Characterization of Nuclear RNases That Cleave Hepatitis B Virus RNA near the La Protein Binding Site†

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Hepatitis B virus (HBV) RNA is downregulated by inflammatory cytokines induced in the liver by adoptively transferred HBV-specific cytotoxic T lymphocytes (CTLs) and during murine cytomegalovirus (MCMV) infections of the livers of HBV transgenic mice. The disappearance of HBV RNA is tightly associated with the cytokine-induced proteolytic cleavage of a previously defined HBV RNA-binding protein known as La autoantigen. La binds to a predicted stem-loop structure at the 5' end of the posttranscriptional regulatory element of HBV RNA between nucleotides 1243 and 1333. In the present study, we searched for nuclear RNase activities that might be involved in HBV RNA decay. Nuclear extracts derived from control livers and CTL-injected and MCMV-infected livers were analyzed for the ability to cleave HBV RNA. Endonucleolytic activity that cleaved HBV RNA at positions 1269 to 1270 and 1271 to 1272, immediately 5' of the stem-loop bound by the La protein (positions 1272 to 1293), was detected. Furthermore, we provide evidence that the cytokine-dependent downregulation of HBV RNA following MCMV infection is temporally associated with the upregulation of the endonucleolytic activity herein described. Collectively, these results suggest a model in which the steady-state HBV RNA content is controlled by the stabilizing influence of La and the destabilizing influence of nuclear RNase activities.

The hepatitis B virus (HBV) is a noncytopathic, hepatotropic virus with a 3.2-kb circular DNA genome that encodes four overlapping 3.5-, 2.4-, 2.1-, and 0.7-kb unspliced messages that terminate at a common polyadenylation site (45). Because HBV is not infectious in tissue culture, except for primary hepatocytes or for genetically or immunologically undefined animals, the development of an HBV transgenic mouse model was very useful to define the host-virus interactions involved in virus clearance and disease pathogenesis (2, 10, 11, 22, 23, 25, 37). Using that model, it has been shown that cytotoxic T lymphocytes (CTLs) inhibit HBV gene expression and replication noncytopathically at the posttranscriptional level (24, 49) by secreting gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) upon antigen recognition (20). Consistent with these results, hepatocellular HBV gene expression and replication are also downregulated noncytopathically by inflammatory cytokines produced during lymphocytic choriomeningitis virus-induced (21) and murine cytomegalovirus (MCMV)-induced (8) hepatitis in these animals. The intracellular mechanism(s) whereby the CTL-induced inflammatory cytokines posttranscriptionally destabilize HBV RNA remains to be determined.

RNA-protein interactions play an important role in the regulation of pre-mRNA processing (32, 47, 50), nuclear export (19), and stabilization and destabilization (14, 42, 48) of mRNAs. In the systems studied thus far, cellular RNA-binding proteins and

RNases influence transcript stability by interacting with sequences and/or structural elements in the RNA. In *Saccharomyces cerevisiae*, several different pathways are responsible for mRNA decay, including deadenylation-dependent and -independent decapping, 5'-to-3' and 3'-to-5' degradation by exoribonucleases, and endonucleolytic cleavage within the mRNA (14). Less is known about the cellular RNases responsible for mRNA degradation in vertebrates, although some vertebrate RNases have been characterized in detail (4, 7, 12, 15, 33, 39, 43, 53). A good example of the coordinated action of RNA-binding proteins, *cis*-acting RNA elements, and endoribonucleases is provided by the posttranscriptional control of transferrin receptor (TFR) mRNA. The interaction of an iron response element in the TFR mRNA with a cellular iron response element-binding protein (36), whose binding activity is induced by low cellular iron concentration (30) and phosphorylation (17), protects the TFR mRNA from endonucleolytic cleavage (3).

Recently we identified the La autoantigen (p45) and La fragments (p39 and p26) as HBV RNA-binding proteins, which bind to a predicted stem-loop structure located between nucleotides (nt) 1243 and 1333 of HBV RNA (26, 27), numbering according Galibert et al. (18). The presence of full-length La protein correlated directly with the presence of HBV RNA, detectable when the viral RNA was abundant and disappearing when the RNA degradation was posttranscriptionally induced in response to IFN- γ and TNF- α (26). In contrast, p26 was inversely related to HBV RNA, detectable only when the viral RNA disappeared following cytokine induction by adoptively transferred HBV-specific CTLs, after MCMV and lymphocytic choriomeningitis virus infection (26). If p45 actually stabilizes HBV mRNA, it might do so by protecting it

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against RNase-mediated degradation at a neighboring cleavage site. To test this hypothesis, we developed an RNase activity assay (RAA) and analyzed liver nuclear extracts for RNases able to degrade (i) HBV oligoribonucleotides, (ii) in vitro-transcribed HBV transcripts, or (iii) full-length HBV RNA prepared from the livers of HBV transgenic mice.

In the present report, we describe the identification of hepatic RNase activities able to cleave all three of these HBV RNA substrates in a site-specific manner. In addition, we show that these activities are transiently upregulated in the livers of HBV transgenic mice when p45 and HBV RNA disappear and p26 appears following the induction of IFN- γ and TNF- α . These results suggest that an interplay between the potential destabilizing activity of these RNases and the potential stabilizing effect of the La protein regulates hepatic HBV RNA content in this model.

MATERIALS AND METHODS

HBV transgenic mice. The HBV transgenic mouse lineages Tg(HBV 1.3 genome)Chi32 (designated 1.3.32) and Tg(HBV 1.3 genome)Chi46 (designated 1.3.46) used in this study have been described previously (25). Lineages 1.3.32 and 1.3.46 express all of the HBV transcripts under the control of their respective promoters and replicate HBV at high levels in the liver and kidney without any evidence of cytopathology (25). Mice were matched for age (8 to 10 weeks), sex (male), and serum hepatitis B e antigen (HBeAg) concentration using a commercially available solid-phase radioimmunoassay (Sorin Biomedica, Saluggia, Italy).

HBsAg-specific CTLs. Ld-restricted, CD3⁺ CD4⁺ CD8⁺ hepatitis B surface antigen (HBsAg)-specific CTL clones that recognize an epitope located between residues 28 and 39 of HBsAg (HBsAg28-29) and secrete IFN- γ and TNF- α upon antigen recognition (20) were used in these studies. In all experiments, 10⁷ CTLs were injected intravenously into transgenic mice 5 days after in vitro stimulation with irradiated P815 cells that stably express the HBV large envelope protein (2). CTL-induced liver disease was monitored by measuring serum alanine aminotransferase levels at various time points after CTL injection. Liver tissue obtained at autopsy was either processed for histological analysis or snap-frozen for subsequent molecular analyses.

MCMV infection. The Smith strain of MCMV (ATCC VR-194; American Type Culture Collection, Manassas, Va.) was used in this study. Lineage 1.3.32 mice were injected intraperitoneally with 5 $\times$ 10⁴ PFU of MCMV (8) and sacrificed at various time points thereafter. Livers were harvested, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent molecular analysis as previously described (8).

RNA analyses. Snap-frozen (liquid nitrogen) liver tissues were mechanically pulverized, and total genomic RNA was isolated for Northern blot analyses exactly as previously described (24, 25).

Preparation of liver nuclear and cytosolic extracts from HBV transgenic mice. Frozen liver tissue (0.2 to 0.5 g) was thawed and homogenized in a fivefold volume of ice-cold homogenization buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 2.5 mM MgCl₂, 1 mM EDTA (buffer A) containing 0.5 mM dithiothreitol (DTT), and 1/25 volume of proteinase inhibitor mixture (Boehringer Mannheim, Indianapolis, Ind.) by five strokes in a glass homogenizer with a loose-fitting motor-driven (50 rpm) Teflon pestle. The homogenate was centrifuged at 2,000 $\times$ g for 20 min, and the resulting supernatant was stored at -80°C. The pellet was resuspended in 6 ml of buffer A containing 0.88 M sucrose (buffer B), loaded onto a 7-ml cushion of buffer B, and centrifuged at 10,000 $\times$ g for 30 min. The supernatant was discarded, and the pellet was dissolved in 5 ml of buffer A containing 2.0 M sucrose (buffer C). The slurry was loaded onto a 7-ml cushion of buffer C and centrifuged at 180,000 $\times$ g for 70 min. The supernatant was discarded, and the nuclei were resuspended in 100 μ l of storage buffer containing 20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 50% glycerol, 0.5 mM DTT, and 1/10 volume of proteinase inhibitor mixture (Boehringer Mannheim). Nuclei were counted by light microscopy and lysed by adding 5 $\times$ lysis buffer containing 100 mM Tris-HCl (pH 8.0), 2.1 M NaCl, 7.5 mM MgCl₂, 1.0 mM EDTA, and 25% glycerol to a final concentration of 33 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT, and 1/10 volume of proteinase inhibitor mixture (Boehringer Mannheim). The viscous lysate was transferred into dialysis tubes (molecular weight cutoff, 6,000 to 8,000) (Spectro/Por; Spectrum Companies, Gardena,

Calif.) and dialyzed three times against 500 ml of dialysis buffer F containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 3 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and proteinase inhibitor mixture (Boehringer Mannheim). The dialyzed nuclear extract was cleared by centrifugation for 10 min at 24,000 $\times$ g, and the protein content was determined by the Bradford dye-binding procedure, with a commercial kit (Bio-Rad Laboratories, Hercules, Calif.).

In vitro transcription and oligoribonucleotides. A plasmid containing the entire HBV genome (*ayw* subtype) was used for the production of DNA templates for generation of HBV transcripts. Two primers were used. Primer 1 (5'-CCATCGATTAAATACGACTCACTATAG-3') contained a restriction site for *Clai*I (shown in italics), the T7 RNA polymerase promoter sequence (shown in boldface), and the sense HBV *ayw* DNA sequences (18) spanning nt 1243 to 1261 (5'-GAACCTTTTCGGCTCTCT-3'). Primer 2 contained antisense HBV sequences from nt 1312 to 1333 (5'-GTCCCCGATAATGTTTGCTCCAG-3') (RNA.B). PCRs for HBV templates were produced with 1 ng of plasmid, and the mixture contained 80 pmol of each primer in 1 $\times$ PCR buffer; 0.2 mM (each) GTP, ATP, TTP, and CTP; and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). PCR was performed as follows: 5 min at 95°C; followed by 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; and finally 1 cycle for 5 min at 72°C. The PCR products were purified by size exclusion with a commercial kit (PCR Purification kit; Boehringer Mannheim), ethanol precipitated, and used as templates to generate transcripts. Transcription reactions were carried out with 0.5 to 1.0 μ g of PCR product in a final volume of 20 μ l in transcription buffer (Promega, Madison, Wis.) containing 0.31 mM ATP, CTP, and GTP; 7.5 mM [³²P]UTP (800 Ci/mmol) (NEN, Boston, Mass.); 5 mM DTT; and 20 U of RNasin (Promega). The reaction was started by the addition of 20 U of T7 RNA polymerase (Promega). After incubation for 45 min at 37°C, another 20 U of T7 RNA polymerase was added to the mixture, and the reaction was continued for 45 min at 37°C. The reaction was terminated by the addition of 10 μ g of yeast tRNA and 1 U of DNase I (Promega) and incubation for 15 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, transcripts were dissolved in 10 mM Tris-HCl (pH 7.4)-diethyl pyrocarbonate (DEPC)-treated water.

RNA.E and RNA.F are synthetic oligoribonucleotides spanning the HBV sequence at nt 1243 to 1281 (RNA.E; 5'-GAACCUUUUCGGCUCUCUGCGAUAUACUCUGCGAAC-3') and nt 1243 to 1271 (RNA.F; 5'-GAACCUUUUCGGCUCUCUGCGAUAUAC-3'), produced by Oligos Etc., Wilsonville, Oreg.

UV cross-linking (UV-C) experiments. Standard binding reactions were carried out with a final volume of 40 μ l with 5 μ g of total nuclear protein and 40 fmol of the ³²P-labeled transcripts in binding buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 1.5 mM EDTA, 450 mM NaCl, 0.01% Triton X-100, 20 μ g of yeast tRNA, and 6 μ g of heparin, incubated for 20 min at room temperature. The reaction mixtures were incubated on ice, irradiated for 10 min with UV light (254 nm) with a Stratagene (Stratagene, La Jolla, Calif.) approximately 3 cm from the bulbs, and then digested with 40 μ g of RNase A and 100 U of RNase T₁ for 45 min at 37°C. Forty microliters of sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 5% mercaptoethanol, 63 mM Tris-HCl [pH 6.8], 10% glycerol, and 0.01% bromophenol blue) was added, and samples were boiled for 5 min, placed on ice, and resolved on an SDS-12.5% polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, the gels were stained with Coomassie blue, destained, dried, and exposed to Biomax (Kodak, Rochester, N.Y.) overnight at -80°C.

RAA. Standard RAA reactions were carried out with a final volume of 40 μ l with 1 μ g of total nuclear protein and 50,000 to 150,000 cpm of the 5' ³²P-labeled HBV oligoribonucleotide E (RNA.E), 40 fmol of unlabeled in vitro transcript B (RNA.B), or 5 μ g of total liver RNA prepared from HBV transgenic mice in a reaction buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 1.5 mM EDTA, 300 mM NaCl, and 0.01% Triton X-100 for 20 min at 37°C. Reactions were stopped by the addition of 150 μ l of 10 mM Tris-HCl (pH 7.4), 20 μ l of 3 M Na acetyl (Ac) (pH 5.2), and 10 μ g of yeast tRNA. Proteins were extracted by the addition of 100 μ l of phenol-chloroform-isoamyl alcohol (1:1:29). Samples were vortexed and centrifuged at maximal speed for 4 min in a tabletop centrifuge (23,000 $\times$ g). Two hundred microliters of supernatant was transferred to a new tube and extracted with 100 μ l of chloroform. After centrifugation as described above, supernatants were transferred into a new tube, and 600 μ l of ethanol (absolute) was added. Samples were mixed, and RNA was precipitated at maximum speed for 15 min at room temperature in a tabletop centrifuge (23,000 $\times$ g). Pellets were washed with 100 μ l of 80% ethanol and vacuum dried. Pellets were used for primer extension or were resuspended in 10 μ l of loading buffer (containing 80% formamide, 1 $\times$ TBE [45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.5], and 0.01% bromophenol blue) and xylene xyanol, loaded, and resolved on a 10% denaturing PAGE gel. After electrophoresis, the gels

were transferred to filter paper, dried under vacuum at 80°C for 2 h, and exposed to Biomax (Kodak) overnight at -80°C or analyzed by phosphorimaging.

5' Labeling and gel purification. Standard labeling reactions were carried out as described in the manufacturer's instructions (Ambion, Austin, Tex.). Briefly, 5 µl of nuclease-free water, 1 µl of oligonucleotide (10 pmol), 1 µl of [γ -³²P]ATP (NEN, Boston, Mass.), 2 µl of 5× forward reaction buffer, and 1 µl of T4 polynucleotide kinase (1 U/µl) were incubated for 30 min at 37°C. After the addition of 10 µl of loading buffer, samples were heated for 10 min at 68°C, cooled on ice, and resolved on a 10% 1.5-mm-thick denaturing PAGE gel. After electrophoresis, the gels were covered with plastic wrap, exposed for 3 min to Kodak Biomax, and developed, and the full-length bands were cut out. The labeled oligonucleotides were eluted in elution buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, and 0.5 mM EDTA for 1 h at 56°C. The elution buffer was replaced, and after the addition of new elution buffer the extraction was continued for 1 h at 56°C. Both eluates were combined, and 1/10 volume of the 3 M NaAc (pH 5.2), 10 µg of tRNA, and 2.5 volumes of ethanol (absolute) were added. Labeled oligonucleotides were recovered by precipitation and resolved in nuclease-free water, and aliquots were counted.

Primer extension. RNA pellets obtained from the RAA reaction mixtures were washed with 70% ethanol-DEPC-treated water, dried, and resuspended in 7 µl of DEPC-treated sterile H₂O. Two microliters of 5× annealing buffer (250 mM Tris-HCl [pH 8.3], 2.7 M KCl, 5 mM EDTA) was added to the RNA along with 1 µl of 5' ³²P-labeled HBV-specific primer (approximately 2 × 10⁵ cpm containing antisense HBV sequences from nt 1312 to 1333 [5'-GTCCCGATA ATGTTTGCTCCAG-3']). RNA was denatured by heating at 70°C for 10 min and annealed to the primer by 56°C for either 2 h or overnight. For extension reactions, the 10 µl of annealing reaction mixture was brought to 40 µl containing a 0.7 mM final concentration of dATP, dCTP, dGTP and dTTP; 50 mM Tris-HCl (pH 8.3); 5.0 mM MgCl₂; 135 mM KCl; 0.25 mM EDTA; 50 ng of actinomycin D/µl; and 10 U of SuperScript reverse transcriptase (Gibco BRL)/µl. The extension reaction mixtures were incubated at 37°C for 3 h. Reactions were terminated with 170 µl of precipitation buffer (370 mM NaAc, 10 mM Tris-HCl [pH 7.4] in DEPC-treated H₂O, 0.5 µg of tRNA/µl), extracted with phenol-chloroform-isoamyl alcohol (24:25:1), ethanol precipitated, and subjected to electrophoresis with 12% polyacrylamide sequencing gels.

RESULTS

Identification of a distinct cleavage event within the in vitro transcript HBV RNA.B. Recently, a tight correlation was found between the cytokine-mediated downregulation of HBV RNA and the disappearance of the full-length HBV RNA-binding protein La, coinciding with the appearance of a smaller La fragment (26). The La binding site was mapped to a computer-predicted stem-loop structure (Fig. 1) (27). It was concluded that this interaction may determine the stability of HBV RNA and that disruption of this interplay allows RNases to attack HBV RNA, thereby accelerating the decay of HBV RNA.

To identify a potential cleavage site within the HBV RNA, we developed an RAA with different HBV RNAs as substrates and nuclear extracts prepared from untreated, MCMV-infected, or CTL-injected HBV transgenic mouse liver as a source for RNases. First, we asked whether distinct HBV RNA cleavage products could be detected by primer extension after incubation of HBV RNA.B with various nuclear extracts. Following incubation of the unlabeled in vitro transcript RNA.B (Fig. 1) with nuclear extracts, remaining RNA was phenol-chloroform extracted, precipitated, and subsequently analyzed by primer extension analysis to detect potential 3' cleavage products (3'-CPs). The 5' ³²P-labeled antisense primer was located at the 3' end of RNA.B, spanning nt 1312 to 1333. As shown in Fig. 2A, two 3'-CPs (3'-CP1 and -2) were detected after incubation of RNA.B with nuclear extracts prepared from CTL-injected or untreated HBV transgenic mice. The background bands probably represent products of nonspecific degradation or pausing sites of the reverse transcriptase. Importantly,

FIG. 1. Predicted secondary structure of HBV in vitro transcript RNA.B bound by the La protein. The secondary structure of the 91-nt-long in vitro transcript RNA.B was calculated with the MFOLD program, version 3 (<http://mfold2.wustl.edu/~mfold/rna/form1.cgi>). The free energy of the structure was calculated to be -25.0 kcal/mol. The stem-loop 2 represents the La binding site. Arrows indicate the 3' ends of synthetic oligonucleotides RNA.E and RNA.F and the identified cleavage sites at positions 1269 to 1270 and 1271 to 1272. The positions for all RNAs are shown according to the HBV ayw subtype sequence.

tantly, the full-length primer extension product was reduced in a time-dependent manner, predominantly with the CTL extracts (Fig. 2, lanes 2 and 3) compared to extracts from untreated mice (lanes 5 and 6). No obvious difference was seen in the amount of 3'-CPs produced by both nuclear extracts. This could be explained by assuming that the cleavage products had already reached a steady state between synthesis and further degradation. Addition of 40 U of RNase inhibitor (RNasin) to the RAA reduced the signal for 3'-CP2 and prevented the overall degradation of the input RNA (Fig. 2, compare lanes 3 and 4 and 6 and 7). These data indicate that the input RNA was cleaved at two positions and that RNA degradation was much more efficient with nuclear extracts prepared 5 days after CTL injection, corresponding to reduced levels of viral RNA and cleavage of La protein (25) in the livers of HBV transgenic mice (24). Additionally, the strong reduction of 3'-CP2 in the presence of RNasin could be explained if we assume that after an initial endoribonucleolytic cleavage of RNA.B (RNasin-resistant 3'-CP1), additional nucleotides were removed by non-specific 5'-to-3' exoribonucleases (RNasin-sensitive 3'-CP2). Also, it remains to be understood how stable the 3'-CP intermediates are, to understand whether the strong degradation by CTL extracts was due to a higher cleavage rate or activation of additional RNases or if the cleavage site was more accessible for the RNase because the La protein was processed.

Next, we asked whether or not the cleavage products were

FIG. 2. RNA.B and RNA.E are cleaved by RNases present in nuclear extracts prepared from HBV transgenic mice. (A) Unlabeled RNA.B was incubated for 30 or 60 min with or without 1 μ g of nuclear extracts (NE) prepared from the livers of HBV transgenic mice, and the cleavage products were analyzed by primer extension as described in Materials and Methods. CTL d5, nuclear extract prepared from the livers of HBV transgenic mice sacrificed on day 5 after CTL administration; Con, nuclear extract prepared from the liver of untreated transgenic mice. RNase inhibitor (40 U) was included in lanes 4 and 7. 3'-CP1 and -2 are indicated. (B) 5' 32 P-end-labeled RNA.E was incubated for 30 min with nuclear extracts (NE) prepared from untreated HBV transgenic mice under standard conditions as described for the RAA, and 5'-CPs were detected. 5'-CP1 was inhibited in the presence of an RNase inhibitor (40 U). RNA was analyzed with a 12% denaturing PAGE gel.

generated by endoribonucleolytic cleavage. We used the 5' 32 P-labeled oligoribonucleotide RNA.E spanning the 5' part (nt 1243 to 1281) of the RNA.B (nt 1243 to 1333) as a substrate for RNase activity present in nuclear extracts derived from untreated transgenic mouse liver (Fig. 1). As shown in Fig. 2B, RNA.E was degraded, and 5'-CPs were detectable. Addition of 40 U of RNasin hindered the appearance of 5'-CP1 (Fig. 2B, lanes 3), suggesting that this product may have been generated by an RNasin-sensitive RNase, while the other product was probably generated by RNasin-insensitive RNases. Note that after primer extension analysis of degraded RNA.B, the shortest 3'-CP (3'-CP2) was reduced in the presence of RNasin (Fig. 2A, lanes 4 and 7), suggesting that 3'-CP2 and 5'-CP1 were produced by the same activity. To map the cleavage sites more accurately, the shorter 5' 32 P-labeled RNA oligoribonucleotide RNA.F (nt 1243 to 1271; 29 nt) was used in the RAA (Fig. 1). RNA.F was cleaved and a 5'-CP similar in size to the 5'-CP2 observed with RNA.E was obtained (Fig. 3). Interestingly, 5'-CP1 was only detectable with RNA.E and not with RNA.F, indicating that 5' cleavage site 1 was located a few bases 3' of the 3' end of RNA.F and that 5' cleavage site 2 was probably located a few nucleotides 5' of the 3' end of RNA.F (Fig. 3). Therefore, it is concluded that HBV RNA.E was cleaved at nucleotides between positions 1265 and 1274.

To show that the cleavage sites were also recognized in full-length HBV RNA and to locate the cleavage sites more precisely, total RNA was prepared from control HBV transgenic mice and subsequently analyzed by RAA and primer extension analysis (Fig. 4). Two 3'-CPs were detected after incubation of full-length HBV RNA with nuclear extracts from livers obtained 5 days after CTL injection. Importantly, the signal for 3'-CP2 was again hindered in the presence of RNasin. The sequencing ladder produced with the same primer (nt 1312 to 1333) used for the primer extension analysis revealed the cleavage sites at position 1269 to 1270 and 1271 to 1272 in

the viral RNA. These positions match very well with the predicted cleavage positions extrapolated from the analysis of the 5'-CPs (Fig. 3).

Taken together, precise mapping of 3' cleavage positions 1

FIG. 3. Identification of cleavage sites using synthetic RNA substrates of different lengths. A standard RAA was performed under conditions described in Materials and Methods. Liver nuclear extracts (NE) (1 μ g) prepared from the livers of untreated HBV transgenic mice were incubated for 30 min at 37°C with 80,000 cpm of 5'-labeled RNA.E or RNA.F. RNA was analyzed with a 12% sequencing gel. RNase inhibitor (40 U) was included in lanes 4 and 8. 5'-CP1 and -2 are indicated.

FIG. 4. HBV RNA is cleaved at positions 1269 to 1270 and 1271 to 1272 by RNase present in nuclear extracts prepared from HBV transgenic mice. HBV RNA (5 μ g) extracted from livers of untreated HBV transgenic mice was incubated with nuclear extract (1 μ g) prepared from the livers of HBV transgenic mice sacrificed on day 5 after CTL administration (CTL NE), processed, and subsequently analyzed by primer extension as described in Materials and Methods. RNase inhibitor (40 U) was included as indicated. The sequencing ladder was produced with a plasmid containing HBV DNA as a template and the same 5' 32 P-labeled oligonucleotide used for the primer extension reaction. Reaction products were loaded on the same sequencing gel. 3'-CP1 and -2 are indicated.

and 2 within full-length HBV RNA and the detection of 5'-CPs most likely indicates that HBV RNA was cleaved by an endoribonuclease. This is further supported by the observation that the longest 5'-CP and the shortest 3'-CP were reduced in the presence of RNasin. In addition, evidence was provided that degradation of RNA.B was more efficient with nuclear extracts prepared from CTL-injected mice.

Next, changes in RNase activity in liver nuclear extracts prepared from MCMV-infected HBV transgenic mice were monitored. RNA.E was used as a substrate because this RNA does not include the complete sequence necessary to form the proposed La binding site and, consequently, cleavage should be independent of endogenous La binding to the substrate. Liver nuclear extracts and total liver RNA were prepared at various time points after MCMV infection. HBV RNA, RNA-binding proteins, RNase activities, and cytokine gene expression were subsequently monitored by Northern blot analysis, UV-C, RAA, and RNase protection analysis (RPA), respectively. As shown in Fig. 5, the disappearance of HBV RNA and p45 coincided with the appearance of the 5'-CP1 and p26 on day 3 after MCMV infection, at which time the inflammatory cytokines (IFN- γ and TNF- α) and 2',5'-oligoadenylate synthase (OAS; a marker for IFN- α/β induction) were strongly induced. These changes were maintained through day 7, after

which HBV RNA and the cytokines returned to baseline levels, followed by the reappearance of p45 coinciding with the disappearance of p26 and 5'-CPs on day 14. These results suggest that at least during the first several days after MCMV infection the cytokine-associated changes in RNase activity and RNA-binding proteins contribute to the disappearance of the viral RNA. The fact that the RNase activity and the HBV RNA-binding protein La take longer to return to baseline than the HBV RNA suggests that other events contribute to the reappearance of the viral RNA. These results were confirmed by the analysis of liver nuclear extracts prepared at various time points after CTL injection of HBV transgenic mice (T. Heise and F. V. Chisari, unpublished observation). The very close relationship between the RNase activity and the presence of La proteins, however, suggests that they may be functionally linked, as is suggested by the physical proximity of the RNase cleavage sites and the La binding site in the viral RNA (Fig. 1). It is important to point out, however, that the increase in RNase activity is independent of the binding of La to the predicted stem-loop (nt 1275 to 1291), since RNA.E (nt 1243 to 1281) does not include the complete sequence necessary to form this structure and was unable to compete for La binding to RNA.B (27). Therefore, it is possible that the increase in RNase activity reflects other cytokine-inducible events such as inactivation of an inhibitor or posttranslational modification of the RNase. These results suggest again a correlation between the changes in RNase activity and RNA-binding proteins, both of which may contribute to the disappearance of the viral RNA.

Because the RNase activity increased in parallel with the disappearance of p45 and the appearance of p26, we asked whether p45, p39, or p26 displayed RNase activity. Therefore, liver nuclear extracts prepared from untreated and CTL-injected mice were mixed, partially purified as recently described (27), and finally subjected to gel filtration. The derivative fractions were analyzed for HBV RNA-binding and HBV-specific RNase activity by UV-C and RAA, respectively. As shown in Fig. 6, the RNase activity responsible for the production of the 5'-CPs eluted later than the appearance of p45, p39, and p26, suggesting that these proteins do not display RNase activity. For unknown reasons, little or no p45 was detected in the gel filtration fractions (Fig. 6, fractions 16 and 17), and thus it remains to be determined whether p45 displays RNase activity. However, the fact that the RNase activity increases as the content of p45 decreases (Fig. 5) makes this possibility quite unlikely. The RNases responsible for the 5'-CPs eluted in fractions 21 and 22, suggesting either that the same RNase produces all of the cleavage products or that different RNases with similar molecular masses are responsible for the observed cleavages.

Characterization of nuclear endoribonuclease activities in extracts from control mice. Additional studies were performed in order to characterize the RNase activities in more detail; to do so, we studied the kinetics and temperature dependence of RNase activity. We first determined the optimal reaction temperature to be 37°C or higher, while the substrate was only partially cleaved at 23°C and no cleavage was observed at 4°C (Fig. 7). Monitoring the cleavage efficiency over time revealed that 5'-CP2 was produced at the highest rate and that after 80 min almost no further increase in cleavage products was ob-

FIG. 5. Kinetics of RNA.E cleavage during MCMV infection. HBV transgenic mice were infected with MCMV, and livers were harvested from groups of mice sacrificed on day 1 (d1), d3, d5, d7, d14, and d28 after infection, as indicated. Total hepatic RNA and liver nuclear extracts were prepared and then analyzed by Northern blotting (NB), UV-C, RAA, and RPA as described in Materials and Methods. Northern blots were probed for the expression of HBV RNA, glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH), and 2',5'-OAS mRNA and compared to total liver RNA prepared from two saline-injected animals. Nuclear extracts (5 μ g) from each mouse were incubated with 40 fmol of in vitro-transcribed RNA.B, processed, and analyzed by SDS-PAGE. Nuclear extracts (1 μ g) from each mouse were incubated with 80,000 cpm of RNA.E, processed as described in Materials and Methods, and analyzed with a 12% sequencing gel. Total RNA (10 μ g) from the same livers was analyzed by RPA for the expression of TNF- α and IFN- γ . The mRNA encoding the ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane. 5'-CP1 and -2 are indicated.

served (Fig. 7). At this time, we do not know whether this reflects a steady state between cleavage and subsequent degradation of the cleavage intermediates or whether the enzymes are destroyed or inhibited by the products. We then analyzed the nature of these RNases and studied the influence of the different components in standard reaction mixtures. Prior digestion of nuclear extracts with proteinase K reduced the appearance of 5'-CP1 (Fig. 8A, compare lanes 2 and 3), while little reduction was observed for 5'-CP2. Furthermore, prior heating of the nuclear extracts at the indicated temperature for 10 min and subsequent centrifugation of precipitated denatured proteins revealed that the activities were quite stable. However, heating the extract at 75 or 95°C partially or completely destroyed the activity, respectively, indicating a protein-dependent cleavage of RNA.E (Fig. 8B). To measure the pH dependency of the RNases, the RAA was performed at pH 9.5, 7.4, and 5.2 (Fig. 8A, lanes 4 through 6). Cleavage was almost completely inhibited at pH 9.5, while 5'-CP1 appeared maximal at pH 7.4 (Fig. 8A, lanes 4 and 7) and 5'-CP2 was produced maximally at pH 5.2 (Fig. 8A, lane 4). Collectively, these results suggest that the RNases in the liver nuclear extracts probably consist of one or several proteins.

In separate experiments (Fig. 9), we demonstrated that the

FIG. 6. p45, p39 and p26 do not display RNase activity. HBV transgenic mice were injected with saline or 10^7 CTLs, and livers were harvested from groups of mice sacrificed on days 3 and 5 and from untreated mice. Liver nuclear extracts were prepared, mixed, partially purified, and subjected to gel filtration as described previously (27). Twenty-microliter aliquots of the indicated gel filtration fractions were analyzed by UV-C (UV) and RAA as described in Materials and Methods. Gel filtration aliquots were incubated with 40 fmol of in vitro-transcribed RNA.B, processed, and analyzed by SDS-PAGE (UV) or subjected to RAA and processed as described in Materials and Methods. The remaining RNA was analyzed with a 12% sequencing gel. 5'-CP1 and -2 are indicated.

FIG. 7. Time and temperature dependencies of RNA.E cleavage. A standard RAA was performed under conditions described in Materials and Methods. Liver nuclear extracts (NE) (1 μ g) prepared from the livers of untreated HBV transgenic mice were incubated with 80,000 cpm of 5'-labeled RNA.E and incubated for different periods of time as indicated or for 20 min at 4, 23, and 37°C. Remaining RNA was analyzed with a 12% sequencing gel. 5'-CP1 and -2 are indicated.

RNase activities were independent of $MgCl_2$ and other divalent ions (data not shown), EDTA, and Triton X-100 but were strongly inhibited at higher sodium chloride salt concentrations (450 mM) (Fig. 9, compare lanes 2 and 6).

To determine the chemical nature of the cleavage products, we attempted to discriminate between a 3'-terminal hydroxyl or 3' phosphate group (Fig. 10). Therefore, an RAA sample was separated on a 10% urea polyacrylamide gel, and the cleavage products were eluted, precipitated, and subsequently treated with phosphodiesterase (PDE, also known as snake venom). PDE is an exonuclease selectively degrading RNA molecules containing a 3' hydroxyl group but not a 3' phosphate (46). In a separate reaction, 5'-labeled RNA.E was treated with PDE under the same conditions. As shown in Fig. 10, PDE was able to degrade the 5'-labeled substrate RNA.E (compare lanes 4 and 5) but not the eluted cleavage products (compare lanes 6 and 7), indicating that the described activities

produce cleavage products containing 3' phosphate and 5' hydroxyl groups.

DISCUSSION

HBV RNA is downregulated by inflammatory cytokines induced in the liver following the injection of HBsAg-specific CTLs or during unrelated viral infections that cause hepatitis in HBV transgenic mice (8, 21–24). 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 fragment of the La protein in the mouse liver following CTL injection or viral infection (26, 27). In the present study we identified endoribonucleolytic activities that cleave the viral RNA close to the binding site of the La protein. These activities were upregulated concurrent with HBV RNA decay and the appearance of a La protein fragment, suggesting that a functional correlation may exist between these two parameters and the endoribonuclease. Several endoribonucleases were recently described, and in some cases it was shown that the cleavage site can be protected by RNA-binding proteins, indicating that a regulatory mechanism for mRNA degradation could be the protection of cleavage sites by protein factors (3, 7, 13, 53, 54). Recently, the La protein was described as stabilizing a histone mRNA decay intermediate, indicating that the La protein prolongs the histone mRNA half-life (33). That such a mechanism may also be operative in the cytokine-mediated downregulation of HBV is reasonable to assume, because the disappearance of HBV RNA coincides with the disappearance of the full-length La protein which was shown to bind to a specific HBV RNA structure. In support of this assumption, preliminary data from our lab suggest that the HBV RNA half-life is reduced when structural features of the La binding site are changed by mutagenesis (T. Heise and I. Ehlers, unpublished observations). That 3'- as well as 5'-CPs

FIG. 8. Dependence of nuclear ribonucleolytic activity on pH, proteinase K, and temperature. A standard RAA was performed under conditions described in Materials and Methods. Liver nuclear extracts (NE) (1 μ g) prepared from the liver of untreated HBV transgenic mice were incubated for 30 min at 37°C with 80,000 cpm of 5'-labeled RNA.E. Remaining RNA was analyzed with a 12% sequencing gel. (A) Pretreatment of the extracts with proteinase K (20 μ g) was performed for 30 min at 37°C (lane 3), or cleavage reactions were performed at pH 5.2, 7.4, or 9.5 (lanes 4, 5, and 6). (B) Nuclear extracts (NE) were heated at 45, 55, 75, and 95°C for 10 min, cleared by centrifugation, and subjected to RAA. 5'-CP1 and -2 are indicated.

FIG. 9. Characteristics of RNA.E cleavage. A standard RAA was performed under conditions described in Materials and Methods (lanes 1 and 2) with the alterations as indicated (lanes 3 through 7). Liver nuclear extracts (NE) (1 μ g) prepared from the livers of untreated HBV transgenic mice were incubated for 30 min at 37°C with 100,000 cpm of 5'-labeled RNA.E. Remaining RNA was analyzed with a 12% sequencing gel. 5'-CP1 and -2 are indicated.

were generated by cleavage at the same positions was shown by mapping of the 3' cleavage sites to positions 1269 to 1270 and 1271 to 1272 and by narrowing down the 5' cleavage sites with substrates of different length. These sites are located immediately upstream of a computer-predicted stem-loop structure identified as a La binding site (Fig. 1) (27). In addition, it was shown that detection of 5'-CP1 and 3'-CP2 was reduced in the presence of RNasin, indicating that the same activity produced the 5'-CP1 and the 3'-CP2.

We observed different cleavage efficiencies in nuclear extracts prepared from untreated and treated mice. The primer extension analysis of HBV RNA revealed strong degradation of RNA.B with nuclear extracts prepared from CTL-injected mice and less-efficient degradation with nuclear extracts from untreated mice (Fig. 2). Note that the extract with increased RNase activity was prepared from liver, harvested 5 days after CTL injection, at a time when HBV RNA levels were reduced and La was fragmented (26). The coincidence of HBV RNA decay, La fragmentation, and efficient cleavage of HBV RNA substrates strongly supports a functional correlation between HBV RNA stability, the presence of a full-length La protein, and lower levels of endoribonucleolytic activity. Since the cleavage site is located immediately 5' to the La binding site, it is possible that La sterically hinders RNases from accessing the cleavage sites.

The cleavage of the 5'-labeled RNA.E was more pronounced after MCMV infection (Fig. 5) at time points when HBV RNA was reduced, cytokines were induced, p45 was absent, and p26 was detectable. The transient increase in cleavage efficiency suggests that the cleavage might be independent of the stem-loop and/or the binding of the full-length La protein to it, indicating an upregulation of these RNase activities. Furthermore, the estimated molecular mass of less than 26 kDa (Fig. 6) excludes the possibility that the La protein or the La fragments detectable by UV-C experiments display the RNase activity detected in these assays. Therefore, it is assumed that the increase in cleavage efficiency is due to an upregulation of endoribonucleases by either the induced ex-

pression of the endoribonucleases, their activation by post-translational modification, or the inactivation of an inhibitor. An increase in RNase activity has been reported after herpes simplex virus infection of Vero cells (40), after human immunodeficiency virus infection of lymphocyte cell lines (1), after insulin treatment of primary rat hepatocytes (28), and after estrogen treatment (15, 41). RNase L is activated by 2'-5'-linked oligoadenylates produced after the induction of 2'-5' oligoadenylate synthetase by IFN or by the appearance of double-stranded RNAs in cells (16, 34). RNase L is thought to be part of a host defense mechanism against viral infections. However, the molecular masses of two RNase L forms in mice were determined as 40 and 80 kDa (44), which differs from the apparent molecular mass of the RNases described in our report.

Obviously, until the identity of the endoribonucleolytic activity described in this study is established and its functional role in the stabilization or destabilization of HBV RNA can be directly tested, we must consider the possibility that this endoribonucleolytic activity is related to known endoribonucleases and that the different cleavage products are related to different endoribonucleolytic activities. Comparison of these activities with known endoribonucleases provides some information about the type of enzymes involved in cleavage. The activities were found to be partially RNasin resistant and proteinase K sensitive and partially inactivated at 75°C; the RNA substrate was cleaved in a time- and temperature-dependent manner (Fig. 7 and 8B).

FIG. 10. Characterization of the 5'-CPs. A standard RAA was performed with or without RNase inhibitor (lanes 2 and 3) under the conditions described in Materials and Methods. Liver nuclear extracts (NE) (1 μ g) prepared from the liver of untreated HBV transgenic mice were incubated for 30 min at 37°C with 100,000 cpm of 5'-labeled RNA.E. Cleavage products were detected by autoradiography, extracted, precipitated, and dissolved in 7 μ l of DEPC-treated water. 5'-labeled RNA.E (lane 5) and purified 5'-CPs (lanes 6 and 7) were treated with PDE (1 μ l) for 5 min at room temperature in 20 μ l of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 14 mM MgCl₂. The remaining RNA was extracted and analyzed with a 12% sequencing gel.

Some other proteins are described to be proteinase K resistant, like the RNA-binding protein Auf (5) and the ferritin L chain, which displays also RNA-binding activity (29). Furthermore, the activities were independent of $MgCl_2$, and the cleavage efficiency was reduced at high concentrations of NaCl (Fig. 9). These features are most consistent with the cleavage properties reported for polysomal RNase 1, which is also independent of divalent ions, inhibited at high salt concentrations, still active after heating at 70°C, and RNasin resistant, but different in molecular mass (9, 15).

The calculated molecular mass of less than 26 kDa indicates that the activities described in this report are different from those of endoribonucleases described cleaving *c-myc* mRNA (~39 kDa) (33), albumin mRNA (~60 kDa) (9, 15), interleukin-2 mRNA (~60 to 70 kDa) (31), and Xihbox2B mRNA (~120 kDa) (6, 7) and from RNase L (~40 and 80 kDa) (44). The molecular masses for the endonucleolytic activities involved in the decay of TFR mRNA (3) and insulin-like growth factor II mRNA (35, 38) are, to our knowledge, currently undefined. Other endoribonucleases described as human equivalents of prokaryotic RNA.E with molecular masses of ~65 (55) and 13.3 (12, 52) kDa have been described. Therefore, the endoribonuclease activity described in this report appears to be different in molecular mass from all previously reported endoribonucleases except for ARD-1 (activator of RNA decay 1) (12).

Another unique feature of the activity described herein is the production of cleavage products carrying a 3' phosphate group, which protected the cleavage product against degradation by PDE (Fig. 10). This observation distinguishes these RNases from the polysomal RNase 1 and ARD-1, because these RNases produced cleavage products with 3' hydroxyl groups (9, 12, 46).

The cleavage position recognized in intact viral RNA prepared from the liver of HBV transgenic mice was mapped to the sequence 5'-CCA/UA/CU-3'. Although we do not know whether the surrounding nucleotides or the structural features of the RNA molecule influence recognition of the cleavage site by the RNase activity described in this report, some endoribonucleases are known to cleave their substrates adjacent to an adenosine (3, 4, 9, 38). We do not know yet how selectively the HBV RNA was cleaved by this endoribonucleolytic activity, and it is possible that the viral RNA was cleaved at additional positions as described for estrogen-regulated endoribonucleases involved in the decay of albumin mRNA (9) and apolipoprotein 2 mRNA (4), which cleaved the respective mRNA at several positions.

In summary, we have identified a novel endoribonucleolytic activity in nuclear extracts prepared from normal and especially inflamed transgenic mouse liver tissue that cleaves HBV RNA in proximity to the proposed La binding site. The up-regulation of this endoribonuclease activity correlates with the disappearance of p45, the full-length La protein, and with the degradation of HBV RNA from the liver in response to CTL injection or MCMV infection. Purification of the endoribonuclease and more-detailed characterization of the cleavage site will be necessary to determine the precise mechanisms whereby this endoribonucleolytic activity regulates the stability of HBV RNA in this model.

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