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A Novel Hepatitis B Virus (HBV) Genetic Element with Rev Response Element-Like Properties That Is Essential for Expression of HBV Gene Products

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Many viruses possess complex mechanisms involving multiple gene products and *cis*-regulatory elements in order to achieve a fine control of their gene expression at both transcriptional and posttranscriptional levels. Hepatitis B virus (HBV) and retroviruses share many structural and functional similarities. In this study, by genetic and biochemical analyses, we have demonstrated the existence of a novel genetic element within the HBV genome which is essential for high-level expression of viral gene products. This element is located 3′ to the envelope coding region. We have shown that this genetic element is *cis* acting at the posttranscriptional level and that its function is exerted at the level of RNA processing as part of transcribed sequences. This RNA element is also functional in the context of a heterologous gene. Similar to the function of Rev-Rev response element interaction of human immunodeficiency virus type 1, this element appears to inhibit the splicing process and facilitate the transport and utilization of HBV transcripts.

Human hepatitis B virus (HBV), a major causative agent of acute and chronic liver disease in humans is the prototype of the family *Hepadnaviridae*. The HBV genome is a partially double-stranded circular DNA of 3.2 kbp in length. Examination of HBV nucleotide sequences reveals four open reading frames (ORFs), all encoded on the positive strand; they are the core, reverse transcriptase-polymerase (RT/Pol), surface, and X genes (16). HBV has a compact genomic organization and exhibits remarkable economy in usage of its limited coding capacity. The overlapping ORFs, the presence of multiple regulatory genetic elements interspersed in various regions of the genome, and the shared common 3′ terminus of the HBV transcripts are the structural features of the genome of hepadnavirus family. Three major species of hepadnavirus-specific mRNAs have been identified (16). The large genomic (3.5-kb) RNA contains all the genetic information encoded by the virus and functions both as an intermediate in viral replication and as a template for translation of viral gene products. In addition, there are two subgenomic RNAs of 2.1 and 2.4 kb in length which direct the synthesis of pre-S1, pre-S2, and S proteins. A third RNA species (0.7 kb) specific for the X gene has been identified in tissue culture as well as in infected liver tissue (24, 43). All these transcripts terminate at the same polyadenylation site within the 5′ region of the core gene.

HBV is distantly related to retroviruses, with many features common to both viruses (36). In addition to certain sequence homology and similar genomic organization, the two viruses share many functional similarities, such as replication through a reverse transcriptase mechanism, persistent and latent infection, integration into host genome, lymphotropism, and nonacute type of oncogenic potential (36). Although the mechanisms underlying each of the similarities are rather distinct between the two viruses, it is reasonable to classify them as related viruses with common evolutionary origin. One salient feature of the retroviral life cycle is the intricate and complicated regulation of gene expression at both transcriptional and posttranscriptional levels. The more complex retroviruses, such as the human immunodeficiency virus type 1 (HIV-1), have evolved an elaborate mechanism involving multiple viral gene products and *cis*-regulatory elements to achieve the fine posttranscriptional controls of viral gene expression (10, 11). Rev and Tat of HIV are the two best-studied examples of viral gene products with such functions. The Tat protein interacts with a *cis*-acting stem-and-loop element (TAR) located near the 5′ end of the HIV-1 RNA transcripts and appears to enhance transcription from the 5′ long terminal repeat by stabilizing the elongation process of transcription (11). The other viral protein, Rev, appears to increase the transport and utilization of all viral mRNA that contains another *cis*-acting element (RRE) within the Env coding region of the viral genome (8, 15, 28).

Although there are numerous splice consensus sequences within the HBV genome, the predominant and essential RNA species coding for all the viral gene products are unspliced. Singly and doubly spliced transcripts involving some of these splice sites have been observed in cultured hepatoma cells transfected with HBV genome as well as in infected liver tissues (45). Elimination of these spliced sites by site-directed mutagenesis apparently did not affect the process of viral replication in tissue culture (49). Therefore, the functions of these spliced forms of viral transcripts remain largely unknown.

In this study, we reported the presence of a Rev-RRE-like genetic element located approximately between nucleotides (nt) 1200 and 1650 within the HBV genome. We showed that this is a *cis*-acting element, functioning at the posttranscriptional level to enhance expression of HBV viral gene products. We further demonstrated that this element exerts its function by inhibiting splicing and facilitating transport and utilization of HBV transcripts from nucleus to cytoplasm.

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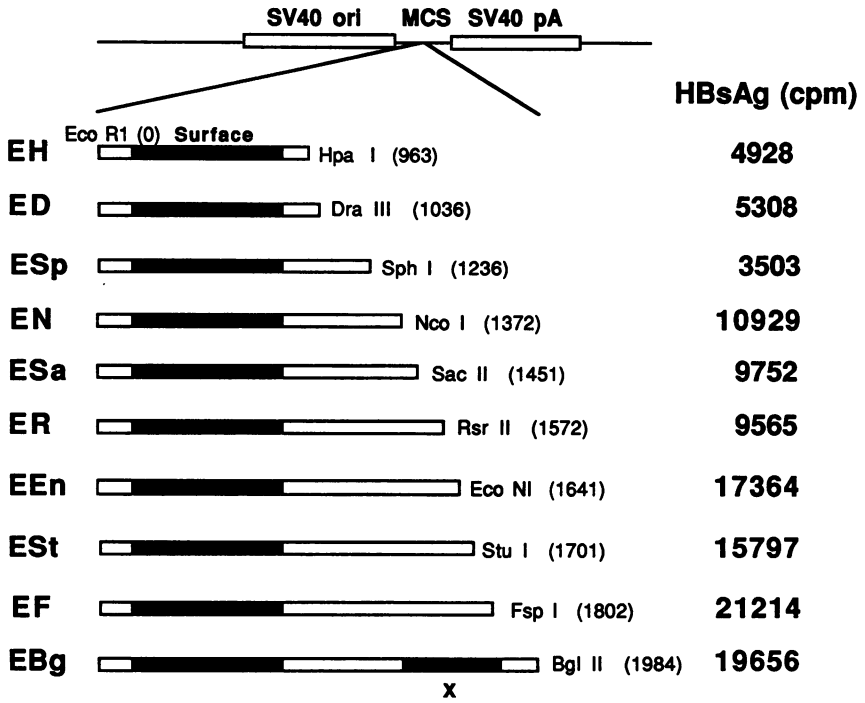

FIG. 1. Production of HBsAg by SV40 expression constructs. Various fragments of HBV genome containing the major envelope ORF were placed under the control of the SV40 enhancer and promoter and polyadenylation signal as described in Materials and Methods. On day 2 after transfection, spent culture medium from each transfected HuH-7 cells was collected and assayed for HBsAg (in counts per minute). The results were representative of at least three separate experiments. The EH+ construct shown here and in Fig. 2 represents insertion of the HBV fragment in the sense orientation in front of SV40 ori. Similar data were observed with a construct containing the antisense insertion. Cotransfection of pTKGH plasmid demonstrated similar levels of transfection efficiency for each transfection.

MATERIALS AND METHODS

Plasmid construction. A series of plasmids for expression of hepatitis B surface antigen (HBsAg) were constructed in the pSV.1 plasmid that was derived from the pJ3Omega (5) expression vector by eliminating the simian virus 40 (SV40) small T intron sequences (tIVS). All the HBV sequences used in this study were derived from an adw strain (5). As shown in Fig. 1, HBV fragments of various lengths, all starting at the *Eco*RI site (nt 0) of the HBV genome and containing the major HBsAg ORF, were cloned into the *Eco*RI and *Hpa*I sites of pSV.1. The 3′ ends of the various constructs were at *Hpa*I (nt 963), *Dra*III# (nt 1036), *Sph*I* (nt 1236), *Nco*I# (nt 1372), *Sac*II# (nt 1451), *Rsr*II# (nt 1572), *Eco*NI# (nt 1641), *Stu*I (nt 1701), *Fsp*I (nt 1802), and *Bgl*II (nt 1984) restriction sites; T4 DNA polymerase was used to remove the 3′ protruding ends (*), and Klenow was used for filling the 5′ protruding ends (#). The constructs made through these sites were named EH, ED, ESp, EN, ESa, ER, EEn, ESt, EF, and EBg, respectively. The construct EH-FH was generated by inverting the region from *Hpa*I (nt 963) to *Fsp*I (nt 1802) of the EF construct. The *Hin*cII fragment of HBV genome from nt 963 to 1684 was inserted in the *Pvu*II site in front of the SV40 promoter and enhancer of the EH construct, resulting in constructs Hinc3-EH in the sense and Hinc13-EH in the antisense orientation, collectively referred to as EH+ in Fig. 1 and 2. An HBX-minus construct, EBgX-, was generated from the EBg by Klenow treatment of the 5′ protruding ends at the *Sac*II site (nt 1451), resulting in a 2-bp frameshift insertion that had been confirmed by DNA sequencing. The constructs EH+tIVS, EF+tIVS, and EHFH+tIVS contained the SV40 tIVS inserted between the HBV sequences and the SV40 polyadenylation site (pA) of the EH, EF, and EH-FH, respectively. The tIVS used here was derived from pSVSPORT.1 plasmid (Bethesda Research Laboratories, Gaithersburg, Md.) that contains a modified tIVS in which a 72-bp

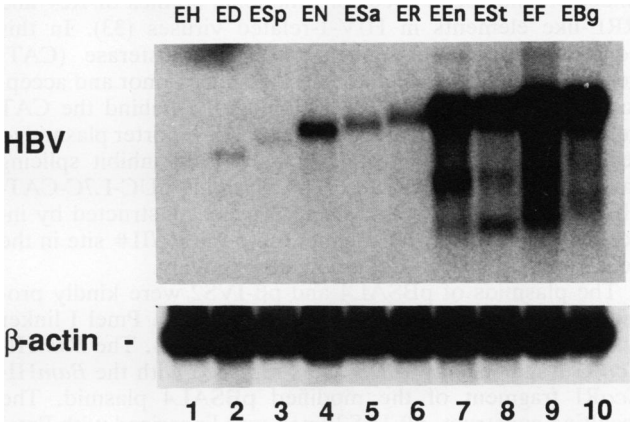

FIG. 2. HBV RNA in cells transfected with SV40 expression constructs. On day 2 after transfection, poly(A)$^+$ RNAs were extracted from HuH-7 cells transfected with various HBsAg expression constructs and analyzed by Northern blot analysis. After hybridization with a ^{32}P-labeled HBV probe, the blot was exposed with an intensifying screen overnight and shown in the upper panel. The blot was then stripped and rehybridized with a β-actin probe to control for RNA loading (lower panel). Lane 1, EH construct; lane 2, ED; lane 3, ESp; lane 4, EN; lane 5, ESa; lane 6, ER; lane 7, EEn; lane 8, ESt; lane 9, EF; lane 10, EBg. Cotransfection of pTKGH plasmid demonstrated similar levels of transfection efficiency for each transfection.

fragment was inserted within the native tIVS in order to produce a longer intron for more efficient splicing (21). A 1.1× genomic length of HBV sequences from *Fsp*I to *Bgl*II site (nt 1802 through 0 to 1984) was cloned in pSV.1 to give rise to the FBg construct. The FBg-FH construct was constructed by inverting the *Hpa*I-*Fsp*I (nt 963 to 1802) fragment in an opposite orientation within the FBg. The construction of HBV adwR9 has been described (5). The adwR9-FH construct was generated by inverting the *Hpa*I-*Fsp*I DNA fragment of adwR9. The frameshift mutation within the X ORF described previously was introduced into the adwR9 as the adwR9X−.

The pSVCAT was constructed by inserting the *Hin*dIII-*Kpn*I* fragment from the pGEM3CAT plasmid into the pSV.1 expression plasmid. pGEM3CAT contains the CAT gene fragment (*Hin*dIII-*Bst*YI) from the pSV2CAT inserted into the *Hin*dIII and *Bam*HI sites of pGEM3Z(+) (Promega, Madison, Wis.). pSVCAT-HR and pSVCAT-HB were constructed by replacing the HBV sequences between *Eco*RI and *Hpa*I of the ER and EBg constructs, respectively, with the same CAT DNA fragment. pHinc3-SVCAT and pHinc13-SVCAT were derived from the Hinc3-EH and Hinc13-EH, respectively, the same way.

The pSVmyc was constructed by inserting the *Eco*RI-*Bam*HI# fragment from pBSKmyc which contains exons 2 and 3 of human c-*myc* cDNA (a generous gift from Anil Rustgi) into the pSV.1 expression plasmid. A Pmel I linker (10-mer) (New England Biolabs, Beverly, Mass.) was also inserted between the c-*myc* and SV40 pA sequences for convenience of subsequent plasmid construction. The HBV *Hin*cII fragment (nt 963 to 1684) was inserted into the *Pme*l I site of pSVmyc, resulting in constructs pSVmyc-Hinc3 in the sense and pSVmyc-Hinc13 in the antisense orientation. The construction of pHinc3-SVmyc and pHinc13-SVmyc containing an HBV *Hin*cII fragment were generated the same way as we did in pHinc3-SVCAT and pHinc13-SVCAT construction.

The plasmid pUC-L7C-CAT (a generous gift from Barbara Shacklett) has been used for functional studies of Rev and RRE-like elements in HIV-1-related viruses (33). In this construct, the chloramphenicol acetyltransferase (CAT) gene was placed between the HIV-1 splice donor and acceptor sites with a unique *Bgl*II cloning site behind the CAT gene (see Fig. 9). We employed this CAT reporter plasmid to test whether HBV *Hin*cII fragment could inhibit splicing involving these splice sites. The plasmids pUC-L7C-CAT-Hinc3 and pUC-L7C-CAT-Hinc13 were constructed by inserting the HBV *Hin*cII fragment into the *Bgl*II# site in the sense and antisense orientation, respectively.

The plasmids of pBSAL4 and pβ-IVS2 were kindly provided by Ben Blencow and Phillip Sharp (8). Pmel I linker was inserted into the *Nco*I# site of pBSAL4. The *Bam*HI-*Eco*RI fragment of pβ-IVS2 was replaced with the *Bam*HI-*Eco*RI fragment of the modified pBSAL4 plasmid. The resulting construct, pβ-IVS2Pmel, was linearized with Pmel I to which the HBV *Hin*cII fragment was ligated. pβ-IVS2-Hinc3 has the sense and pβ-IVS2-Hinc13 has the antisense orientation of the inserted HBV sequences.

pGEM3HBVBal2 was constructed by ligating HBV sequences from nt 2988 to 303 (*Bal*I sites) to pGEM3Z(+) linearized by *Hin*cII. By using T7 polymerase, antisense RNA transcripts were generated for RNase protection experiment. pGEM3GH-BE was constructed by ligating the *Bgl*II-*Eco*RI DNA fragment of the human growth hormone (hGH) gene from plasmid pTKGH (39) to pGEM3Z(+) digested with *Bgl*II and *Eco*RI. Antisense riboprobe was generated by using T7 polymerase.

Tissue culture and transfection. Human hepatoma cells HuH-7 were maintained in plastic dishes containing Dulbecco's modified minimal essential medium (GIBCO, Grand Island, N.Y.) plus 10% fetal bovine serum at 37°C and 5% CO_2 (4). HuH-7 cells were grown to 70% confluence and transfected with DNA by using a modified $CaPO_4$ transfection method (5). For 10-cm-diameter dishes, 20 μg of plasmid DNA was transfected, and 2 μg of plasmid pTKGH was cotransfected to control for transfection efficiency. On day 2 after transfection, total cellular RNAs were purified and culture media were collected for radioimmunoassays to measure growth hormone, HBsAg, and HBV e antigen (HBeAg).

RNA analysis. On day 2 after transfection, total cellular RNAs were purified with the RNAzol-B reagents (Biotecx, Houston, Tex.), and poly(A)$^+$ RNAs were isolated by using the Polytract RNA kit (Promega). RNAs were subjected to Northern (RNA) blot analysis on a 1% agarose gel containing formaldehyde. The blots were hybridized with ^{32}P-labeled DNA probes. Nuclear transcription run-on assay was carried out by methods described previously (3). Briefly, 5 μg of linearized plasmid DNA was immobilized on a Nytran membrane and hybridized with the radioactively labeled RNAs generated from the run-on reaction.

For analysis of RNA stability, cells were treated with 10 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml on day 2 after transfection. At different time points, total cellular RNAs were purified for Northern blot analysis as described above. The nuclear and cytoplasmic RNAs were isolated from transfected cells on day 2 after transfection (3). RNase protection assay was performed as described previously (3). A total of 10 μg of cytoplasmic and 5 μg of nuclear RNAs were used for each RNase protection analysis. The riboprobes were generated by using the In Vitro Transcription Kit from Promega. The digestion products were separated on a denaturing 6% polyacrylamide gel containing 8 M urea. Radioactive molecular weight markers were included for size determination.

CAT assay and radioimmunoassays. HuH-7 cells were harvested 2 days after transfection, and cell lysates were analyzed for CAT activity as described elsewhere (3). Culture medium was assayed for HBsAg and HBeAg by using Ausria II and HBe (rDNA) kits (Abbott Laboratories, Chicago, Ill.), respectively. Secreted hGH in medium was measured by using a radioimmunoassay kit from Nichols Institute Diagnostics (San Juan Capistrano, Calif.).

RESULTS

High-level expression of HBsAg is dependent on a *cis*-acting element in HBV genome. In order to assess the possible presence of a *cis*-regulatory element(s) on the HBV genome important for the expression of HBsAg, we generated a series of HBsAg expression constructs containing the HBsAg ORF (nt 157 to 835) and progressively longer 3′ untranslated region. The shortest construct, EH, contained predominantly the HBsAg ORF. The longest construct, EBg, included HBV sequences up to the HBV polyadenylation site (nt 1936). These constructs were transfected into HuH-7 cells, and the results are shown in Fig. 1. Analysis of the spent culture medium revealed that HBsAg accumulation was markedly low in constructs containing HBV sequences up to nt 1236 (constructs EH, ED, and ESp). A significant increment in HBsAg production was observed in

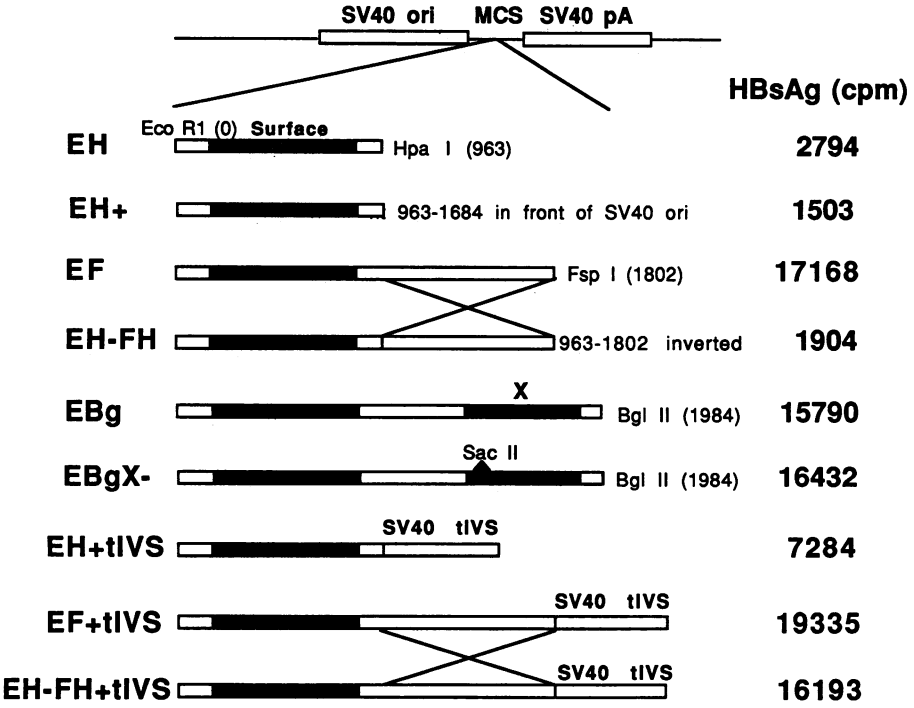

FIG. 3. Effects of HBV Enh 1, HBX, and SV40 tIVS on HBsAg expression. Additional HBsAg expression constructs were generated as described in Materials and Methods. EH+ represents the EH(Hinc3) construct. Similar data were also observed but not shown for the antisense construct of EH(Hinc13). On day 2 after transfection, spent culture medium from transfected HuH-7 cells was collected and assayed for HBsAg. The results were representative of at least three separate experiments. Transfection efficiencies as controlled by GH production were similar in this experiment.

constructs containing downstream HBV sequences between nt 1236 and 1572 (constructs EN, ESa, and ER). A further increase was noted in constructs EEn, ESt, EF, and EBg. The EEn construct appeared to be the shortest construct capable of expressing the highest level of HBsAg. Because of the nonlinear nature of the HBsAg radioimmunoassay at a high HBsAg concentration (>10,000 cpm), we measured HBsAg in serially diluted culture medium and showed that HBsAg accumulation in EBg-transfected cells was estimated to be more than 50-fold greater than that of the EH construct. Cotransfection of hGH expression plasmid demonstrated similar transfection efficiency in this experiment. In order to establish that selective retention of HBsAg in the cytoplasm of cells transfected with some of the constructs was not the basis for the observed differences, we analyzed the intracellular contents of HBsAg in cells transfected with each of the constructs. A parallel trend of increase in HBsAg contents was observed, as in Fig. 1 (data not shown), suggesting that the observed disparity in HBsAg accumulation was most likely due to a difference at the level of synthesis.

mRNA was isolated from cells transfected with these constructs and analyzed by Northern blot hybridization (Fig. 2). The results demonstrated that the difference in HBsAg production was similarly observed at the RNA level. We also transfected these constructs into other hepatic and nonhepatic cell lines of human or nonhuman origin, such as HepG2, Hep3B, and SK-Hep1 (human hepatoma); HeLa (human cervical cancer); COS (monkey kidney); F-111 (rat fibroblast); and NIH 3T3 (mouse fibroblast) cells, and obtained similar results of HBsAg production (data not shown). In addition, we generated similar data for primary rat hepatocytes, suggesting that this phenomenon is not a unique observation in cell lines. These findings suggest that there is no species or tissue specificity with respects to the function of this element.

HBV enhancers and X ORF are not accountable for the observed differences in HBsAg production. Since construct EEn, the shortest construct capable of high-level HBsAg expression, contains enhancer (Enh) I and part of the HBX ORF, we needed to assess the potential roles of these elements in our constructs. To address this issue, we constructed two additional plasmids, one (EH+) with HBV sequences from nt 963 to 1684 in front of the SV40 ori, and the other (EH-FH) with the HBV sequences between nt 963 and 1802 inverted in the EF construct (Fig. 3). We reasoned that the enhancer element, by definition, should work in a position- and orientation-independent manner. Figure 3 shows that the enhancer element(s) appeared not to be important for the observed differences in HBsAg production. To answer the question regarding the importance of X ORF, we introduced a frameshift mutation around nt 1451 in the X ORF (construct EBgX−). This construct also directed high-level HBsAg production, suggesting that the function of X protein is dispensible in these expression constructs (Fig. 3). In addition, cotransfection of an X expression construct (6) to provide wild-type X protein in *trans* did not result in any significant increase in HBsAg production in cells transfected with EH, EN, or EBg constructs (data not shown).

Previous studies have shown that proper splicing is necessary for high-level eukaryotic gene expression (7, 19, 31). Since our expression constructs did not contain an intron with authentic splicing sequences, such as the SV40 tIVS, we generated three additional constructs to evaluate the

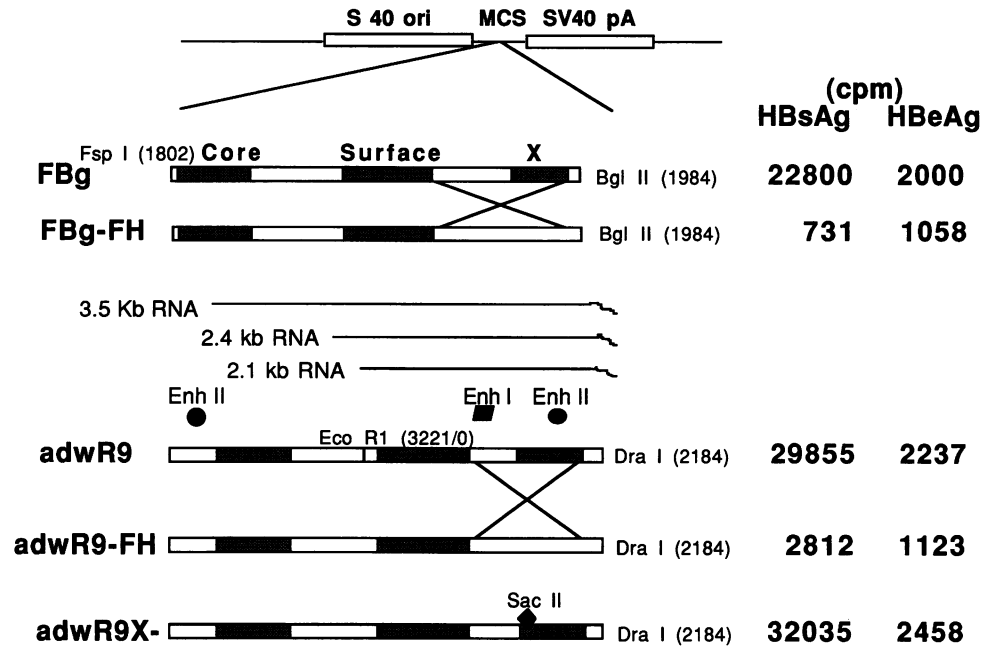

FIG. 4. Expression of HBsAg and HBeAg by full-length HBV constructs. HBV expression constructs containing the entire HBV genome were generated as described in Materials and Methods. On day 2 after transfection, spent culture medium from transfected HuH-7 cells was collected and assayed for HBsAg. The results were representative of at least three separate experiments. Transfection efficiencies as controlled by GH production were similar in this experiment.

effects of splicing on the level of HBsAg RNA. The SV40 tIVS was placed between the HBV and SV40 pA sequences in the EH, EF, or EH-FH construct (Fig. 3). A significant increase in HBsAg production, but not to the same level as the EF construct, was observed in the EH-tIVS construct compared with that in the EH construct. On one hand, the inclusion of tIVS in the EH-FH construct was able to enhance HBsAg synthesis to almost the same level as that observed in the EF construct. On the other hand, no appreciable increase was noted in the EF construct with the addition of tIVS (Fig. 3). In order to corroborate the findings of HBsAg synthesis at the RNA level, we performed Northern blot analysis on cells transfected with the various constructs described in Fig. 3. Results similar to those of the HBsAg synthesis were observed at the level of HBV transcripts (not shown).

The HBV element is also important in maintaining a high level of 3.5-kb transcripts. The previous findings suggested that a *cis*-acting element 3′ to the HBsAg ORF is important for the efficient expression of HBsAg at the RNA level. Since the other predominant HBV transcripts, the 3.5-kb RNAs, also contain the same 3′ HBV sequences, we generated several additional expression constructs to examine the effect of this element on the level of 3.5-kb transcripts. A fragment containing a 1.1× genomic length of HBV sequences from nt 1802 to 1984 was cloned into the SV40 expression plasmid as construct FBg. A comparable construct, FBg-FH, containing the inverted HBV sequence as in construct EH-FH was also generated. Parallel to our previous findings, a major disparity in HBsAg production between the two constructs was again demonstrated (Fig. 4). Similarly, a significant difference was also evident in HBeAg synthesis, but the difference was not as dramatic as those of HBsAg production (Fig. 4). Since all the above experiments utilized a strong heterologous enhancer and promoter to drive the expression of HBV transcripts, we needed to address the question of whether HBV transcription directed by the endogenous HBV enhancer and promoter elements would be similarly affected. We have previously generated an HBV construct, adwR9, which contains a 1.2× genomic length of HBV DNA and is capable of directing complete viral replication after transfection into human hepatoma cells (4). As with the EH-FH construct, we inverted the *Hpa*I-*Fsp*I fragment in the adwR9 construct to generate the adwR9-FH construct (Fig. 4). Although this manipulation alters sufficiently the RT/Pol gene product and likely affects viral replication, we reason that viral transcription can proceed normally because all the enhancer elements (Enh 1 and 2) are present and the promoter elements for either the 3.5- or 2.4- and 2.1-kb RNAs are also intact in the adwR9-FH construct. In addition, we created a adwR9X− construct with a frameshift mutation in the N terminus of X ORF to assess the importance of X in HBV transcription. The results are shown in Fig. 4. The inversion of the *Hpa*I-*Fsp*I fragment in the adwR9 construct again resulted in much lower levels of HBsAg and HBeAg synthesis, with the HBsAg synthesis being affected much more. Similar to our above finding and previous observations (6), X protein is dispensable for the expression of other HBV gene products.

Northern blot analysis of cells transfected with these constructs were performed (not shown). Higher levels of 3.5- and 2.4- plus 2.1-kb RNA species were evident in the FBg construct compared with those in the FBg construct. Parallel to the protein data, the 3.5-kb RNA species appeared to be affected less than the 2.4- and 2.1-kb species. Compared with those of wild-type and X-minus adwR9 construct, cells transfected with the adwR9-FH construct contained a significantly lower level of both RNA species.

The function of this *cis*-acting element is not exerted at the level of transcription or RNA stability. Although our previous

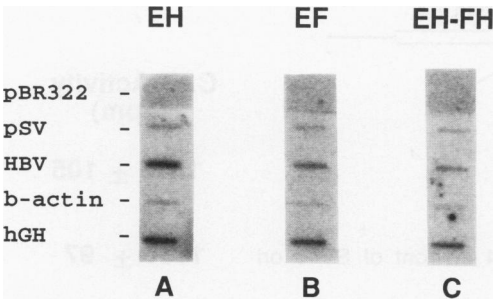

FIG. 5. Nuclear run-on assay of HBV expression constructs. Two days after transfection, HuH-7 cells were harvested and nuclei were isolated for nuclear run-on assay as described in Materials and Methods. Linearized pBR322, pSV.1 (pSV), pGEM7HBV (HBV), pGEM3β-actin (b-actin), and pTKGH (hGH) plasmids (5 μg) were immobilized on Nytran membrane in three sets in the positions indicated on the figure. One set of membrane was incubated with the run-on ^{32}P-labeled RNA from EH-transfected cells, the second set was incubated with EF RNA, and the third set was incubated with EH-FH RNA. The membrane was washed and exposed overnight with an intensifying screen.

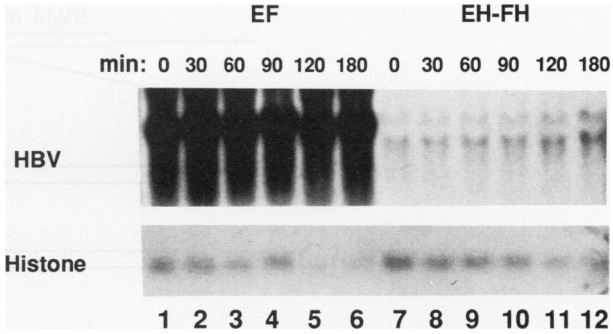

FIG. 6. HBV RNA stability in cells transfected with HBV expression constructs. Two days after transfection of HuH-7 cells in 10-cm-diameter dishes with either EF or EH-FH constructs, 10 μg of actinomycin D was added to the cells, and one dish of cells was harvested immediately at time 0. Cells were collected further at 30, 60, 90, 120, and 180 min. Total RNA was isolated from cells at each time point and subjected to Northern blot analysis. Overnight exposure with an intensifying screen was obtained after hybridization with ^{32}P-labeled HBV probe (top panel). The blot was then stripped and rehybridized with a β-histone probe. An overnight exposure was shown in the bottom panel. Lanes 1 to 6, EF transfection; lanes 7 to 12, EH-FH transfection.

genetic analysis of this element 3′ to the HBsAg ORF suggested that enhancer elements were not involved, we cannot absolutely rule out the possible existence of another as-yet-unidentified HBV enhancer element(s) in this region. In order to address this issue, we performed nuclear run-on assay in cells transfected with the EH, EF, and EH-FH constructs (Fig. 5). Using the transcription rates of β-actin and hGH as controls, we demonstrated similar levels of transcription rate among all the three constructs. In order to eliminate the possibility that a unique interaction of SV40 enhancer and promoter with this HBV element may result in our initial findings, we generated similar expression constructs by using a cellular enhancer and promoter of the mouse albumin gene as well as another strong viral enhancer and promoter, the cytomegalovirus immediate-early promoter, to direct the expression of HBsAg. Experiments with these additional enhancer and promoter constructs resulted in findings similar to those with the SV40 constructs (data not shown).

Since transcription was not affected, we proceeded to assess the importance of RNA stability as a determining factor in the observed disparity of HBV RNA levels. Cells transfected with either EF or EH-FH constructs were first treated with actinomycin D to block cellular transcription and then harvested at various times for RNA purification. Northern blot analysis was performed to assess the time course of HBV RNA degradation (Fig. 6). Histone RNA was used as a control for measurement of RNA stability in this experiment. No difference in the rates of HBV RNA degradation between the two constructs was observed, whereas the histone RNA disappeared gradually, with a half-life of <1 h. Interestingly, all the HBV RNA species were remarkably stable with equally strong signals 12 h after addition of actinomycin D (data not shown). An additional species of HBV RNA was noted in both the EH-FH- and EH-FH-tIVS-transfected cells. Reverse transcription-polymerase chain reaction was performed to analyze these aberrant transcripts further. DNA sequencing of the products revealed that a cryptic 5′ splice site at nt 460 and a 3′ splice site at nt 1742 in the minus strand were spliced together to give these additional RNA species in both samples (data not shown).

The HBV RNA element is functional in the context of heterologous genes. In order to demonstrate that the HBV RNA genetic element, which functions most likely as an RNA element, can exert its effect in genes other than the HBV genes, we generated several constructs to address this issue. First, we inserted two HBV fragments, one from nt 963 to 1572 and the second from nt 963 to 1984, behind the CAT gene in the pSVCAT expression construct. We also placed HBV sequences from nt 963 to 1684 in front of the SV40 ori of the pSVCAT plasmid in either orientation. The results are shown in Fig. 7. The CAT activity was enhanced greater than fourfold by the 3′ addition of HBV sequences from nt 963 to 1984 (construct pSVCAT-HBg), whereas the addition of HBV sequences from nt 963 to 1572 resulted in an intermediate level of CAT activity (pSVCAT-HR). Insertion of HBV sequences in front of the SV40 ori did not lead to any increment of CAT activity. These data paralleled those of HBsAg production, with the exception that a much less dramatic effect was seen in the CAT expression construct. This can possibly be explained on the basis that the CAT transcript may be an efficiently processed and intrinsically stable mRNA, and this *cis*-acting HBV element is only able to enhance its RNA level to a certain level.

It is well known that expression of the *myc* gene family is tightly modulated at both transcriptional and posttranscriptional levels, and *cis*-acting elements present on their transcripts are important in the regulation of RNA stability (23). Since the *myc* mRNA transcripts are rather unstable, with short half-lives, we elected the c-*myc* gene as our second reporter gene to assess the effect of this HBV element. As in our previous construction, HBV sequence from nt 963 to 1684 was placed either behind the *myc* cDNA or in front of the SV40 ori in either orientation in a SV40-driven c-*myc* cDNA expression construct. These constructs were transfected into HuH-7 cells, and purified RNAs were analyzed by Northern blot analysis. The results are shown in Fig. 8. Only the *myc* construct with HBV sequences in the sense orientation within the transcribed sequences exhibited a high level of *myc* transcript. After correction for RNA loading, the signal of pSVmyc-Hinc3 is at least 20 times greater than that of the pSVmyc-Hinc13 (antisense) and 10 times greater

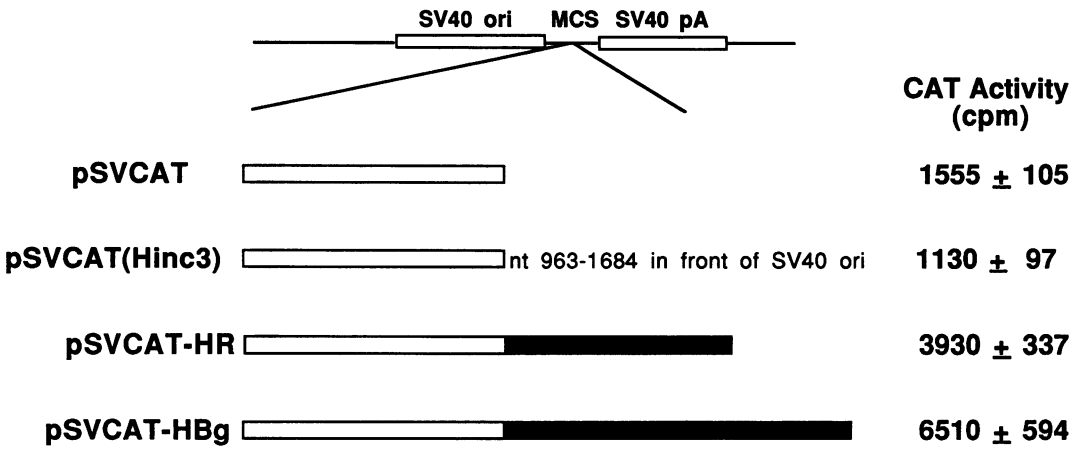

FIG. 7. Effects of HBV RNA element on CAT expression. The various CAT constructs depicted at the left were transfected into HuH-7 cells. On day 2 after transfection, cells were harvested and lysed for CAT activity analysis. The results are averages of triplicates ± standard errors of the mean and are shown at the right.

than those of the other constructs by densitometry quantitation. In addition, we demonstrated that this HBV element is also functional behind the gene of human insulin response substrate 1 (20a) which belongs to an unusual class of intronless mammalian genes (46).

This HBV element exhibits Rev-RRE-like functions by inhibiting splicing. Retroviruses contain important *cis* and *trans* elements regulating the splicing and processing of their transcripts. HIV-1, being a prototypic virus exhibiting such a regulation, harbors both of these elements, the Rev gene product and the RRE. These two elements work in concert to regulate HIV gene expression at the posttranscriptional level. The basic mechanism has been attributed to their effects on the splicing and/or transport of various HIV transcripts (8, 15, 28). In order to evaluate the functions of this HBV element, we inserted HBV sequences from nt 963 to 1684 in either orientation in the pUC-L7C-CAT plasmid which has been used to demonstrate the inhibitory effect of Rev- and RRE-like elements on splicing in HIV-related viruses (33). The resulting constructs were transfected into HuH-7 cells, and the resulting CAT activities are shown in Fig. 9. A significant increase in the CAT activity was observed in construct pUC-L7C-CAT-Hinc3, suggesting that the HBV sequences in the sense orientation inhibited splicing significantly and resulted in production of unspliced mRNA capable of directing CAT expression. HBV sequences, in the antisense orientation, did not lead to an increase in CAT activity and, on the contrary, resulted in a reduced level of CAT activity. This latter observation was reproducible. At present, we have no definitive explanation for this observation. We can only surmise that the HBV sequence in the antisense orientation probably stimulated splicing by providing a more favorable 3′ splice site than that of HIV-1 and resulted in an even lower level of unspliced RNA species than that of the parental plasmid. RNAs were isolated from cells transfected with these three constructs, and Northern blot hybridization was performed by using either the CAT gene or HBV DNA as a probe (Fig. 9B). A major unspliced species hybridizable to both CAT and HBV probes was seen only in cells transfected with pUC-L7C-CAT-Hinc3 (Fig. 9B, lanes 2). A weak signal which represents the unspliced RNA of pUC-L7C-CAT was visible in lanes 1.

Studies with HIV-1 demonstrated that regulation by the Rev-RRE interaction depends on recognition of splice sites (8). The RRE in the presence of Rev inhibits splicing of a weaker splicing site, such as the one in HIV-1. On the other hand, a stronger splicing site, such as the one in intron 2 (IVS2) of the rabbit β-globin gene, is minimally affected by the presence of Rev and RRE. Our above experiment demonstrated that the HBV element inhibited splicing in-

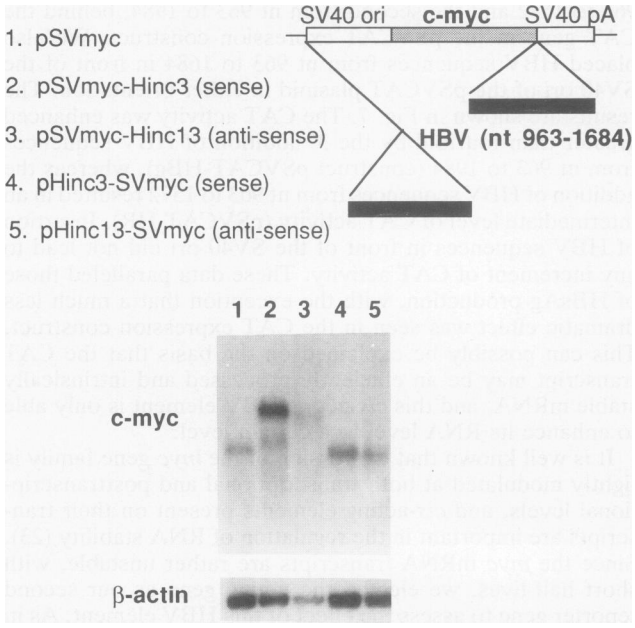

FIG. 8. Effects of HBV RNA element on the levels of c-*myc* RNA. On day 2 after transfection, poly(A)$^+$ RNAs were extracted from HuH-7 cells transfected with the c-*myc* expression constructs described in Materials and Methods and were analyzed by Northern blot analysis. After hybridization with ^{32}P-labeled c-*myc* probe, autoradiogram was obtained with an intensifying screen overnight and is shown in the upper panel. The blot was then stripped and rehybridized with β-actin probe to control for RNA loading (lower panel). Lane 1, pSVmyc; lane 2, pSVmyc-Hinc3; lane 3, pSVmyc-Hinc13; lane 4, pHinc3-SVmyc; lane 5, pHinc13-SVmyc. Cotransfection of pTKGH plasmid demonstrated a similar level of transfection efficiency for each transfection.

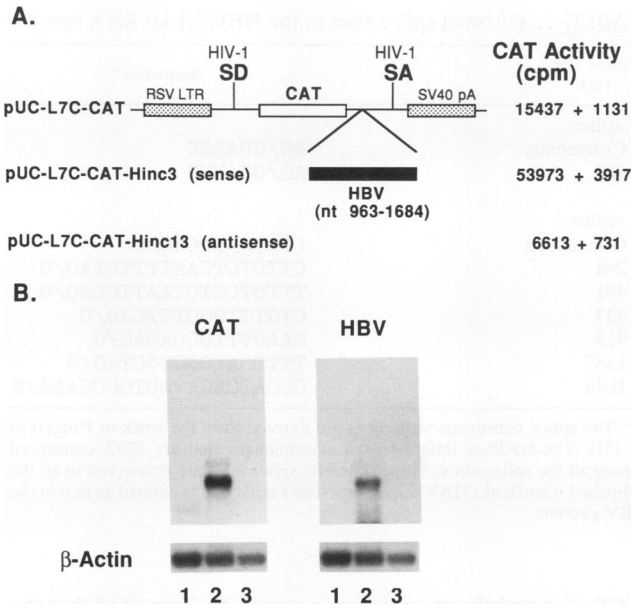

FIG. 9. Inhibition of HIV-1 splicing by HBV element. (A) Constructs containing HBV sequences inserted into the pUC-L7C-CAT plasmid were described in Materials and Methods. Transfected HuH-7 cells were harvested on day 2, and cell lysates were analyzed for CAT activity. A total of 10 μg of protein was used for each CAT assay. The results are averages of triplicates ± standard errors of the mean and are shown at the right. (B) In the same experiment, poly(A)$^+$ RNA was also purified from each transfection and subjected to Northern blot analysis in duplicate. The first blot was hybridized with CAT probe and the second was hybridized with HBV probe. The resulting autoradiograms (overnight exposure with an intensifying screen) are shown. The expected size of unspliced mRNA for pUC-L7C-CAT is 1.23 kb, which is faintly visible on the blot hybridized with CAT probe, and those for the others is 2.05 kb, while the spliced species for all constructs is 0.3 kb. After the hybridization, the blots were stripped and rehybridized with β-actin probe to control for RNA loading (bottom panels). Lanes 1, pUC-L7C-CAT; lanes 2, pUC-L7C-CAT-Hinc3; lanes 3, pUC-L7C-CAT-Hinc13.

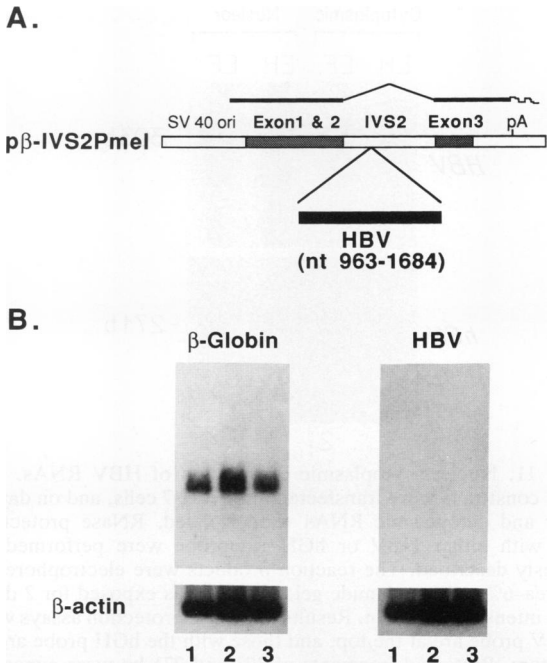

FIG. 10. Lack of inhibition on β-globin splicing by HBV element. (A) Constructs containing HBV sequences inserted into the pβ-IVS2 plasmid were described in Materials and Methods. Transfected HuH-7 cells were harvested on day 2, and total RNA was subjected to Northern blot analysis in duplicate. (B) Resulting autoradiograms (overnight exposure with intensifying screen). Left panel, hybridization with the β-globin probe; right panel, hybridization with the HBV probe. The expected size of spliced mRNA species for all constructs is 0.71 kb. After the hybridization, the blots were stripped and rehybridized with the β-actin probe to control for RNA loading (bottom panels). Lanes 1, pβ-IVS2; lanes 2, pβ-IVS2-Hinc3; lanes 3, pβ-IVS2-Hinc13.

volving the HIV-1 splicing sites. We performed similar experiments with the pβ-IVS2 plasmid expressing a partial β-globin cDNA containing the second β-globin intron (IVS2) (8). The HBV fragment was placed within the intron in either orientation: pβ-IVS2-Hinc3 in the sense and pβ-IVS2-Hinc13 in the antisense orientation. RNAs were isolated from transfected cells, analyzed by Northern blot in duplicate, and hybridized by using the β-globin cDNA probe for one set and HBV probe for the other. The results are shown in Fig. 10. In the β-globin-hybridized blot, all the RNA species appeared to be in the spliced form. The HBV probe that should detect unspliced β-globin RNA species appeared not to hybridize to any RNA species, confirming our interpretation that this HBV element does not inhibit splicing involving a strong splicing site.

The HBV element enhances transport of HBV transcripts. One of the major effects of Rev-RRE is to facilitate nuclear export of unspliced or partially spliced transcripts of HIV. In order to examine the effect of this HBV element on this process, we purified nuclear and cytoplasmic RNAs and performed RNase protection experiment to quantitate the distribution of HBV RNA. hGH expression plasmid was cotransfected in this experiment, and nuclear and cytoplasmic distribution of hGH transcripts were analyzed as controls. The results are shown in Fig. 11. Similar to what we have shown previously on Northern blot analysis, the level of cytoplasmic RNA was much higher in the EF-transfected cells, whereas the levels of nuclear RNA in cells transfected with either construct were similar. As a consequence, the ratio of nuclear to cytoplasmic levels of HBV RNA was much less in the EF-transfected cells, suggesting that a much more efficient nuclear export of HBV transcripts occurred in cells transfected with the EF construct. On the other hand, the hGH controls demonstrated a uniformly low nuclear/cytoplasmic ratio, consistent with the hypothesis that efficiently spliced mRNA is rapidly transported out to the cytoplasm (8, 18, 22).

DISCUSSION

Studies on the regulation of HBV gene expression have concentrated on the elucidation of interaction between transcription factors and *cis*-acting elements at the transcriptional level. Two enhancer elements, Enh 1 and 2, have been studied in detail with respects to their effects on HBV transcription as well as their potential interactions with nuclear transcription factors (2, 14, 18, 32, 47). Several promoter elements, each of them specific for the initiation of its corresponding transcript(s), have also been the focus of intense investigation (26, 30, 34, 43). Finally, the X gene product was thought to be not only a transactivator of a variety of viral and cellular promoters but also a potential

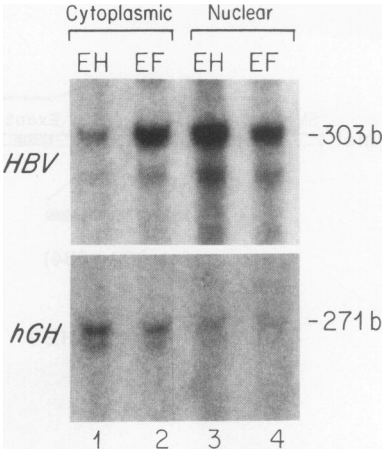

FIG. 11. Nuclear-cytoplasmic distribution of HBV RNAs. EH and EF constructs were transfected into HuH-7 cells, and on day 2, nuclear and cytoplasmic RNAs were isolated. RNase protection assays with either HBV or hGH riboprobe were performed as previously described. The reaction products were electrophoresed on a urea–6% polyacrylamide gel. The gel was exposed for 2 days with an intensifying screen. Results of RNase protection assays with the HBV probe are at the top, and those with the hGH probe are at the bottom. Protected fragments of 303 and 271 bp were expected from RNase protection analysis of HBV and hGH transcripts, respectively.

regulator of its own and other HBV gene transcription (9, 20, 25, 27, 40, 41). In this study, by genetic and biochemical analysis, we demonstrated the existence of a heretofore unrecognized genetic element within the HBV genome which is important for the efficient expression of viral gene products. We have shown that this genetic element is *cis* acting at the posttranscriptional level and that its function is exerted at the level of RNA processing as part of transcribed sequences. The expression of the 2.4- and 2.1-kb HBV transcripts appeared to be affected most by this *cis*-acting element. The level of the 3.5-kb RNA was also affected, but to a lesser extent. At present we have no explanation for this discrepancy. We can only speculate that perhaps some additional *cis*-acting element(s) is present in the 5′ region of the 3.5-kb RNA. Another possibility is that because of its interaction with core and RT/Pol proteins as part of the encapsidation process, the 3.5-kb RNA may be subjected less to regulation by this genetic element.

By deletion analysis, this element is located 3′ to the envelope coding region approximately between nucleotide positions 1200 and 1650. This region overlaps with the Enh 1 element (12). It is interesting to note that Vannice and Levinson (47), while studying the properties of Enh 1, also made the observation that the Enh 1 element, unlike the classical definition of enhancers, is position and orientation dependent with respects to its enhancer function. The possibility of posttranscriptional regulation at the RNA processing or stability level was discussed but not analyzed further. Therefore, it is possible that we are describing the same phenomenon. The clarification of these two genetic elements will require further analysis of their functional domains. In addition, our genetic element is rather different from the HBV enhancers in that it exhibits no cell type specificity with respects to its function.

The genetic element described by us also functions in the context of heterologous genes as well, such as the bacterial CAT gene and the mammalian c-*myc* gene, provided that the element is within the transcribed sequences in the correct orientation. This HBV element appears to affect the levels of these transcripts differently, suggesting that each RNA species possesses its own unique feature(s) of regulation. The c-*myc* transcript, being an unstable RNA species, is affected more by the presence of this HBV element.

TABLE 1. Potential splice sites in the HBV 2.1-kb RNA species

Splice site (nt)	Sequence[a]
5′ splice	
Consensus	AG/GUAAGU
460	AG/GUAUGU
3′ splice	
Consensus	$(Py)_{8-10}$NCAG/G
284	CTTCTCTCAATTTTCTAG/G
491	TTTGTCCTCTAATTCCAG/G
737	CTGTTTGGCTTTCAG/C
918	GAACTTTGCCACAG/G
1387	TTTCCATGGCTGCTAG/G
1649	CCCATCAGATCCTGCCCAAG/G

[a] The splice consensus sequences are derived from the work of Padgett et al. (31). The boldface letters represent sequences that are 100% conserved among all the splice sites. These potential splice sites are conserved in all the published (GenBank) HBV sequences. The *Eco*RI site is defined as nt 0 in the HBV genome.

Similar to the function of Rev-RRE interaction in HIV-1, this element appears to inhibit the splicing process in the context of a weak splicing signal, such as the one in HIV-1 (8). A stronger splicing signal such as the one existing in β-globin IVS2 can overcome such an inhibitory effect (8). By sequence analysis, at least one 5′ and six 3′ splice consensus sites exist within the HBV 2.1-kb RNA (Table 1). It is surprising that they are not utilized more frequently. The presence of this element likely inhibits the occurrence of splicing in order to provide functional mRNAs for expression of viral gene products, more specifically, the envelope proteins. We have preliminary data that spliced HBV RNAs were frequently observed in our HBV expression constructs without this element in proper position. A commonly used 5′ splice site which matches closely to the splice consensus sequence occurs at nucleotide position 460. Recent studies showed that this 5′ splice site was utilized by chimeric HBV-cellular gene transcripts in human hepatoma tissues with HBV integration (17, 48). In one example, the junction of HBV integration occurred at a site upstream of our genetic element, juxtaposing the 5′ HBsAg transcript with a cellular transcript. This integration led to the splicing of the 5′ splice site at nt 460 with a 3′ splice site of the cellular gene.

This element also functions to facilitate the transport and utilization of HBV transcripts. In contrast to the Rev-RRE interaction, our genetic and biochemical analyses suggest that this HBV element appears to be *cis* acting and does not require a *trans*-acting protein component. In addition, rather than actively promoting RNA transport as the Rev-RRE does, the HBV element may merely function to stabilize unspliced mRNA so they are exported to the cytoplasm by a default mechanism. Furthermore, introduction of frameshift mutations at nt 247, 537, 679, 1236, 1372, 1451, and 1641 in order to inactivate any other potential ORF in the EF construct did not affect the levels of HBV transcripts (data not shown). It is likely that this RNA element interacts with a cellular factor(s) in order to accomplish its function.

Computer-generated secondary structure of this HBV sequence predicts a complex stem-and-loop structure, which is characteristic of many RNA species that interact with RNA binding proteins. Evidence that complex interactions exist between RNAs and proteins in modulating a variety of cellular processes, including posttranscriptional regulation of mRNAs, has been accumulating (4, 29, 44). A recent report described a provocative finding that the 3′ untranslated region of several muscle-specific structural genes plays an important role in regulating growth and differentiation of muscle cells (35). This *trans* effect was thought to be mediated through the transcribed RNA sequences of this region.

Addition of an intron with intact splice sites, such as the SV40 tIVS, appeared to compensate partly for the lack of this element in HBsAg expression constructs. This observation is consistent with our data regarding the function(s) of this element as well as some of the known mechanisms of Rev-RRE interaction (8, 14, 28, 38). The presence of this element in a RNA species probably diverts the RNA from the splicing pathway and facilitates the transport of the RNA species from nucleus to cytoplasm. In contrast, the export of RNA species containing introns, like most mRNA transcripts in eukaryotic cells, is coupled efficiently to the splicing and polyadenylation pathway (18, 22). RNA species without either element, such as that transcribed from the EH construct, are retained in the nucleus and prevented from export into the cytoplasm for translation. This finding raises an interesting question regarding the presence of a negative *cis*-acting element(s) within the HBsAg coding region of the HBV transcripts. Studies on posttranscription regulation of HIV-1 demonstrated the existence of distinct RNA sequences in the *gag* and *env* regions of HIV-1 destabilizing and inhibiting the expression of these mRNAs in the absence of Rev (37, 38). These inhibitory effects were counteracted by the positive effect mediated by the Rev-RRE interaction. To define the possible existence of such a negative element within the HBsAg coding region, HBV sequence from nt 247 to 963 was placed behind the CAT gene of pSVCAT in either sense or antisense orientation. No difference in the CAT gene expression was observed with these constructs (data not shown). This observation suggests that such a negative element probably does not exist within the HBsAg transcripts. At present, it is unclear to us as to how the EH transcripts are retained in the nucleus.

In conclusion, we present evidence arguing that HBV contains a novel genetic element with properties similar to the Rev-RRE components of its distant relative, HIV-1. An analogous *cis*-acting element involved in splicing and nucleocytoplasmic transport of the influenza virus NS1 mRNA has also been reported (1). The presence of this element adds another level of complexity to the regulation of gene expression in HBV. It is reasonable to hypothesize that this regulatory sequence, not previously described in the genomic organization of hepadnaviruses, has an important function in the life cycle of HBV and other related viruses. By interacting with host cellular factors, this genetic element may offer the virus several advantages, including the ability to enter a persistent state of low or latent expression in the host.

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