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The Hepatitis B Virus Posttranscriptional Regulatory Element Is Composed of Two Subelements

JOHN E. DONELLO, ARLYNE A. BEECHE, GEORGE J. SMITH III, GINGER R. LUCERO,† AND THOMAS J. HOPE*

Infectious Disease Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037

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The RNAs of the hepatitis B virus (HBV) contain a *cis*-acting regulatory element which facilitates the cytoplasmic localization of unspliced transcripts (J. Huang and T. J. Liang, Mol. Cell. Biol. 13:7476–7486, 1993, and Z. M. Huang and T. S. Yen, J. Virol. 68:3193–3199, 1994). Such localization is presumed to be mediated by cellular factors which interact with the element. The HBV posttranscriptional regulatory element (HBVPRE) can efficiently activate an RNA export reporter system in an orientation-dependent and position-independent manner. Deletion analysis reveals that the HBVPRE consists of two subelements which function synergistically. A synergistic effect was also observed when the 5′ (PREα) or 3′ (PREβ) subelements were duplicated. The bipartite structure of the HBVPRE is reminiscent of reports that the high-affinity binding sites of the Rev-like proteins must be duplicated to function efficiently (M. Grone, E. Hoffmann, S. Berchtold, B. R. Cullen, and R. Grassmann, Virology 204:144–152, 1994; X. Huang, T. J. Hope, B. L. Bond, D. McDonald, K. Grahl, and T. G. Parslow, J. Virol. 65:2131–2134, 1991; and D. McDonald, T. J. Hope, and T. G. Parslow, J. Virol. 66:7232–7238, 1992).

The hepatitis B virus (HBV) is a small double-stranded DNA virus that replicates via reverse transcription. The 3.2-kb HBV genome contains four open reading frames which encode the polymerase, core, surface, and X proteins (9, 14, 28). The four HBV mRNAs originate from different promoters yet have a common 3′ end. The majority of HBV RNAs are exported from the nucleus unspliced (4, 12, 32).

Two recent studies have identified an orientation-dependent RNA element that is required for the efficient expression of the HBV surface protein. This *cis*-acting element posttranscriptionally facilitates the cytoplasmic localization of surface protein mRNA (17, 19). Since all HBV RNAs have a common polyadenylation site, the posttranscriptional regulatory element (HBVPRE) is at least partly contained within the 3′ end of all HBV mRNAs (Fig. 1). The current model suggests that the HBVPRE binds cellular factors which mediate export of unspliced RNAs (19). Although HBV and the complex retroviruses are phylogenetically distinct, the ability of the HBVPRE to direct the cytoplasmic localization of unspliced RNAs is functionally similar to those of the Rev-like proteins and their RNA targets (5, 14).

The Rev-like proteins of the complex retroviruses are required for the efficient cytoplasmic localization of incompletely spliced and unspliced viral RNAs. Rev-like proteins bypass the normal cellular requirement for the complete splicing of mRNAs by interacting with a specific viral RNA structure and directly exporting the RNA from the nucleus. Rev has two essential domains: (i) an RNA binding domain which specifically interacts with structural elements contained within the viral mRNAs and (ii) an effector domain which interacts with an endogenous nuclear export pathway (15, 26, 34). It has recently been demonstrated that the effector domain of human immunodeficiency virus type 1 (HIV-1) Rev acts as a nuclear export signal (7).

An additional posttranscriptional regulatory element has recently been identified within the type D retroviruses (3, 37). Bray et al. (3) identified a 219-base RNA element that mediates nuclear export of unspliced Mason-Pfizer monkey virus mRNAs. Similar to the HBVPRE, the Mason-Pfizer monkey virus constitutive transport element is a *cis*-RNA element that associates with cellular proteins which export the unspliced RNA.

Previous studies reported that partial 3′ deletions of the HBVPRE diminished but did not completely abolish the cytoplasmic localization of the surface protein mRNA, suggesting that the PRE may be structurally complex. We undertook this study to further characterize the primary structure of the PRE and to identify the minimal regions of the PRE that facilitate the cytoplasmic localization of unspliced RNAs. Elucidation of the minimal PRE regions will aid the identification of the cellular protein(s) binding sites and may reveal constraints within which the cellular proteins associate with the HBVPRE.

MATERIALS AND METHODS

Plasmids. The chloramphenicol acetyltransferase (CAT) reporter plasmids pDM138, p138RRE, and p138XRE have been previously reported (15, 18). The HBV fragments tested in this study were amplified by 25 to 30 cycles of PCR with an HBV clone (ATCC 31518) as a template. Ten percent of the PCR product was gel isolated, purified by GeneClean (Bio 101), digested with the appropriate restriction enzymes, ethanol precipitated, and ligated into the pDM138 vector. The PCR-generated HBV fragments were inserted into a unique *Cla*I site located within the intron of pDM138, 3′ of the CAT reporter gene. For the experiments shown in Fig. 2D, the fragments were inserted into the unique *Bgl*II within the 3′ exon of pDM138. All fragments were sequenced after insertion into the pDM138 vector. The PCR primers contained a GCG clamp for efficient restriction enzyme digestion and either *Cla*I and *Bam*HI or *Bgl*II restriction sites. The following PCR primers were used to generate the fragments utilized in this study (HBV sequences are lowercase): 5′ primer at position 963 (CGCAGATC TATCGATaaacaggcctattgattg); 5′ primer at 1051 (CGCGGATCCATCGATtta atgcctttatatgcatg); 5′ primer at 1151 (CGCGGATCCATCGATttgctcggcaacggc ctg); 5′ primer at 1215 (CGCGGATCCATCGATggcttggccataggccat); 5′ primer at 1352 (CGCGGATCCATCGATtcccgcaagtatacatcg); 3′ primer at 1684 (CGCA

* Corresponding author. Mailing address: Infectious Disease Laboratory, Salk Institute, P.O. Box 85800, San Diego, CA 92186-5800. Phone: (619) 453-4100, ext. 1559. Fax: (619) 554-0341. Electronic mail address: hope@qm.salk.edu.

† Present address: Grinnell College, Grinnell, IA 50112.

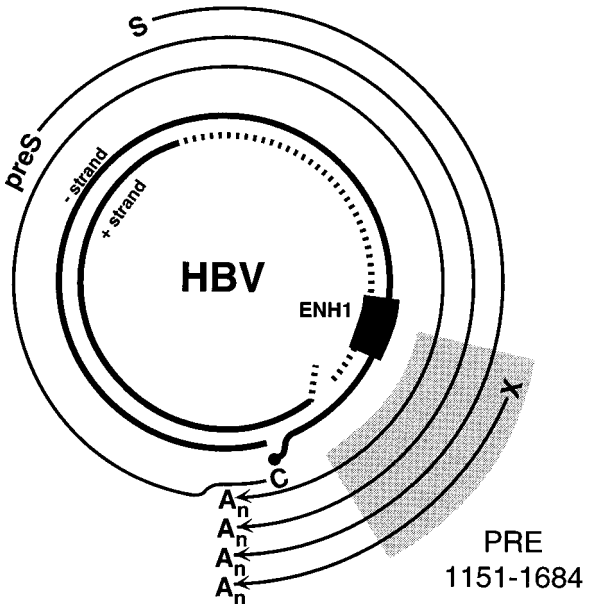

FIG. 1. Schematic map of the HBV genome. The negative and discontinuous positive strands of HBV relaxed DNA are shown in the center circle (bold). The tissue-specific enhancer I (ENH1) is shown as a filled rectangle. The four classes of HBV RNAs (3.4, 2.4, 2.1, and 0.8 kb) are represented by curved arrows. These mRNAs encode core (C), presurface (preS), surface (S), and protein X (X), respectively. The HBV mRNAs all have a common polyadenylation site (A_n). The shaded region within these RNAs indicates the position of the minimal HBVPRE, which is at least partially included in all of the HBV RNAs.

GATCTATCGATgacattgctgaaagtcca); and 3′ primer at 1413 (CGCGGATCC ATCGATaggatccagttggcagca). An additional 1352-to-1684 derivative was generated with an alternative 5′ primer at position 1352 (CGCGGATCCGGCG CCtcccgcaagtatacatcg) that contained a *Nar*I site instead of a *Cla*I. This fragment was inserted into the *Cla*I site of pDM138 to generate a derivative of $p138HBV_{(1352–1684)}$. This derivative was the precursor of $p138PRE_{(switch)}$, $p138PRE_{(switch:anti)}$, and p138PREβ:α. The pCH110, pUC118, pRSV-Rev, pRSV-Rex, and pRSV (Rous sarcoma virus promoter) plasmids have been described elsewhere (16).

Cell culture and transfection. CV-1 and 293T cells were grown at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 293T cells are a derivative of the human kidney fibroblast line 293 and stably express the simian virus 40 large T antigen. To transfect, a confluent plate of cells was split 1:8 approximately 24 h before transfection. A total of 10 μg of plasmid DNA was diluted in 220 μl of 0.1× Tris-EDTA and mixed with 250 μl of 2× Hanks balanced salt solution. While vortexing, 31 μl of 2 M $CaCL_2$ was added dropwise. CV-1 cells were transfected by applying the $CaPO_4$-DNA mixture directly to the naked cells. 293T cells were transfected by adding the $CaPO_4$-DNA mixture to the medium. Fresh medium was added after 24 h. Approximately 48 h after transfection, the cells were harvested with calcium- and magnesium-free 5 mM EDTA phosphate-buffered saline.

CAT assay. CAT assays were performed as previously described (16). Cell lysates were prepared and normalized to β-galactosidase activity. CAT activity was quantitated by thin-layer chromatography. Each construct was transfected in duplicate or triplicate (as indicated in the figure legends) within a single experiment. To calculate the mean percent acetylation, the gross percent conversion of each point was first determined. The gross mean of the repeated points was then calculated, from which the gross mean percent acetylation of the sham transfection was subtracted. The standard error of the mean was calculated by dividing the standard deviation of a datum set by the square root of the number of samples within the datum set. Each experiment was repeated at least three times, and the results are representative of the repeated experiments.

RNA analysis. Cytoplasmic and total RNA was extracted from 293T cells and subjected to Northern (RNA) analysis as previously described (16). Lysis buffer was modified to contain 0.05% Nonidet P-40. Total RNA was prepared with an RNeasy kit (Qiagen). Derivatives of the reporter plasmids containing the cytomegalovirus immediate-early promoter were used to facilitate detection of RNA. Cytoplasmic RNA (10 μg) was electrophoresed on a 0.8% agarose–formaldehyde gel, blotted, and hybridized for 18 h at 42°C. RNA was identified with an *Xba-Bam*HI probe derived from pDM138. This probe primarily hybridizes with unspliced pDM138 RNA. The probe was labeled with [α-^{32}P]dCTP by random priming. The hybridization mixture contained 50% formamide.

Numbering of sequences. All sequence numbering presented in this report is based on the following GenBank sequences: for HBV, D00329; for HIV-1, K03455; and for human T-cell leukemia virus (HTLV), J02029.

RESULTS

To further characterize the HBVPRE, we chose to employ a versatile and well-characterized reporter system that has previously been utilized to study the RNA export elements of the complex retroviruses (2, 16, 25, 30, 36). Briefly, the pDM138 reporter is derived from the second intron of HIV-1, into which the CAT gene was inserted to occupy the position of the envelope (Fig. 2A) (10). When the pDM138 reporter is transiently transfected, RNAs transcribed from the reporter are either spliced, which removes the CAT coding region, or exported from the nucleus unspliced. The appearance of unspliced RNAs in the cytoplasm is dependent upon the presence of an RNA export element (16, 18). When the unspliced RNAs are exported from the nucleus, CAT is translated and can be accurately quantitated.

To avoid any transcription effects of the liver-specific HBV enhancer I, which is within the region of the HBVPRE, the HBVPRE constructs were analyzed in fibroblast cell lines.

The HBVPRE can efficiently localize unspliced reporter RNAs to the cytoplasm. The HBVPRE fragment 963 to 1684, originally identified by Huang and Liang (17), was inserted into pDM138 in the sense and antisense orientations. These two derivatives, designated p138HBVPRE and $p138HBVPRE_{(anti)}$, were transiently transfected into CV-1 cells. To determine the strength of the HBVPRE, constructs containing the HIV-1 Rev-responsive element (RRE) and HTLV-1 Rex-responsive element (XRE) were cotransfected with their respective transactivators (1). The results illustrate that the HBVPRE increases levels of CAT activity, independent of any viral proteins, in an orientation-specific manner (Fig. 2B). Compared with Rev-RRE or Rex-XRE, the HBVPRE is 15 to 30% as efficient at facilitating the cytoplasmic localization of unspliced RNA.

To verify that the increase in CAT activity levels was due to the appearance of unspliced RNA within the cytoplasm, 293T cells were transiently transfected with p138RRE, p138RRE+ Rev, p138HBVPRE, or $p138HBVPRE_{(anti)}$. Cytoplasmic and total RNAs were isolated from the transfected cells and subjected to Northern analysis. In addition, these cells were also assayed for CAT activity, which verified that the HBVPRE was functional and orientation dependent in 293T cells (data not shown). The Northern analysis results, illustrated in Fig. 2C, confirm that the observed induction of CAT activity is the result of the element-dependent cytoplasmic localization of unspliced mRNAs. The controls p138RRE and $p138HBVPRE_{(anti)}$ did not have significant amounts of spliced or unspliced RNA within the cytoplasmic or total RNA fractions, suggesting that these RNAs were degraded.

HBVPRE can function within the intron or exon of unspliced RNA. It has been previously demonstrated that the complex retrovirus response elements are functional within an exon or intron of unspliced RNA (24). To determine whether the HBVPRE is also position independent, the PRE was removed from the intron and inserted into a *Bgl*II site located within the 3′ exon of pDM138. The $p138HBVPRE_{(BglII)}$ and $p138HBVPRE_{(BglII:anti)}$ (the sense and antisense derivatives, respectively) were transiently transfected into CV-1 cells. As controls, pDM138, p138HBVPRE, and $p138HBVPRE_{(anti)}$ were also included in this experiment. The results, shown in Fig. 2D, indicate that the HBVPRE can function within the introns or exons of unspliced RNAs. Compared with the

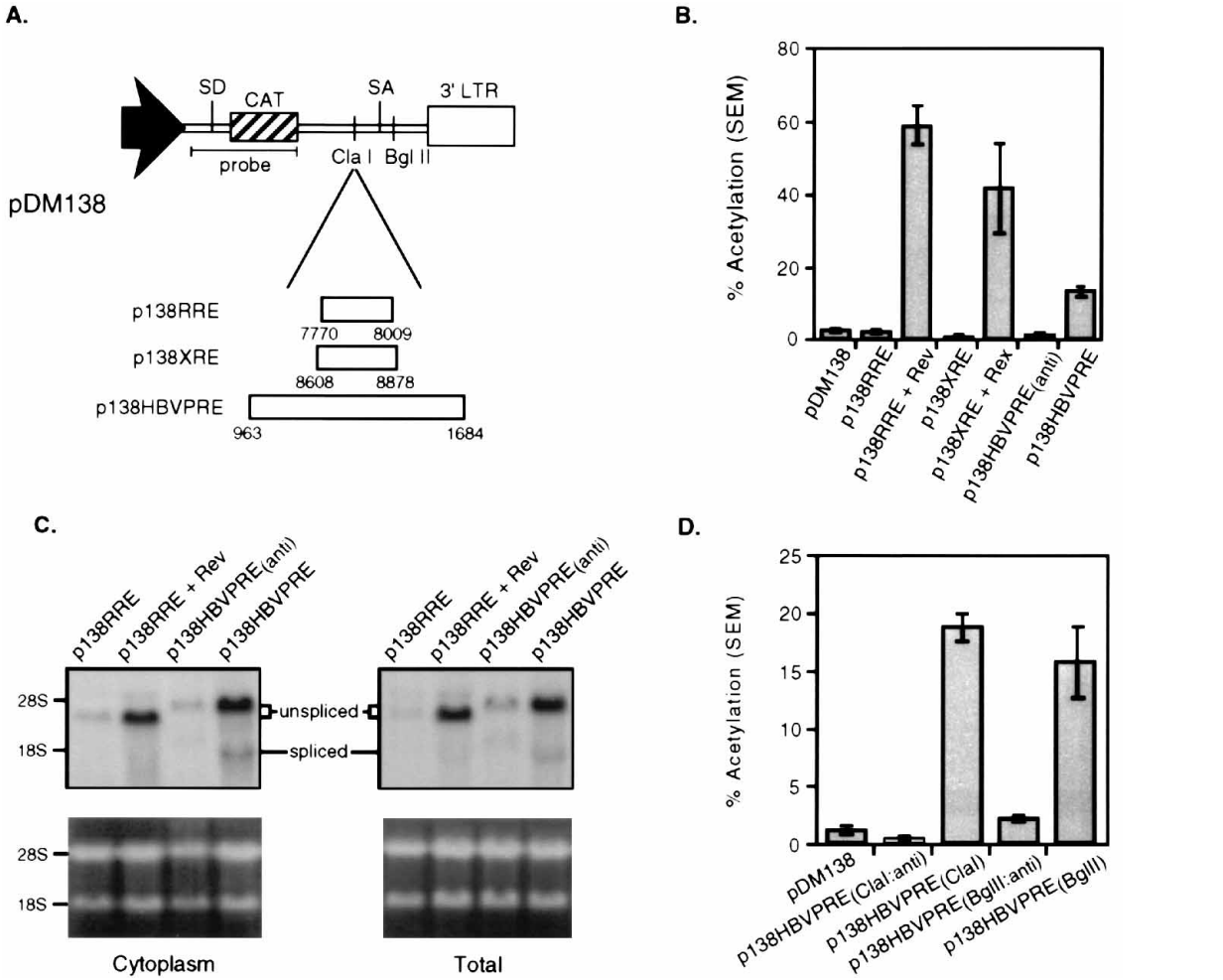

FIG. 2. The HBVPRE facilitates cytoplasmic localization of unspliced RNA in an orientation-dependent and position-independent manner. (A) Schematic representations of the pDM138 reporter and the constructs utilized in Fig. 2B to D. The pDM138 reporter is derived from the second intron of HIV-1. The filled arrow represents the early simian virus 40 promoter (or the immediate-early cytomegalovirus promoter [Fig. 2C]) from which the RNAs are transcribed. The CAT gene (hatched box), which is efficiently expressed only when unspliced RNA is exported, is located within the intron. The unique *Cla*I and *Bgl*II restriction sites are indicated. SD, splice donor; SA, splice acceptor; 3′ LTR, 3′ long terminal repeat. The RRE, the XRE, and the HBVPRE were inserted into the pDM138 *Cla*I site. The GenBank nucleotide numbers are labeled underneath each construct. (B) Comparison of the cytoplasmic localization activities among Rev-RRE, Rex-XRE, and HBVPRE. CV-1 cells were transiently transfected with 2 μg of reporter DNA, 2 μg of transactivator DNA (pRSV-Rev, pRSV-Rex, or pRSV), 0.5 μg of pCH110 (an internal β-galactosidase control), and 5.5 μg of pUC118. For each construct, three individual plates were transfected and assayed for CAT activity 36 to 48 h later. The shaded bars represent percent acetylation. SEM, standard error of the mean. (C) Northern analysis of cytoplasmic and total RNAs, which are labeled. The locations of the 28S and 18S ribosomal RNAs are also indicated. In the top panels, 293T cells were transiently transfected, in triplicate, with 10 μg of DNA of the immediate-early cytomegalovirus promoter-driven p138RRE or p138HBVPRE constructs. 293T cells were utilized to increase the amounts of RNA obtained from the preparations. CAT assay results from transfected 293T cells indicated that there are no cell-type-specific effects (data not shown). The p138RRE derivative was cotransfected with pRSV-Rev or pRSV. The RNA was electrophoresed, blotted, and probed with a random-primed P32 labeled *Xba*I-*Bam*HI fragment of pDM138 (depicted in Fig. 2A) which primarily hybridizes with unspliced transcripts. In the bottom panels, ethidium bromide staining of 28S and 18S ribosomal RNAs is shown. (D) The HBVPRE is functional within an intron or exon. CV-1 cells were transfected with 2 μg of the reporter DNA, 0.5 μg of pCH110, and 7.5 μg of pUC118. Each construct was transfected in triplicate. The transfected cells were assayed for CAT activity 48 h after transfection. Shaded bars represent the mean percent acetylation and are labeled with the appropriate construct. ClaI, HBVPRE placed within the intron of pDM138; BglII, HBVPRE placed within the 3′ exon of pDM138; (anti), antisense orientation of the HBVPRE.

pDM138 control, the antisense derivatives did not have an appreciable level of CAT activity.

Deletion analysis of the HBVPRE. Huang and Liang initially defined the HBVPRE 3′ boundary by different truncations of the 3′ end of the HBV genome (17). This study reported an inverse relationship between the size of the 3′ truncation and HBVPRE-dependent surface protein expression. A truncation from the *Eco*NI site (1641) to the 3′ end of the HBV genome had no effect upon HBVPRE activity. A series of truncations from the *Rsr*I site (1572) to a *Nco*I site (1372) significantly decreased the level of expression of the HBV surface protein to approximately 50% that of the wild type. Further deletion to the *Sph*I site (1236) reduced HBVPRE activity to baseline levels (17).

To define the HBVPRE 5′ boundary, we generated a series of 5′ deletions which complement the 3′ deletions described above. HBV fragments 1051 to 1684, 1151 to 1684, 1215 to 1684, and 1352 to 1684 were PCR amplified and cloned into the pDM138 reporter. As illustrated in Fig. 3, CV-1 cells transfected with construct $p138_{(1051–1684)}$ exhibited a level of CAT activity equal to that of p138HBVPRE, while cells transfected with $p138_{(1151–1684)}$ exhibited a slightly greater level of activity. Cells transfected with $p138_{(1215–1684)}$ and $p138_{(1352–1684)}$ exhibited a stepwise decrease in CAT activity levels. The observed

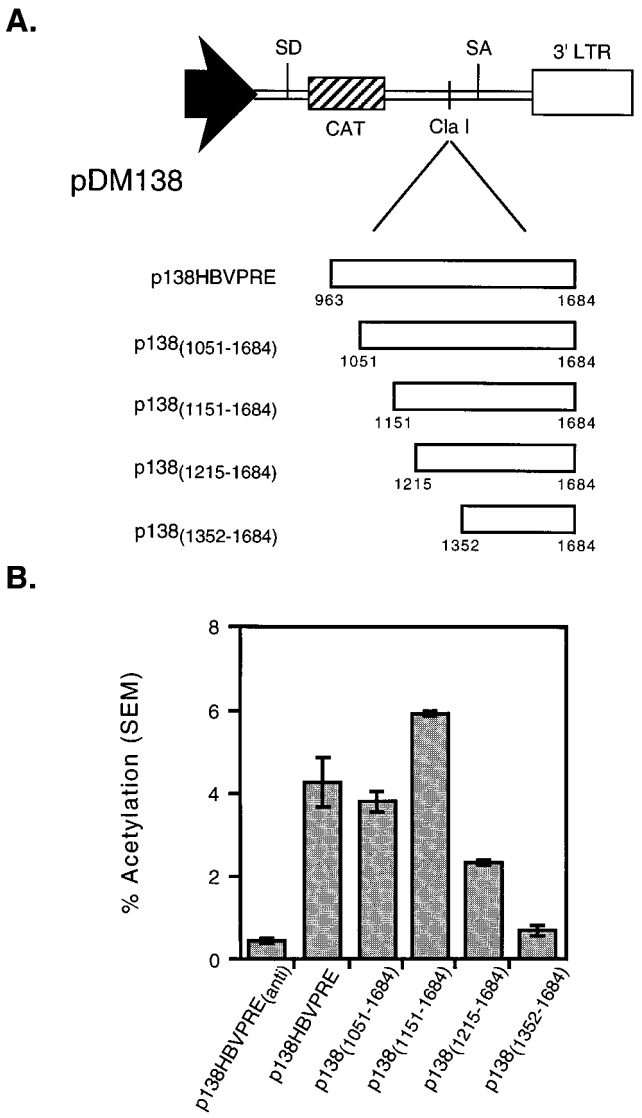

FIG. 3. Deletion analysis of the HBVPRE. (A) Schematic representations of the 5′ deletions of the HBVPRE. A series of HBVPRE 5′ deletions were generated by PCR and inserted into the *Cla*I site of pDM138. The resulting constructs are labeled accordingly. (B) The minimal HBVPRE is contained within nucleotides 1151 to 1684. CV-1 cells were transiently transfected, in duplicate, with the derivatives described above and assayed for CAT activity 48 h later. The shaded bars represent the mean percent acetylation of the duplicate transfected plates. CV-1 cells were transfected with 2 μg of reporter DNA, 0.5 μg of pCH110, and 7.5 μg of pUC118. Abbreviations are as described in the legend to Fig. 2.

induction of CAT activity was orientation dependent (data not shown). These results indicate that the functional HBVPRE is contained within nucleotides 1151 to 1684.

The HBVPRE is a bipartite structure. The results of the 5′ and 3′ HBVPRE deletions suggest that the HBVPRE is structurally complex, possibly consisting of two or more subelements. To define the possible HBVPRE subelements, the HBV fragments encompassing nucleotides 963 to 1412, 1151 to 1413, and 1412 to 1684 were PCR amplified and inserted into pDM138. To test the possibility that the putative subelements are interchangeable, a fragment encompassing nucleotides 963 to 1413 was inserted, in the sense and antisense orientations, 3′ of the 1352-to-1684 fragment. These constructs were transiently transfected into CV-1 cells. The results, shown in Fig. 4, indicate that the 5′ subelement deletions, $p138_{(963–1413)}$ and $p138_{(1151–1413)}$, are approximately 33 and 28% as active as p138HBVPRE. The 3′ subelement deletions, $p138_{(1352–1684)}$ and $p138_{(1412–1684)}$, exhibited activity levels 24 and 26% that of p138HBVPRE. The construct within which the relative orders of the 5′ and 3′ subelements were inverted, $p138PRE_{(switch)}$, displayed a level of activity approximately 55% that of p138HBVPRE. The controls, pDM138, $p138HBVPRE_{(anti)}$, and $p138_{(switch:anti)}$, displayed background activities approximately 11, 5, and 10%, respectively, that of p138HBVPRE. These results confirm that the 5′ subelement is encompassed by nucleotides 1151 to 1413 and that the 3′ subelement is

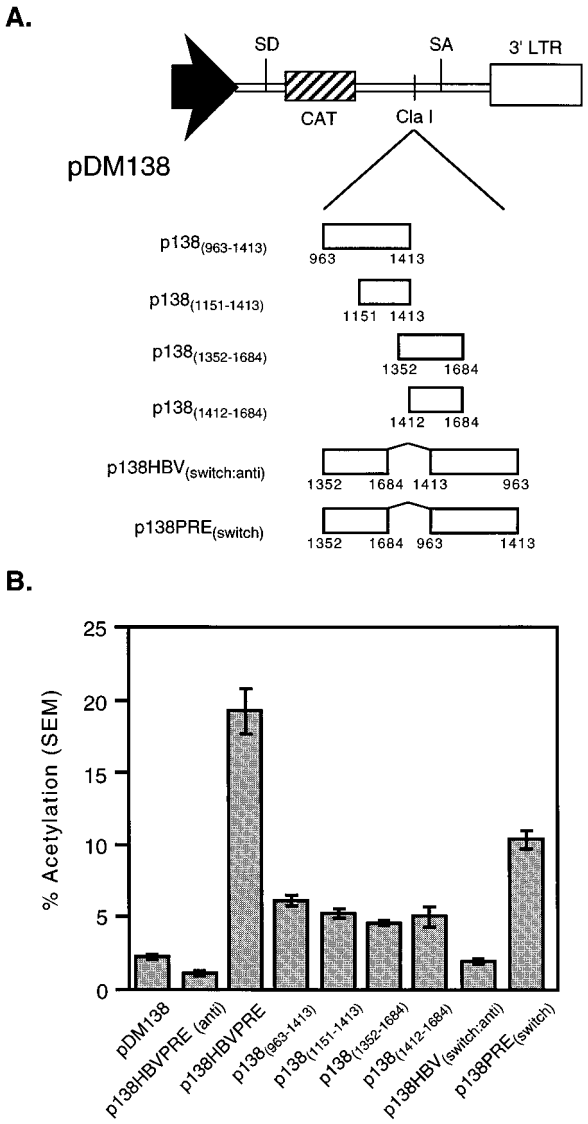

FIG. 4. The HBVPRE has a bipartite structure. (A) Schematic representations of $p138_{(963–1413)}$, $p138_{(1151–1413)}$, $p138_{(1352–1684)}$, $p138_{(1412–1684)}$, $p138PRE_{(switch)}$, and $p138PRE_{(switch:anti)}$. The nucleotide numbers, shown below the diagrammed fragments, are relative to the HBV sequence. (B) The HBVPRE subelements can function independently or synergistically. CV-1 cells were transiently transfected, in triplicate, with the derivatives described above and assayed for CAT activity 48 h later. The shaded bars represent the mean percent acetylation, calculated from the triplicates. CV-1 cells were transfected with 2 μg of reporter DNA, 0.75 μg of pCH110, and 7.25 μg of pUC118. Abbreviations are as described in the legend to Fig. 2.

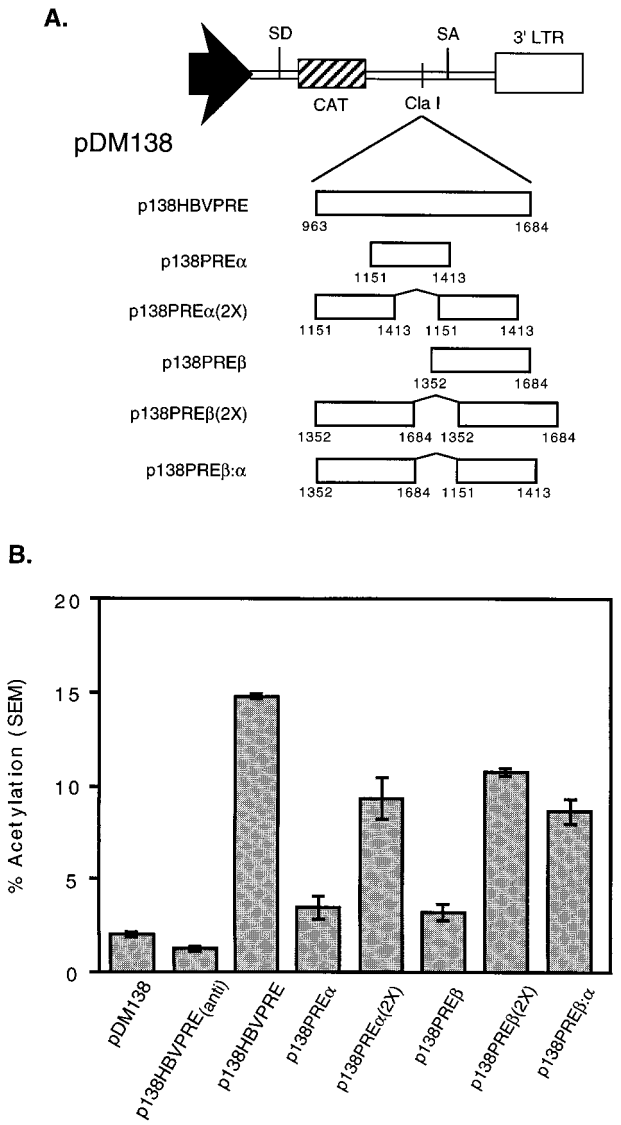

FIG. 5. Synergistic activities of PREα and PREβ. (A) Schematic representations of p138PREα, p138PREα(2X), p138PREβ, p138PREβ(2X), and p138PREβ:α. The derivatives are labeled accordingly. The nucleotide numbers, shown below the diagrammed fragments, are relative to the HBV sequence. (B) PREα and PREβ function synergistically. CV-1 cells were transiently transfected, in triplicate, with the derivatives described above and assayed for CAT activity 48 h later. The shaded bars represent the mean percent acetylation, calculated from the triplicates. CV-1 cells were transfected with 2 μg of reporter DNA, 0.75 μg of pCH110, and 7.25 μg of pUC118. Abbreviations are as described in the legend to Fig. 2.

located within nucleotides 1412 to 1684. We have designated the 5′ and 3′ subelements PREα and PREβ, respectively. The results also suggest that the 1352-to-1684 and 963-to-1413 fragments function synergistically even if their normal positions are inverted.

Duplication of PREα or PREβ restores near-wild-type activity. To further examine the nature of the PREα and PREβ subelements, the 1151-to-1413 and 1352-to-1684 HBV fragments were duplicated and inserted into pDM138. We also generated a derivative in which the minimal PREα (nucleotides 1151 to 1413) was inserted 3′ of PREβ (nucleotides 1352 to 1684). These constructs were transiently transfected into CV-1 cells, which were subsequently assayed for CAT activity. The results, shown in Fig. 5, suggest that duplication of PREα or PREβ results in an element that is more than twice as active as a single subelement. In this assay, p138HBVPRE displayed a 14.8% mean acetylation. The background activity levels of the controls, p138HBVPRE$_{(anti)}$ and pDM138, were 8 and 13.4% that of p138HBVPRE. The mean values of p138PREα and p138PREβ were 22 and 24% that of p138HBVPRE. Duplication of either PREα [p138PREα(2X)] or PREβ [p138PREβ (2X)] yielded elements that were 63 and 73% as active as the full-length HBVPRE. Finally, p138PREβ:α displayed an activity level that was 59% that of p138HBVPRE.

DISCUSSION

The splicing and transport of mRNAs are critical checkpoints for eukaryotic gene regulation, ensuring that only correctly processed mRNAs will be translated. The HBVPRE enables surface protein mRNAs to bypass the cellular mRNA splicing machinery, presumably by directly mediating export of the unspliced RNA (17, 19, 20). To identify the minimal functional HBVPRE and the putative RNA binding sites, we constructed a number of HBVPRE derivatives and assayed these derivatives for their abilities to localize unspliced RNA to the cytoplasm.

The exact mechanism by which the HBVPRE facilitates the cytoplasmic localization of unspliced RNAs remains unknown. Our data are consistent with previous reports that the HBVPRE mediates nucleocytoplasmic export of unspliced HBV RNAs, although we have not ruled out a possible role for the HBVPRE in RNA stability (17, 19, 20). There is a slight increase in the amount of spliced p138HBVPRE RNA that is not present in the p138HBVPRE$_{(anti)}$ control (Fig. 2C). This difference may be the result of the HBVPRE stabilizing unspliced RNAs, which are then spliced before they are exported. It has previously been reported that Rev will also stabilize RNAs, indicating that export and stability of RNAs may be tightly associated (6, 23). Although there was no observed difference between the cytoplasmic and total RNAs, it is now well established that Rev directly exports unspliced RNAs (7, 8). This indicates that the contribution of nuclear RNA to the total RNA was minimal. The similar patterns of cytoplasmic and total RNAs of the Rev-RRE and HBVPRE systems suggest that they are functionally similar.

The data suggest that the HBVPRE is a bipartite RNA structure encompassed by nucleotides 1151 to 1684. We have mapped a 5′ (PREα) subelement to nucleotides 1151 to 1413 and a 3′ (PREβ) subelement to nucleotides 1412 to 1684. As shown in Fig. 5, a single copy of PREα or PREβ functions inefficiently and is only 22 to 24% as active as the complete HBVPRE. If two copies of PREα or PREβ are present, the cytoplasmic localization of unspliced RNA is more efficient, 63 or 73%, respectively, of HBVPRE activity. When the order of the PREα and PREβ subelements is reversed, the resulting element was 59% as active as the HBVPRE.

These results indicate that the cellular factor(s) which interacts with PREα and PREβ functions synergistically to mediate export via the HBVPRE. If the most conservative background activity level of the control, p138HBVPRE$_{(anti)}$, is subtracted, the data suggest that the PREα- and PREβ-associated proteins can function synergistically when the order of PREα and PREβ is reversed. Synergy is also observed when PREα and PREβ are duplicated, confirming that the synergistic interaction is dependent upon the presence of two subelements. Although not large, the synergistic effect was observed in all of the experiments.

None of the HBVPRE derivatives exhibit wild-type

HBVPRE activity. Two possible explanations for this observation exist. First, the HBVPRE may contain an uncharacterized third RNA binding site which contributes to the efficiency of RNA export. Alternatively, PREα and PREβ may be optimally presented within the HBVPRE, which would enable PREα and PREβ to function most efficiently. We favor the latter model, since smaller 5′ or 3′ HBVPRE deletions do not exhibit an intermediate level of activity.

Although the nature of the HBVPRE is not completely understood, our data support a model in which PREα and PREβ represent RNA binding sites for cellular RNA export factors. It is not known whether PREα and PREβ are recognized by a single cellular protein or if different proteins associate with each subelement. The functional equivalence and synergistic activities of PREα and PREβ support the notion that the transactivating factor is a single cellular protein. However, sequence analysis of PREα and PREβ does not reveal any obvious sequence similarities and preliminary computer models of the subelements' secondary structures reveal no striking secondary structure similarities (data not shown). These results strengthen the possibility that more than one cellular protein mediates HBVPRE function.

The HBVPRE bipartite structure is reminiscent of results from studies of the minimal RNA binding sites of HIV-1 Rev and HTLV-1 Rex. These minimal RNA binding sites have been identified in vitro yet do not function efficiently in vivo (13). We have previously shown that two copies of the minimal Rev RNA binding site restore Rev function to near wild-type levels (18). Similar results have recently been published for the minimal HTLV-1 Rex binding site (11). In this artificial context, both Rev and Rex require a duplication of their minimal binding sites to efficiently export unspliced RNA. We have obtained similar results using fusion proteins consisting of the bacteriophage MS2 RNA-binding protein and HIV-1 Rev or HTLV-1 Rex. To be fully functional, these chimeric proteins require a duplication of the MS2 binding site (27). The HBVPRE is the first natural example within which two separate RNA binding sites function synergistically to export RNA.

Although the HBVPRE does not direct export of unspliced reporter RNAs as efficiently as Rev-RRE or Rex-XRE, the functional similarities of the HBVPRE and the HIV-1 RRE-Rev complex suggest that the HBVPRE-associated cellular proteins may be functionally similar to Rev. Recent reports suggest that Rev directly exports RNA bearing the RRE. In oocytes, Rev directs export of a spliced lariat structure which contains the RRE (8). Furthermore, studies suggest that Rev utilizes an export pathway normally used by ribosomal 5S RNA and the small nuclear RNAs (7). Finally, the effector domain of Rev has been demonstrated to be a nuclear export signal (7, 35). The functional similarities between Rev-RRE and HBVPRE suggest that the HBVPRE-associated proteins will be similar to Rev.

cis-acting posttranscriptional regulatory elements are being found in a growing number of virus families. A recent report indicates that the human papillomavirus contains such an element (32). Likewise, a specific RNA export signal has been found in the intronless thymidine kinase gene of the human herpes simplex virus (22). The presence of such elements suggests that intronless mRNAs require a mechanism by which they can bypass the cellular mRNA splicing pathway (23).

The exact role of the HBVPRE in the biology of HBV has yet to be elucidated. A recent report demonstrated that the HBVPRE can functionally replace an efficiently spliced intron (20). It is well known that the presence of a functional intron enhances cDNA expression in stable cell lines and transgenic animals (21, 29). Interestingly, the 3′ end of HBV has previously been used to enhance transgenic mice cDNA expression (31). The PREα and PREβ subelements may enhance the expression of HBV proteins by directly mediating the efficient export of unspliced mRNAs. Before the potential regulatory roles of PREα and PREβ can be elucidated, the subelements' functions must be better characterized.

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