Molecular Characterization of the Human La Protein-Hepatitis B Virus RNA.B Interaction in Vitro*

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The La protein was recently identified as a host factor potentially involved in the cytokine-induced post-transcriptional down-regulation of hepatitis B virus (HBV) RNA. The La binding site was mapped to a predicted stem-loop structure within a region shared by all HBV RNAs, and it was concluded that the La protein might be an HBV RNA-stabilizing factor. To characterize the RNA binding mediated by the different RNA recognition motifs (RRMs) of the human La protein, several La deletion mutants were produced and analyzed for HBV RNA binding ability. The data demonstrate that the first RRM is not required for binding, whereas the RNP-1 and RNP-2 consensus sequences of the RRM-2 and RRM-3 are separately required for binding, indicating a cooperative function of these two RRMs. Furthermore, the results suggest that monomeric La disassembles into dimeric La upon binding of HBV RNA.B. By gel retardation assay the affinity of the wild type human La-HBV RNA.B interaction was determined in the nanomolar range, comparable to the affinity determined for the mouse La-HBV RNA.B interaction. This study identified small regions within the human La protein mediating the binding of HBV RNA. Hence, these binding sites might represent targets for novel antiviral strategies based on the disruption of the human La-HBV RNA interaction, thereby leading to HBV RNA degradation.

The human La protein is a 47-kDa phosphoprotein predominantly localized in the nucleus. It was first discovered as an autoantigen recognized by antibodies present in sera of patients suffering from systemic lupus erythematosus and Sjögren’s syndrome (1, 2). The La protein is a member of a large group of RNA-binding proteins containing RNA recognition motifs (RRMs) (3–8) and is implicated in several steps of RNA metabolism. Among the different La proteins identified in a variety of organisms, the N-terminal part is highly conserved (9). La was shown to co-immunoprecipitate with a number of small RNA molecules (10). A role for La in the termination of RNA polymerase III transcription has been described. It was shown that La interacts with RNA polymerase III transcripts such as pre-tRNA by binding to a small stretch of uridines at the 3’-end common to these transcripts and might be necessary for proper processing of these precursors (11–17). In addition, La is known to interact with a variety of viral and other cellular RNAs (18–26). La is also suggested to be involved in the cap-independent translation initiation of several viruses, including polio virus and hepatitis C virus (19, 27–29), and more recently evidence is growing that La stabilizes various RNAs, such as histone and hepatitis C and B virus RNA (22, 23, 25, 30, 31). At this time point it is not clear yet how La fulfills all of these different functions, however, assuming that this protein acts as a RNA chaperone, thereby stabilizing RNA structures, a function in these varied processes might be envisaged.

The human La protein contains three RNA recognition motifs (RRM) involved in the binding of RNAs (9), although the RRMs do not match very well to the RRM core structure identified by comparison of 70 known RRMs (7). The RNA recognition motif is one of the best characterized RNA binding motifs present as single or as multiple copies in a multitude of RNA-binding proteins. The three-dimensional structure was resolved for some RRMs, and the secondary structural elements composing the three-dimensional structure are β1α1β2/β3α2β4 folding (32, 33). Two conserved amino acid signatures referred to as RNP-1 and RNP-2 (RNP consensus sequence) are described as essential motifs for RNA binding. Maraia and coworkers established a model for La binding interactions of RNA with pre-tRNA (9). In this model the N-terminal RRM interacts with the 3’-end of pre-tRNA by binding to the poly-U stretch, the second and third RRM interacts with the pre-tRNA molecule, and the C-terminal part of La binds the 5’-end of the pre-tRNA (9). This C-terminal region contains a basic amino acid stretch and a Walker-A motif (34–36). After complete processing of tRNA, La is unable to interact with mature tRNA, signifying that the 5’- and 3’-ends of the pre-tRNA are essential for the recognition by La.

Studying the immune response against the hepatitis B virus using the HBV transgenic mouse model (37), Chisari and coworkers (38–42) have shown that injection of hepatitis B virus surface antigen-specific cytotoxic T lymphocytes into HBV transgenic mice led to suppression of all viral products by an non-cytotoxic pathway. This as of yet unresolved process was mediated by the cytokines interferon-γ and tumor necrosis factor-α. Furthermore, it was shown that these cytokines lead to post-transcriptional degradation of the viral RNA (43). In an attempt to identify host factors involved in degradation of the viral RNA, the mouse La protein (mLa) was identified as an HBV RNA-specific binding protein. A strong correlation between the cytokine-mediated disappearance of HBV RNA and the cytokine-induced processing of full-length mLa was ob-
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served, indicating that full-length mLa is involved in stabilizing HBV RNA (22, 23). The binding site of mLa was mapped to a predicted stem-loop structure within an element 91 nucleotides long (referred to as HBV RNA.B) located at the 5′-end of the post-transcriptional regulatory element of HBV, and the specificity of the interaction was confirmed by competition experiments using a variety of competitors (22, 23). In addition, the affinity of the interaction was determined in the nanomolar range, further indicating a specific mLa-HBV RNA.B interaction (23). The idea that this viral RNA element might represent a stabilizing element was further supported by a recent publication showing that the viral RNA was accessible to endoribonuclease cleavage near the mLa binding site (31). The endoribonuclease activity present in nuclear extracts of HBV transgenic mice was characterized, and it was shown that the HBV RNA substrates were more efficiently cleaved after induction of HBV RNA degradation and mLa processing. These data imply that the mLa protein is involved in the HBV RNA metabolism.

To gain better insight into the role of human La protein (hLa) in the viral RNA metabolism, we characterized the hLa-HBV RNA interaction in more detail. We evaluated the optimal conditions for the interaction between recombinant hLa protein and in vitro transcribed HBV RNA.B and determined the binding affinity. Our results indicate that multimeric hLa disassembles into monomers upon binding of HBV RNA.B. Furthermore, we investigated the requirement of each of the three RRM domains for binding by deleting the whole RRM-1 and the RNP-2 and RNP-1 motifs located in the second and third RRM. Deletion of short amino acid stretches gives us the ability to discover a cooperative binding mechanism between RRM-2 and RRM-3 and reduces the possibility of major structural changes triggered by this kind of manipulation. Our study shows that HBV RNA.B is bound by the RNP-2 motifs of RRM-2 and RRM-3, suggesting an interplay goes on between those two RRM domains. We identified short amino acid stretches that might be useful as targets for specific disruption of the hLa-HBV RNA interaction to destabilize the viral RNA.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Mutagenesis—A plasmid containing the hLa sequence cloned into the pET28b (+) (Novagen, Madison, WI) encoding the hLa sequence under the control of a isopropyl-1-thio-β-D-galactopyranoside-inducible T7 promotor with an N-terminal hexa-histidine tag was a kind gift of E. Chan (The Scripps Research Institute). Mutations were introduced into the hLa coding sequence by PCR according to the site-directed mutagenesis method (Stratagene, La Jolla, CA) using proofreading Pfu DNA polymerase (Roche Molecular Biochemicals, Germany). To introduce the different mutations the following oligonucleotides were used: for mutant hLa-D1, sense primer M19 (5′-GTG ACT GAC TAT AAA AAT G-3′) and antisense primer M20 (5′-CTT TTC ATT ATC ACC-3′); for mutant hLa-D2, sense primer M21 (5′-CCA ACT GAT GCA ACT TTC-3′) and antisense primer M22 (5′-AGA TCT GTT TAT TTC-3′); for mutant hLa-D3, sense primer M23 (5′-GAT GAC ATT GAAT CTC ACC-3′) and antisense primer M24 (5′-AAA AAT GAC TAG ACC-3′); for mutant hLa-D4, sense primer M25 (5′-TTG GAT GTG ACC GAG-3′) and antisense primer M26 (5′-AGA TCT GCC TCT TTC-3′); for mutant hLa-D5, sense primer M27 (5′-GTA CAG ACT TCC TTT AAC-3′) and antisense primer M28 (5′-GTA CAG ACT TCC TTT AAC-3′); for mutant hLa-D6, sense primer M29 (5′-AAA TGC TTT ATGCAA TGT TC-3′) and antisense primer M30 (5′-AAA TGC TTT ATGCAA TGT TC-3′); for mutant hLa-D7, sense primer M31 (5′-TGG CCA GCC AAG GAA GC-3′) and antisense primer M32 (5′-AGG CCA GCC AAG GAA GC-3′); for mutant hLa-D8, sense primer M33 (5′-TAC TTG GAT GCC ACC GAG-3′) and antisense primer M34 (5′-TTG GAT GCC ACC GAG-3′); for mutant hLa-D9, sense primer M35 (5′-TGG CCA GCC AAG GAA GC-3′) and antisense primer M36 (5′-TGG CCA GCC AAG GAA GC-3′).

PCR products were purified using the QiAquick PCR purification kit (Qiagen, Germany), phosphorylated at the 5′-end with 10 units of T4 polynucleotide kinase (Roche Molecular Biochemicals, Germany), subsequently ethanol-precipitated, and resuspended in an appropriate volume of H2O. The PCR products were ligated with the Rapid DNA Ligation kit (Roche Molecular Biochemicals, Germany) following the manufacturer’s instructions. Finally, the ligated DNA was digested with 10 units of DpnI enzyme (New England BioLabs, Beverly, MA) for 1 h at 37 °C with appropriate buffers and transformed into Escherichia coli strain DH5α. The introduced mutations were checked by sequencing using the Licor 4000L cycles Kit and MWG Biochemicals, Germany.

Expression and Purification of Recombinant Proteins—Recombinant proteins were expressed in 100 ml of E. coli BL21 cultures containing the appropriate antibiotic. 100 ml of bacteria cultures was grown at 37 °C to a density of A600 = 0.5, treated with isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and shaken for 5 h at 37 °C. The cells were subsequently precipitated by centrifugation. All purification steps were carried out with chilled buffers at 4 °C, and all solutions contained 1% protease inhibitor mix Complete (Roche Molecular Biochemicals, Germany). Cells were lysed in 2 ml of lysis buffer containing 50 mM NaH2PO4, 10 mM imidazole, 300 mM NaCl and sonicated three times for 10 s. His-tagged hLa protein was purified with Ni-NTA Spin columns (Qiagen, Germany) as follows. The supernatant containing the soluble proteins was loaded onto the spin columns and centrifuged for 2 min at 700 × g. Nonspecifically bound proteins were removed by washing the column four times each with 2 ml of wash buffer (50 mM NaH2PO4, 1 M NaCl, 0.1% Triton X-100). The His-tagged hLa protein was eluted with 3 × 200 µl elution buffer (50 mM NaH2PO4, 300 mM imidazole, 300 mM NaCl).

For large scale hLa preparations, 1 liter of bacteria solution was treated as above, and the pellet was resuspended in 20 ml of lysis buffer, sonicated, and centrifuged for 15 min at 4 °C with 20,000 × g. The supernatant was then incubated with 5–8 ml of equilibrated Ni-NTA-agarose (Qiagen, Germany) for 30–60 min with gentle stirring at 4 °C and loaded to a 1.5–15 cm column. Nonspecifically bound proteins were removed with 50 ml of wash buffer and His-tagged hLa was eluted with elution buffer in 1 ml fractions. Eluted hLa was dialyzed three times against 500 ml of buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, and 5% glycerol). When higher protein concentrations were desired, the protein solution was concentrated with Amicon Centricon YM-10 concentrators (Millipore, Germany) following the manufacturer’s instructions. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Germany). Correct size and purity of the expressed and purified recombinant hLa were analyzed by 12.5% SDS-PAGE minigels using the MiniProtean III System (Bio-Rad, Germany) followed by Coomassie Blue staining and Western blot analysis (see Fig. 5 below). The latter was achieved according to standard methods, briefly, by electrotransfer (90 min, 4 °C, 65 V) to a nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) in a wet-blot system using the Bio-Rad Trans-Blot Cell (Bio-Rad, Germany) and immunoblotting with primary mouse monoclonal oLA antibodies 3B9, 4B6, SW5 (M. Bachmann, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma) and monoclonal α-LA antibody 1982 and horseradish peroxidase-conjugated secondary goat-anti-mouse IgG antibody (H+L) or goat-anti-rabbit IgG antibody (H+L) (Dianova, Germany), respectively. Visualization of immunodecorated proteins was achieved with the SuperSignal chemiluminescent peroxidase solution system (Pierce, Rockford, IL), followed by x-ray film development. To verify the results, at least two to three different protein preparations were tested that gave similar results.

Gel Filtration—As a further step of protein purification, we performed preparative gel filtration analysis using a HiPrep 26/60 Sephacryl S100-HR column (Amersham Bioscience, Germany), which had been equilibrated with buffer A (see above). To maintain constant flow rates of 0.5 ml/min and for sensitive detection of eluted protein by monitoring the absorbance at 280 nm, the column was connected to a high-performance liquid chromatography BioCad Sprint System (Per-Septive Biosystems Inc./Applied Biosystems, Germany) using the BioCad Perfusion Chromatography Workstation software (version 3.0.0), including an external fraction collector. To establish a calibration curve for the column, the void volume was determined using 1 mg of bovine serum albumin (M, 66,000), ovalbumin (M, 44,720), and carbonic anhydrase (M, 29,000) were loaded separately, and the elution volume for each protein was detected. A standard curve was obtained for all calibration proteins with the y-axis showing the log of molecular weight and the x-axis showing Kav, where Kav is calculated as Kav = (Ve − Vo)/(Ve − V0), where Ve is bed volume of the column, Vo is void volume, and V0 is elution volume. For protein
purification at least 1 mg of Ni-NTA-purified His-tagged hLa protein in a volume of 1–2 ml was loaded to the equilibrated column, the elution volume of the different peaks was determined and compared with those of the standard proteins thereby calculating the K_S and molecular weight of each peak.

Characterization of the oligomerization of WT hLa protein and of the synthesized mutants was performed by gel filtration analysis using an analytical Superdex 200 HR 10/30 column (Amersham Bioscience, Germany). This column was connected to the same high-performance liquid chromatography-system as mentioned above and calibrated with the same proteins (0.2 mg each, loaded separately) with a flow rate of 0.5 ml/min. The buffer used corresponded to buffer A but contained 10 mM EDTA to prevent cation-dependent oligomerization of His-tagged recombinant hLa. For analysis of hLa proteins 0.05 mg of Ni-NTA-purified His-tagged hLa protein in a volume of 0.5 ml was loaded to the equilibrated column. The elution volume was determined, K_S was calculated, and the molecular weight was then derived from the standard curve of this column.

In Vitro Transcription—The in vitro transcript HBV RNA.B was generated by in vitro transcription as previously described (22, 23). For competition experiments, actin and glyceraldehyde-3-phosphate dehydrogenase RNA were produced as described elsewhere (23). An Fspl-linearized plasmid (pHTR1) containing the human telomerase RNA coding sequence (kindly provided by W. Filipowicz) was used for the generation of human telomerase competitor RNAs by in vitro transcription.

Gel Retardation Assay—The standard binding reaction was carried out in a final volume of 40 μl with 200 ng or, as indicated, Ni-NTA spin columns or, if indicated, preparative gel filtration-purified (Peak-2, 85–90 KDa) recombinant hLa protein and about 200,000 cpm or molar concentrations, as indicated, of 32P-radiolabeled HBV RNA.B in binding buffer containing 10 mM Tris-HCl, pH 7.4, 3 mM MgCl_2, 100 mM NaCl, 0.5 mM EDTA, and 0.5% Nonidet P-40 (unless otherwise stated). The in vitro transcribed 32P-labeled RNA.B was denatured at 75 °C for 10 min and renatured by cooling slowly to RT prior to addition to the reaction mixture. Samples were incubated for 10 min at RT. After addition of 5 μl of electrophoresis buffer containing 10% glycerol and 0.01% bromophenol blue, reaction mixtures were separated on an 8% native polyacrylamide gel (18 × 18 cm) for 3–4 h at 200 V at room temperature. The gels were prerun at 240 V for 1 h in 1 × TBE containing 45 mM Tris, 45 mM boric acid, 1 mM EDTA. Gels were dried for 1.5 h at 80 °C on Whatman paper (Whatman, UK) using a Bio-Rad Slab Dryer Model 483 (Bio-Rad, Germany), and signals were evaluated using a FujiX BAS 2000 phosphorimaging system (Fuji, Germany) or documented via exposure to x-ray films. All EMSAs with wild type and deletion mutants of hLa protein were performed at least three to five times with at least two different protein preparations, and similar results were obtained in all cases.

For saturation experiments and calculation of dissociation constants (K_D), increasing amounts of protein were added to constant amounts of RNA as indicated in the figures. The signal intensity of the hLa protein was quantified using TINA2.09d software provided by the supplier (Raytest, Germany). The band intensities of monomeric hLa monomer, was the most prominent one, and the values were transformed to relative ratios calculated as

RESULTS

Characterization of the hLa-HBV RNA.B Interaction—To study the interaction between hLa and HBV RNA.B, an RNA gel retardation assay was established using recombinant hLa and in vitro transcribed HBV RNA.B as interaction partners. In the first experiments, we tested whether the specificity and the binding conditions for the complex formation between recombinant hLa and HBV in vitro transcribed RNA.B were similar to the previously shown specific interaction and optimized binding conditions between HBV RNA.B and endogenous mLα prepared from HBV transgenic mice livers and HBV RNA.B.

The recombinant hLa protein used in this study was fused to a hexa-histidine tag, expressed in E. coli, and purified by Ni-NTA affinity chromatography or gel filtration as indicated under “Experimental Procedures.” HBV RNA.B was synthesized by in vitro transcription using [α-32P]UTP for uniform labeling of the transcript. Incubation of recombinant hLa with labeled HBV RNA.B leads to the formation of hLa-RNA.B complexes with different electrophoretic mobility as shown by RNA gel retardation assays (Fig. 1A, lane 1). The signal intensities of these hLa-RNA.B complexes varied during different experiments, but the complex with the highest mobility (Fig. 1A, hLa monomer) was consistently predominant, suggesting a preferential formation of this complex. To confirm that these complexes were formed between hLa and HBV RNA.B, a supershift analysis was performed using two La-specific antibodies. The monoclonal antibody SW5 and the polyclonal rabbit anti-hLa serum shifted all of the hLa RNA.B complexes (Fig. 1A, lanes 2 versus 3 and 4), whereas the rabbit pre-immune serum had no effect on complex formation or on complex mobility (Fig. 1A, lanes 2 versus 5). These results show that hLa forms RNA-binding competent multimers, although the hLa-HBV RNA.B complex with the highest mobility, referred to as monomer, was the most prominent one, and the formation of higher molecular weight complexes varied from experiment to experiment as seen in this study.

Next we asked whether recombinant hLa binds HBV RNA.B as specific as it was shown recently for the interaction between endogenous mLα present in nuclear protein extracts of HBV transgenic mice and HBV RNA.B (22, 23). Competition exper-
Addition of EDTA (5 mM) strongly increased the formation of HBV RNA.B, whereas in the presence of 1 M NaCl, indicating non-electrostatic interactions. The efficiency of RNA binding was only slightly reduced in the presence of 100 mM NaCl, 3 mM MgCl₂, 0.5 mM EDTA, and 0.1% Nonidet P-40. Taken together, these data show similar binding characteristics of recombinant hLa to HBV RNA.B compared with the binding conditions reported earlier for the interaction between endogenous mLa protein and HBV RNA.B (22), signifying a similar method of RNA binding for recombinant hLa and endogenous mLa.

To determine the molecular weight of the hLa protein and potential hLa multimers in our preparation, purified recombinant hLa was submitted to preparative gel filtration chromatography. Gel filtration analysis of the wild type hLa protein revealed two major peaks with estimated molecular masses of >150 kDa (peak 1) and 85–90 kDa (peak 2). The presence of full-length hLa (47 kDa) in each of these protein peaks was confirmed by Western blot analysis using hLa-specific antibody 3B9 (Fig. 3B). We conclude from this analysis that peak 1 contains hLa multimers and peak 2 hLa dimers, however, we did not observe a peak corresponding to the molecular mass of hLa monomers (47 kDa), indicating that hLa prepared under our conditions exists preferentially as dimers and multimers. These observations confirmed the previous assumption that the different hLa-HBV RNA.B complexes detectable during gel retardation assay analysis consist of hLa multimers and monomers. At this point it was still not possible to decide whether the complex with the highest mobility represents hLa monomers or multimers bound to HBV RNA.B. Previously, it was shown that hLa contains a dimerization domain located in the C-terminal part between amino acids 298 and 348 of La (45). To test to which extent this domain contributes to the formation of the different complexes, we deleted the dimerization domain. This deletion mutant (hLaΔ6, deletion of amino acids 274–354) and wild type hLa were purified by nickel affinity chromatography (see Fig. 5, C and D) and subsequently submitted to analytical gel filtration analysis. To exclude the formation of recombinant hLa multimers via the interaction of divalent cations and the His-tags, 10 mM EDTA was included in the buffer for the analytical gel filtration. Under this condition separation of wild type hLa revealed a major peak of molecular mass of ~100 kDa and one smaller peak of ~50 kDa representing hLa dimers and monomers, respectively. Analysis of hLaΔ6 revealed one major peak of ~45 kDa, indicating that the deleted region was required for hLa dimerization. The presence of full-length hLaΔ6 (approximately 40 kDa) in these protein peaks was confirmed by Western blot analysis using hLa-specific antibody 3B9 (Fig. 3C).

Next, we submitted wild type and Δ6 hLa proteins to gel retardation analysis. Although wild type hLa forms several hLa-HBV RNA.B complexes of different mobilities (Fig. 3A, lane 2), hLaΔ6 leads to the formation of a single complex (Fig. 3A, lane 3). The electrophoretic mobility of this complex was similar to the wild type hLa-HBV RNA.B complex with the highest mobility, but the signal intensity appears to be less intense. Because in analytical gel filtration analysis hLaΔ6 was only eluted as a monomeric protein, the results of the gel retardation assay indicate that the complex with the highest mobility consists of hLa monomers bound to HBV RNA.B. Additionally, these results indicate that, upon binding of HBV RNA.B, the state of hLa oligomerization changes: gel filtration analysis revealed a multimeric form of the protein, whereas gel retardation assays predominantly revealed monomers bound to RNA. This signifies that hLa monomers preferentially bind to HBV RNA.B, although the original fraction contains hLa multimers, sug-
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Fig. 3. A, the dimerization domain and the C-terminal part of hLa are required for efficient interaction with HBV RNA.B. Standard gel retardation assay was performed under conditions described under “Experimental Procedures.” Wild type recombinant hLa purified by Ni-NTA spin columns (WT, lane 2) was analyzed for HBV RNA.B binding. The hLa dimerization domain (Δ6, as 274–354) was deleted in mutant hLa-Δ6 and the C-terminal region (Δ7, as 353–393) of hLa was deleted in mutant hLa-Δ7. Recombinant hLa-Δ6 (lane 3) and hLa-Δ7 (lane 4) were purified by Ni-NTA spin columns and analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.” B, detection of full-length hLa WT in gel filtration peaks by Western blot analysis. hLa WT was submitted to preparative gel filtration chromatography, and the start material for gel filtration (lane 1), protein of peak 1 (>150 kDa, lane 2), and peak 2 (85–90 kDa, lane 3) (200 ng each) were electrophoresed, blotted, and detected by antibody 3B9 as described under “Experimental Procedures.” C, detection of full-length hLa WT in gel filtration peaks by Western blot analysis. hLa WT (lane 1), start material (lane 2) for analytical gel filtration, and proteins of the minor peak (>150 kDa, lane 3) and the major peak (40 kDa, lane 4) (200 ng each) were electrophoresed, blotted, and detected by antibody 3B9 as described under “Experimental Procedures.” Positions of hLa WT and hLa Δ6 are indicated by arrows at the right, molecular size marker (kDa) is depicted on the left side of the blot.

In this context it is also very important to calculate how much HBV RNA.B binding competent WT hLa protein exists in our gel filtration fraction. We determined that 100 ng (50 nM) gel filtration-purified recombinant hLa (Fig. 3B, GF peak-2, 85–90 kDa) was able to bind ~2 nM HBV RNA.B (see below). Assuming that the hLa WT purified by preparative gel filtration (peak-2, 85–90 kDa) was not contaminated with monomeric hLa, then approximately 1 of 12 hLa dimers was able to bind one HBV RNA.B transcript. On the other hand, we cannot ultimately exclude a minor contamination of monomers in gel filtration peak 2 (hLa dimers, 85–90 kDa), although rechromatography of the hLa dimer fraction showed no monomeric hLa peak (data not shown).

To test the influence of additional regions in the C-terminal part of hLa on RNA binding, amino acids 353–393 were deleted, referred to as mutant hLa-Δ7 (Fig. 5, A, C, and D). This mutation reduced the efficiency of the interaction with HBV RNA.B relative to WT hLa (Fig. 3A, compare lanes 2 and 4), indicating that amino acids in this region were also partially required for binding or that structural changes account for the reduced binding activity. This may also be true for the reduced RNA binding activity of hLa-Δ6 (Fig. 3A, compare lanes 2 and 3). Taken together, it was shown that hLa monomers preferentially interact with HBV RNA.B and that amino acids and/or structural features of the C-terminal part might be involved in binding.

Affinity of the Wild Type hLa-HBV RNA.B Interaction—Experiments were performed to determine the binding affinity of recombinant wild type hLa to HBV RNA.B. Protein purified by Ni-NTA affinity chromatography and subsequent preparative gel filtration chromatography was used for the calculation of the binding affinity. First, increasing amounts of preparative gel filtration-purified (peak 2, 85–90 kDa) wild type hLa proteins were added to constant concentrations of RNA.B to determine the optimal protein concentration required for titration of RNA.B (not shown). Gel retardation assay results were analyzed by phosphorimaging with arbitrary units to quantify the ribonucleoprotein complexes formed at varying hLa protein concentrations. We observed a linear increase in RNA.B binding at hLa protein concentrations between 25 (50 ng) and 100 nM (200 ng) at 0.1 nM RNA.B per binding reaction (data not shown). Based on these results, 50 nM (100 ng) of preparative gel filtration-purified WT hLa (peak 2, 85–90 kDa) was used to determine the binding affinity at increasing RNA.B concentrations. Gel retardation results were analyzed by phosphorimaging to quantitate RNA-protein complex formation at increasing RNA.B concentrations (Fig. 4A). The apparent affinity (Kp) was calculated according to the mass action equation. The maximal monomeric hLa-HBV RNA.B complex formation as determined by phosphorimaging was set as 100%, and the percentage of complex formation was plotted against the corresponding RNA.B concentration. By using this method, we determined the apparent affinity for the formation of the monomeric wild type hLa-HBV RNA.B complex as Kp = 0.8 nM (Fig. 4B), which represents the mean of five independent experiments. Note the intense formation of monomeric hLa-HBV

suggesting a shift from hLa multimers to monomers following RNA binding.
Analysis of the HBV RNA.B binding activity of hLa- 

representative gel retardation assays are shown in this study. Furthermore, at least two different protein purifications of each of the recombinant hLa mutants were applied to the gel analysis was performed to confirm that equal amounts (200 ng) versus increasing HBV RNA.B concentrations. The band intensities of RNA complexes were measured, background intensities were subtracted, the resulting values were transformed to relative ratios calculated as a percentage of maximum signal, and data were fitted to non-linear regression curves. Data are expressed as the mean out of five independent experiments.

Contribution of the Different RNA Recognition Motifs to the Binding of HBV RNA.B—The hLa protein contains three RRM motifs mediating the recognition of HBV RNA.B. To gain better insight into the contribution of the individual domains to HBV RNA.B binding, we introduced several deletions within the different RRM motifs. The RRM-1 was completely deleted, whereas the RNP-2 and RNP-3 motifs were deleted in the mutant hLa-(WT) purified by Ni-NTA spin columns and subsequent preparative gel filtration chromatography (GF, peak 2, 85–90 kDa) was analyzed for HBV RNA.B binding (lanes 2–11). The concentration of labeled HBV RNA.B was increased as indicated in lanes 2–11. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.” B, plot of the percentage of complex formation versus increasing HBV RNA.B concentrations. The band intensities of monomeric hLa-RNA complexes were measured, background intensities were subtracted, the resulting values were transformed to relative ratios calculated as a percentage of maximum signal, and data were fitted to non-linear regression curves. Data are expressed as the mean out of five independent experiments.

Fig. 4, A, apparent affinity of the hLa-HBV RNA.B interaction determined in gel retardation assays. Standard gel retardation assay was performed under conditions described under “Experimental Procedures.” In all binding reactions 100 ng of wild type recombinant hLa (WT) purified by Ni-NTA spin columns and subsequent preparative gel filtration chromatography (GF, peak 2, 85–90 kDa) was analyzed for HBV RNA.B binding (lanes 2–11). The concentration of labeled HBV RNA.B was increased as indicated in lanes 2–11. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.” B, plot of the percentage of complex formation versus increasing HBV RNA.B concentrations. The band intensities of monomeric hLa-RNA complexes were measured, background intensities were subtracted, the resulting values were transformed to relative ratios calculated as a percentage of maximum signal, and data were fitted to non-linear regression curves. Data are expressed as the mean out of five independent experiments.

RNA.B complexes, although dimeric hLa protein was applied. The affinity for the interaction between recombinant WT hLa is in accordance to the previously determined affinity (Kd ~ 1.4 nM) for the interaction between endogenous mLa protein and HBV RNA.B (23).

Recruitment of the Different RNA Recognition Motifs to the Binding of HBV RNA.B—The hLa protein contains three RRM motifs mediating the recognition of HBV RNA.B. To gain better insight into the contribution of the individual domains to HBV RNA.B binding, we introduced several deletions within the different RRM motifs. The RRM-1 was completely deleted, whereas the RNP-2 and RNP-3 motifs were deleted in the mutant hLa-(WT) purified by Ni-NTA spin columns and subsequent preparative gel filtration chromatography (GF, peak 2, 85–90 kDa) was analyzed for HBV RNA.B binding (lanes 2–11). The concentration of labeled HBV RNA.B was increased as indicated in lanes 2–11. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.” B, plot of the percentage of complex formation versus increasing HBV RNA.B concentrations. The band intensities of monomeric hLa-RNA complexes were measured, background intensities were subtracted, the resulting values were transformed to relative ratios calculated as a percentage of maximum signal, and data were fitted to non-linear regression curves. Data are expressed as the mean out of five independent experiments.

During a search for hepatocellular proteins that mediate the cytokine-induced degradation of HBV RNA, we recently demonstrated a correlation between the disappearance of the viral RNA and the appearance of a La protein fragment in the mouse liver following cytotoxic T lymphocytes injection or viral infection (22, 23). In the present study, we extended the knowledge about the interaction between recombinant hLa and HBV RNA.B by characterization of the binding conditions, determination of the affinity, and identification of hLa motifs mediating the interaction with HBV RNA.B. It is shown that the binding conditions for the interaction between endogenous mLa and recombinant hLa were very similar, in particular it was observed that the mouse and hLa-HBV RNA.B complexes were stable under high salt conditions (Fig. 2) and that binding is independent of MgCl2. Other protein-RNA complexes are destabilized at increasing ionic strength (44, 46, 47), presumably due to competition between salt anions and nucleic acid anions for protein interaction sites. Therefore, the stability in presence of high ionic strength might
indicate that non-electrostatic interaction (i.e. hydrophobic forces) contributes to the hLa/H18528 RNA.B interaction. To verify this assumption structural information about the hLa protein and hLa-RNA complexes will be needed.

The binding affinity of monomeric wild type hLa was determined to be ~0.8 nM (Fig. 4), which is similar to the affinity calculated by UV cross-linking experiments for the interaction between mLa and HBV RNA.B (~1.4 nM (23)), signifying a high affinity interaction between recombinant human or endogenous mLa protein with the HBV RNA.B. The monitored value for the affinity is in the range of other ribonucleoprotein complexes described, such as has been calculated for the interaction between the HIV Tat protein and the TAR element (~0.14 nM (48)), hnRNP proteins (10 pm to 10 nM (49)), and the binding of the hLa protein and the TAR element (17 nM (50)). Previously the specific interaction between endogenous mLa protein and HBV RNA.B was confirmed by competition experiments using different RNA substrates (23). In contrast we were not able to show a specific interaction between HBV RNA.B and recombinant hLa. Therefore, we assume that auxiliary factors are a prerequisite for a specific interaction between hLa and HBV RNA.B, as contained in nuclear mouse liver protein extracts, where the interaction was shown to be specific (22, 23). The first experiments addressing this point suggest that the complex formation between recombinant hLa and HBV RNA.B could indeed be modulated by addition of nuclear extracts prepared from Huh-7 cells (not shown).

The formation of several hLa-HBV RNA.B complexes was revealed by gel retardation assays (Fig. 1A). As shown in this study these complexes were composed of monomeric and multimeric hLa proteins bound to HBV RNA.B, and evidence is provided that these complexes were not stabilized by disulfide bridges, because the reducing agent DTT did not change the

**Fig. 6.** hLa RRM-1 is not required for interaction with HBV RNA.B. Standard gel retardation assay was performed under conditions as described under “Experimental Procedures.” Wild type recombinant hLa purified by Ni-NTA spin column (WT, lane 2) was analyzed for HBV RNA.B binding. hLa RRM-1 (Δ1, aa 11–99) was deleted, and recombinant hLa-Δ1 (lane 3) purified by Ni-NTA spin column was analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.”
RNA.B binding.

Lane 1 analyzed as described under "Experimental Procedures." Wild type recombinant hLa purified by Ni-NTA spin columns (WT, lane 2) was analyzed for HBV RNA.B binding. The hLa RNP-2 signature (Δ2, aa 113–119) of RRM-2 was deleted in mutant hLa-Δ2 and the RNP-1 signature (Δ3, aa 151–158) of RRM-2 was deleted in mutant hLa-Δ3. Recombinant hLa-Δ2 (lane 3) and hLa-Δ3 (lane 4) purified by Ni-NTA spin columns were analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. Binding reactions were analyzed as described under "Experimental Procedures.”

Fig. 7. Interaction between hLa and HBV RNA.B strongly depends on the RNP-2 signature of RRM-2. Standard gel retardation assay was performed under conditions described under "Experimental Procedures." Wild type recombinant hLa purified by Ni-NTA spin columns (WT, lane 2) was analyzed for HBV RNA.B binding. The hLa RNP-2 signature (Δ2, aa 113–119) of RRM-2 was deleted in mutant hLa-Δ2 and the RNP-1 signature (Δ3, aa 151–158) of RRM-2 was deleted in mutant hLa-Δ3. Recombinant hLa-Δ2 (lane 3) and hLa-Δ3 (lane 4) purified by Ni-NTA spin columns were analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.”

Fig. 8. Interaction between hLa and HBV RNA.B strongly depends on the RNP-2 signature of RRM-3. Standard gel retardation assay was performed under conditions described under “Experimental Procedures.” Wild type recombinant hLa purified by Ni-NTA spin columns (WT, lane 2) was analyzed for HBV RNA.B binding. The hLa RNP-2 signature (Δ4, aa 235–242) of RRM-3 was deleted in mutant hLa-Δ4 and the RNP-1 signature (Δ5, aa 266–276) of RRM-3 was deleted in mutant hLa-Δ5. Recombinant hLa-Δ4 (lane 3) and hLa-Δ5 (lane 4) purified by Ni-NTA spin columns were analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. Binding reactions were analyzed as described under “Experimental Procedures.”

It was shown in this study that deletion of amino acids 274–348, covering the dimerization domain, was involved in the oligomerization of hLa, however, in addition it was observed that complex formation between hLa-Δ6 and HBV RNA.B was diminished (Fig. 3A). Therefore, additional amino acids in the deleted regions were required for high affinity binding. On one hand it might be due to the deletion of the C-terminal part of the RRM-3, including the last two amino acids of RNP-1. This RNP-1 motif contributes partially to the binding as discussed below. On the other hand the Walker-A motif (amino acids 333–340 (34)) was completely removed as well as a partial stretch of basic amino acids (amino acids 328–363) located in this region. These elements might also contribute to the binding, because the 5’-processing of pre-tRNA was dependent on these elements (9). However, La-RNA binding studies have shown that deletion mutants missing the C-terminal part of La are still able to bind to HIV TAR and H1 RNA (50) as well as different single- and double-stranded RNA substrates (35, 53). In contrast, the binding of La to the HCV internal ribosome entry site was diminished after substituting amino acids in the basic region between aa 328 and 344 (27), thus indicating that different La domains are involved in the recognition of various subsets of RNAs described in the literature. Further work will be needed to discriminate between the specific amino acids in the C-terminal part of hLa necessary for direct binding or stabilization of a structure allowing interaction with HBV RNA.B. Structural changes within the hLa protein may be induced by the deletion in hLa-Δ7 (amino acids 353–393, Fig. 3A), which might artificially reduce the binding
capability of this mutant. However, we cannot rule out the possibility that the deleted acidic amino acid stretch (amino acids 367–375) and/or the casein kinase II phosphorylation site at position 366 contribute to a certain extent to the binding of HBV RNA.B. The casein kinase II phosphorylation was shown to control the maturation of pre-tRNA by releasing the 5′-end of the RNA after phosphorylation (15) without rendering the RNA binding activity to hY1 RNA (13). Recently, it was shown that phosphorylation of recombinant hLa by casein kinase II reduced the interaction with synthetic RNA oligomers carrying a poly-U stretch at their 3′-end (35). This suggests that serine 366 phosphorylation did not regulate the general RNA binding activity of hLa but does effect the interaction with certain RNA substrates and controls other functions mediated by hLa. It is likely that the interaction between hLa and HBV RNA.B is regulated by phosphorylation, because dephosphorylation of mouse nuclear extracts prior to UV-cross-linking abolished binding of mLa protein to HBV RNA.B (23). Therefore, future work will address the question as to what extent potential phosphorylation sites are involved in the regulation of the hLa-HBV RNA.B interaction.

Another deletion mutant hLa-Δ1, in which the RRM-1 was deleted (amino acids 11–99, Fig. 6), bound HBV RNA.B very intensively, indicating that deletion of the RRM-1 did not contribute to the binding at all and did not induce unfavorable structural changes. This mutant is of special interest, because the RRM-1 is clearly implicated in the interaction with pre-tRNA (15). Recently, a model for the interaction between La and pre-tRNA was established (9). In this model the RRM-1 mediates the binding of the typical 3′-UUU signature of RNA polymerase III transcripts, whereas the RRM-2 and RRM-3 motifs are required for general pre-tRNA binding. In addition, the 5′-flank of the immature tRNA is bound by the Walker A motif located in the C-terminal part of hLa. Therefore, the RRM-1 and the Walker-A motifs are essential determinants for a stable interaction between La and pre-tRNA, because mature tRNA is not bound by La. In comparison to this method of binding, the recognition of HBV RNA.B differs in the way that the RRM-1 is not involved and that the high affinity binding might be established by a cooperative binding mode of RRM-2 and RRM-3 as discussed below.

RRMs are well described RNA binding motifs found in a variety of RNA-binding proteins. These motifs are between 70 and 80 amino acids long and contain the RNP-2 and RNP-1 amino acid signatures required for RNA binding. The structure of RRMs was determined e.g., for the human hnRNP C protein, the small nuclear ribonucleoprotein A and the poly(A)-binding protein (32, 33, 54) and revealed the secondary structure of the RRMs composed of βα1β2β3β2α2β4 folding structure. The RNP-2 is located in the β1 and the RNP-1 is located in the β2 structural element. In the three-dimensional organization, the RNP-2 and RNP-1 are placed between helices 1 and 2, assembling a RNA binding surface. RRNs are required for general RNA binding activity, but they do not necessarily determine the binding specificity. In some cases the amino acids leading to the specificity for the interaction are located in the loop 3 between β2/β3 or in the C-terminal region of the RRM (49). The comparison of 70 different RRM-containing proteins leads to the formulation of a RRM core sequence (7). In that study only the RRM-3 of the hLa protein was used for comparison and interpreted as an atypical RRM. Although the RNPs are not very typical, deletion of the RNP-2 and RNP-1 sequences clearly show that each of these motifs were required for RNA.B binding. Interestingly, separate deletion of the RNP-2 motifs of RRM-2 and RRM-3 identified these elements as the most important ones, because the RNA-binding activity was almost completely abolished (Figs. 7 and 8). These results strongly indicate that the binding of HBV RNA.B is not only mediated by a single RRM but that RRM-2 and RRM-3 are required. Deletion of the RNP-1 signatures located in the RRM-2 and RRM-3 partially reduces binding (Figs. 7 and 8). Hence, all RNPs of RRM-2 and RRM-3 contribute to binding, suggesting that the RRM-2 and RRM-3 domains are functionally linked. Many RRM-containing proteins carry not only one RRM but multiple RRMs. For the HuD protein and the Sex-lethal protein, it was shown that two RRMs are forming a cleft in which the RNA was bound (55, 56). This study provides evidence that the RRM-2 and RRM-3 motifs of hLa are the central requirements for HBV RNA.B binding, most likely by forming an RNA binding domain composed of RRM-2 and RRM-3, as is shown for the binding of AU-rich elements by the HuD protein (55).

In conclusion, the La protein interacts with a variety of RNA molecules with a wide diversity of structures and sequences. It is likely that La interacts with these subsets of RNAs by establishing varying binding modes accomplished by different La domains. For binding HBV RNA.B the RRM-1 is redundant, whereas the RRM-2 and RRM-3 domains function presumably in a cooperative manner. Whether the C-terminal part directly interacts with HBV RNA.B or indirectly stabilizes a structure necessary for a stable interaction remains to be clarified. Furthermore, we assume that the equilibrium between oligomeric and monomeric hLa is regulated by RNA binding. This is of general interest to understand the variety of hLa functions. The RNP-2 motifs of RRM-2 and RRM-3 were identified as the most important regions for the hLa-HBV RNA.B interaction. It is tempting to speculate that the selection of molecules specifically interfering with the hLa-HBV RNA interaction might induce HBV RNA degradation.

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