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Hepatitis B Virus RNA Element That Facilitates Accumulation of Surface Gene Transcripts in the Cytoplasm

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Hepatitis B virus enhancers I and II are critical for high-level expression from the viral major surface gene promoter. These enhancers are in an unusual position, since both are entirely contained within the downstream transcribed region of the surface gene. In this report, we present data showing that a fragment of the viral genome encompassing enhancer II activates accumulation of surface gene transcripts at the posttranscriptional level. Specifically, the total steady-state amount of surface gene transcripts in the cell drops by more than fourfold when enhancer II is displaced to a position downstream of the transcription termination site. There is a similar decrease in the amount of cytoplasmic surface gene transcripts but not of nuclear transcripts. These changes in steady-state transcript levels do not result from a decrease in the rate of transcriptional initiation or from an increased rate of degradation in the cytoplasm. Reinsertion of enhancer II in the correct orientation into the surface gene transcribed region partially restores transcript levels. From these data, we conclude that a hepatitis B virus RNA element functions in *cis* to increase the steady-state levels of surface gene transcripts by facilitating cytoplasmic accumulation of these transcripts.

Hepatitis B virus (HBV) is an unusual DNA virus that replicates via reverse transcription of an RNA intermediate and is the only member of the hepadnavirus family known to infect human beings (reviewed in reference 7). As its name implies, HBV efficiently replicates in hepatocytes and causes hepatitis as the main symptomology (reviewed in reference 10). While acute hepatitis B is usually mild and can be even asymptomatic, a significant percentage of infected people fail to clear the virus and become chronic carriers. There is at present no effective therapy for chronic HBV infection. This is an important public health problem, as there are estimated to be well over 200 million people worldwide who are chronically infected with HBV and therefore at risk for liver failure, cirrhosis, and hepatocellular carcinoma. Therefore, we have been investigating the regulation of HBV gene expression, in the hope that these studies will facilitate the formulation of strategies to down-regulate viral gene expression and hence prevent HBV-associated diseases.

Four classes of HBV transcripts, specified by four promoters on the minus strand and terminating at the same polyadenylation signal, are translated into the various viral proteins (Fig. 1A; reviewed in references 19, 22, and 30). The major surface (virion envelope) protein is synthesized from the major surface (S) gene transcripts. The amount of S gene transcripts is known to be up-regulated in *cis* by the viral enhancers I and II (EnI and EnII, respectively). However, both enhancers are in an unusual position, being present within the S gene transcribed region, downstream of the S coding region (Fig. 1). We have previously demonstrated that when the enhancers were deleted from this position and replaced upstream of the S promoter, S gene transcript levels dropped by more than fourfold (12). This result raised the possibility that one or both enhancers function optimally only when present within the transcribed region of the S gene. We present data here showing that RNA transcribed from the region encompassing EnII activates the accumulation of S gene transcripts by a posttranscriptional mechanism, since removal of this RNA segment from the transcribed region does not have an effect on the transcriptional initiation rate of the S promoter. Instead, there is a *cis*-acting element, overlapping EnII, that promotes the accumulation of S gene transcripts in the cytoplasm. Therefore, these observations point to an additional layer of regulation of gene expression in HBV that hitherto has not been well characterized.

MATERIALS AND METHODS

Plasmid constructions. HBV fragments were derived from pHBV2, which contains a head-to-tail dimer of the HBV genomic DNA, strain adw (25). The plasmid pSAgΔHin contains the *Hin*cII-to-*Bgl*II fragment of HBV DNA (Fig. 1B) inserted into the plasmid pTZ19U (34); we have previously demonstrated that this subgenomic fragment contains the *cis* elements necessary for high-level expression of the S gene in hepatoma cells (34). To generate plasmids giving rise to truncated S gene transcripts, a DNA fragment containing three copies of the simian virus 40 (SV40) polyadenylation signal was excised from the plasmid pTAG-1 (6) with *Sal*I and inserted separately into four restriction sites in pSAgΔHin: *Hpa*I, *Sph*I, *Nco*I, and *Sma*I (Fig. 1). The names of these plasmids are, respectively, pSHpA-HpaI, pSHpA-SphI, pSHpA-NcoI, and PSHpA-SmaI. In addition, a fragment similar in size to the SV40 polyadenylation signal, excised from the vector sequences in pTAG-1 by digestion with *Eco*RI and *Xba*I, was inserted into the *Hpa*I site to generate pSHNS-HpaI.

The plasmids pSHpA-NcoI(160bpC) and pSHpA-NcoI (160bpR) were constructed by synthesizing a fragment of EnII (map positions 1600 to 1760, flanked by *Sph*I sites) by PCR and inserting this fragment into the *Sph*I site of pSHpA-NcoI in the wild-type and reverse orientations, respectively.

The plasmid pXGH5 contains the human growth hormone

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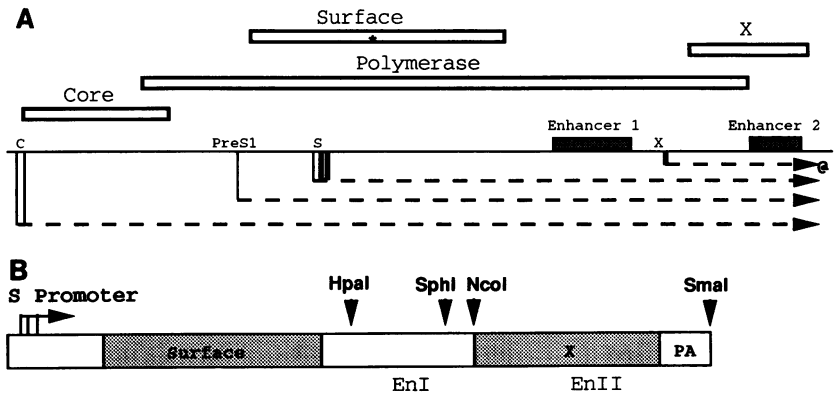

FIG. 1. (A) Map of the HBV genome (central solid line). The open reading frames are shown as boxes, while the transcripts are represented as dashed arrows. Note that the major surface protein is translated from an internal ATG codon (*) of the S open reading frame, since the S promoter is embedded within this open reading frame. (B) Map of the *Hin*cII-*Bgl*II fragment of HBV present in the plasmid pSagΔHin. The relevant restriction sites used to generate the pA series of plasmids (Fig. 2) are indicated. In this diagram, only the portion of the surface open reading frame that is translated into the major S protein is shaded. pA, HBV polyadenylation signal.

gene driven by the mouse metallothionein II promoter (21). The plasmid pMT-S (also called p30A8) contains the HBV surface gene under the control of the mouse metallothionein II promoter (32).

Cell culture and transfection. HuH-7 well-differentiated human hepatoma cells (16), which are free of endogenous HBV sequences, were cultured in DME-H21 medium (Cell Culture Facility, University of California San Francisco) with 10% fetal bovine serum, at 37°C under an atmosphere of 7% carbon dioxide–93% air. Cells were transfected at 50% confluency for 15 h by the calcium phosphate coprecipitation method, as described previously (8, 34). Five to ten micrograms of plasmid DNA was used per 100-mm-diameter dish, and the cells and media were harvested approximately 48 h following transfection.

In experiments measuring RNA stability, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma Chemicals) was added to culture media to a final concentration of 25 μg/ml at 40 h after transfection (20). The cells were then harvested at various time points after the addition of the drug.

RNA analyses. Total RNA was extracted from transfected cells with RNAzolB (obtained from Biotecx) by the manufacturer's suggested protocol. Nuclear and cytoplasmic RNA fractions were obtained by the protocol of Daar and Maquat (5); this protocol was chosen because the extensive washing prevents significant contamination of the nuclear fraction by cytoplasmic fragments. Briefly, cells on the second day after transfection were trypsinized and lysed in Nonidet P-40. Nuclei were removed by centrifugation, and the supernatant was extracted with RNAzolB to produce the cytoplasmic RNA fraction. The crude nuclear pellet was then washed twice with buffer containing Nonidet P-40 and once with buffer containing Nonidet P-40, Tween 80, and sodium deoxycholate. The rinsed nuclei were then centrifuged through a 2.1 M sucrose cushion and extracted with RNAzolB to produce the nuclear RNA fraction.

For primer extension analysis (18), 10 μg of total RNA, 1 μg of cytoplasmic RNA, or 1 μg of nuclear RNA was used in each reaction. The primer for S gene transcripts has been previously described (33) and yields five major products ranging from 105 to 136 bases long for the transcripts arising from the S promoter (33) but a major product of 203 bases for the transcript arising from the metallothionein promoter (32). The primer for β-actin transcripts (5'GGAGTCCTTCTGGCCC ATGCCCACCAT) is designed to produce extension products ~230 bases long (9).

For Northern (RNA) blotting (18), 20 μg of RNA per lane was electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (obtained from Amersham), and probed with a ^{32}P-labeled fragment of HBV DNA extending from the *Eco*RI site to the *Bgl*II site and hence capable of detecting all portions of the S gene transcripts.

Nuclear run-on transcription. Run-on transcription was performed as described by Almendral et al. (1). Briefly, on the second day after transfection cells were lysed by shearing through a needle in Nonidet P-40 and the nuclei were collected through a sucrose cushion. Nascent transcripts within the nuclei were extended in the presence of [α-^{32}P]UTP, and the labeled RNA was partially purified and used to probe a dot blot of various plasmid DNAs (4 μg per dot). Prehybridization was performed in 1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's reagent, 0.1% sodium dodecyl sulfate, and 0.3 mg of salmon sperm DNA per ml at 42°C for 2 h (18). Hybridization was performed in the same buffer at 65°C for 16 to 24 h.

RESULTS

Effect of enhancer position on S gene transcript levels. Previously, we had demonstrated that when the HBV enhancers were removed from their usual position and replaced upstream of the S promoter, the amount of S gene transcripts dropped by approximately fourfold (12). This result raised the possibility that the enhancers functioned optimally only when present within the transcribed region of the S gene. However, other explanations such as distance and/or position effects could not be conclusively ruled out with those constructs used in previous experiments. Therefore, we constructed an additional series of plasmids to address this issue.

The parent plasmid, pSAgΔHin, contains the entire S gene with its promoter, both enhancers, and the polyadenylation signal (Fig. 1). The derivatives contain three copies of the SV40 polyadenylation signal inserted into various restriction sites (specifically, the *Nco*I and *Sph*I sites between EnI and EnII, and *Hpa*I upstream of EnI) (Fig. 1). As a consequence,

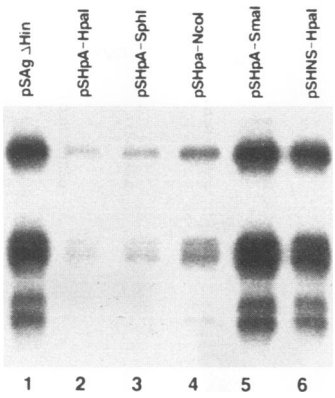

FIG. 2. Primer extension analysis of S gene transcripts synthesized in HuH-7 cells transfected with pSAgΔHin or derivatives. The pA series of plasmids contains three tandem copies of the SV40 polyadenylation signal inserted into the restriction sites indicated (see Fig. 1). The pSHNS-HpaI plasmid contains a randomly selected plasmid sequence inserted into the *Hpa*I site. Similar results were obtained in three independent transfections. The five major bands represent the five major start sites of the S gene transcripts, which show 5′ end microheterogeneity (34).

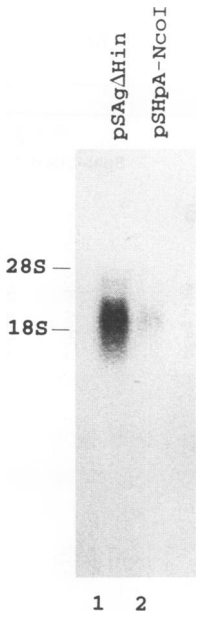

FIG. 3. Northern blot analysis of S gene transcripts synthesized in HuH-7 cells transfected with pSAgΔHin or pSHpA-NcoI (see Fig. 1B for map). Similar results were obtained with three independent transfections. The migration positions of the cellular 18S and 28S rRNA are indicated.

the S gene transcripts synthesized from these plasmids are truncated at those sites, while the topographic relationship of the enhancers to the S promoter is preserved. These plasmids were individually transfected into HuH-7 hepatoma cells, and the amount of S gene transcripts was measured by primer extension analysis. Indeed, the amount of S transcripts dropped significantly when the polyadenylation signal was inserted into the *Nco*I site (compare lanes 1 and 4, Fig. 2). In several duplicate experiments, this decrease was four- to fivefold, as quantitated by phosphor imaging. A further small drop (less than twofold) was seen in the transcript levels with insertion of the SV40 polyadenylation signal further upstream into the *Sph*I site (Fig. 2, lane 3), but no further decrease was seen with insertion into the *Hpa*I site (Fig. 2, lane 2). This decrease in transcript levels was not an artifact of primer extension analysis, since Northern blotting confirmed that insertion of the SV40 polyadenylation signal into the *Nco*I site markedly decreased the amount of S gene transcripts synthesized by the transfected cells (Fig. 3).

To ensure that these changes were not due to the inserted polyadenylation signal increasing the distance between the enhancers and the S promoter, we inserted a randomly chosen plasmid sequence of similar length into the *Hpa*I site. As seen in Fig. 2, lane 6, this insertion did not result in any detectable change in S gene transcript levels. Furthermore, we ruled out the possibility that the SV40 polyadenylation signal fortuitously contained a transcriptional repressor element, as insertion of the SV40 sequences downstream of the HBV polyadenylation signal did not decrease the amount of S gene transcripts and even had a small positive effect (Fig. 2, lane 5).

In summary, truncation of the S gene transcripts by insertion of a heterologous polyadenylation signal resulted in decreased steady-state levels of these transcripts. This decrease cannot have been due to the loss of a *cis*-acting DNA element, since no HBV sequences were deleted or rearranged in these plasmids. Rather, these results suggested that an RNA element downstream of the S coding region functioned in *cis* to increase accumulation of S gene transcripts. From the results shown in Fig. 2, this element appears to overlap largely with the X gene/EnII region (Fig. 1). Therefore, it seemed probable that part of the positive effect of EnII on S promoter strength was due to its functioning at the RNA level. To test this supposition, we inserted a second copy of EnII, in either orientation, into pSHpA-NcoI upstream of the SV40 polyadenylation signal (Fig. 4A). As shown in Fig. 4B, the amount of steady-state S gene transcripts synthesized from this plasmid was increased almost to wild-type levels when EnII was inserted in the native orientation (compare lane 3 with lane 1 containing the wild type). In contrast, insertion of EnII in the reverse orientation did not increase S gene transcript levels (Fig. 4B, lane 4). Insertion of EnII in the native orientation, but downstream of the SV40 polyadenylation signal, also did not have any effect on S gene transcript levels (data not shown). Therefore, EnII was able to up-regulate the steady-state levels of the S gene transcripts only when it was present in the correct orientation within the transcribed region of the S gene.

The above results, taken together, show that a region of the HBV genome (the EnII/X gene region; see Fig. 1) acts in *cis* at the RNA level to increase the steady-state levels of S gene transcripts. For convenience, hereafter we will refer to this region as the posttranscriptional regulatory element (PRE).

PRE acts posttranscriptionally. Enhancers are commonly identified as DNA elements. However, there is recent evidence that some transcriptional *trans* activators can function when tethered via RNA elements (24). To determine whether the HBV PRE was acting as an RNA enhancer, we performed nuclear run-on analysis to measure the rate of transcriptional initiation from the S promoter, using both the parent plasmid (pSAgΔHin) and pSHpA-NcoI, the plasmid with the SV40 polyadenylation signal inserted in the NcoI site (Fig. 1B) which gives rise to truncated S gene transcripts devoid of the PRE. For these experiments, one plate of HuH-7 cells was transfected with pSAgΔHin, while a parallel plate was transfected

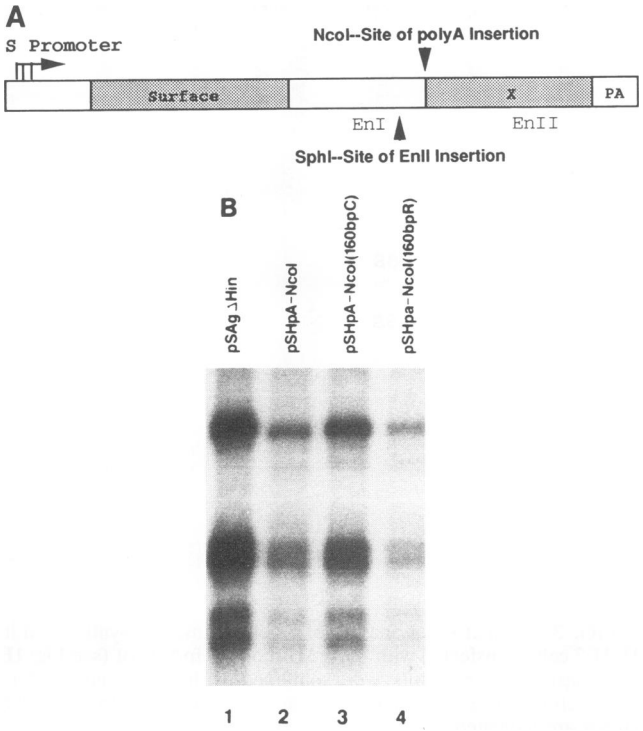

FIG. 4. (A) Map of the HBV fragment in pSAgΔHin. Also indicated are the *NcoI* site into which the SV40 polyadenylation signal is inserted in the plasmids pSHpA-NcoI, pSHpA-NcoI(160bpC), and pSHpA-NcoI(160bpR) and the SphI site into which a 160-bp fragment of EnII is inserted in the plasmids pSHp-NcoI(160bpC) and pSHpA-NcoI(160bpR), in the wild-type and reverse orientations, respectively. PA, HBV polyadenylation signal. (B) Primer extension analysis of S gene transcripts synthesized from the indicated plasmids. Similar results were obtained in three independent transfections.

with pSHpA-NcoI. On the second day after transfection, the cells were permeabilized and nascent RNA transcripts were elongated in the presence of labeled UTP. The labeled RNA was then used to probe a dot blot of HBV S gene DNA. As

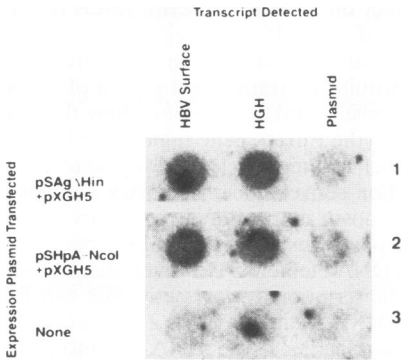

FIG. 5. Nuclear run-on analysis of HuH-7 cells transfected with the plasmids indicated on the left. The three dots on the left contain immobilized HBV S gene DNA, the three dots in the middle contain human growth hormone (HGH) DNA, while the three dots on the right contain the plasmid pTZ19U. See the text for a detailed description of experimental methods. Similar results were obtained in three independent transfections.

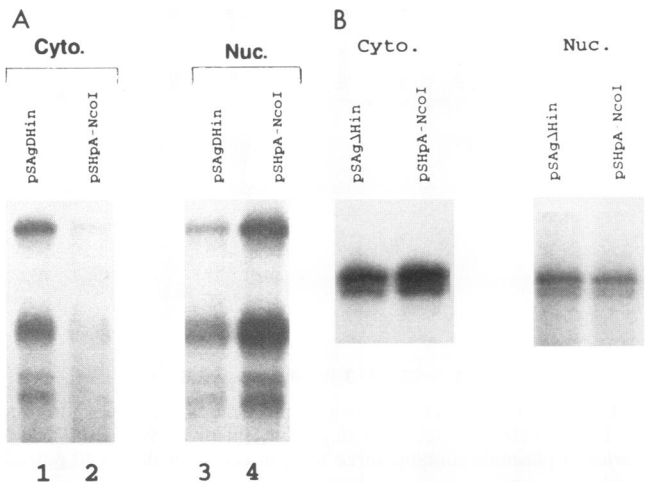

FIG. 6. (A) Primer extension analysis of cytoplasmic (Cyto.) and nuclear (Nuc.) RNA for S gene transcripts in HuH-7 cells transfected with the indicated plasmids. Similar results were obtained in three independent transfections. Note that the fraction of S gene transcripts in the nucleus is small, since nuclear RNA from approximately an entire plate of cells was used for primer extension analysis, while less than 20% of cytoplasmic RNA from each plate was used. Therefore, the slightly increased amount of nuclear S gene transcripts seen in cells transfected with pSHpA-NcoI was not sufficient to compensate for the loss of transcripts in the cytoplasm. Hence, the amount of total S gene transcripts was decreased in cells transfected with pSHpA-NcoI (Fig. 2). (B) Primer extension analysis of β-actin transcripts in the same RNA samples analyzed in panel A.

seen in Fig. 5, there was no significant difference in the amounts of hybridization to the S gene between the RNA transcripts synthesized from these two plasmids (compare the dots labeled HBV Surface in rows 1 and 2). The transfection efficiencies of the two plates of cells were similar, since the amount of transcription from a cotransfected plasmid with the human growth hormone gene under the control of the metallothionein promoter (pXGH5) was the same in both sets of cells (compare the dots labeled HGH in Fig. 5, rows 1 and 2). The hybridization was specific for either S or growth hormone genes, since there was only a low background level of hybridization to plasmid DNA sequences (dots labeled Plasmid in Fig. 5). Furthermore, RNA from untransfected cells also failed to hybridize significantly to either gene (Fig. 5, lane 3). From these results, we conclude that the HBV PRE (the sequences downstream of the *NcoI* site) could not have been elevating the steady-state levels of the S gene transcripts by increasing the rate of transcriptional initiation from the S promoter. Therefore, it appears that PRE increases S gene transcript accumulation by a posttranscriptional mechanism.

PRE facilitates RNA accumulation in the cytoplasm without affecting RNA stability in the cytoplasm. Since the 3′ untranslated regions of many transcripts contain determinants of RNA stability (reviewed in reference 14), it seemed possible that the HBV PRE might stabilize the S gene transcripts in the cytoplasm. To test this possibility, we transfected cells with either pSAgΔHin or pSHpA-NcoI and separately examined the nuclear and cytoplasmic fractions for S gene transcript levels. Indeed, the amount of S transcripts in the cytoplasmic fraction was much smaller in cells transfected with the plasmid with the displaced PRE (Fig. 6A, lane 2) than in cells transfected with the wild-type plasmid (Fig. 6B, lane 1). However,

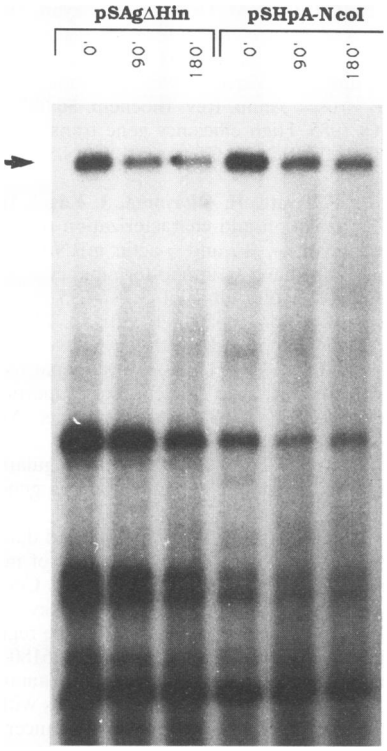

FIG. 7. Analysis of S gene transcript stability in the cytoplasm. HuH-7 cells were transfected with the plasmids indicated at the top and after 2 days were treated with DRB to stop transcription. At the indicated times after drug treatment, RNA was harvested and analyzed with primer extension. Similar results were obtained in three independent transfections. As an internal control for transfection efficiency and RNA recovery, the cells were all cotransfected with pMT-S, which contains the S gene under the control of the mouse metallothionein II promoter. The S gene transcripts synthesized from this plasmid give rise to a single extended product of ~203 bases (arrow).

contrary to expectations, the amount of nuclear transcripts was slightly but consistently larger in cells transfected with the plasmid containing the displaced PRE (Fig. 6A, lanes 3 and 4). In all cases, the amount of β-actin RNA was the same in the two sets of RNA fractions (Fig. 6B), ruling out differential RNA recovery as a source of variability between different samples. It is difficult to envision that destabilization of transcripts in the cytoplasm could lead to a change in nuclear transcript levels. Rather, these results suggested that the PRE may increase S gene transcript levels by facilitating an intranuclear event that subsequently leads to accumulation of the transcripts in the cytoplasm. To measure directly the rate of degradation of S gene transcripts in the cytoplasm, we transfected cells with either pSAgΔHin or pSHpA-NcoI and after 40 h added DRB, an inhibitor of RNA polymerase II (20). At various times after drug treatment, cytoplasmic RNA was extracted from the cells and the amount of remaining S transcripts was quantitated by primer extension. The S transcripts synthesized from both plasmids indeed had similar half-lives of approximately 3 h (Fig. 7). Therefore, we conclude that the PRE does not prevent S gene transcript degradation in the cytoplasm but must instead facilitate the accumulation of these transcripts in the cytoplasm, presumably by activating their transport from the nucleus.

DISCUSSION

The region of the HBV genome downstream of the S open reading frame contains two DNA segments that can function as transcriptional enhancers when coupled to heterologous promoters (reviewed in references 19, 22, and 30). Previously, we had demonstrated that when this region was placed upstream of the S promoter, it did not function optimally to increase the steady-state amount of HBV S gene transcripts (12). This result raised the possibility that this region also has an effect on gene expression at the posttranscriptional level. The data presented here confirm that the X gene/EnII region of HBV acts in *cis* at the RNA level to increase S gene transcript levels. As we showed, there was a markedly decreased amount of S gene transcripts when they were terminated before this region. Insertion of EnII back into the transcribed region largely restored the transcript levels in an orientation-dependent manner. Since previous reports clearly demonstrated transcriptional regulation by EnII (27, 29, 31, 33), the region of the HBV genome encompassing EnII must be able to activate gene expression at both the transcriptional and postranscriptional levels. We have named this region, when it functions at the RNA level, the postranscriptional regulatory element (PRE). Interestingly, Vannice and Levinson (26) have previously shown that in a heterologous context, EnI functions better when it is within the transcribed region. Therefore, it is possible that there are other similar RNA elements within the HBV genome.

Changes in steady-state RNA levels induced by the PRE were correlated neither with any change in transcriptional initiation rate nor with a change in the rate of RNA degradation in the cytoplasm. Therefore, the PRE influences a step in RNA metabolism between initial synthesis and eventual degradation. This is presumably an intranuclear event, since RNA processing takes place primarily in the nucleus. Indeed, removal of the PRE from the S gene transcripts led to the accumulation of the transcripts in the nucleus to a level slightly higher than the wild-type level. The most parsimonious interpretation of these results is that in the absence of the PRE, a large fraction of these transcripts is not transported out of the nucleus but is instead degraded within the nucleus at a rate slightly slower than the normal rate of export out of the nucleus. Unfortunately, we could not directly test this inference, since both actinomycin D and DRB appeared to block the transport of S gene transcripts out of the cytoplasm and stabilize the intranuclear transcripts (data not shown). Nevertheless, our data taken together are consistent with the model that the PRE functions to facilitate the nuclear-to-cytoplasmic transport of the S gene transcripts, although further experiments are needed to confirm this model.

We note that the behavior of PRE-minus transcripts is similar to that of intron-containing transcripts that do not undergo proper splicing. One class of such transcripts appears to be retained in the nucleus because they are recognized as being incompletely spliced. A prominent example is the human immunodeficiency virus structural protein transcripts, which contain inefficiently utilized splice sites (2) that lead to nuclear retention and degradation (15). This retention is antagonized by the *cis*-acting RNA sequence known as the Rev response element (RRE), which activates the transport of RRE-containing transcripts into the cytoplasm (reviewed in references 4, 17, and 28). These characteristics are similar to those we have found for the PRE. Indeed, the PRE can partially substitute for the RRE to effect the cytoplasmic transport of human immunodeficiency virus transcripts (11, 13). However, while it has been reported that there are potentially functional splice

acceptor sites in the S gene transcripts (3, 23), no spliced S gene transcripts have been reported. Furthermore, removal of these acceptor sites from these transcripts did not relieve the requirement for the PRE (13). Therefore, at this time we cannot ascertain the reason for the nuclear retention of PRE-minus S gene transcripts.

In addition, we speculate that a *trans*-acting factor interacting with the PRE is involved in releasing S gene transcripts into the cytoplasm. The RRE is known to depend on the Rev protein of the human immunodeficiency virus to function. However, the HBV X protein, the only known regulatory gene product of HBV, does not appear to be this factor, since the PRE functions in its absence (12). Therefore, it is likely that the factor is of cellular origin and may also be responsible for posttranscriptional regulation of cellular genes.

In summary, we have obtained data showing that a region in the HBV S gene transcripts encompassing enhancer II facilitates cytoplasmic accumulation of these transcripts. Further characterization of this *cis*-acting region, and the *trans*-acting factors that interact with it, may lead to novel means of down-regulating HBV gene expression in infected hepatocytes and to new insights into the control of cytoplasmic transport of cellular genes.

During the revision of this report, Huang and Liang (11) published a paper describing findings similar to ours. They, too, came to the conclusion that an HBV RNA element is important in allowing the nuclear-to-cytoplasmic transport of HBV transcripts.

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ADDENDUM IN PROOF

A similar RNA element in the Mason-Pfizer monkey virus genome has recently been described (M. Bray, S. Prasad, J. W. Dubay, E. Hunter, K. T. Jeang, D. Rekosh, and M. L. Hammarskjold, Proc. Natl. Acad. Sci. USA **91**:1256–1260, 1994).

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