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Woodchuck Hepatitis Virus Contains a Tripartite Posttranscriptional Regulatory Element

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The hepatitis B virus posttranscriptional regulatory element (HBVPRE) is a *cis*-acting RNA element that partially overlaps with enhancer I and is required for the cytoplasmic accumulation of HBV surface RNAs. We find that the closely related woodchuck hepatitis virus (WHV), which has been shown to lack a functional enhancer I, also contains a posttranscriptional regulatory element (WPRE). Deletion analysis suggests that the WPRE consists of three independent subelements. Comparison of the bipartite HBVPRE and tripartite WPRE activities reveals that the tripartite WPRE is two to three times more active than the bipartite HBVPRE. Mutation of a single WPRE subelement decreases WPRE activity to the level of the HBVPRE. Bipartite and tripartite chimeras of the WPRE and HBVPRE possess activities which suggest that elements containing three subelements are posttranscriptionally stronger than those containing two. These data demonstrate that the posttranscriptional regulatory element is conserved within the mammalian hepadnaviruses and that its strength is determined by the number of subelements within the RNA.

The *Hepadnaviridae* family consists of closely related yet species-specific DNA viruses which replicate via reverse transcription (8, 19). Studies of human hepatitis B virus (HBV) and woodchuck hepatitis virus (WHV) have shown that both viruses are mainly hepatotropic and contain four open reading frames that encode the major viral proteins: core, polymerase, surface, and X. The two viruses show approximately 59% nucleotide identity and have similar physical maps (7). Although spliced HBV RNAs have been reported, the major HBV and WHV proteins are translated from unspliced RNAs (28). The viral RNAs terminate at the same polyadenylation site and have a common 3′ terminus (Fig. 1A).

The correlation of HBV infection with an increased risk of hepatocellular carcinoma has stimulated investigation of the virus-host interactions and gene regulation of *Hepadnaviridae*. Transcription of the major viral proteins is mediated by four promoters which are partially regulated by HBV enhancers I and II. HBV enhancers I and II have been shown to upregulate heterologous promoters and are believed to be key determinants of HBV hepatotropism (10, 27). Both enhancers are liver specific, although enhancer I retains lower activity levels in some nonhepatic cells (25, 33, 35). HBV enhancer I maps upstream of the X open reading frame and consists of a modulatory domain, a core enhancer domain, and a basal X promoter domain (3, 30); enhancer II maps to the core promoter region and is thought to influence levels of genomic RNA (34).

The transcriptional regulatory elements of WHV are not as thoroughly characterized. Mapping studies have confirmed that WHV contains promoters analogous to the major HBV promoters (2, 29). Recent studies have shown that WHV enhancer II is a strongly liver-specific enhancer that regulates the production of pregenomic RNAs, which is an important rate-limiting step of hepadnavirus replication (6, 32). Surprisingly, the WHV region homologous to HBV enhancer I lacks enhancer activity in the three human liver cell lines tested (2, 6, 31). This region failed to activate transcription of the four viral promoters as well as a heterologous thymidine kinase (*tk*) promoter. The authors suggest that either the human liver cells do not express the required transcription factors or major differences exist in the transcriptional control of HBV and WHV (2).

The HBV posttranscriptional regulatory element (HBVPRE) is an orientation-dependent *cis*-acting RNA element that partially overlaps with enhancer I and is required for the cytoplasmic localization of HBV surface RNAs (Fig. 1B) (13, 15). The HBVPRE function is independent of virally encoded proteins, and it is believed that cellular proteins interact with the HBVPRE and mediate the HBVPRE posttranscriptional effect. The HBVPRE can functionally substitute for the human immunodeficiency virus type 1 (HIV-1) Rev–Rev-responsive element (RRE) complex in a transient transfection reporter assay (13, 16). In addition, the HBVPRE can increase the amount of intronless cytoplasmic RNAs of a normally intron-dependent β-globin cDNA, consistent with the notion that the HBVPRE functionally replaces an intron during RNA processing (16). Deletion analysis suggests that the HBVPRE consists of two independent subelements within nucleotides 1151 to 1684 (4). These subelements, termed HBVPREα (HPREα) and HBVPREβ (HPREβ), are encompassed by nucleotides 1151 to 1412 and 1413 to 1684, respectively. A single HBVPRE subelement displays a low level of posttranscriptional activity, and both subelements function cooperatively when duplicated. In addition, the order of HPREα and HPREβ can be switched, suggesting that the subelements are modular (4). The subelements most likely represent distinct binding sites for cellular RNA binding proteins.

A number of reports have suggested that the posttranscriptional mechanism of the HBVPRE may be RNA export (4, 13, 16). The first-identified and best-characterized viral export system is the HIV-1 Rev-RRE complex. HIV-1 Rev has been shown to directly mediate RNA export via its nuclear export signal (5). The Mason-Pfizer monkey virus (MPMV) encodes a *cis*-acting RNA export element, termed the constitutive transport element (CTE), which is required for the export of the intron-containing genomic RNA (1). An additional element

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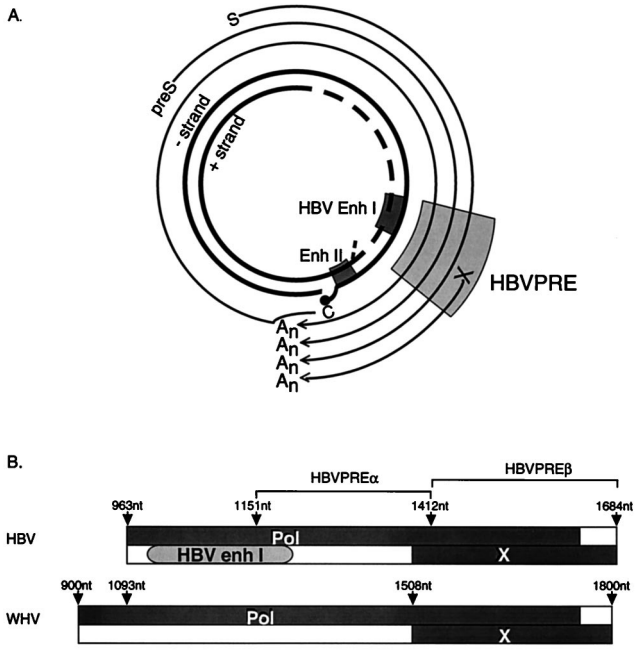

FIG. 1. Schematic representation of the HBV and WHV genomes. (A) The negative and discontinuous positive strands of HBV relaxed DNA are shown in the center circle (bold). The HBV liver-specific enhancer (Enh) I and the WHV and HBV liver-specific enhancer II are shown as rectangles. The four classes of hepadnavirus RNAs are represented by the curved arrows. The RNAs encode core (C), presurface (preS), surface (S), and X proteins. The shaded region within these RNAs indicates the position of the HPRE. (B) Comparison of the PRE and enhancer I regions of HBV and WHV. The darkened regions correspond to the open reading frames of the polymerase (Pol) and X proteins. The regions containing the HPREα and HBVPREβ subelements are indicated. Homologous nucleotides (nt) are aligned, and the fragments are drawn to scale. The HBV enhancer (enh) is indicated.

has been found in the intronless *tk* gene of herpes simplex virus type 1 (18). Liu and Mertz reported that hnRNP L binds to a site within the *tk* gene and, using mutants of the *tk* gene, showed a correlation between hnRNP L binding and cytoplasmic RNA accumulation. All of these *cis*-acting elements are essential for the cytoplasmic localization of viral RNA and, with the exception of the complex retrovirus elements, are thought to interact with cellular RNA binding proteins.

A posttranscriptional regulatory element (PRE) has not yet been identified in WHV. The similarities between WHV and HBV suggest that a PRE is present, yet the lack of a WHV enhancer I raises the possibility that functional differences exist in this region. To address whether the role of the PRE is conserved between HBV and WHV, we sought to map the putative WHV PRE (WPRE) and determine whether the gross structure is conserved between WPRE and HBVPRE. We find that the WPRE consists of three subelements, termed WPREα, WPREβ, and WPREγ. The PREα and PREβ subelements are homologous between the two viruses, while the WPREγ region corresponds to the region containing the HBV enhancer I. The tripartite WPRE displays significantly stronger activity than the bipartite HBVPRE, demonstrating that the strength of the posttranscriptional effect is determined by the number of subelements in the RNA.

MATERIALS AND METHODS

Construction of reporter plasmids. The cytomegalovirus (CMV) surface expression construct was synthesized by amplifying nucleotides 135 to 1685 from GenBank accession no. D00329. The amplified fragment was digested with *Sac*I and *Bgl*II and ligated into a *Sac*I-*Bgl*II-digested CMV expression construct. The HBVPRE was then removed from this construct by digestion with *Eco*RV. The vector was religated to yield the ΔHBVPRE surface expression vector. The HPRE(963-1684) and WPRE(1093-1684) fragments were then ligated into the *Cla*I site. The pDM138 vector system has been previously described (11). To construct the pDM138 reporter derivatives, 32-base oligonucleotides were synthesized and used to PCR amplify the fragment of interest from the DNA template. The oligonucleotides consisted of the 5′ sequence GCGGGATCCAT CGAT followed by 20 bases of the HBVPRE or WPRE sequence. The WPRE fragments were amplified from the viral DNA template of WHV accession no. J04514. The amplified fragments were purified on a 2% agarose gel, digested with *Cla*I, and subsequently ligated into the *Cla*I site of pDM138. The pGL3 vector (Promega) was digested with *Sma*I, and the *Cla*I-digested WPRE and HBVPRE fragments were Klenow enzyme-treated and ligated into the pGL3 vector. The mCC1 mutant was also synthesized via PCR. The HPREα/WPREβ and WPREγα/HPREβ constructs were constructed by PCR mutagenesis. Briefly, a mutant WPRE was synthesized with a single nucleotide change at nucleotide 1533, which produces a *Bam*HI site (WPRE *Bam*HI), and cloned into the *Cla*I site of pDM138. The mutant and the WPRE activities were identical (data not shown). p138HBVPRE(963-1684) and p138WPRE *Bam*HI were digested with *Cla*I and *Bam*HI. The 5′ and 3′ fragments from both digestions were gel isolated. The fragments were then ligated with the corresponding fragment into the pDM138 vector.

Tissue culture and transfections. CV1 cells were grown in 10-cm-diameter plates containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Before the cells were transfected, the medium was removed and the DNA-$CaPO_4$ mix was added directly to the naked cells. After 10 min, 5 ml of medium was placed back onto the cells. The medium was changed 16 h after transfection. The cells were harvested 36 to 48 h later. For the chloramphenicol acetyltransferase (CAT) assays, CV1 cells were transfected in triplicate with 2 μg of reporter plasmid, 1 μg of pCH110, and 7 μg of pUC118 by the $CaPO_4$ method. For the luciferase assays, CV1 cells were transfected when the 10-cm-diameter dish was approximately 30% confluent. The cells were transfected in triplicate with 2 μg of the luciferase reporter, 1 μg of pCH110, and 7 μg of pUC118. Luciferase activity was determined by standard methods. To assay for surface expression, CV1 cells were transfected in duplicate with 25 μg of surface expression vector and 5 μg of CMV secreted alkaline phosphatase. The medium was changed approximately 16 h after transfection. The spent medium was harvested 48 h later.

RNA isolation and analysis. HEK 293 cells were transfected with 10 μg of p138 vector, 1 μg of pCH110, and 2 μg of pGL3. Cells were resuspended in cytoplasmic lysis buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.5% Nonidet P-40). The lysed cells were spun at 8,000 × *g*; the supernatant was recovered and spun for an additional 5 min at 14,000 × *g*. The supernatant was then transferred to 1 ml of RNA Stat-50LS (Tel-Test). The nuclear pellet from the first spin was resuspended in 1 ml of cytoplasmic lysis buffer and then spun at 8,000 × *g* for 3 min. The supernatant was discarded, and the pellet was resuspended in 800 μl of nuclear buffer (10 mM Tris [pH 8.4], 1.5 mM $MgCl_2$, 140 mM NaCl, 20% glycerol). The sample was centrifuged at 8,000 × *g*, and the supernatant was discarded. The pellet was then resuspended in 300 μl of nuclear buffer and lysed with 1 ml of RNA Stat-50LS. The manufacturer's RNA Stat-50 protocol was followed. After RNA purification, the samples were DNase treated for 15 min at 37°C. Five micrograms of nuclear RNA and 10 μg of cytoplasmic RNA were loaded onto a 1% agarose/formaldehyde gel.

Surface expression radioimmunoassay. The spent medium from duplicate transfections was assayed for the presence of surface antigen with an Ausria II kit (Abbott Laboratories) and quantitated in a gamma counter. As an indicator of transfection efficiencies, the medium was also assayed for the presence of secreted alkaline phosphatase.

CAT assays. CV1 cells were lifted by using phosphate-buffered saline and 5 mM EDTA and resuspended in 150 μl of reporter lysis buffer (Promega). The lysates were spun briefly to pellet insoluble cell debris. An aliquot of each lysate was assayed for β-galactosidase activity, which was then used to normalize each lysate for transfection efficiency. The normalized lysates, equalized with reporter lysis buffer, were incubated at 37°C for 30 min to several hours with 1.5 nCi of [^{14}C]chloramphenicol (50 to 60 mCi/mmol) per μl and 1 mM acetyl coenzyme A in 50-μl volumes. Substrate and products were resolved by thin-layer chromatography and quantitated by a PhosphorImager (Molecular Dynamics).

RESULTS

In this study, the nucleotide numbering schemes of accession no. J04514 (WHV) and D00329 (HBV) were used. Consequently, homologous nucleotides are offset by 130 bases. For example, WHV nucleotide 1093 is homologous to HBV nucleotide 963 (Fig. 1B). The schematic in each figure is drawn to scale, and the homologous nucleotides of WPRE and HBVPRE are aligned. The posttranscriptional activity of an element will be discussed as a percentage of the WPRE activity.

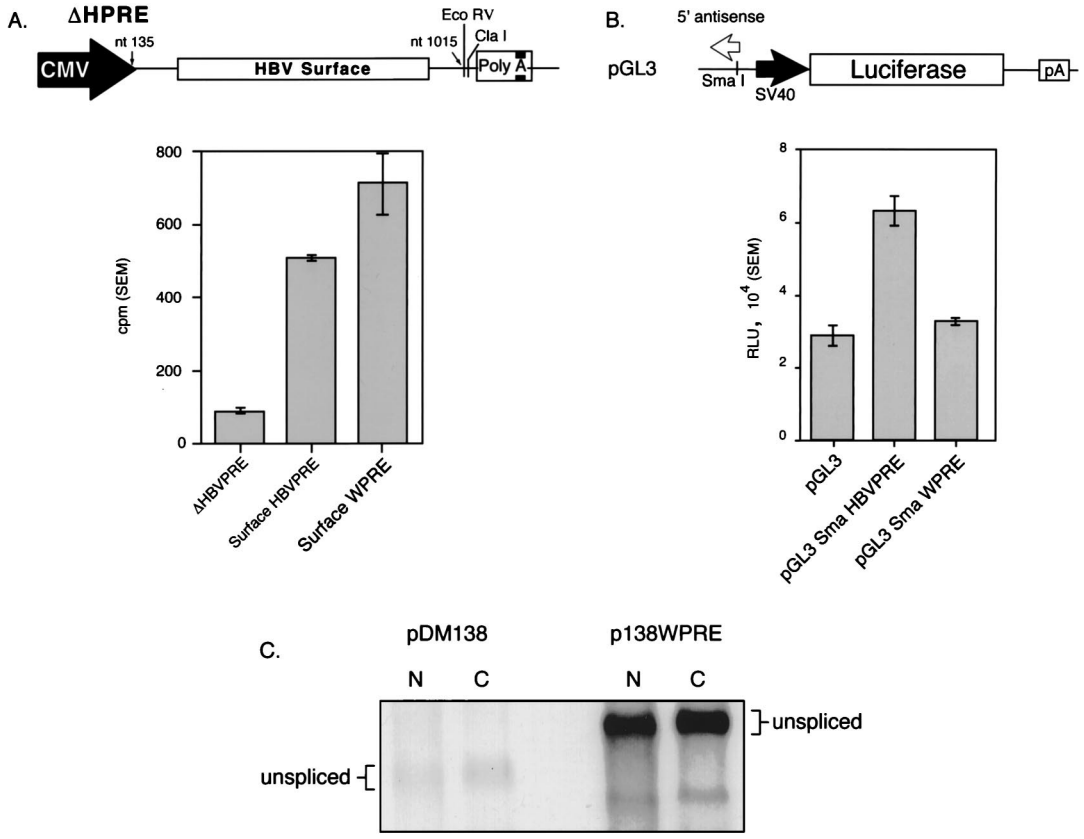

FIG. 2. WHV contains a PRE. (A) The WPRE can rescue HBV surface expression. The shaded bars represent the mean counts per minute from the media of duplicate transfections of CV1 cells. A schematic representation of the CMV HBV surface expression construct is shown at the top. The large black arrow represents the immediate early CMV promoter upstream of the HBV surface protein open reading frame. nt, nucleotide; Poly A, polyadenylation signal. The HPRE and WPRE were cloned into the *Cla*I site. (B) The WPRE does not exhibit enhancer activity in CV1 cells. A schematic representation of the pGL3 vector is shown at the top. The light shaded arrow represents the orientation of the inserted WPRE(1093-1684) and HBVPRE(963-1684) fragments. The black arrows represent the simian virus 40 (SV40) promoter; the luciferase gene is labeled. pA, polyadenylation site. The shaded bars represent mean luciferase activities of triplicate transfections. RLU, relative light units. (C) The WPRE can increase the cytoplasmic accumulation of unspliced pDM138 reporter RNAs.

WHV contains a PRE. To determine whether WHV contains a PRE similar to the HBVPRE, the HBV surface expression constructs depicted in Fig. 2A were transiently transfected into CV1 cells. CV1 cells were used to avoid any liver-specific transcriptional effects of enhancer I. The results (Fig. 2A) confirm that ΔHBVPRE exhibits background levels of surface protein expression. Surface protein expression was increased 6.1-fold by the HBVPRE, while the WPRE induced a 8.6-fold increase. Hence, the region in WHV homologous to the HBVPRE can rescue HBV surface expression.

It has been reported that WHV lacks enhancer I activity in liver cells, but this region has not yet been tested in nonliver cell types (2, 6, 31). To determine whether a functional transcriptional enhancer overlaps with the WPRE, the HBVPRE and WPRE were inserted in the antisense orientation upstream of a simian virus 40 promoter which drives transcription of the firefly luciferase gene (Fig. 2B). These constructs were transiently transfected into CV1 cells, which were subsequently assayed for luciferase activity. Figure 2B illustrates that the fragment containing the HBVPRE induced a 2.2-fold increase in transcription whereas the WPRE had no effect. Similar results were observed when the PREs were inserted downstream of the pGL3 polyadenylation signal (data not shown). In liver HepG2 cells, the WHV fragment had no effect on transcription whereas the HBV fragment increased luciferase expression 6.6-fold (data not shown). These results confirm that the putative WHV enhancer I does not display significant enhancer activity in liver or nonliver cells and that the observed WPRE effect occurs posttranscriptionally.

The HBVPRE has been shown to induce the appearance of unspliced pDM138 reporter RNAs in the cytoplasm of transfected cells. This reporter assay has been used to characterize the gross structure of the HBVPRE (4, 16). The pDM138 reporter is derived from the second intron of HIV-1, into which the CAT gene has been inserted (12). 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. When the unspliced RNAs are exported from the nucleus, CAT is translated and can be accurately quantitated. To determine whether the WPRE could also mediate the appearance of unspliced cytoplasmic reporter RNA, the WHV fragment from nucleotides 900 to 1800 was inserted into the intron of the pDM138 reporter. HEK 293 cells were transiently transfected with pDM138 and p138WPRE. Nuclear and cytoplasmic RNA fractions were isolated from the transfected cells and analyzed by Northern blotting. Figure 2C demonstrates that relative to the empty pDM138 vector, the WPRE increases the amount of unspliced nuclear and cytoplasmic RNA. An additional band is present in the p138WPRE(900-1800) RNA, but this band did not utilize the 3′ long terminal repeat and was not present in RNA prepared from a p138WPRE(1093-1684) transient trans-

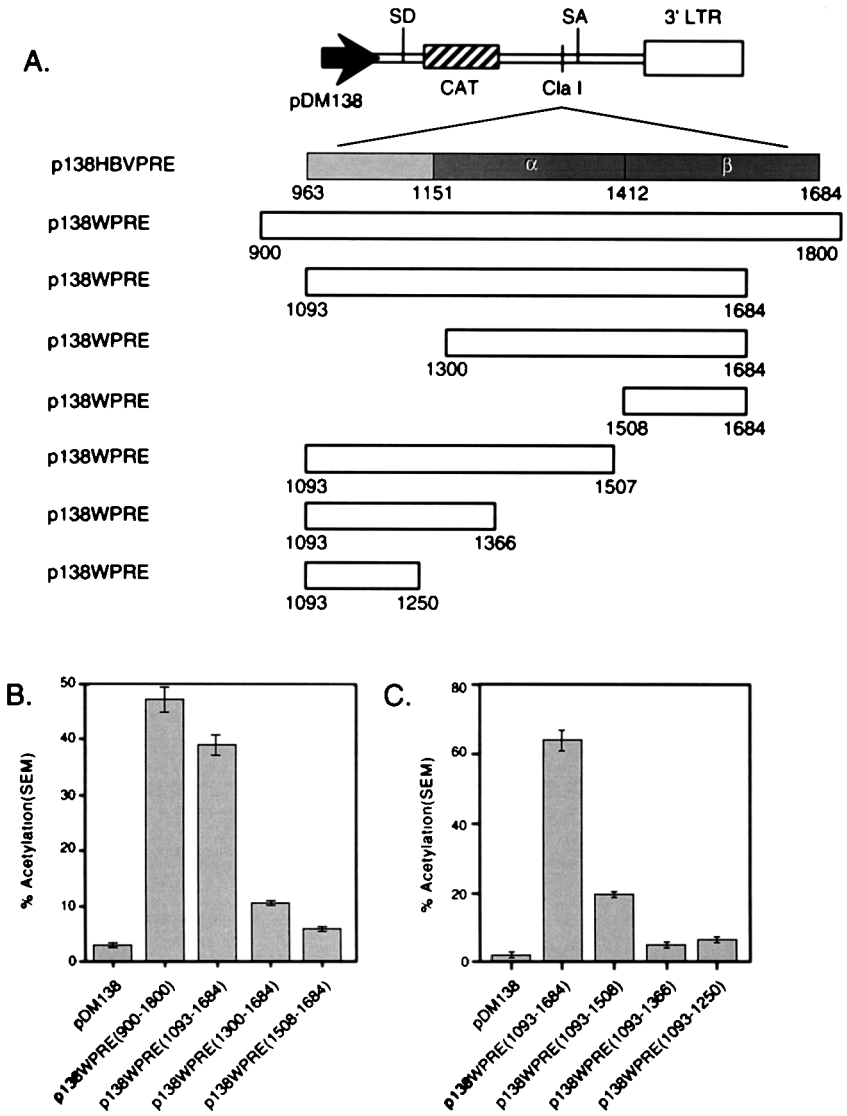

FIG. 3. Deletion analysis of the WHV PRE. (A) Schematic of the pDM138 vector system. The fragments of HBV and WHV are labeled according to the nucleotide numbers of WHV accession no. J04514 and HBV accession no. D00329, respectively. Homologous nucleotides are aligned, and the fragments are drawn to scale. The darkened regions correspond to the HPREα and HPREβ subelements. The shaded region of HBVPRE partially contains the enhancer modulatory domain and is not required for HBVPRE function. The filled arrow represents the simian virus 40 promoter from which the RNAs are transcribed. The CAT (hatched box), which is expressed only when unspliced RNA is exported, is located within the intron. The unique *Cla*I site is indicated. SD, splice donor; SA, splice acceptor; 3′ LTR, HIV-1 3′ long terminal repeat. (B) The 5′ end of the WPRE is sensitive to deletion. The shaded bars represent mean CAT activities of CV1 cells transfected in triplicate. (C) The 5′ end of the WPRE has minimal activity. The shaded bars represent mean CAT activities of CV1 cells transfected in triplicate.

fection (data not shown). Thus, the WPRE increases the level of both nuclear and cytoplasmic unspliced pDM138 RNA.

The WPRE contains three subelements. To identify the critical functional regions of the WPRE, a series of 5′ and 3′ deletions of nucleotides 900 to 1800, shown in Fig. 3A, were assayed for posttranscriptional activity in the pDM138 reporter assay. Figure 3B illustrates that p138WPRE(900-1800) can induce the expression of CAT in the pDM138 assay. This activity is orientation dependent (data not shown). 138WPRE(1093-1684) possesses 85% of the activity of p138WPRE(900-1800), while p138WPRE(1300-1684) is only 22% as active as p138WPRE (900-1800). p138WPRE(1508-1684) exhibits 12% of p138WPRE(900-1800) activity. To continue the analysis of the WPRE, we assayed a series of 3′ deletions of nucleotides 1093 to 1684 for posttranscriptional activity. Figure 3C illustrates that p138WPRE(1093-1508) is 30% and p138WPRE (1093-1250) is approximately 9% as active as p138WPRE (1093-1684).

The stepwise decrease in activity of the 5′ and 3′ deletions suggests that, like the HBVPRE, the WPRE consists of multiple subelements, each of which displays a low level of activity by itself. The homology and activity displayed by p138WPRE (1508-1684) is consistent with it containing the WPREβ subelement. The 30% activity of p138WPRE(1093-1508) suggests that WPRE nucleotides 1093 to 1508 may contain more than one subelement. One of these, given the sequence homology of the region, is most likely the WHV homolog of HPREα. A third subelement, which we term WPREγ, is encompassed by nucleotides 1093 to 1250. These results demonstrate that the subelements of the HBVPRE and WPRE are similarly organized but that the 5′ end of the WPRE, specifically nucleotides 1093 to 1250, and HBVPRE are functionally different.

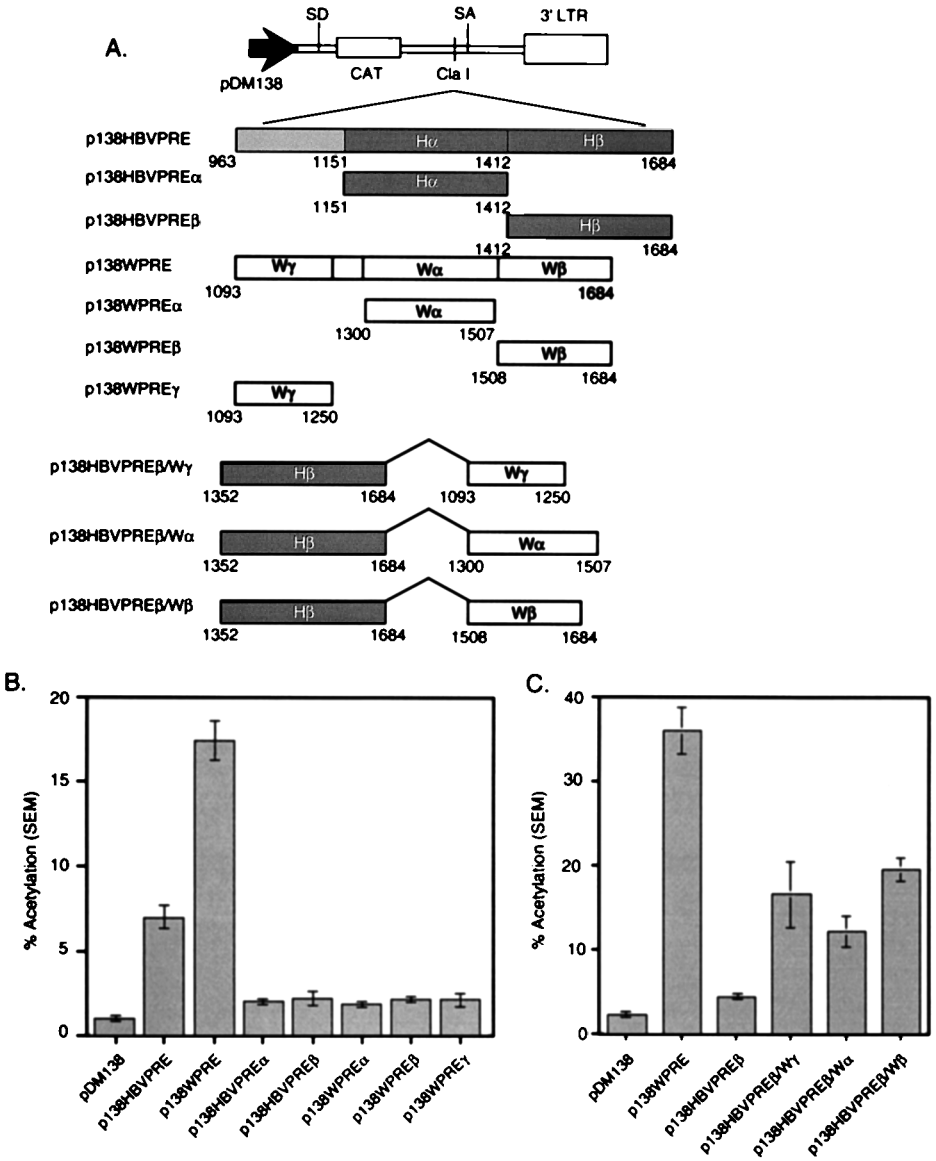

FIG. 4. The WPRE and HBVPRE subelements have similar levels of activity and can be combined. (A) Schematic representation of transfected constructs. The fragments are labeled, and nucleotide numbers correspond to those of the GenBank submissions. The other labels correspond to the descriptions in the legend to Fig. 2A. (B) The WPRE and HBVPRE subelements have similar levels of activity. The shaded bars represent mean CAT activities of CV1 cells transfected in triplicate. (C) WPREγ, WPREα, and WPREβ function in a greater than additive fashion with HBVPREβ. The shaded bars represent mean CAT activities of CV1 cells transfected in triplicate.

The three WPRE subelements function cooperatively. To compare the activities of the WPRE and HBVPRE and their respective subelements, CV1 cells were transiently transfected with the constructs depicted schematically in Fig. 4A. The results (Fig. 4B) demonstrate that p138HBVPRE(963-1684) is significantly (39%) weaker than p138WPRE. The p138WPREα, p138WPREβ, p138WPREγ, p138HBVPREα, and p138HBVPREβ reporters display approximately 12% of p138WPRE activity and twice the activity of the pDM138 background control. The low but statistically significant activities of the WPRE and HBVPRE subelements suggest that the subelements are functionally equivalent and that the increased WPRE activity is not due to the presence of an especially strong subelement.

To confirm that each of the WPRE subelements was functional, chimeras consisting of HBVPREβ and WPREγ, WPREα, or WPREβ were constructed (Fig. 4A). The data (Fig. 4C) indicate that p138HBVPREβ was 12% as active as the WPRE whereas the HBVPREβ/Wγ, HBVPREβ/Wα, and HBVPREβ/Wβ chimeras exhibited 46, 34, and 54% of WPRE activity, respectively. Hence, each of the WPRE subelements functions in a greater than additive fashion with the HBVPREβ subelement.

The posttranscriptional strength of the HBVPRE and WPRE is determined by the number of subelements. The functional conservation of PREα and PREβ within HBV and WHV suggests that the subelement structures are also conserved. To identify the conserved and variable regions of the PRE, 22 HBV, 5 WHV, and 2 ground squirrel hepatitis virus (GSHV) isolate nucleotide sequences were manually aligned. A phylogenetic comparative analysis highlighted two covarying base

pairs between HBV and WHV and one covarying base pair between WHV and GSHV in the WPREα region. Specifically, a C-G base pair between WHV nucleotides 1428 and 1443 changes to a U-A base pair in both HBV and GSHV. In addition, a U-A base pair between WHV nucleotides 1432 and 1440 changes to an A-U base pair in HBV (Fig. 5A). An RNA secondary structure prediction algorithm, Mulfold, was used to generate secondary structure models of WHV nucleotides 1396 to 1475 (17). The secondary structure model consists of an extended stem loop with a G-residue bulge 3 bp from a 5-base loop. The predicted WPREα secondary structure has a free energy of −29.4 kcal/mol. The covarying nucleotides are base paired in the predicted secondary structure model, suggesting that the distal stem-loop is biologically relevant. The covarying nucleotides are also based paired in the predicted secondary structure models of HPREα (data not shown). The covariational analysis did not highlight any conserved base pairs in the WPREβ subelement (data not shown).

The greater posttranscriptional activity of the tripartite WPRE than of the bipartite HBVPRE may be due to the presence of the third WPRE subelement. To test whether mutating a single subelement would reduce WPRE activity to HBVPRE levels, the predicted WPREα stem-loop structure was disrupted by mutating the C residues at nucleotides 1429 and 1431 to G residues to create p138WPREmCC1 (Fig. 5A). In addition, to test whether the predicted stem-loop structure encompassed the entire WPREα, nucleotides 1396 to 1475 were inserted into the pDM138 vector (p138WPREαmin). CV1 cells were transiently transfected with the reporters shown in Fig. 5B, and the results are shown in Fig. 5C. Consistent with previous experiments, p138HBVPRE(963-1684) was 41% as active as p138WPRE(1093-1684). The activity of p138WPRE mCC1, 57% of the WPRE activity, was closer to the activity of the bipartite HBVPRE. p138WPREα(1300-1507) and p138WPREαmin(1396-1475) were both 9% as active as the WPRE. The data indicate that nucleotides 1396 to 1475, which encompass the predicted stem-loop structure, are sufficient for WPREα activity. Disruption of the predicted WPREα structure decreases WPRE activity by over 40%. The large decrease in activity suggests that the three WPRE subelements function cooperatively to increase WPRE activity.

These data imply that the posttranscriptional strength of the hepadnavirus PREs is determined by the number of subelements present within the RNA. To test whether the number of subelement determines the posttranscriptional strength of an element, chimeric bipartite and tripartite elements were constructed. These constructs, depicted schematically in Fig. 6A, were transiently transfected into CV1 cells, which were subsequently assayed for CAT activity. In this experiment (Fig. 6B), the bipartite p138HBVPRE(963-1684) was 41% as active as 138WPRE(1093-1684). The bipartite p138HBVPREα/WPREβ chimera was 27% as active as WPRE, while the tripartite p138 WPREγα/HBVPREβ chimera was 76% as active as p138 WPRE. These data demonstrate that the subelements of the HBVPRE and WPRE are interchangeable. In addition, the results provide further evidence that the posttranscriptional strength of the hepdnavirus PREs is determined by the number of subelements within the RNA.

DISCUSSION

This is the first report of a PRE within WHV. The HBVPRE is required for the efficient expression of HBV surface protein, and its deletion abrogates surface expression (13, 15). Figure 2A demonstrates that the homologous region of WHV can also mediate expression of HBV surface protein.

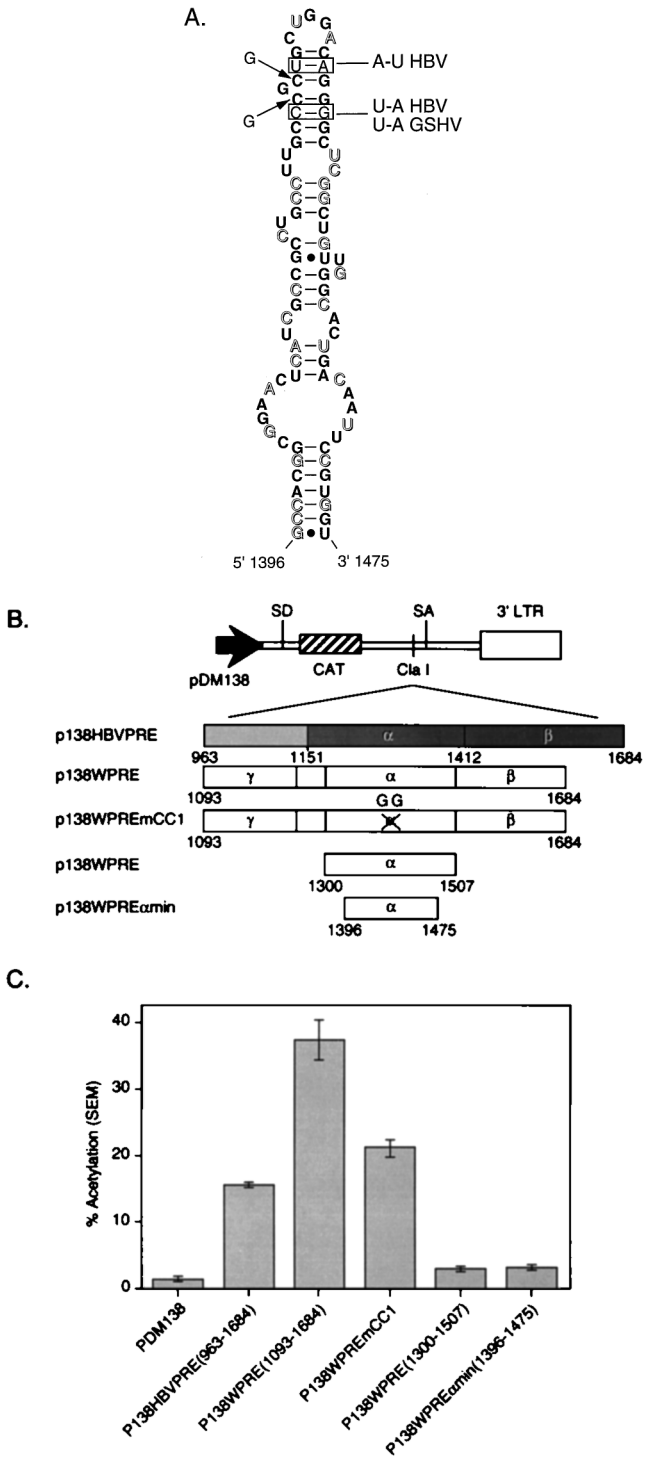

FIG. 5. Putative structure and mutation analysis of the WPREα subelement. (A) Predicted structure of the WPREα subelement. Bold nucleotides indicate residues that are completely conserved between HBV and WHV. Outlined nucleotides vary between HBV and WHV. The boxed bases pairs are those that covary among WHV, HBV, and GSHV. The covarying base pairs in HBV and GSHV are shown. In this region, there is 67.5% nucleotide identity between WHV and HBV. The arrows indicate the two G residues which were mutated in the PREα subelement for Fig. 5C; other labels correspond to those described in the legend to Fig. 2A. (C) Mutating the WPREα subelement decreases WPRE activity. The shaded bars represent mean CAT activities of CV1 cells transfected in triplicate.

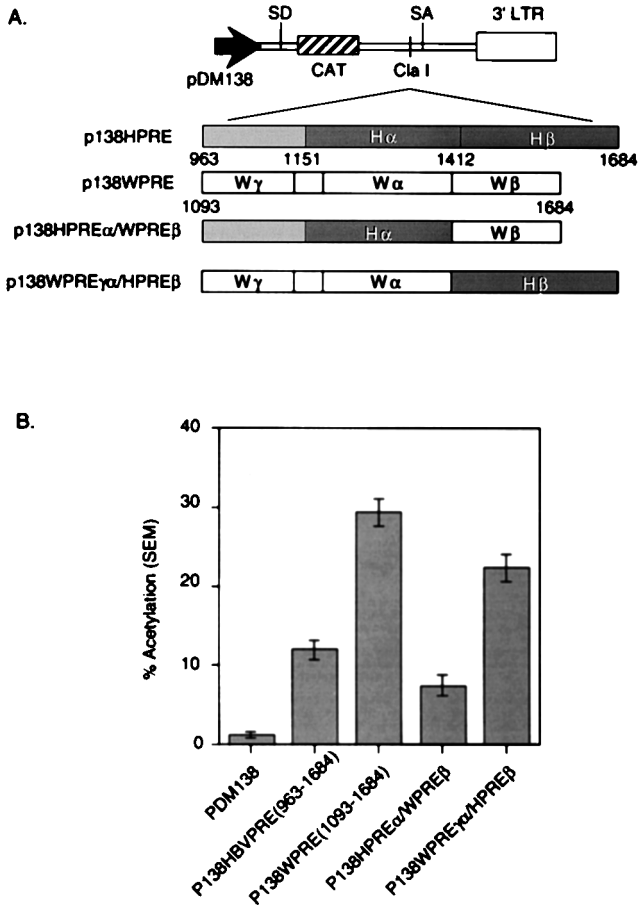

FIG. 6. Tripartite PRE are stronger than bipartite elements. (A) Schematic representation of transfected constructs. Fragments, chimeric elements, and HBVPREα (Hα), HBVPREβ (Hβ), WPREα (Wα), WPREβ (Wβ), and WPREγ (Wγ) subelements are marked; other labels correspond to those described in the legend to Fig. 2A. (B) The shaded bars represent mean CAT activities of triplicate transfections.

Although the WPRE encompasses a region that is homologous with HBV enhancer I, the results in Fig. 2B confirm previous reports that putative WHV enhancer I does not upregulate transcription activity. Hence, the WPRE effect on HBV surface expression is entirely posttranscriptional. The HBVPRE can mediate the cytoplasmic accumulation of unspliced RNA in the pDM138 assay, and the gross structure of the HBVPRE has been characterized by using this assay system. The WPRE can also mediate the cytoplasmic accumulation of unspliced pDM138 RNA (Fig. 2C). The WPRE increases the amount of unspliced RNA in both the nuclear and cytoplasmic compartments.

The PREα and PREβ subelements are evolutionarily conserved between WHV and HBV. The data presented in Fig. 3B suggest that WHV nucleotides 1508 to 1684 encompass the minimal WPREβ subelement. The HPREβ subelement was originally mapped to HBV nucleotides 1412 to 1684, which are homologous to WHV nucleotides 1542 to 1814. In Fig. 3C, the drop in activity between p13WPRE(1093-1508) and p13WPRE (1093-1250) is consistent with the WPREα being contained within nucleotides 1250 to 1507. This fragment is homologous to HBV nucleotides 1120 to 1377, which encompass the HPREα region. Figure 4B indicates that WPREα(1300-1507), WPREβ (1508-1684), HPREα, and HPREβ are comparable and display approximately 12% of WPRE activity.

The functional conservation of the HPREα and WPREα subelements suggests that PREα structure is also conserved. Phylogenetic comparative analysis highlighted two base pairs that covary within a possible stem-loop structure in WPREα. Two covarying base pairs within a helix is considered a nominal proof of a secondary structure model (21). RNA secondary structure predictions provided further support for the covarying bases since the lowest-energy model predicted that the covarying bases were paired (Fig. 5A). Figure 5C shows that mutating two residues within the stem decreased WPRE activity by greater than 40%. In addition, the predicted stem-loop structure of WPREα, nucleotides 1396 to 1475, displayed the same level of activity as p13WPRE(1300-1507). Other studies in our laboratory demonstrate that the stem-loop structure is critical for HBVPREα activity (26). These data support the hypothesis that WHV nucleotides 1396 to 1475 encompass the hepadnavirus PREα protein binding site.

Despite their similarities, the HBVPRE and WPRE exhibit significantly different posttranscriptional activities. The difference between the HBVPRE and WPRE activities maps to the presence of a third WPRE subelement within nucleotides 1093 to 1250. Figures 3C, 4B, and 4C demonstrate that the third subelement, termed WPREγ, exhibits 12% of the WPRE activity and can function in a greater than additive fashion with the HBVPREβ subelement. Previous studies have shown that in HBV, this region does not display posttranscriptional regulatory activity (4, 15). The functional difference between the HBVPRE and WPRE is surprising since this region is evolutionarily well conserved between HBV and WHV. The high degree of conservation is most likely due to the fact that these regions contain two partially overlapping open reading frames, the X promoter, the PRE, and HBV enhancer I. The WPREα- and WPREβ-encoding regions share 66.7% nucleotide identity with HBV. The HBV core enhancer domain is almost completely conserved between the two viruses, but the 5′ HBV enhancer modulatory domain is more divergent. This region, which has 60.7% nucleotide identity between HBV and WHV, encodes the WPREγ subelement in WHV. It is striking that WHV, which lacks enhancer I activity, has a third PRE in the physical location of the inactive enhancer I region.

Several observations suggest that the hepadnavirus PRE subelements function cooperatively and that the cooperativity of a third WPRE subelement results in greater WPRE activity. Each of the PRE subelements, shown in Fig. 4B, displays approximately 12% of the WPRE activity. In Fig. 4C, each of the three mapped WPRE subelements functions cooperatively with HBVPREβ. The HBVPREβ/WPREγ, HBVPREβ/WPREα, and HBVPREβ/WPREβ chimeras exhibited 34, 46, and 54% of the WPRE activity. The bipartite chimera activities are comparable to the 39% activity of the bipartite HBVPRE (Fig. 4B). In all of the constructs assayed, the tripartite elements are significantly stronger than bipartite elements. Figure 5C illustrates that mutation of a single WPRE subelement, WPREα, decreases the tripartite WPRE activity by over 40%. In Fig. 6B, the tripartite WPREγα/HBVPREβ chimera was 76% as active as the tripartite WPRE, but the bipartite HBVPRE and HBVPREα/WPREβ chimeras were 41 and 27% as active, respectively. The increased WPRE activity has also been observed in NIH 3T3 cells and chicken embryo fibroblasts (data not shown). These data suggest that compared to two subelements, the presence of three subelements greatly increases the posttranscriptional strength of an element. It is interesting to speculate that the presence of a third WPRE subelement compensates for the lack of WHV enhancer I activity.

These data support a model in which the PRE-interacting

proteins function cooperatively and the posttranscriptional strength of an element may be modulated by the number of proteins interacting with the RNA element. The cellular proteins that interact with the WPRE and HBVPRE have not yet been identified, but the characterized PRE subelements most likely represent protein binding sites. A single cellular protein may contact all of the PRE subelements or, alternatively, distinct cellular proteins may bind to each. The PRE binding protein(s) are most likely well conserved since the PREs are functional in every cell line tested (data not shown). The functional conservation of PREα and PREβ subelements suggests that the PREα and PREβ binding proteins will interact with both the HBVPRE and WPRE. Excluding the PREα subelement, the secondary structures of the subelements have not been determined, and the predicted secondary structure models of WPREγ, WPREα, and WPREβ do not show any striking similarities. In addition, the WPREγ and WPREβ subelements do not contain any PREα-like secondary structures or sequence similarities (data not shown). Since the subelements are modular and can function irrespective of order, we postulate that multiple cellular proteins mediate hepadnavirus PRE function.

The greater than additive effect of the PRE subelements is reminiscent of previous results obtained with the high-affinity binding sites of HIV-1 Rev and human T-cell leukemia virus type 1 Rex (9, 14). It has previously been suggested that the HBVPRE interacts with cellular proteins that directly export intronless RNAs, similar to the HIV-1 Rev-RRE system (4, 15, 23). However, we find that leptomycin B, a drug which specifically blocks Rev export, does not inhibit the activity of the HBVPRE or WPRE (20). Therefore, the HBVPRE and WPRE are not elements that directly interact with the Rev export pathway. However, the MPMV CTE can directly export intron lariats in *Xenopus* oocytes but is not inhibited by Rev-nuclear export signal peptides (22, 24). Hence, the HBVPRE may be more similar to the MPMV CTE in this respect. Alternatively, the HBVPRE may stimulate HBV RNA processing prior to the export of the RNA. The concise functional mapping of the WPRE will be useful for elucidating the mechanism of the hepadnavirus PREs and identifying their transactivating cellular proteins.

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