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Interleukin-2 Downregulates Hepatitis B Virus Gene Expression in Transgenic Mice by a Posttranscriptional Mechanism†

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We have recently demonstrated that tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) downregulate the hepatic steady-state content of hepatitis B virus (HBV) mRNA in vivo in HBV-transgenic mice and that the IL-2 effect is mediated by TNF-α. In the current study, we demonstrate that IL-2-induced downregulation of hepatic HBV 2.1-kb mRNA is not due to changes in the transcription rate or the intranuclear maturation or export of this transcript but that it is selectively and profoundly depleted from the cytoplasm of the liver cells in vivo following IL-2 administration. Collectively, these results suggest that IL-2 alters the steady-state content of hepatic HBV mRNA by a posttranscriptional mechanism in vivo, that this effect is mediated by TNF-α, and that it probably reflects increased cytoplasmic degradation of the viral transcript.

Hepatitis B virus (HBV)-induced viral hepatitis is a necroinflammatory liver disease that is thought to be initiated by a class I-restricted cytotoxic T-lymphocyte (CTL) response to HBV antigens expressed by infected hepatocytes (4, 23, 25). It has been suggested that the same T-cell response may also play an important role in viral clearance (15, 21). Indeed, it is possible that the immunopathological and antiviral effects of the CTL response could be due either to the direct cytopathic effect of the CTL for HBV-infected hepatocytes or to the action of antigen-nonspecific mediators that they and other inflammatory cells contribute to the inflammatory milieu in the infected liver.

We have recently begun to explore the relative contributions of these direct and indirect pathways to viral clearance and disease pathogenesis in HBV-infected patients and in HBV-transgenic mice. We have shown that patients with acute viral hepatitis who successfully clear the virus develop a strong class I- and class II-restricted T-cell response to multiple HBV-encoded antigens (23, 25), while these responses are absent or greatly diminished in patients with chronic hepatitis who do not clear the virus (23, 25, 28). We have also shown that all of the histopathological manifestations of acute viral hepatitis can be reproduced experimentally following the injection of class I-restricted, hepatitis B surface antigen (HBsAg)-specific CTL into HBsAg-positive transgenic mice and that both direct and indirect cytokine-mediated mechanisms play important roles in the natural history of this disease (1, 2, 24).

Accordingly, we became interested in the possibility that hepatocellular HBV gene expression might be altered by one or more of the inflammatory cytokines that are likely to be produced by the antigen-specific T cells in the liver or by the antigen-nonspecific inflammatory cells that they recruit. Using a transgenic mouse model in which the HBV 2.1-kb major envelope antigen transcript is expressed at high levels by hepatocytes under the transcriptional control of its own promoter-enhancer elements, we recently reported that tumor necrosis factor alpha (TNF-α) administration causes a substantial, noncytopathic decrease in the intrahepatic steady-state content of HBV mRNA (15). In an extended search for other cytokines capable of influencing HBV mRNA biosynthesis, we have recently shown that interleukin-2 (IL-2) is also capable of downregulating HBV mRNA in the transgenic mouse liver and that the IL-2 effect can be blocked completely by the prior administration of antibodies to TNF-α (18). In the current study, we examined the intracellular mechanisms that might be responsible for this effect.

MATERIALS AND METHODS

Transgenic mice. The HBV transgenic lineage pFC80-219 used in these studies was produced by microinjection of a 12.9-kb *Hin*dIII-*Pst*I fragment excised from plasmid pFC80 that contains four complete HBV genomes (*ayw* subtype) linked in a tandem head-to-tail orientation at the HBV *Eco*RI site of pBR322 (15). Structural analysis of the integrated transgene reveals that it is integrated at a single site in the mouse genome and that at least one complete, uninterrupted HBV genome is present. These animals express highly reproducible levels of the HBV 2.1-kb mRNA in their hepatocytes under the transcriptional control of the HBV surface promoter. As illustrated in Fig. 1, this transcript covers approximately two-thirds of the viral genome and it overlaps all of the other known viral transcripts, including the 3.5-kb pregenomic RNA. No other HBV transcripts are detected by Northern (RNA) blot analysis of total liver RNA in these animals. The reason for this is unclear, but it is a common feature of most transgenic lineages that we have produced with greater-than-genome-length fragments of HBV DNA (5a). We also selected this particular lineage because it displays minimal animal-to-animal variation in HBV gene expression, presumably because of integration site effects or the fact that the mice are fully inbred (C57BL/6). To further assure homogeneity, all experiments were performed with mice that were carefully matched for age, sex, and levels of HBsAg in serum.

IL-2. Recombinant murine IL-2 was provided by Steven Gillis (Immunex, Seattle Wash.). In all experiments, 6- to 8-week-old male transgenic mice in groups of two to four received a single intraperitoneal (i.p.) injection of 100,000 U of IL-2 (5×10^7 U/mg; endotoxin, <0.1 EU/mg by the *Limulus* amebocyte lysate assay) diluted in 0.8% NaCl solution (saline) (endotoxin, <0.25 EU/ml by *Limulus* amebocyte assay) as described previously (15, 18). This dose of IL-2 has been shown to cause a profound decrease of HBV steady-state mRNA within 16 h of i.p. injection without any histological or bio-

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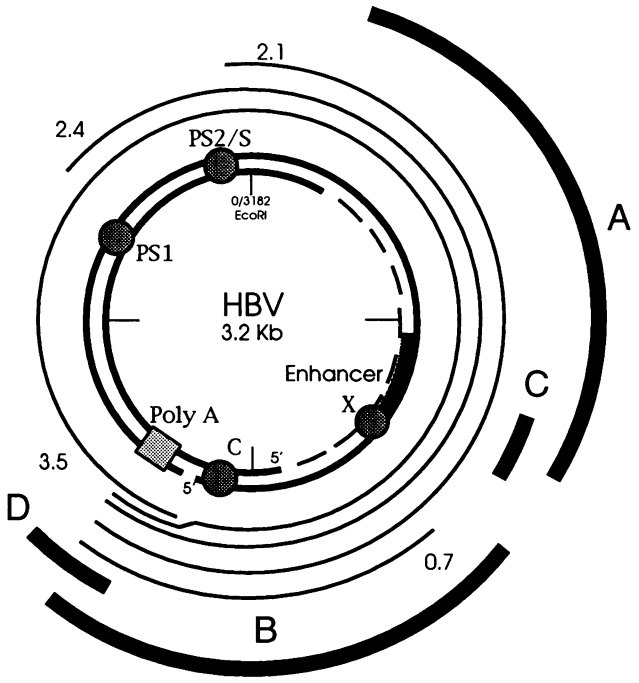

FIG. 1. Schematic representation of the HBV genome (*ayw* subtype) used in these studies. The positions of the HBV probes used for the run-on (A and B) and RNase protection (C and D) assays are indicated.

chemical evidence of liver cell injury or intrahepatic inflammation (18). Livers were harvested 2, 6, and 16 h after injection. Control animals were injected with saline diluent only, and their livers were harvested after 16 h.

RNA preparation. (i) Total RNA. Total RNA was prepared by mechanically pulverizing frozen livers as previously described (15), followed by extraction by the acid-guanidium phenol-chloroform method (8). Northern blot analysis of total RNA was performed by standard procedures as previously described, and RNA was hybridized to HBV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences as described elsewhere (15).

(ii) Preparation of nuclear RNA. Nuclei were prepared according to the procedure of Schibler et al. (35). Briefly, liver samples were homogenized by 10 strokes in a Potter-Elvehjem tissue grinder in 5 ml of buffer A {60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid], 2 mM EDTA, 0.3 M sucrose, 15 mM Tris Cl (pH 7.5)} and centrifuged over a 5-ml cushion of buffer B (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 0.85 M sucrose, 15 mM Tris Cl [pH 7.5]) at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in 3 ml of buffer C (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2 M sucrose, 15 mM Tris Cl [pH 7.5]) and centrifuged over an 8-ml cushion of buffer C in a Beckman SW40 rotor at 36,000 rpm for 1 h at 4°C. Nuclei were resuspended in 1 ml of storage buffer (20 mM Tris-Cl [pH 8], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride [PMSF], 50% glycerol) and stored at −80°C.

Nuclear RNA was extracted from purified nuclei. Nuclei (5×10^6) were pelleted by centrifugation and resuspended in 50 μl of TE buffer (10 mM Tris Cl [pH 8.0], 1 mM EDTA) containing 100 μg of proteinase K and 0.5% sodium dodecyl sulfate (SDS). After incubation at 37°C for 1 h, RNA was extracted by the hot acid-phenol method (34) and ethanol precipitated. RNA was resuspended in TM buffer (10 mM Tris Cl [pH 8.0], 10 mM $MgCl_2$) containing 1 mM dithiothreitol, 10 U of RNasin (Promega, Madison, Wis.), and 2 U of DNase I, and the suspension was incubated for 30 min at 37°C. RNA was extracted by the hot acid-phenol method, ethanol precipitated, and resuspended in water.

(iii) Preparation of cytoplasmic RNA. Livers were homogenized by 10 strokes in a Potter-Elvehjem tissue grinder in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 0.3 M sucrose, 15 mM Tris Cl [pH 7.5]). The homogenate was immediately centrifuged at 10,000 × *g* for 2 min to precipitate the nuclei. The supernatant was diluted fivefold with acid-guanidium solution, and the RNA was purified by the acid-guanidium phenol-chloroform method (8).

RNase protection assay. (i) Plasmids. Short HBV DNA fragments derived from the HBV enhancer region (E) and the HBV polyadenylation signal (PA) region were subcloned into pBluescript II SK+ plasmid (Stratagene, La Jolla, Calif.). The two oligonucleotide pairs—GGTCTTTTGGGTTTTGCTGC and GTTGCCGGGCAACGGGGTAA and ctcggattcCTGTA GGCATAAATTGGT and TTGCCTGAGTGCAGTATGGT GAGG—were used to generate (by polymerase chain reaction amplification with pFC80 [15] plasmid DNA as template) the 160-nucleotide-long E DNA fragment corresponding to HBV (*ayw*) (14) nucleotides 1001 to 1160 and the 289-nucleotide-long PA DNA fragment corresponding to HBV (*ayw*) nucleotides 1782 to 2070, respectively.

The E and PA DNA fragments were subcloned into pBluescript II SK+ at the *Sma*I site. The orientation of the inserts into the pBluescript II SK+ plasmid was determined by sequencing, and the clones pBSII-E/D7 and pBSII-PA, for which the insert orientation allows HBV antisense transcription from the pBluescript II SK+ T7 promoter, were selected.

Assembly of the pBSII-GAP/B6 construct was performed in a similar fashion by polymerase chain reaction amplification (oligonucleotides GCCCAGAACATCATCCCTGC and ACA TTGGGGGTAGGAACACG) of a 113-nucleotide-long DNA fragment corresponding to nucleotides 646 to 760 of the mouse GAPDH cDNA (32) and subcloned into pBluescript II SK+ at the *Sma*I site.

(ii) Probe preparation. Synthetic RNAs were transcribed in vitro with T7 RNA polymerase. The transcribed fragments always included plasmid sequences to allow size distinction in the RNase protection assay between the effectively protected fragment and the residual undigested input probe. Linearized plasmids (1 μg) were incubated in a 25-μl transcription reaction solution (40 mM Tris Cl [pH 8], 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 750 mM dithiothreitol, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM UTP, 50 μCi of [α-^{32}P]CTP, 1 U of RNasin, 10 U of T7 RNA polymerase) for 1 h at 37°C. DNase I (10 U) was added, and the incubation continued for another 30 min. The full-length transcribed fragments were separated by electrophoresis into a denaturing (8 M urea) 6% polyacrylamide gel in Tris-borate–EDTA buffer according to standard procedures. Bands visualized under UV illumination after ethidium bromide staining were excised, and the RNA fragments were eluted overnight at room temperature in 1 ml of 100 mM sodium acetate (pH 5.2).

(iii) Hybridization. Cytoplasmic RNA (10 μg) or nuclear

RNA (5 μg) was ethanol coprecipitated with 2×10^5 cpm of each probe. RNAs were resuspended in 50 μl of hybridization buffer (10 mM Tris Cl [pH 8], 5 mM EDTA, 300 mM NaCl), denatured for 5 min at 85°C, and incubated overnight at 62°C. Samples were diluted with 250 μl of hybridization buffer containing 4 mg of RNase A and T1 per ml and incubated for 1 h at room temperature. Hybridization buffer (50 μl) containing 50 μg of proteinase K and 4% SDS was added, and incubation was performed for 1 h. After addition of 1 μg of *Escherichia coli* RNA, the samples were extracted with a mixture of 450 μl of chloroform and 150 μl of phenol (saturated in 0.1 M Tris Cl [pH 8]). After ethanol precipitation, RNAs were separated by electrophoresis into a denaturing 6% polyacrylamide gel. Gels were fixed in 10% methanol–10% acetic acid, dried, and exposed for autoradiography.

Nuclear run-on. (i) Nascent transcript labeling. Equal numbers of nuclei from different time points were labeled in each experiment. Typically, 7×10^6 to 10^7 nuclei in storage buffer were centrifuged at 4°C for 2 min at 10,000 × *g*. Nuclei were resuspended in 100 μl of transcription reaction mixture (10 mM Tris Cl [pH 8], 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 1 mM UTP, 250 μCi of [α-^{32}P]CTP [400 to 800 Ci/mmol], 10 U of RNasin, 0.1 mM PMSF, 8% glycerol) for 15 min at 30°C. Reactions were terminated by addition of 50 μl of SDS (2%) containing 100 μg of proteinase K, and the reaction mixtures were incubated for 30 min at 45°C. After addition of 350 μl of sodium acetate (10 mM, pH 5.2). RNAs were extracted at 50°C with 400 μl of phenol equilibrated with sodium acetate (1 M, pH 5.2). Nonincorporated nucleotides were removed by filtration through a Sephadex G-50 spin column.

(ii) Filter hybridization. Hybridization membranes for run-on analysis were prepared with the following plasmids: pHcGAP (GAPDH) (36), EBO-X and EBO-S (19), and pBluescript II SK+ (Stratagene). Denatured plasmid DNA (5 μg) was loaded onto nylon membranes (Hybond; Amersham, Arlington Heights, Ill.) with a slot blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.) and UV cross-linked to the membrane. Membranes were prehybridized at 65°C for at least 4 h in hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt solution, 50 mM sodium phosphate buffer [pH 7.5], and 100 ng of *E. coli* RNA per ml). SSC and Denhardt solutions were prepared according to standard protocols (33). Membranes were hybridized with the labeled transcripts in 5 ml of fresh hybridization buffer for 20 h at 65°C. Equal numbers of counts per minute per membrane (typically between 5×10^6 and 10^7) were used for each set of hybridizations. Membranes were washed first in 2× SSC–0.1% SDS and then in 0.2× SSC–0.1% SDS at 65°C and exposed for autoradiography.

RESULTS

Kinetics of the effect of IL-2 on hepatic HBV 2.1-kb mRNA in vivo. Total liver RNA prepared from animals injected with IL-2 or saline was analyzed by Northern blotting with an HBV-specific probe. As we have previously shown (18), the steady-state content of the HBV 2.1-kb mRNA in the liver is reduced by approximately 70 to 80% (estimated densitometrically by comparison with saline-injected controls) relative to GAPDH mRNA, which is unaffected by IL-2 administration (Fig. 2A). These livers served as the source of total, cytoplasmic, and nuclear RNAs employed in all subsequent analyses.

Analysis of HBV S promoter activity. The activity of the HBV S gene promoter that controls the 2.1-kb HBV mRNA transcription rate was assessed by nuclear run-on analysis.

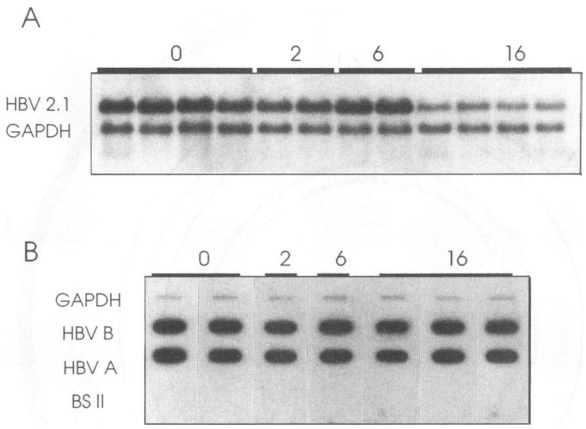

FIG. 2. (A) Northern blot analysis of 20 μg of total liver RNA. Livers were harvested 2, 6, and 16 h after the i.p. administration of 100,000 U of IL-2. Control animals (0) were injected with saline, and the livers were harvested after 16 h. Probes were prepared by random priming of HBV and GAPDH DNA fragments as described in the text. (B) Nuclear run-on transcription analysis of liver nuclei prepared from transgenic mice that had been injected i.p. with 100,000 U of IL-2 2, 6, or 16 h earlier or with saline (0) as described in the text. Radiolabeled nuclear RNAs from the transcription assay were hybridized to GAPDH, HBV, and pBluescript II (BSII) sequences bound to nitrocellulose as described in the text. HBV A and HBV B divide the HBV 2.1-kb mRNA region into approximately equal halves, as illustrated in Fig. 1.

Liver nuclei from IL-2- and saline-injected animals were isolated, and native transcripts were elongated in vitro in a run-on experiment. The newly elongated transcripts were hybridized to HBV S-region, HBV X-region, GAPDH, and control plasmid pBluescript II SK+ sequences prebound to the hybridization membranes.

Despite the major decrease in hepatic HBV mRNA content observed by Northern blot analysis 16 h after IL-2 injection, the HBV-specific signal was unchanged at all time points in the run-on experiments (Fig. 2B), indicating that the transcriptional activity of the HBV S promoter was not affected by the injected cytokine. Furthermore, no differences between the hybridization signals of the HBV S region and the HBV X region were observed at any time point after cytokine injection, indicating that the elongation of the 2.1-kb transcript was not blocked along the HBV genome. As expected since there were no changes in GAPDH transcript abundance, hepatic GAPDH promoter activity was also unaffected when examined in the same experiment (Fig. 2B). The absence of a signal with the control plasmid pBluescript II SK+ indicates that the experiment was performed under sufficiently high stringency conditions to minimize nonspecific cross-hybridization with cellular sequences in this experiment.

Analysis of the intracellular processing of the 2.1-kb HBV mRNA. To analyze the influence of IL-2 administration on the intracellular processing of the 2.1-kb HBV mRNA, we examined nuclear and cytoplasmic RNA from treated and control animals for the relative contents of HBV and GAPDH transcripts by RNase protection with antisense RNA probes. As illustrated in Fig. 3, the 160-nucleotide-long HBV-specific protected fragment was selectively reduced in the cytoplasmic compartment 16 h after injection of IL-2, corresponding to the decrease in the 2.1-kb HBV mRNA detected in total liver mRNA at that time point (Fig. 2A). In contrast, the 113-nucleotide-long GAPDH-protected fragment was unchanged

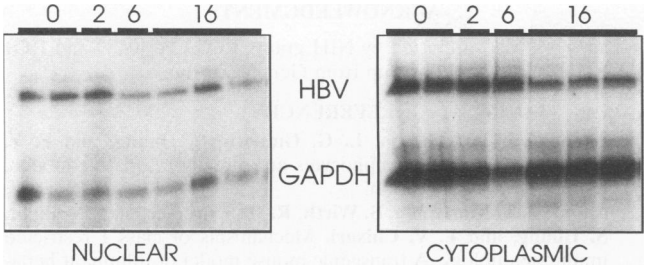

FIG. 3. Intracellular distribution of the HBV mRNA. Cytoplasmic and nuclear RNAs prepared from livers harvested 2, 6, and 16 h after i.p. injection of 100,000 U of IL-2 or 16 h after saline injection (0) were analyzed by RNase protection assay with GAPDH and HBV C antisense riboprobes as illustrated in Fig. 1.

in either compartment. The slight sample-to-sample variation in the nuclear compartment is probably due to technical factors, since it was not observed in a duplicate experiment (data not shown). The unchanged nuclear content of HBV mRNA observed by the RNase protection assay independently confirms the results of the run-on experiments which, together with the profound decrease in HBV cytoplasmic mRNA, suggest that the effect of IL-2 is posttranscriptional and that it occurs in the cytoplasm.

Analysis of HBV 3′-end formation. Having demonstrated differential intracellular responsiveness of HBV transcripts to IL-2 administration when compared with cellular GAPDH transcripts used as an internal standard, we evaluated the relative efficiency of HBV transcript maturation in IL-2-treated and control mice. To this end, we assessed the relative efficiency of 3′-end formation of the 2.1-kb HBV transcript by comparing the amount of HBV mRNA terminating at the HBV polyadenylation site with the amount of HBV mRNA that extends past that site, representing read-through transcripts, in nuclear and cytoplasmic RNA from the IL-2-treated animals and saline-treated controls (Fig. 4). To distinguish between these two RNA species, we generated a 401-nucleotide (including plasmid flanking sequences) antisense RNA probe that overlaps the polyadenylation site of the HBV genome. This probe, when used in an RNase protection assay, can protect a 158-nucleotide (short) HBV RNA fragment if the HBV transcripts terminate at the polyadenylation site or a 289-nucleotide (long) RNA fragment if HBV transcripts extend beyond (read through) the HBV polyadenylation site. If IL-2 causes decreased recognition of the HBV polyadenylation signal, the abundance of the short fragment should decrease in the nuclear compartment, while the amount of the long RNA should increase to a commensurate degree. In fact, however, no changes were observed in either the long fragment or the short fragment in the nuclear compartment at any time after IL-2 administration. In contrast, the short fragment was greatly reduced in the cytoplasmic compartment 16 h after IL-2 injection. The data indicate that recognition of the HBV polyadenylation signal is not affected by IL-2 administration. The reduced abundance of the short fragment in the cytoplasm confirms the observations presented on Fig. 3 with a different HBV-specific probe and further suggests that posttranscriptional events located in the cytoplasm mediate the negative effect of IL-2 on hepatic HBV mRNA. Note that this assay addresses the relative efficiency of the polyadenylation site recognition. Absolute evaluation would require the determination of the individual stability of both read-through and polyadenylated species, which is not achievable in this system.

DISCUSSION

We have previously shown that TNF-α downregulates the steady-state content of hepatic HBV 2.1-kb mRNA in vivo in HBV transgenic mice whose HBV gene expression is controlled by the natural viral promoter (15). Recently, we have shown that IL-2 has the same effect as TNF-α in vivo but that it is more efficient even though it operates by a TNF-α-dependent pathway (18). We have also reported (15, 16) that in HBV transgenic mice, lipopolysaccharide (LPS) downregulates the hepatic steady-state content of a slightly longer (2.4-kb) HBV envelope region mRNA, the transcription of which is controlled by cellular metallothionein or albumin rather than HBV promoters (6, 7).

The mechanism(s) responsible for these changes is a matter of considerable interest for fundamental reasons and, potentially, for therapeutic purposes as well. In our early studies (15), we observed that the transgene-derived, metallothionein-driven HBV transcript decreased in the transgenic mouse liver after LPS administration at the same time that the endogenous cellular metallothionein transcript was induced, as it does during the hepatic acute-phase response that is known to be activated by LPS (9). The observation that HBV transcripts could be similarly downregulated while under the transcriptional control of different promoters (15) suggested to us that HBV gene expression might be susceptible to regulation by inducible, posttranscriptional mechanisms that could lead to changes in HBV mRNA stability. Accordingly, in the current studies using transgenic mice that express the same HBV mRNA under the transcriptional control of the natural viral regulatory elements, we systematically examined the various steps involved in hepatic HBV mRNA synthesis following IL-2 administration in order to identify the molecular basis for this effect.

Since nuclear run-on analysis revealed that the transcription rate of the HBV S promoter was unaffected at all time points after IL-2 injection (Fig. 2B), despite a major decrease in the abundance of the corresponding mRNA (Fig. 2A), it would appear that the negative effect of IL-2 on HBV gene expression occurs at one or more steps after transcription initiation.

Accordingly, we asked whether the decreased HBV mRNA might reflect abortive transcript elongation due to polymerase stalling or arrest, which would cause a block in transcription. This phenomenon has been observed for several viral (20, 30) and cellular genes (3, 5, 13, 26), especially when extreme changes are required in the level of a particular transcript, such as occurs with c-*myc* during cellular differentiation (22, 27) or with c-*fos* during cellular proliferation (13). The evidence suggests that IL-2 does not induce a block in elongation of the 2.1-kb HBV transcript. Specifically, the run-on signals generated by HBV-specific probes which divide the 2.1-kb transcription unit into approximately equal upstream and downstream portions are both unaffected by IL-2 (Fig. 2B). Additionally, there was no change in the amount of intranuclear HBV RNA detected by RNase protection analysis when either an internal probe (Fig. 3) or an extreme 3′-end HBV probe was used (Fig. 4), suggesting that transcript elongation extended to the polyadenylation site despite the decrease in hepatic HBV mRNA content following IL-2 injection.

The next step in HBV mRNA biosynthesis that we examined was maturation of the RNA by the proper recognition of its 3′ extremity, polyadenylation, and removal of intervening sequences. The possibility that IL-2 might impair HBV mRNA polyadenylation is interesting because recognition of the HBV polyadenylation signal is conditional upon the presence of upstream *cis*-acting sequences in the transcript (31) and be-

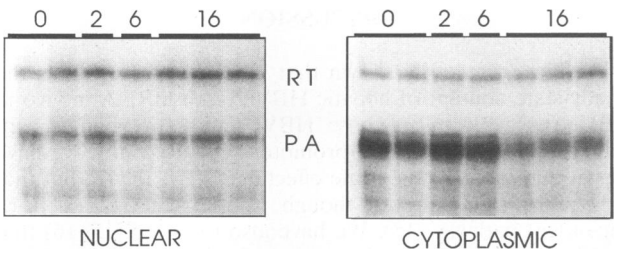

FIG. 4. Analysis of 3′-end formation. Cytoplasmic and nuclear RNAs prepared from livers harvested 2, 6, and 16 h after i.p. injection of 100,000 U of IL-2 or 16 h after saline injection (0) were analyzed by RNase protection assay with an HBV D (Fig. 1) antisense riboprobe overlapping the polyadenylation signal. Polyadenylated HBV RNA protects a 158-nucleotide fragment (PA) and read-through HBV RNA protects a 289-nucleotide probe fragment (RT).

cause differential recognition of a polyadenylation signal has also been observed for other transcription units (for examples, see references 10 through 12). Although the HBV 2.1-kb transcript is normally not spliced, it is conceivable that cryptic splice sites, which would result in deletion of these *cis*-acting sequences or the polyadenylation signal itself, might be activated after IL-2 administration. We do not think this occurs for two reasons. First, we did not observe transcripts smaller than the full-length 2.1-kb transcript that would suggest that otherwise cryptic splice sites are used after cytokine administration. Second, we did not observe decreased recognition of the HBV polyadenylation signal by RNase protection analysis when we compared the relative usage of the polyadenylation site before and after IL-2 administration (Fig. 4).

Thus, we believe the results of these experiments suggest that the transcriptional and maturation processes of the HBV 2.1-kb RNA are not affected by administration of IL-2. Consequently, the reduced level of the HBV 2.1-kb transcript in the cytoplasmic compartment is likely to be the result of an increased rate of the degradation of this transcript. Unfortunately, direct assessment of the mRNA degradation rate is not achievable in vivo by the traditional in vitro approaches such as pulse-chase labeling with radiolabeled nucleotide (17) or global transcription shutoff with actinomycin D (29).

In related in vitro experiments, we have shown that the regulatory effects of IL-2 and TNF-α are not observed when they are added to primary cultures of transgenic hepatocytes (unpublished observations), thereby precluding a direct assessment of HBV message stability in response to these cytokines. The reason for the striking difference between the in vivo and in vitro effects of these cytokines remains unclear. One possibility is that they induce another cytokine(s) in vivo that ultimately activates the hepatocellular gene(s) that presumably decreases the HBV mRNA content of the cell. Alternatively, the altered differentiation state that rapidly occurs when primary hepatocytes are placed in culture might make them refractory to signals to which they are normally responsive in vivo. Thus, direct demonstration that IL-2 and TNF-α trigger increased HBV mRNA degradation must await the development of alternative assays or tissue culture systems that are permissive for detection of this effect.

Nonetheless, we believe that the current observations strongly suggest that the HBV 2.1-kb mRNA molecule carries information that is recognized by the cellular machinery to specifically target this molecule for destruction in the cytoplasm. The precise nature of this signal in the HBV transcript and the identity of the IL-2-inducible cellular element(s) involved in its recognition remain to be determined.

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