Functional Characterization of the Interaction between Human La and Hepatitis B Virus RNA*

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The La protein is a multifunctional RNA-binding protein and has also been suggested to be involved in the stabilization of hepatitis B virus (HBV) RNA. Here we demonstrate that antibodies against the human La protein specifically precipitate HBV RNA from HBV ribonucleoprotein-containing mammalian cell extracts, providing evidence for the association between human La and HBV RNA. Moreover, we report that the turnover of HBV RNA depends on structural features and less on the primary sequence of the La-binding site on the viral RNA. In addition we show that the interaction between human La and HBV RNA in vitro is modulated by accessory factor(s) in a phosphorylation-dependent manner. Taken together these data indicate that both structural features, the composition of La/HBV ribonucleoprotein particles as well as interacting cellular factors, are critical determinants in the regulation of the stability of the HBV RNA.

RNA metabolism depends on the formation of ribonucleoprotein particles mediating diverse processes such as splicing, polyadenylation, nuclear export, and the regulation of mRNA stability (1, 2). The formation of RNP's is a tightly controlled process, potentially regulated by several stimuli, including hormones and cytokines. Such stimuli can alter the RNA binding activity of proteins on the post-translational level by phosphorylation or dephosphorylation and thereby the processing and stability of RNAs (3–5). In addition, RNA processing depends on various cis-acting elements including splice sites, export elements, and endoribonucleolytic cleavage sites recognized by RNA-binding proteins. To understand fully the regulation of processing of a specific RNA, both trans-acting factors and cis-acting elements as well as their functions need to be known. The same applies for a detailed understanding of the metabolism of viral RNA. Such studies could lead to the identification of novel cellular targets valuable for the development of innovative antiviral strategies when focused on the post-transcriptional control of RNAs of viruses with global medical importance. This applies to hepatitis B virus (HBV) with more than 300 million chronically infected carriers worldwide who await more effective antiviral therapies.

HBV is a noncytopathic, hepatotropic virus with a 3.2-kb circular DNA genome. After conversion into a covalently closed circular DNA, this genome serves in the nucleus as a template for transcription of all viral RNAs. Synthesis of these transcripts is driven by at least four promoters, leading to a large size heterogeneity with many different 5'-ends, whereas they all have very similar 3'-ends because of processing at the same polyadenylation site (6). The so-called pregenomic RNA (slightly longer than genome length) is encapsidated into nucleocapsids where it is reverse-transcribed into viral DNA. This RNA serves also as messenger for synthesis of the viral P protein as well as for the core protein. The viral surface proteins as well as a regulatory protein with a role in hepatocarcinogenesis, designated HBx, are translated from subgenomic mRNAs at 2.4, 2.1, and 0.7 kb in length.

Recently, several factors and cis-acting elements involved in the post-transcriptional control of the HBV gene expression were described. A number of publications highlighted the potential of cytokines to induce the post-transcriptional down-regulation of HBV RNA (for review see Ref. 7) in an HBV transgenic mouse model (8), in human hepatoma cells (9, 10), as well as the inhibition of duck HBV replication (11–13). In the HBV transgenic mouse model, the cytotoxic T lymphocyte (CTL) response to HBV antigens was shown to inhibit HBV replication by a post-transcriptional, noncytotoxic mechanism, leading to effective viral RNA degradation as induced by interferon-γ and tumor necrosis factor-α (8, 14, 15). While trying to evaluate the intracellular mechanism(s) responsible for the cytokine-mediated post-transcriptional destabilization of HBV RNA, the mouse La autoantigen (homologue to human La autoantigen) has been identified. The La protein interacts with a small cis-acting element located within the viral RNA between nt 1275 and 1291 (16, 17). The tight temporal correlation between the cytokine-mediated down-regulation of HBV RNA and the cytokine-induced fragmentation of full-length La led to the assumption that full-length La stabilizes HBV RNA by interacting with the cis-acting element (18). Recently, it has been shown that HBV RNA is cleaved close to the La-binding site by an endoribonucleolytic activity present in nuclear ex-
tracts prepared from HBV transgenic mice (19). Up-regulation of this activity coincided with the cytokine-induced fragmentation of La and the degradation of HBV RNA, supporting the assumption that HBV RNA is more accessible to endoribonucleolytic cleavage after the disappearance of full-length La protein. More specifically, we hypothesize that La, in concert with additional trans-acting factors, forms an HBV RNA ribonucleoprotein complex stabilizing HBV RNA.

The La protein has been described as important cellular factor involved in the RNA metabolism of a variety of viruses. Most often a function of La was attributed to the translational factor involved in the RNA metabolism of a variety of viruses. Collectively, the studies described above suggest that disruption of the proposed La-binding site diminishes the HBV RNA half-life. Furthermore, we show that recombinant hLa binds WT and mutant HBV RNA with the same affinity but with lower specificity, thus arguing for the requirement of accessory factors for a specific interaction. Collectively, these results support the concept that hLa contributes to HBV RNA stability.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Mutagenesis—The HBV expression plasmid pCH-9/9091 (kind gift of H. Schaller, Heidelberg, Germany) referred to as pHBV-WT contains a more than cellular histone mRNA (26) but also RNA of hepatitis C virus (27).

We studied whether hLa was associated with full-length HBV RNA, and we evaluated the importance of the La-binding site for the half-life of HBV RNA. We show that HBV RNA is co-precipitated with hLa, a strong indication for a physical interaction between La and HBV RNA in living cells. In addition, we demonstrate in transient transfection experiments that disruption of the proposed La-binding site diminishes the HBV RNA half-life. Furthermore, we show that recombinant hLa binds WT and mutant HBV RNA with the same affinity but with lower specificity, thus arguing for the requirement of accessory factors for a specific interaction. Collectively, these results support the concept that hLa contributes to HBV RNA stability.

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Cell Culture and Transfection Procedure—1.3 × 10⁶ Huh-7 human hepatoma cells were grown as monolayers in 4 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum per 60-mm dish. All plasmids used for transfection were purified by using the Maxiprep kit (Qiagen). Transfections were performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s protocol, ethidium bromide stained, and used for transfection. Transfection reactions were carried out with 0.5–1.0 μg of linearized plasmid or PCR product in a final volume of 20 μl in transfection buffer (Promega) containing 0.31 mM ATP, CTP, and TTP, 0.25 mM UTP, and 5 μM α-<sup>32</sup>PdUTP (800 Ci/mmol) (Hartmann Analytic, Braunschweig, Germany), 5 mM DTT, and 20 units of RNasin (Promega). The reaction was started by the addition of 20 units of T7 RNA polymerase (Promega) and after incubation for 45 min at 37 °C, another 20 units of T7 RNA polymerase were added, and the reaction was continued for 45 min at 37 °C. The reaction was terminated by adding 10 μg of yeast RNA and 1 unit of DNase I (Promega) and incubated for 15 min at 37 °C. Unincorporated nucleotides were removed by using Microspin G-25 columns.

Detection of Pregenomic HBV RNA Half-life by Using the Tet-Off System—Huh-7 cells were transfected with 1.5 μg of pHBV-WT or pHBV-M2, and 0.5 μg of pUr-UTD, expressing tTA, the tetracycline-controlled transactivator. Synthesis of pregenomic RNA was blocked by addition of 500 ng/ml doxycycline to the culture medium 24 h before transfection. Doxycycline was harvested in 10% trichloroacetic acid, 3, 6, and 8 h after transfection. Total RNA was prepared and analyzed by Northern blot analysis.

The La protein has been described as important cellular factor involved in the RNA metabolism of a variety of viruses. Collectively, these results support the concept that hLa contributes to HBV RNA stability.

Co-immunoprecipitation and RT-PCR—For immunoprecipitation of endogenous hLa-RNA complexes, about 10<sup>6</sup> HepG2 2.15 cells were harvested, and cell lysates were prepared as described previously (20). 10<sup>7</sup> HepG2 2.15 cells were washed twice with ice-cold PBS, and 1 ml of ice-cold polyinosinic polyribocytidylic acid (100 μg/ml) in 0.1% Triton X-100 was added. After 30 min on ice, the cells were harvested in 1 ml of ice-cold polysomal lysis buffer containing 10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.5% Nonidet P-40. 120 units of RNasin (Promega), 4 μl of ribonuclease vanadyl complex (200 μg/ml, Sigma), and protease inhibitor mixture (Roche Applied Science) were added. The lysate was incubated on ice for 30 min, and then the supernatant was obtained by centrifugation at 2000 × g for 10 min at 4 °C. The supernatant was immunoprecipitated using 2 μg of rabbit anti-La polyclonal antibody (kindly provided by Dr. Christoph Seeger and described in Ref. 28) and a goat anti-rabbit IgG coupled to 1 μl of protein A-Sepharose (Amersham Biosciences). The immune complex was washed 3 times with 1 ml of washing buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100) and then co-transfected with 0.5 μg of pHBV-M2 and 0.5 μg of pUr-UTD, expressing tTA, the tetracycline-controlled transactivator. Synthesis of pregenomic RNA was blocked by addition of 500 ng/ml doxycycline to the culture medium 24 h before transfection. Doxycycline was harvested in 10% trichloroacetic acid, 3, 6, and 8 h after transfection. Total RNA was prepared and analyzed by Northern blot analysis.
freshly added. Cells were pipetted up and down and incubated for 10 min on ice. The lysate was centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatant was removed and centrifuged again. The final supernatant was stored on ice until use. Protein A-Sepharose 4 fast flow beads (Amersham Biosciences) were blocked with bovine serum albumin and pre-coated with specific antibodies as described previously (29). Mouse anti-La antibody (about 7 μg of SWS, kindly provided by M. Bachmann) or a purified mouse IgG (7.5 μg per reaction) were used. The antibody-coated beads were washed six times with 900 μl of NT2 buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40. Finally, the pellets were resuspended in 850 μl of NT2 buffer, supplemented with 4 μl of vanadyl ribonucleoside complex, 160 units of RNAsin, and 20 μl EDTA, and 100 μl of cell lysate. The immunoprecipitation reactions were rotated head over head overnight. Beads were collected by centrifugation at 800 × g for 2 min, and pellets were washed six times with ice-cold NT2 buffer. Pellets for Western blot analysis were resolved in 100 μl of SDS denaturing buffer, and pellets for RNA analysis were resuspended in 100 μl of NT2 buffer supplemented with 0.01% SDS, 40 μg of proteinase K (Roche Applied Science) and incubated at 50 °C for 30 min in a thermomixer (1000 rpm); subsequently, RNA was extracted by using the TriPure isolation reagent (Roche Applied Science) and precipitated in the presence of 20 μg of glycogen. For Northern blot analysis, the complete RNA pellet was solved in 40 μl of loading buffer, loaded onto a 1.2% agarose-formaldehyde gel, and processed as described above. Western blot analysis was performed by standard methods, using 12.5% polyacrylamide gels and nitrocellulose membranes.

For RT-PCR, the RNA pellets (starting material, pellets, and supernatants) were solved in 43 μl of diethyl pyrocarbonate-treated water; subsequently, 5 μl of RNase-free DNase reaction buffer (Promega) and 2 units of RNase-free DNase (Promega) were added and incubated for 30 min at 37 °C. DNase was inactivated by heating the sample for 10 min at 70 °C. 2 μl of DNase-treated RNA was used as template for RT-PCR. RT-PCR was carried out using the Titan One-Step RT-PCR kit (Roche Applied Science), and the following primer pairs were used: HBV sense, 5′-GGAGCCCTCCCTGGGTGTCG-3′, and antisense, 5′-GTGGTGTTGGAGGATCTCGG-3′; L-37 sense, 5′-ACATGTCGGTCATTATGACCAGG-3′, and antisense, 5′-CTCAAGGAACATTTCTCTTGGAG-3′. Expression, Purification, Phosphorylation, Dephosphorylation of Recombinant La Protein, and Preparation of Nuclear Extracts—Recombinant hLa protein was prepared as described previously (17). Phosphorylation of recombinant hLa was performed for 3 h at 37 °C in 30 μl reactions containing 1 μg of hLa, 0.2 μl of casein kinase II (New England Biolabs), 3 μl of 10X reaction buffer, and 6 μl of 1 mM ATP. Dephosphorylation occurred with shrimp-alkaline phosphatase (Roche Applied Science) or α-protein phosphatase (New England Biolabs) according to the manufacturer’s instructions. All reactions were centrifuged at 3,000 × g for 5 min, and corresponding protein containing flow cells 1–3, and results were fitted (Biacore); curves of flow cell 4 without protein were subtracted from the loaded and prepared for successive measurements. RNAs were supplied in at least four different concentrations, which resulted in consistent data where the level of response units after RNA loading increased with the amount of RNA loaded. Data were evaluated with the BIAevaluation 3.0.2 software (Biacore); curves of flow cell 4 without protein were subtracted from the corresponding protein containing flow cells 1–3, and results were fitted into Langmuir’s model of 1:1 binding. As for the fit, software internal χ2 standards never exceeded values above 0.8, and Rmax values were in the expected ranges, and the residuals had a mean deviation from fitted graphs of less than 1.0 (not shown). Estimated half-life of a given RNA-protein complex was calculated as t1/2 = −ln(0.5)/k1.

RESULTS

The hLa Protein Is Associated with HBV RNA—Recently, it has been shown that the cytokine-induced post-transcriptional degradation of HBV RNA tightly correlates with the cytokine-dependent processing of the mouse La protein (18). In addition it has been shown that recombinant hLa interacts with in vitro transcribed HBV RNA-B (17). These data suggest an association of La and HBV RNA in living cells. Accordingly, we asked whether it would be possible to co-precipitate HBV RNA with hLa-specific antibodies for immunoprecipitations (IP). For this purpose HepG2 2.15 cells, stably expressing HBV RNA (31), were harvested and lysed, and subsequently the cleared cell lysate was incubated with either anti-La antibodies or mouse IgG 2A-coated protein A-Sepharose beads. As shown in Fig. 1, A and B, hLa was specifically precipitated and not detectable in the pellet of the control IP. To detect viral RNA in the IP pellets, RNA was extracted from additional pellets immunoprecipitated with hLa-specific as well as unspecific antibodies and analyzed by RT-PCR (Fig. 1A) and Northern blotting (Fig. 1B). RT-PCR was performed with specific primers for HBV RNA, TOP RNA L-37, and the hRNp E2 RNA. Recently, the co-precipitation of L-37 mRNA but not of the hRNp E2 with La was shown (32) and used as positive and negative controls in our study, respectively. As shown by RT-PCR, HBV RNA and

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It has been reported earlier that mouse La present in nuclear extracts binds a predicted stem loop structure with high affinity \((K_d \approx 1 \text{ nmol})\) and with high specificity \((16)\) in vitro. In contrast highly purified recombinant hLa binds HBV RNA in vitro with high affinity but with low specificity \((17)\). It was concluded that accessory factors are required to specify the association between hLa and HBV RNA and suggest a physical contact between hLa and HBV RNA in living cells.

Mutagenesis of the La-binding Site Reduces HBV RNA Levels—It has been reported earlier that mouse La present in nuclear extracts binds a predicted stem loop structure with high affinity \((K_d \approx 1 \text{ nmol})\) and with high specificity \((16)\) in vitro. In contrast highly purified recombinant hLa binds HBV RNA in vitro with high affinity but with low specificity \((17)\). It was concluded that accessory factors are required to specify the interaction between La and HBV RNA. To verify further the association between hLa and HBV RNA, we performed an additional co-immunoprecipitation experiment (Fig. 1B), and we analyzed the RNA extracted from the IP pellets for HBV RNA by Northern blot analysis. This experiment reveals that full-length HBV pregenomic RNA was co-precipitated with HBV RNA by Northern blot analysis. This experiment reveals that full-length HBV pregenomic RNA was co-precipitated with hLa but not with IgGs, although to a very small extent. Taken together both experiments clearly show the association between hLa and HBV RNA, but not with IgGs, although to a very small extent. Taken together both experiments clearly show the association between hLa and HBV RNA.

First we monitored HBV RNA levels transcribed from pHBV-WT, pHBV-M2, pHBV-M7, pHBV-M12, and pHBV-M13 at 16 and 24 h post-transfection. Differences in the levels of the HBV RNA in cells transfected with pHBV-WT, pHBV-M2, pHBV-M7, pHBV-M12, and pHBV-M13 (Fig. 2C) were prepared 48 h post-transfection. Differences in the levels of the HBV RNA in cells transfected with pHBV-M13, pHBV-M7, pHBV-M12, and pHBV-M13 at 16 and 24 h post-transfection. Differences in the levels of the HBV RNA in cells transfected with pHBV-WT, pHBV-M2, pHBV-M7, pHBV-M12, and pHBV-M13 (Fig. 2C) were observed at both time points when pHBV-M13 (Fig. 2C) was transfected. To study the influence of these mutations on HBV RNA levels, wild type and mutant HBV expression plasmids were transiently transfected into HuH-7 cells, and RNA was prepared at the indicated time points thereafter and analyzed by Northern blotting. Quantified HBV mRNA levels were normalized against transfection efficiency controlled by co-expression of GFP or serum alkaline phosphatase, and RNA loading was standardized by detection of GAPDH or histone 2A mRNAs.

First we monitored HBV RNA levels transcribed from pHBV-WT, pHBV-M2, pHBV-M7, pHBV-M12, and pHBV-M13 at 16 and 24 h post-transfection. Differences in the levels of the HBV RNA in cells transfected with pHBV-M13, pHBV-M7, pHBV-M12, or pHBV-WT were not detectable (data not shown), indicating that the respective mutations did not affect mRNA levels. In contrast, lower HBV RNA levels compared with pHBV-WT-transfected cells were observed at both time points when pHBV-M2 was transfected (data not shown). Very similar results were obtained when the RNA levels transcribed from pHBV-WT, pHBV-M2, pHBV-M7, pHBV-M12, and pHBV-M13 were prepared 48 h post-transfection and analyzed by Northern blotting, again only RNA levels transcribed from pHBV-M2 were lower compared with HBV RNA levels transcribed from pHBV-WT (Fig. 3A, lanes 1 and 2 versus 3 and 4). Quantification of six unrelated experiments performed in duplicate and with different plasmid preparations revealed an average de-
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Natural nucleotide substitutions found in the stem-loop

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Mutations introduced in the predicted stem-loop

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<td>HBV M13</td>
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**Fig. 2.** A, predicted secondary structure of HBV RNA B (16). B, the sequence of the proposed La-binding site is highly conserved. For sequence alignments of the stem loop sequence the MacVector software was used and following sequences applied: genotype A IDs: 59455, 59418, 59416, 59429, 329633, 260397, genotype B IDs: 221497, 221499, 329628, 329621; genotype C IDs: 59455, 452622, 452641, 329616, 56585, 329621, 59404, 221494, 59402, 29409, 329649, 329621; genotype D IDs: 59429, 62280, 288927, 59434, 329667, 313780, 451966, 59439, 59448; genotype E IDs: 452617, 452627; genotype F IDs: 452683, 452637, 59422. Nucleotide substitutions are shown in boldface. Mutation/sequence indicates in how many of the sequences analyzed the indicated substitution was found. C, outline of nucleotide substitution introduced into the predicted stem loop region. Nucleotide substitutions are shown in boldface.

crease both of the pregenomic RNA and of the 2.4- and 2.1-kb subgenomic RNAs by 60% when comparing transcript levels in pHBV-WT- and pHBV-M2-transfected cells (Fig. 3B), indicating a substantial importance of this cis-acting element for HBV RNA expression.

**Mutagenesis of the La-binding Site Reduces HBV RNA Half-life**—Next we asked whether reduced HBV RNA-M2 levels compared with HBV-WT RNA were because of an accelerated turnover rate. Therefore, the half-life of wild type and pHBV-M2 pregenomic RNA was determined using a tetracycline-controlled HBV expression system. Mutation M2 was introduced into the tetracycline-regulated HBV expression vector (pHBV-WT) referred to as pHBV-M2. This system allows the specific shut-off of pregenomic HBV RNA transcription without affecting the general cellular transcription machinery that would be blocked by the use of inhibitors such as actinomycin D. Hence, potential ambiguous results due to secondary effects caused by inhibiting general transcription were prevented. In this system synthesis of the pregenomic RNA was under the control of a CMV promotor inactivated after addition of the tetracycline analogue doxycycline. Huh-7 cells were transiently transfected with pHBV-WT and pHBV-M2 plasmids, and HBV RNA half-life was determined by Northern blot analysis. A substantial amount of pregenomic RNA levels was raised 24 h after transfection at which time doxycycline was added to abolish further HBV RNA synthesis (Fig. 4, A, lanes 8h). Three, six, and eight hours thereafter cells were harvested, and RNA was prepared and analyzed. Fig. 4A shows a representative Northern blot analysis of the half-life determination of pregenomic RNA. As expected from the reduced HBV-M2 RNA levels observed in our previous experiments, the pregenomic RNA levels of WT HBV were higher than that of HBV-M2 24 h post-transfection immediately before doxycycline addition to the cell culture medium (Fig. 4A, lanes 1 and 2 versus 9 and 10). Three hours after doxycycline treatment, pregenomic RNA levels were decreased to about 30% for WT HBV and to about 50% for HBV M2 (Fig. 4, A, lanes 9h versus lanes 3h, and B). The pregenomic RNA levels decreased further to about 30 and 20% during the following observation period up to 8 h post-addition of doxycycline (Fig. 4, A, lanes 6h and 8h, and B). By comparing the kinetics of the decline of the pregenomic RNA levels, a half-life of pHBV-M2 pregenomic RNA, which is shorter by about 2 h compared with HBV WT, became apparent (Fig. 4B). The quantification is based on PhosphorImaging of the signals from four independent experiments and each performed with duplicate values, by setting the pregenomic RNA levels at time 0 h to 100% and by normalizing transcription efficiency as well as RNA loading. From this careful quantitative analysis of the half-life of HBV RNA, two major conclusions can be made. First, pregenomic RNA of HBV M2 has a higher turnover than that of HBV WT and, therefore, is considerably less stable. Second, the half-life of the pregenomic RNA of WT HBV, so far not reported elsewhere, is about 5 h. These data imply that nucleotide substitutions GC and CC at positions 1275/76 and 1287/88, respectively, by adenosines, which weaken the base pairing of the predicted stem (Fig. 1B, HBV-M2), lead to reduced HBV RNA levels and a shorter half-life of the corresponding pregenomic RNA. Therefore, this region appears to contribute to pregenomic HBV RNA stability.

**Recombinant hLa Interacts with HBV RNA B-WT and -M2 with Similar Specificity and Affinity in Vitro**—Next, we compared the binding specificity of recombinant hLa to in vitro transcribed HBV RNA B-WT and HBV RNA B-M2 in gel shift analysis. Competitive gel retardation assays revealed that both cold competitors HBV RNA B-WT (Fig. 5A, lanes 3–5) and -M2 (Fig. 5A, lanes 7–9) were similarly efficient in competing for the binding of recombinant hLa to labeled HBV RNA B WT. Moreover, no difference in complex formation was observed when direct binding of recombinant hLa to labeled HBV RNA B WT and -M2 was compared (data not shown). Taken together, in gel retardation assays no significant difference between the binding specificity of hLa for HBV RNA B-WT and that of HBV RNA B-M2 was observed.

To define the specific affinities of the interaction between recombinant hLa and HBV RNA B-WT, -M2, and -M7, we measured binding parameters by using surface plasmon resonance technology. In these experiments, recombinant His-
For test whether additional cellular factors modulate the interaction between highly purified recombinant hLa and HBV RNA, we analyzed the binding in the presence of nuclear extracts prepared from Huh-7 cells. Different amounts of recombinant hLa or nuclear extracts were incubated with labeled HBV RNA. B-WT and analyzed by electrophoretic mobility shift assay. As shown in Fig. 6, 50 and 100 ng of recombinant hLa without nuclear extract form two major complexes with HBV RNA.B indicated as monomers and multimers (Fig. 6A, lanes 2–7). Next, different amounts of nuclear extracts were tested revealing the formation of a major complex of low mobility (indicated by an arrowhead, Fig. 6B, lanes 2–7) and a minor complex of higher mobility (indicated by an asterisk, Fig. 6B, lanes 2–7). The nature of these complexes is unknown, but it might be possible that the complex indicated by an asterisk represents a complex between endogenous La and HBV RNA.B because this band disappeared in supershift experiments (Fig. 6C, lanes 4 and 5 versus 10 and 11). Addition of increasing amounts of nuclear extract to 50 ng of recombinant hLa led to reduced signals of the multimeric and to increased signals of the monomeric hLa-HBV RNA.B complex (Fig. 6A, lanes 4 and 5 versus 8–13). The predominant formation of the monomeric hLa-HBV RNA.B complex in presence of nuclear extracts was confirmed if increasing amounts of recombinant hLa were added to 2.0 µg of nuclear extract (Fig. 6B, compare lanes 8–13 with 14 and 15). To verify that hLa-specific RNPs were formed, supershift experiments were performed. As shown in Fig. 6C, La-specific antibodies shifted the multimeric monomeric hLa-HBV RNA.B complexes (lanes 2 and 3 versus 8 and 9) as well as the dominant monomeric hLa-HBV RNA.B

Fig. 3. Northern blot analysis of HBV RNA transcribed from wild type and mutated HBV expression plasmids revealed that M2-RNA levels were reduced significantly. A, Huh-7 cells were co-transfected with 1.5 µg of plasmids pHBV-WT, pHBV-M2, pHBV-M13, pHBV-M7, or pHBV-M12. To monitor transfection efficiency, 0.07 µg of pEGFP-N1 were co-transfected. 48 h thereafter, RNA was prepared, and 10 µg of total RNA was analyzed by Northern blotting. The pregenomic HBV RNA (3.5 kb) and the subgenomic HBV RNAs (2.4/2.1 kb) were detected by using the HBV probe 1. To normalize RNA loading and transfection efficiency, the same blots were hybridized with histone H2A and GFP mRNA-specific probes, respectively. B, the pregenomic and subgenomic HBV RNA levels were quantified by PhosphorImaging. Six independent experiments in performed in duplicate were quantified, and HBV RNA levels were normalized against RNA loading and transfection efficiency. The standard deviations are indicated (± S.D.; n = 6).
complex formed in presence of nuclear extracts (lanes 6 and 7 versus 12 and 13). In contrast an unrelated antibody was unable to shift the hLa-HBV RNA-B complex (Fig. 6C, lanes 14 and 15). These data demonstrate that components present in nuclear extracts modulate the binding behavior by preferentially supporting the formation of monomeric hLa HBV RNA-B RNPs. These findings indicate that additional factors are involved in specific formation of hLa/HBV RNA RNPs.

As shown earlier, dephosphorylation of mouse nuclear extracts strongly diminished the ability of mouse La to bind to HBV RNA-B (16). In that study it was not possible to discriminate whether the complex formation was abolished because of the dephosphorylation of endogenous mouse La or of accessory factors. La is mainly phosphorylated by casein kinase II at serine 366, and it was shown earlier that this modification did not alter the binding of hY1-RNA (33). We now tested whether phosphorylation of recombinant hLa by casein kinase II or dephosphorylation of hLa by alkaline phosphatase affected binding of HBV RNA-B. As shown in Fig. 7A, untreated, dephosphorylated, and phosphorylated hLa bound HBV RNA-B with similar efficiency, indicating that recombinant hLa interacts with HBV RNA-B in a phosphorylation-independent manner. Although in this experiment a decrease in multimers was observed with phosphorylated hLa (compare lanes 3 and 4 and lanes 6 and 8), this effect was not reproducible. Two-dimensional gel electrophoresis analysis of the different hLa preparations verified successful phosphorylation and dephosphorylation of recombinant hLa used in the gel retardation assays (not shown). These results suggest that the strong reduction in HBV RNA-B binding of mouse La after dephosphorylation of nuclear extracts (16) was because of a phosphorylation-dependent activity present in the nuclear extract used. Next we asked whether dephosphorylated nuclear extract still predominantly led to the formation of a monomeric hLa-HBV RNA-B complex as visualized by gel retardation assays. Addition of untreated nuclear extracts led almost exclusively to the formation of a
monomeric complex (Fig. 7B), whereas after addition of phosphatase-treated nuclear extracts, multimeric complexes were still clearly visible, and the monomers were much less abundant (Fig. 7C). In summary, we show by gel retardation assays and with surface plasmon resonance technology that hLa binds HBV RNA.B-WT and -M2 with comparable affinity but with low specificity and that nuclear extracts module the complex formation between hLa and HBV RNA.B in a phosphorylation-dependent manner, suggesting that accessory factors increase phosphorylation-dependently the specificity of the interaction between La and HBV RNA.

**DISCUSSION**

HBV gene expression is not only regulated at the transcriptional level but also at the post-transcriptional level. The cytokine-induced processing of the La protein that coincides with the cytokine-induced degradation of HBV RNA suggests that the La protein stabilizes HBV RNA by a post-transcriptional mechanism. Recently, a predicted stem loop structure was reported to be bound by the La protein, which is thought to confer HBV RNA stability. The present study was conducted to gain experimental evidence for the interaction of human La with HBV RNA in cell culture, the requirement of the predicted La-binding site for HBV RNA stability, and the role of accessory host factors in regulating the interaction between human La and HBV RNA.

Here, for the first time, we provide experimental data suggesting an association between hLa and HBV RNA in human hepatoma cells. The co-precipitation of full-length HBV RNA with hLa as shown in this report strongly suggests that La physically interacts with HBV RNA in living cells. Although we cannot rule out the formal possibility that the interaction between hLa and HBV RNA is mediated by a bridging factor, the
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Co-precipitation of hLa and HBV RNA would still mean that hLa is part of HBV RNPs, presumably stabilizing the viral RNA. The association of hLa with HBV RNA or HBV stabilizing RNPs combined with previous results showing that HBV RNA degradation coincides with mouse La fragmentation and that mouse La interacts specifically with the predicted stem loop structure (16, 18) strongly support the hypothesis that hLa is involved in the post-transcriptional regulation of HBV RNA. Notably, the putative regulatory element bound by mouse and human La is located in the 3'-region of all viral RNAs targeted by the cytokine-induced post-transcriptional mechanism. To prove the significance of the La-binding site for viral RNA stability, several mutations were introduced, and the half-life of the pregenomic RNAs was determined. These experiments revealed that only mutations disrupting the 4 bp in the stem diminished HBV RNA half-life. Conversely, changing the nucleotides in the stem but maintaining base pairing does not affect HBV RNA levels. These findings indicate that probably structural rather than sequential features of the RNA element are important to uphold HBV RNA half-life. In this context, it is important to note that the sequence of the stem loop is highly conserved between the diversity of HBV genotypes and isolates (Fig. 1B). Our study raises the interesting question whether the nucleotide substitutions within the predicted structure involved in La protein binding as present in various HBV genotypes and isolates have positive or negative consequences for the stability of the viral RNAs and efficiency of virus propagation.

It was shown that mutation M2 diminishes pregenomic RNA half-life by ~2 h, indicating that HBV RNA stability depends in part on the structural features of this element. Note, however, with the current data we cannot exclude the possibility that the half-life of pregenomic RNA M2 was reduced by La-independent factors. Therefore, it is reasonable to consider that additional regulatory elements within the viral RNA and cellular factors interacting with HBV RNA and/or the La protein have to be integrated to fully understand the decay of HBV RNA under steady state conditions like in our experiments. Furthermore, the variety and remarkable changes in gene expression profiles observed after CTL injection into HBV transgenic mice (34, 35), probably inducing the inhibition of viral replication and inducing HBV RNA decay, suggest that additional cellular factors and cellular events have to be identified in order to understand the induced decay mechanism specific for the HBV RNA. It is reasonable to speculate that structural features of the La-binding site determine the interaction with trans-acting factors, and it might therefore be possible that mutation M2 leads to disruption of hLa containing RNPs normally protecting HBV RNA against endoribonucleolytic cleavage. However, changing the structure of this RNA element and therefore the HBV RNP composition might be not enough to induce efficient degradation as observed after CTL injection in the mouse system in which HBV RNA degradation was accompanied by the processing of mouse La and the augmentation of an endoribonuclease activity (19). Hence, the identification of factors involved in La-HBV RNP formation is of great importance and might shed light on the mechanism regulating HBV RNA decay under different cellular conditions.

Fig. 7. A, binding of recombinant hLa is independent of its phosphorylation state. Recombinant hLa purified by NiNTA spin columns was phosphorylated by casein kinase II, dephosphorylated by shrimp alkaline phosphatase, or untreated and applied for gel retardation assays. Efficient phosphorylation/dephosphorylation was verified by two-dimensional gel electrophoresis (data not shown). Standard gel retardation assay was performed under conditions described under “Experimental Procedures.” 100 ng of untreated (lanes 3 and 4), dephosphorylated (dephos., lanes 5 and 6), or phosphorylated (phos., lanes 7 and 8) recombinant hLa was analyzed for binding of 400,000 cpm HBV RNA.B-WT. Reaction without hLa is shown in lanes 1 and 2. B and C, nuclear factors affecting the formation of hLa-HBV RNA.B complexes are phosphorylation-dependent. B, standard gel retardation assay was performed under conditions described under “Experimental Procedures.” 100 ng of recombinant hLa (lanes 2 and 3) purified by NiNTA spin columns and 2 μg of nuclear extracts (lanes 4 and 5) were analyzed separately or in combination (lanes 6 and 7) for binding of 140 fmol of HBV RNA.B-WT. Reaction without hLa is shown in lane 1. C, 100 ng of recombinant hLa (lanes 2 and 3) purified by NiNTA spin columns and 2 μg of nuclear extracts (lanes 4 and 5) were separately or in combination (lanes 6 and 7) analyzed for binding of 140 fmol of HBV RNA.B-WT. Prior to the addition of HBV RNA.B-WT samples were treated for 30 min at 37°C with 10 μg of alkaline phosphatase. Reaction without hLa is shown in lane 1. < and * indicate RNPs formed between HBV RNA.B and nuclear extracts.
group of proteins called AU-rich binding proteins (38). In most cases these proteins destabilize RNAs by interacting with the AU-rich regions, but stabilizing effects have also been reported (39–41). To our knowledge, it is not known how exactly this interaction destabilizes/stabilizes RNA. The stable α-globin mRNA has been described to interact with several proteins forming the so-called α-complex associated with the 3'-untranslated region. Changes in the composition of this complex lead to endoribonucleolytic cleavage of this RNA (42, 43), demonstrating that a variety of trans-acting factors are involved. The requirement of several trans-acting factors was also described for interleukin-2 mRNA stability (44). The transferrin receptor mRNA is stabilized by the iron-regulatory protein, which interacts with the 3'-end of the RNA if cellular iron levels are low, thereby protecting the RNA against endoribonuclease cleavage (45). An increase in cellular iron concentration leads to reduced affinity of the iron-regulatory protein to the transferrin receptor mRNA and subsequently to the degradation of the RNA (reviewed in Ref. 46). Furthermore, intensive studies on the post-transcriptional control of albumin and vitellogenin mRNA have shown that albumin mRNA is destabilized after estrogen treatment in parallel with the activation of the polysomal ribonuclease 1 (PMR-1 (47, 48)), whereas vitellogenin mRNA is stabilized by binding of vigilin, protecting the mRNA against PMR-1 cleavage (49). These reports clearly demonstrate and support our assumption that a complex regulatory mechanism determines HBV RNA stability by integrating the action of regulatory trans-acting factors interacting with mRNA stability determinants. Notably, in competitive UV cross-linking experiments, the binding of mouse La in nuclear extracts was highly specific for HBV RNA.B-WT but not for the mutant M2 (16), indicating that under such experimental settings mouse La was able to discriminate between WT and mutant HBV RNA-M2. In contrast highly purified, recombinant hLa binds HBV RNA.B WT and M2 in an unspecified manner, suggesting that additional components, which were present in mouse nuclear extracts, are required for a specific interaction. Until those factors are characterized, it is probably not feasible to explore a difference in binding specificity of recombinant hLa to HBV RNA.B-WT versus -M2 in vitro and to show more directly that the half-life of pregenomic RNA depends on the association with hLa. We recently identified an hLa-interacting protein, and preliminary data show that this RNA-binding protein interacts specifically with HBV RNA.B, suggesting that this protein might modulate the association between HBV RNA.B and hLa.

Previously, it has been shown that binding of mouse La to HBV RNA in vitro was abolished after dephosphorylation of the La-containing nuclear extract (16). In this previous study, it was not possible to distinguish whether impaired RNA binding activity was because of dephosphorylation of the phosphoprotein La or because of other factors present in the nuclear extracts. Our observation that phosphorylation or dephosphorylation of recombinant hLa had no effect on binding HBV RNA.B, whereas dephosphorylation of nuclear extracts partially inactivated the activity-promoting formation of the monomeric La complex, showed that a phosphorylation-dependent activity modulates hLa binding to HBV RNA.B. We believe that the initial data support our view that the binding specificity of hLa to HBV RNA.B is mediated by phosphorylation-dependent host factors. In the absence of those factors recombinant hLa binds HBV RNA.B with low specificity. In this context it is important to note that the newly identified hLa-interacting protein is also a phosphoprotein and that it will of interest to study whether the interaction between hLa and this protein might be regulated by phosphorylation. Furthermore, it will be interesting to find out which cellular signaling pathway affects the proposed phosphorylation-dependent composition of HBV RNA's thereby potentially regulating the turnover of viral RNAs. Most importantly, it has been shown recently that Janus kinase activity is required for the antiviral effect of interferons on HBV replication (55), indicating that cell signaling pathways and thereby phosphorylation/dephosphorylation events are required. This might also apply for cytokine-induced degradation of HBV RNA decay as supported by our finding that binding of mouse and human La to HBV RNA is modulated by accessory factors in a phosphorylation-dependent manner. Previously, it has been shown that a variety of cellular factors involved in the regulation of mRNA stability, like RNA-binding proteins, was regulated by phosphorylation/dephosphorylation (50, 51) and that ribonuclease activities were elevated by hormones (48, 52, 53), indicating that post-translational modification and signal transduction pathways are important cellular mechanisms determining mRNA levels.

As a model, we assume that cytokine-induced HBV RNA degradation is initiated at the La-binding site, and this concept is supported by the following findings. First, the cytokine-induced HBV RNA degradation occurring in the mouse system in parallel with La processing is associated with increased endoribonuclease activity that leads to cleavage of the HBV RNA close to the La-binding site (18, 19). Second, mutagenesis of the La-binding site reduces HBV RNA half-life from 5 to 3 h in cell culture experiments. Third, binding of the mouse protein as well as of the human La protein to HBV RNA.B can be modulated by phosphorylation/dephosphorylation of accessory factors. The cytokine-induced HBV RNA decay might be initiated by the loss of protective factors (e.g. La) inducing structural changes and/or exposing the cleavage site located close to the predicted stem loop to endoribonuclease (19), thereby initiating HBV RNA degradation.

We conclude that the La-binding site together with the cleavage sites compose a destabilizing element leading to HBV RNA degradation under specific cellular conditions. It will be of major importance to identify host factors interacting with hLa and/or HBV RNA in a phosphorylation-dependent manner to understand in more detail the initiation of HBV RNA degradation.

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REFERENCES

‡ G. Tettweiler and T. Heise, unpublished data.
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