthermore, GFP-hLa proteins are likely prone to minor degradation during preparation, as additional bands appear in the pellets. Compareable amounts of IgGs were applied in specific IP’s, as is visible by levels of IgG<sub>H</sub> and IgG<sub>L</sub> that were co-detected due to antibody species.

In parallel, RNAs were prepared from all fractions and blotted and detected by multiple hybridizations with labeled oligo-nucleotides (Fig. 4B). As expected, GFP-hLa-WT associated with Y1 RNA and precursors of tRNA<sub>Tyr</sub> and tRNA<sub>Val</sub> (and tRNA<sub>Aeu</sub> and tRNA<sub>Aan</sub>, data not shown) but not with the respective mature forms. Hence, GFP-hLa-WT interacts with the same RNAs described to be bound by endogenous hLa.

As shown previously (29), deletion of RNP2 in RRM1 (GFP-hLa-D2) causes a loss of detectable pol III transcript binding activity in vivo, showing the general importance of this motif. We did not observe any co-precipitation of pre-tRNA<sub>Tyr</sub> or pre-tRNA<sub>Val</sub> (and tRNA<sub>Aeu</sub> or tRNA<sub>Aan</sub>, data not shown). Further,
signals for associated Y1 RNA were drastically reduced and hardly above background levels. We conclude that this motif is required for association of hLa with RNA pol III transcripts in vitro and in vivo. Although virtually no RNA was precipitated in co-IPs with hLa-H90042 or IgG-control, signal intensities of pre-tRNAs in supernatants were found to be weaker than those in starting materials (Fig. 4B), probably a result of minor degradation during the experiment.

Deletion of the La motif does not completely abolish the ability to interact with pol III products in vivo. The amounts of GFP-hLa-H90041 co-precipitated Y1 RNA and various pre-tRNAs were strongly reduced (pre-tRNAVal) or undetectable (pre-tRNAVal) when compared with RNA levels associated with GFP-hLa-WT (Fig. 4B). In contrast to the in vitro results shown above, the La motif is thus critical for pre-tRNA binding in vivo.

Fig. 3. In vitro real-time binding kinetics of human La proteins with pre-tRNAVal reveal different functions of La motif and RRMs 1 and 2. BIAcore experiments were performed and evaluated according to instructions described under “Experimental Procedures.” Representative sensorgrams are shown for hLa-WT (A), hLa-Δ1 (B), hLa-Δ2 (C), and hLa-Δ4 (D). Molar concentrations of the supplied RNAs are indicated. Response units of empty flow cell (loaded with 9.4 nM RNA) is shown as a comparison. The summary of evaluated data is shown in E. Standard deviations are given for each rate of $k_a$, $k_d$, and calculated $K_D$. $n$ is the number of individual repetitions. For the fitting according to Langmuir’s 1:1 ratio binding, software-internal $R^2$ standards never exceeded values above 0.8 (not shown). $t$ gives the estimated half-life of given RNA-protein complexes. $K_D$ of hLa-WT for mature tRNAVal is $1.43 \times 10^{-7}$ (not shown). Note that after the deletion of the La motif (hLa-Δ1), residual amounts of RNA remain attached to the protein, as indicated by the curves (compare dissociation phases in A and B), in addition to a prolonged half-life of the complex.

The RNA Binding Surfaces Are Differently Involved in Subcellular Localization of GFP-hLa Proteins—Human La may facilitate subnuclear transport of pre-tRNAs, which are synthesized in the nucleoplasm (44) and processed at least partially in nucleoli (6, 45, 46). Thus, a feasible assumption would be that RNA binding may affect the localization of hLa, which is substantiated by the fact that RRM1 is of importance not only for RNA binding but also for subnuclear localization of hLa (29). With this report, we compared the effects of all three RNA binding surfaces on cellular distribution of GFP-hLa, thereby studying whether localization is mediated by RNA binding itself or restricted to functions of, specifically, RRM1.

HeLa cells were transiently transfected with GFP-hLa plasmids, and subcellular localization was analyzed by confocal laser-scanning microscopy of living cells. The majority of GFP-hLa-WT appeared evenly nucleoplasmic with varying intensities in nucleoli (Fig. 5A), indistinguishable from the distribution of endogenous hLa (29). Localization of GFP-hLa-Δ1 (Fig. 5B) was identical to GFP-hLa-WT, revealing that interactions or functions mediated by the La motif are without influence on subcellular localization. Deletion of RNP2 in RRM1 caused a prominent nucleolar accumulation of GFP-hLa-H90042 (Fig. 5C; see also Ref. 29). From these data, it appears as though parts of nucleoli do not contain GFP-hLa-Δ2, which was unnoticed before (29). In contrast to this RNP2 domain in RRM1, deletion of RNP1 did not cause nucleolar accumulation (Fig. 5D). Similarly, deletion of RNP2 (but not RNP1 in RRM2) caused a nucleolar accumulation (Fig. 5, E and F). However, as GFP-hLa-Δ4 had a moderate effect on RNA binding, its involvement in localization appeared diverse, because we observed both
nucleolar accumulation as well as a punctuated nucleolar appearance (Fig. 5E, upper and lower panels). These data show that nucleolar accumulation of hLa is caused by the interactions of RNP2 signatures of RRMs 1 and 2, whereas other domains seem insignificant for subnuclear localization.

**DISCUSSION**

With this report, we show that the different RNA binding surfaces of hLa contribute individually to the binding of precursor RNAs as well as to subnuclear localization. Human La-WT associated with pre-tRNA in all assays *in vitro* and *in vivo*, and SPR studies revealed a high affinity interaction. As shown elsewhere and herein, RRM1 is most critical for RNA binding, because mutation of this motif abolished association of various pre-tRNAs and hY1 RNA *in vivo* and hepatitis B virus RNA, hY4 RNA, and human immunodeficiency virus transactivation response element *in vitro* (28, 29, 41, 47). Remarkably, neither the La motif nor RRM2 was able to compensate for the loss of RRM1-mediated binding both *in vitro* and *in vivo*, indicating that a functional RRM1 is a prerequisite for the La motif and RRM2 binding to e.g. pre-tRNA.

The strong complex formation in BIAcore analysis and EMSAs reveals that the La motif is dispensable for pre-tRNA binding *in vitro*. Furthermore, the dynamics and affinities of pre-tRNA *Val* binding remained very similar to hLa-WT, indicating that this domain does not contribute significantly to the high affinity of this interaction *in vitro*. However, we cannot exclude the possibility that our BIAcore measurements are not sensitive enough to monitor the interaction between pre-tRNA and some of the aromatic residues located in the La motif found to be important for pre-tRNA binding (27). Importantly, co-IPs clearly show that the La motif strongly contributes to the productive association between GFP-hLa and pre-tRNA binding *in vivo*. From these data, we conclude that this domain confers specificity rather than affinity of hLa binding and/or protects bound pre-tRNA against ribonucleolytic degradation. The latter is in line with the RNase sensitivity of hLa-Δ1-associated RNAs in UV cross-links. These observations verify the importance of this motif for specific RNA binding of hLa but concurrently indicate that only the interplay of RNA binding surfaces allows the stable association with pre-tRNA.

Although in EMSAs, a loss of pre-tRNA binding was observed with hLa-Δ4, UV cross-links specified that an interaction occurred, suggesting a short-lived complex formation. This occurrence was entirely verified by SPR analysis. However,
co-IPs showed a moderate contribution of this motif to the association of hLa with pre-tRNA and hY1 RNA. Hence, in contrast to the La motif and RRM1, a minor involvement of RRM2 in pol III transcript binding is indicated.

The aforementioned data are used to generate a model of sequential association of RNA binding surfaces with pre-tRNA (Fig. 6). The starting point clearly is binding of RRM1 to RNA, because the presence of this domain is an indispensable prerequisite for the association. Because no other domain is able to compensate for loss of RRM1 functionality, this domain performs the critical step in association. As soon as this contact is provided in full-length hLa, RRM1 binding may control the duration of the interaction and allow other domains to bind specifically and with high affinity. This is implied by a prolonged half-life of the complex with hLa-Δ1. We cannot discriminate if association of the La motif or RRM2 is the next stage in complex formation. However, we believe that La motif-mediated selectivity of RNA binding is a critical aspect and demands its involvement in the initial stages of complex formation and thus may represent a quality control of associated RNAs.

In line with this model, deletion of the La motif has different effects: (i) it noticeably prolongs $t$ of formed complexes, (ii) it causes RNA to remain associated with the hLa-Δ1 in SPR experiments, and (iii) leads to much weaker binding of pre-tRNA in vivo. A two-step mechanism of RNA recognition is described for other RRM-containing proteins, such as spliceosomal U1A and neuronal HuD proteins (48) and may be a common feature of some members of this protein family. Likewise, RRM2 is required to establish pre-tRNA binding with full affinity in vitro, but it is not crucial for this interaction in vivo.

This finding is of importance, because this motif is not present in yeast La homologue Lhp1p, indicating that it has gained additional functions performed exclusively by vertebrate La, as e.g. recognition of LA95 RNA (49) or a role in internal ribosome entry site-mediated translation of hepatitis C virus RNA (30).

Although it was suggested that Lhp1p binds pre-tRNA at more than one site (10), the La motif and the RRM1 of the Trypanosoma brucei La homologue may act in a cooperative manner (27), and it is supposed that both motifs of hLa lie linearly across flanking surfaces or form a V-shaped binding pocket (26). Our data and those by others (10) imply that the La motif may regulate specificity and therefore discriminate RNA substrates, whereas RRM1 decides for overall binding. To our knowledge, the direct molecular association of the isolated La motif with UUUOH termini has not been shown and may therefore allow for speculation that the La motif and RRM1 form a V-shaped binding crevice to interact with RNA precursors. Importantly, it will be of interest to determine whether the La motif directly interacts with RNA or whether it protects RNA bound by RRM1. The latter case is supported by our in vivo and in vitro studies. However, both scenarios would fit with an RNA chaperone function of hLa. Although this model would demand structural changes of hLa in the course of RNA binding, our SPR-derived data suggest that structural changes, if occurring, are not rate-limiting.

Previously we have shown that mutation of RRM1 led to nucleolar accumulation, and we correlated that RNA binding of hLa may modulate its subcellular localization (29). Accordingly, the individual RNA binding domains of hLa may contribute differently to subcellular localization. Only deletions of RNP2 signatures in RRM1s 1 or 2 affected subnuclear localizations and caused enrichment in nucleoli, although to differing extents and probably to different subnuclear regions. However, GFP-hLa-Δ4 efficiently co-precipitated precursor RNAs, whereas GFP-hLa-Δ2 did not co-precipitate any precursor RNA monitored, implying that localization of hLa is influenced rather by specific functions of RNP2 sequences in RRM1 and 2 than by the general RNA binding activity. These observations offer the following possibilities for nucleolar accumulation of
mutant hLa proteins. (i) GFP-hLa-Δ2/-Δ4 are indirectly retained in nucleoli, potentially because the introduced mutations cause critical malfunctions in processes that affect nucleolar functions of hLa; (ii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is triggered by failure to interact with RNPs exclusively via RNP1 proteins in RRM1 and/or RRM2; or (iii) hLa interacts with other proteins via these domains required to traffic hLa-Δ2/-Δ4/nucleolar functions of hLa; (ii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is retained in nucleoli, potentially because the introduced mutations cause critical malfunctions in processes that affect nucleolar functions of hLa; (iii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is mediated by two RRRMs simultaneously (51). Taken together, our study indicates that the La motif and the RRRMs 1 and 2 differ in their contributions to precursor RNA binding and that the La motif and the RRM1 act in a sequential mode to interact with a specific RNA substrate.

Acknowledgments—we are grateful to E. Chan for the prokaryotic expression plasmid pET-human La, M. Bachmann for La monoclonal antibodies 3B9, 4B6, and SW5; H. Beier for tRNAVal plasmids; I. Ehlers expression plasmid pET-human La, M. Bachmann for La monoclonal mutant hLa proteins. (i) GFP-hLa-Δ2/-Δ4 are indirectly retained in nucleoli, potentially because the introduced mutations cause critical malfunctions in processes that affect nucleolar functions of hLa; (ii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is triggered by failure to interact with RNPs exclusively via RNP1 proteins in RRM1 and/or RRM2; or (iii) hLa interacts with other proteins via these domains required to traffic hLa-Δ2/-Δ4/nucleolar functions of hLa; (ii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is retained in nucleoli, potentially because the introduced mutations cause critical malfunctions in processes that affect nucleolar functions of hLa; (iii) nucleolar accumulation of GFP-hLa-Δ2/-Δ4 is mediated by two RRRMs simultaneously (51). Taken together, our study indicates that the La motif and the RRRMs 1 and 2 differ in their contributions to precursor RNA binding and that the La motif and the RRM1 act in a sequential mode to interact with a specific RNA substrate.

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