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Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes

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ABSTRACT **Using transgenic mice that replicate the hepatitis B virus (HBV) genome, we recently demonstrated that class I-restricted, hepatitis B surface antigen-specific cytotoxic T lymphocytes (CTLs) can noncytolytically eliminate HBV pregenomic and envelope RNA transcripts from the hepatocyte. We now demonstrate that the steady-state content of these viral transcripts is profoundly reduced in the nucleus and cytoplasm of CTL-activated hepatocytes, but their transcription rates are only slightly reduced. Additionally, we demonstrate that transcripts covering the HBV X coding region are resistant to downregulation by the CTL. These results imply the existence of CTL-inducible hepatocellular factors that interact with a discrete element(s) between nucleotides 3157 and 1239 within the viral pregenomic and envelope transcripts and mediate their degradation, thus converting the hepatocyte from a passive victim to an active participant in the host response to HBV infection.**

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis and hepatocellular carcinoma. Clearance of HBV is thought to be mediated by the destruction of infected cells by a major histocompatibility complex class I-restricted cytotoxic T lymphocyte (CTL) response to HBV-encoded antigens (1). Using HBV transgenic mice, we have recently shown that the cytodestructive properties of the CTL response may not be the only mechanism for HBV clearance from the liver. In particular, we have shown that HBV envelope transcripts are specifically degraded in the cytoplasm by a posttranscriptional mechanism when the hepatocytes are activated by the administration or activation of tumor necrosis factor α (TNF-α) (2). We have also shown that injection of hepatitis B surface antigen (HBsAg)-specific CTLs into HBV transgenic mice causes a much more profound reduction in hepatocellular HBV gene expression than TNF-α (3). Furthermore, we have demonstrated that this effect is noncytolytic and that it is mediated by interferon γ (IFN-γ) as well as by TNF-α, both of which are secreted by the CTLs after antigen activation (3).

Recently, we have produced transgenic mouse lineages that express high levels of the HBV 3.5-kb pregenomic RNA as well as HBV envelope transcripts. These animals replicate the HBV genome in the liver and secrete HBV virions into the blood (4). Administration of HBsAg-specific CTLs into these mice causes a profound reduction of all of these HBV products in the liver and serum by a process that is inhibitable by the prior administration of antibodies to IFN-γ and TNF-α (L.G.G. and F.V.C., unpublished results). This suggests that the CTLs deliver one or more cytokine-mediated signals to the hepatocyte that induce or activate specific cellular gene products that either inhibit the synthesis, destabilize, or actively degrade these viral gene products, including the viral mRNA, thereby precluding viral replication.

We now report that the HBV pregenomic and envelope RNAs are profoundly reduced in the nucleus, as well as the cytoplasm, of the hepatocyte after CTL administration, whereas the transcription rates of these transcripts are either unaffected or marginally reduced, and we demonstrate that the HBV X mRNA is resistant to this effect. This suggests that CTL-derived cytokines activate posttranscriptional mechanisms in the hepatocyte nucleus, as well as in the cytoplasm (2), that target one or more element(s) between nucleotides 3157 and 1239 in the viral RNA and mediate their destabilization or destruction.

MATERIALS AND METHODS

Transgenic Mice. HBV lineages pFC80-219 and 1.3.32 have been described (2, 4–6). Lineage 1.3.32 mice express the 3.5- and 2.1-kb HBV RNAs in their hepatocytes under the control of the natural viral promoters, and they produce hepatitis B core antigen, hepatitis B e antigen (HBeAg), and HBsAg as well as replicative HBV DNA intermediates and virus particles. Lineage pFC80-219 expresses high levels of the 2.1-kb envelope RNA under the control of its natural promoter. Mice from lineages 1.3.32 and pFC80-219 were extensively backcrossed onto the C57BL/6 parental background and mated with B10.D2 mice to generate H-$2^{b \times d}$ F_1 hybrids that have the appropriate genetic background necessary for recognition by H-2^d-restricted, HBsAg-specific CTLs. Selected H-$2^{b \times d}$ F_1 hybrids were backcrossed a second generation against B10.D2 to produce transgenic mice in which the pFC80-219 transgene existed on an homozygous H-2^d background as determined by flow cytometry using H-2^d- and H-2^b-specific antibodies (PharMingen).

Lineages MUP precore 5 (MPC-5) (official designation Tg[MUP HBV precore]Chi5) and MUPX-760 (official designation Tg[MUP X]Chi760) were used to analyze the CTL effect on HBV transcripts covering these regions. The details of the production and characterization of these animals will be reported separately (L.G.G. and F.V.C., unpublished results). Briefly, lineages MPC-5 and MUPX-760 were generated by inserting HBV fragments containing nucleotides 1802–2800 and 1239–1984, respectively, into plasmid 11AS-SV40-7 (7) downstream of the mouse major urinary protein (MUP) promoter and upstream of the simian virus 40 polyadenylylation site. The corresponding transcription units were excised from these plasmids by restriction endonuclease digestion, and the purified fragments were microinjected into (C57BL/6 × SJL)F_2 embryos. Transgenic mice from lineage MPC-5 were identified by detection of HBV precore protein (HBeAg) in the serum (Abbott) as described (4). Transgenic mice from lineage MUPX-760 were identified by PCR, using primers that amplify the MUP–HBV junction. Both lineages were expanded by extensive backcrossing against the C57BL/6 parental strain. The level of RNA expression was assessed by

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Abbreviations: CTL, cytotoxic T lymphocyte; HBV, hepatitis B virus; IFN-γ, interferon γ; MUP, major urinary protein; TNF-α, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

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Northern blot analysis. MPC-5 produces a 1.7-kb HBV transcript, and MUPX-760 produces a heterogeneous population of HBV-specific transcripts that average 0.7 kb. Both transcripts are detectable in the vast majority of the hepatocytes as determined by *in situ* hybridization. These animals were mated with lineage pFC80-219 transgenic mice that were homozygous for H-2^{d} (as described above). Double transgenic H-$2^{b\times d}$ progeny were identified by monitoring serum HBsAg content and the presence of MUP–HBV DNA sequences in tail DNA by PCR. These hybrids coexpress the MUP–HBV transcripts with the native 2.1-kb viral RNA.

HBsAg-Specific CTLs. An L^{d}-restricted, $CD3^{+}$, $CD4^{-}$, $CD8^{+}$ HBsAg-specific CTL clone, designated 6C2 (8, 9), was used for these studies. These CTLs recognize an epitope located between residues 28 and 39 of HBsAg, and they secrete IFN-γ and TNF-α upon recognition of antigen (8, 9). In all experiments, 1×10^{7} 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 (8). CTL-induced liver disease was monitored by measuring serum alanine aminotransaminase levels at various time points after CTL injection. Liver tissue obtained at autopsy was processed either for histological analysis or snap frozen for subsequent RNA analysis.

Northern Blot Analysis. Total liver RNA was extracted by the acid guanidium/phenol/chloroform method (10) from snap-frozen mouse livers taken at various time points after CTL injection. Twenty micrograms of RNA was fractionated on a 0.8% formaldehyde/agarose gel, transferred to nylon membranes (Hybond-N; Amersham), and hybridized with radiolabeled DNA probes prepared by random primer labeling (Boehringer Mannheim) of the complete HBV genome (ayw subtype) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes as described (6).

Nuclear Run-on Analysis. Nuclei were isolated from livers of uninjected or CTL-injected mice as described (2), and 1×10^{7} were used for each nuclear run-on assay. Nascent transcript labeling was performed as described (2) except that 0.01 μM creatine phosphate was included in the transcription reaction. The reaction was carried out at 26°C for 20 min, and subsequently 20 μg of proteinase K (Boehringer Mannheim) and SDS (final concentration, 1%) were added for 30 min at 45°C. Next, 0.35 ml of sodium acetate (10 mM, pH 3.5) was added, followed by extraction with H_2O-saturated phenol. Radiolabeled transcripts were ethanol precipitated, and the nonincorporated nucleotides were removed by Sephadex G-50 filtration.

Approximately 1×10^{7} cpm of labeled transcripts were hybridized to filter strips containing 2 μg each of probes corresponding to HBV nucleotides 2139–2800 (Galibert sequence) (probe A; Fig. 1), 44–967 (probe B), and 1243–1988 (probe C) and mouse GAPDH (full-length cDNA) and Bluescript SK II+ plasmid DNA (Stratagene), which served as a negative control. Probe A spans the HBV core gene and will exclusively detect the 3.5-kb transcript. This was generated by excising the *Xba* I fragment (nucleotides 2139–2800) from a Bluescript vector containing core sequences spanning nucleotides 1887–2800. Probe B spans the 5′ half of the S gene transcript and will hybridize both the 3.5-kb and 2.1-kb transcripts. Probe C spans the X gene and can hybridize to all HBV RNA species. GAPDH served as an RNA loading control. To produce these probes, the fragments were cloned into the Bluescript SK II+ vector (Stratagene). The plasmid was linearized by restriction enzyme digestion, denatured in 0.2 M NaOH for 30 min at room temperature, and neutralized in 10 volumes of 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) at 4°C. The filter strips were prepared by loading the probes onto nitrocellulose membranes (Schleicher & Schuell) using a slot blot apparatus (Schleicher & Schuell) and UV crosslinked (Stratagene). The membranes were prehybridized

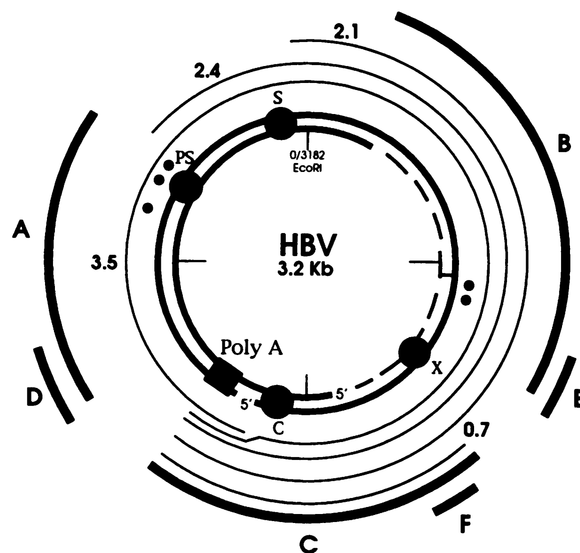

FIG. 1. Schematic map of the HBV genome showing the locations of the DNA probes used for nuclear run-on assays (probes A, B, and C) and RNA probes used for RNase protection assays (probes D, E, and F). Dots represent AUUUA elements at nucleotides 828, 870, 2552, 2669, and 2744.

at 60°C for 4 h in 5× SSC, 5× Denhardt's solution, 50 mM sodium phosphate (pH 7.5), and 100 ng of *Escherichia coli* RNA. Membranes were then hybridized in 2 ml of fresh hybridization buffer containing 1×10^{7} cpm of radiolabeled transcripts for 36 h at 60°C. Membranes were washed in 2× SSC/0.1% SDS three times at room temperature for 20 min each and once in 0.2× SSC/0.1% SDS at 60°C for 20 min.

RNase Protection Analysis. Nuclei and cytoplasmic fractions from the same livers were isolated as described (2). Nuclear RNA was prepared by lysing 1×10^{7} pelleted nuclei in 0.5 ml of acid guanidium/sodium acetate/H_2O-phenol [1:0.1:1 (vol/vol)]. The nucleic acids were sheared by passing the sample through a 20-gauge needle 10 times, following which 0.1 ml of chloroform was added. The aqueous phase was collected, and nuclear RNA was precipitated with 2-propanol. The cytoplasmic fraction was diluted 5-fold with acid guanidium and extracted by the acid guanidium/phenol/chloroform method (10). RNA probe preparation and RNase protection analyses were performed according to the procedure of Hobbs *et al.* (11) using RNA probes derived from the HBV S gene (probe E, nucleotides 1001–1160), the HBV X gene (probe F, nucleotides 1261–1400), and GAPDH gene (nucleotides 646–760) (described in ref. 2). Probe D containing HBV nucleotides 2133–2332, which spans exclusively the core region, was used to detect the HBV 3.5-kb transcript. A *Bss*HII restriction fragment from the BluescriptII vector containing the appropriate gene insert was gel purified and used as a template for making radiolabeled transcripts using the Promega transcription kit (Promega).

RESULTS

Effect of HBsAg-Specific CTLs on Total Hepatic HBV 3.5-kb and 2.1-kb mRNA Content. The regulatory effect of HBsAg-specific CTLs on the HBV 3.5-kb pregenomic RNA and the 2.1-kb envelope transcript was examined by Northern blot analysis of total hepatic RNA. To begin this study, transgenic mice from lineages 1.3.32 and pFC80-219 were injected with 1×10^{7} CTLs from clone 6C2, and the steady-state content of total hepatic HBV and GAPDH RNA was determined 4 h, 2 days, and 5 days later. As shown in Fig. 2 *Upper*, both HBV transcripts were slightly reduced (relative to the GAPDH control) in lineage 1.3.32 by day 2, and they were virtually

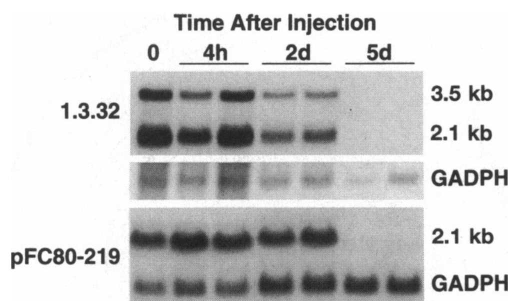

FIG. 2. Northern blot analysis of total liver RNA after administration of HBsAg-specific CTLs. Mice from lineages 1.3.32 or pFC80-219 were injected intravenously with 10^7 CTLs from clone 6C2. Total RNA was isolated from livers harvested at 4 h, 2 days, and 5 days after CTL injection. ^{32}P-labeled DNA probes for HBV and GAPDH were prepared by random priming.

undetectable by day 5 after CTL injection. Similarly, in lineage pFC80-219, the HBV 2.1-kb mRNA was reduced almost 50-fold (relative to the GAPDH control) at the same time point (Fig. 2 *Lower*). In both lineages, the HBV transcripts remained undetectable for up to 14 days after CTL injection (data not shown). We have previously reported that virtually 100% of the hepatocytes express the HBV mRNAs before CTL injection, and <20% of the hepatocytes are killed by the CTLs under these conditions (ref. 3; L.G.G. and F.V.C., unpublished results).

Effect of HBsAg-Specific CTLs on Hepatic Nuclear and Cytoplasmic HBV RNA Content. To determine whether the 3.5-kb and 2.1-kb HBV RNA species were reduced in the nuclear or the cytoplasmic compartments of the hepatocyte or both, RNase protection experiments (Fig. 3) were performed on the same livers using RNA probes that specifically detect the 3.5-kb and 2.1-kb transcripts. The 3.5-kb HBV transcript was examined using a probe that spans HBV nucleotides 2133–2332 (probe D), and the steady-state level of the 2.1-kb HBV transcript was examined using probes that span HBV nucleotides 1001–1160 (probe E) and nucleotides 1261–1400 (probe F). As shown in Fig. 3, the 3.5-kb and 2.1-kb HBV transcripts were profoundly reduced relative to GAPDH in the nuclear and cytoplasmic compartments in both lineages on day 5 after CTL administration.

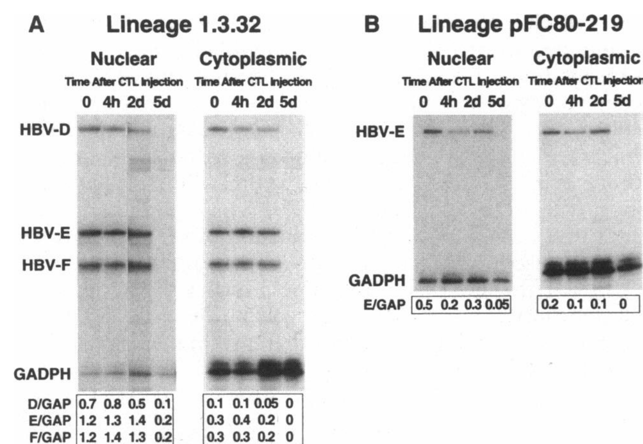

FIG. 3. HBV RNA steady-state levels in the hepatic nuclear and cytoplasmic compartments. Nuclear and cytoplasmic RNA fractions were prepared from the same livers described in the legend to Fig. 2 and analyzed by RNase protection assay using RNA probes E and F, which will detect the 2.1- and 3.5-kb RNAs, and probe D, which will exclusively detect the 3.5-kb RNA (see Fig. 1). Ratios represent the relative band intensities of HBV to GAPDH transcripts as determined by densitometric analysis. For lineage 1.3.32, D/GAP represents the ratio of the 3.5-kb RNA/GAPDH RNA, and E/GAP and F/GAP represent ratios of all HBV RNAs/GAPDH RNA. For lineage 219, probe E detects only the 2.1-kb RNA; thus, E/GAP represents the ratio of 2.1-kb/GAPDH RNAs.

Effect of HBsAg-Specific CTLs on HBV 3.5-kb and 2.1-kb RNA Transcription Rates. To determine whether the profound decrease in nuclear and cytoplasmic HBV RNA was due to a decrease in the rates of transcription of the 3.5- and 2.1-kb RNAs, nuclear run-on experiments (Fig. 4) were performed on liver nuclei isolated from the same transgenic mice at the same intervals after CTL injection. The transcription rate of the 3.5-kb HBV transcript was examined in lineage 1.3.32 using a probe that spans HBV nucleotides 2139–2800 (probe A), and the transcription rate of the 2.1-kb HBV transcript was examined in both lineages using probes that span HBV nucleotides 44–967 (probe B) and nucleotides 1243–1988 (probe C). As shown in Fig. 4, the transcription rates of the 3.5-kb and 2.1-kb HBV RNAs decreased <2-fold during the entire observation period. These results are compatible with a slight reduction in the transcription rates of the 3.5-kb and 2.1-kb RNAs in response to the CTLs, but the changes are too small to account for the profound reduction in the steady-state content of HBV RNA seen in Figs. 2 and 3.

HBV RNA Target Region. Based on the foregoing, it appears that the steady-state content of HBV RNA is regulated posttranscriptionally by a CTL-induced process in the nucleus, and perhaps the cytoplasm, of the hepatocyte. To begin to define the putative response element(s) in the HBV transcripts, we examined the ability of the CTLs to downregulate subgenomic transcripts derived from different regions of the HBV genome that overlap the 3.5- and 2.1-kb RNAs in double-transgenic mice that coexpress the 2.1-kb RNA, which encodes the CTL target antigen and is downregulated by the CTL. To this end, we crossed HBV lineages MPC-5 and MUPX-760, which express HBV transcripts that span the precore region (nucleotides 1802–2800) and the X region (nucleotides 1239–1984), respectively, with lineage pFC80-

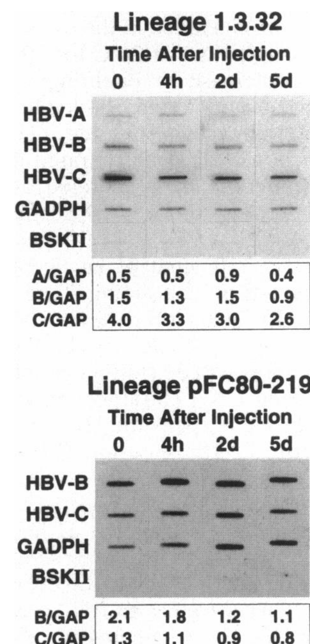

FIG. 4. Analysis of HBV transcription rates after administration of CTLs. Nuclear run-on assays were performed on liver nuclei isolated from the same transgenic mice shown in Figs. 2 and 3. Radiolabeled transcripts were hybridized to slot blots containing HBV probes A, B, and C (see Fig. 1) as well as GAPDH and BluescriptSKII (BSKII). Ratios represent the relative band intensities as described in the legend to Fig. 3. In addition, A/GAP represents the ratio of 3.5 kb/GAPDH RNAs.

219, which expresses the 2.1-kb RNA that spans nucleotides 3157–1984. The F_1 hybrids were injected with 1×10^7 6C2 CTLs, and changes in hepatic expression of the various HBV transcripts were evaluated by Northern blot 5 days later. As shown in Fig. 5, the steady-state content of the MUP precore and MUP X transcripts did not change after injection of HBV-specific CTLs, whereas the HBV 2.1-kb transcript was profoundly reduced. These results suggest that the HBV MUP precore and MUP X transcripts lack elements required for CTL-mediated downregulation of the 3.5- and 2.1-kb HBV RNA species, implying that the element(s) responsible for posttranscriptional downregulation of the 3.5- and 2.1-kb viral RNAs is located between nucleotides 3157 and 1239, the transcriptional start site of the 2.1-kb pre-S2 RNA (12) and the 5′ HBV nucleotide in the MUP X transgene (L.G.G. and F.V.C., unpublished results), respectively.

To determine if the failure of the MUP precore and MUP X transcripts to be downregulated by the CTLs could reflect the combined effects of CTL-induced activation of the MUP promoter balanced by posttranscriptional destabilization of the transcripts, we performed nuclear run-on analysis on CTL-injected pFC80-219 × MUP precore hybrids using probe A (Fig. 1), which recognizes only the MUP precore promoter-driven transcripts, and probe B, which detects only the 2.1-kb transcripts generated from viral promoter elements in the pFC80-219 transgene. No change in the transcription rates from either promoter was observed in this experiment (data not shown).

DISCUSSION

Collectively, these results demonstrate that HBsAg-specific CTLs can clear virtually all traces of the HBV 3.5-kb pregenomic RNA and the 2.1-kb HBV envelope mRNA from the liver of transgenic mice and that they do so principally by activating one or more posttranscriptional pathways in the nucleus of the hepatocyte (and possibly also in the cytoplasm). Furthermore, the results suggest that these putative CTL-inducible factors destabilize HBV RNA by targeting an element(s) located between the start sites of the 2.1- and 0.7-kb HBV transcripts. By inference, we conclude that the 2.4-kb envelope RNA is also downregulated by this mechanism since, like the 3.5-kb RNA, it completely overlaps the 2.1-kb transcript. Also, we have shown previously that the 2.4-kb transcript is downregulated by CTLs (3) and by inflammatory cytokines (5) in an independent transgenic lineage not included in the current study.

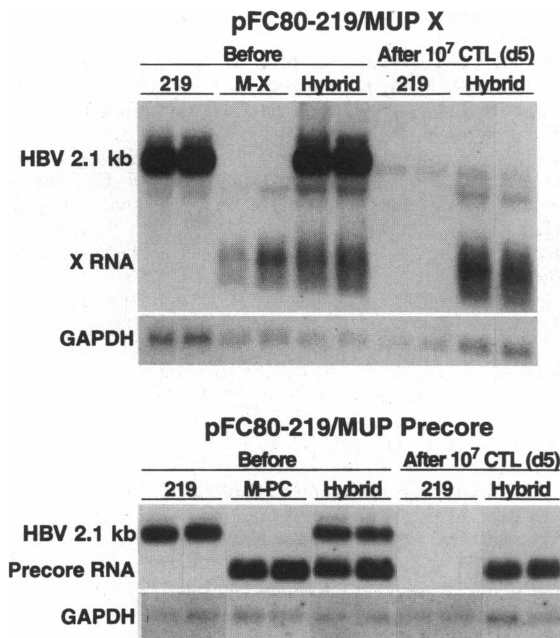

FIG. 5. Localization of the putative posttranscriptional regulatory element on the HBV RNA. Northern blot analysis of 20 μg of total liver RNA isolated from transgenic mice of lineages MPC-5 (MUP Precore) or MUPX-760 (MUP X) which were crossed with lineage 219. Single and double transgenics were injected either with saline or with 10^7 HBV-specific CTLs, and livers were harvested 5 days later as indicated.

We have previously shown that the regulatory effect of these CTLs in lineage 1.3.32 can be almost completely blocked by a cocktail of monoclonal antibodies to IFN-γ and TNF-α, but not by the same antibodies administered separately (L.G.G. and F.V.C., unpublished results). This suggests that the impressive strength of the CTL effect may derive in part from additive or synergistic aspects of the complex response that these cytokines induce in the transcriptional and translational programs of the hepatocyte.

We know from previous studies that CTLs release both of these cytokines after antigen recognition (3), that systemically administered recombinant mouse IFN-γ has no effect on hepatic HBV RNA content in these animals (5), that TNF-α destabilizes HBV RNA in the *cytoplasm* of the hepatocyte and not in the nucleus (2), and that TNF-α has no effect on HBV RNA at the transcriptional level (2). In light of these observations, it is possible that the profound reduction of *nuclear* HBV RNA content induced by CTLs in the current study (Fig. 3) may be mediated by CTL-derived IFN-γ since the CTLs can probably deliver much higher concentrations of this cytokine to the hepatocyte than can be achieved when it is administered systemically. We assume that TNF-α-mediated posttranscriptional events are also induced in the cytoplasm after CTL administration, but that they may be eclipsed by the destabilizing events induced in the nucleus by the CTLs.

The molecular basis(es) for these CTL-inducible pathways is (are) not yet known. Since HBV does not naturally infect the mouse liver, we assume that these pathways evolved to limit the abundance of one or more normal hepatocellular transcripts that share currently unknown target elements with HBV. We also assume that they reflect the activity of multiple, CTL-inducible hepatocellular factors that interact negatively with one or more steps in HBV RNA processing, transport and stabilization, and possibly to a small extent with the HBV transcriptional apparatus.

Several motifs are present in the HBV RNA that could serve as targets for posttranscriptional regulation. For example, the AUUUA consensus sequence, which is found in the 3′-untranslated region of many short-lived RNAs, including several cytokine genes and oncogenes, and is thought to be involved in RNA destabilization (13), is present at several sites in the 3.5-kb and 2.1-kb HBV transcripts (see Fig. 1). Several groups have identified cellular proteins that recognize this motif and play a role in RNA stability (14–20). Whether they are inducible by IFN-γ and TNF-α has not been determined. Five such motifs exist in the HBV genome (see Fig. 1). Three of these are located in the 5′ end of the 3.5-kb RNA and two others are present in the 3.5-, 2.4-, and 2.1-kb HBV transcripts just downstream of the envelope protein stop codon and upstream of the start sites of the 0.7-kb HBV X mRNA. Therefore, it is possible that the stability of all HBV transcripts except the X mRNA could be regulated posttranscriptionally by the interaction of CTL-induced, RNA-destabilizing hepatocellular proteins and one or more of these motifs.

Since the subgenomic MUP precore transcript is resistant to downregulation by the CTL (Fig. 5), we can eliminate the AUUUA motifs at nucleotides 2552, 2669, and 2744 as potential target sequences for destabilization of HBV RNA. Since the MUP-X transcript is also resistant to CTL-induced downregulation, the target element(s) must be located between the start sites of the 2.1-kb HBV envelope RNA

(nucleotide 3157) and the 0.7-kb HBV X RNA (nucleotide 1239). Interestingly, this region includes two AUUUA motifs at positions 828 and 870 that are potential candidate response elements for this effect. Obviously, additional studies are needed to map the target elements with greater precision, but the current observations provide an important starting point for those experiments.

An AU-specific endoribonuclease V has been shown to be responsible for the decay of RNAs containing this motif (21). It is interesting that HBsAg can prevent the shift of this enzyme from its matrix-associated active form to a free inactive form (21). The presence of AUUUA motifs followed by an AU-rich element in the 3′-untranslated region of the human papilloma virus (HPV) late mRNAs have been shown to be involved in posttranscriptional repression of HPV gene expression (22). In that study, it was suggested that the HPV RNAs containing this inhibitory element may enter a nonproductive pathway in the nucleus, resulting in mRNA degradation in the nuclear compartment (22). A similar process may be occurring in the HBV system as well.

Another posttranscriptional regulatory element (PRE) has recently been defined (23, 24). This element partially overlaps the area mapped in the current studies since it is located between nucleotides 1200 and 1650. The HBV PRE is similar to the HIV Rev response element in that it is essential for the efficient expression of HBV genes at the posttranscriptional level *in vitro*. If the HBV PRE plays a similar role at the level of HBV RNA processing and/or nuclear transport, negative interactions between this element or the cellular proteins that normally influence its function and CTL-inducible hepatocellular factors could be responsible for the current observations. Additional experiments are needed to test this hypothesis.

We believe the current observations are important for several reasons. First, they provide insight into a series of pathways that are potentially available to the immune system for viral clearance, in addition to CTL-mediated destruction of infected cells and antibody-mediated neutralization of extracellular virus. Second, they illustrate that these processes can be activated quite readily under the physiological conditions of antigen recognition *in vivo*. Third, they create a physiological framework for interpretation of a large body of data demonstrating responsiveness of HBV (3, 25–27) and several other viruses (28–32) to type I and type II IFNs and TNF-α under pharmacological conditions *in vitro* and *in vivo*, suggesting that similar immunologically activated intracellular events might also contribute to the clearance of these other viruses as well as HBV.

Finally, they illustrate the important concept that infected cells are not necessarily passive victims during a viral infection. Instead, the data suggest that certain cells can be activated by CTL-derived signals to an antiviral state in which they can rid themselves of certain viruses, at least in some viral infections, and be restored to their normal pre-infected condition—i.e., cured.

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