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Effects of Mutations within and Adjacent to the Terminal Repeats of Hepatitis B Virus Pregenomic RNA on Viral DNA Synthesis

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The viral polymerase and several *cis*-acting sequences are essential for hepadnaviral DNA replication, but additional host factors are likely to be involved in this process. We previously identified two sequences, UBS and DBS (upstream and downstream binding sites), present in multiple copies in and adjacent to the pregenomic RNA (pgRNA) terminal redundancy, that were specifically recognized by a 65-kDa host factor, p65. The possible roles of these two sequences in hepatitis B virus (HBV) replication were investigated in the context of the intact viral genome. UBS is contained within the terminal redundancy of pgRNA, and the 5′ copy of this sequence is essential for viral replication. Mutations within the central core of UBS ablate p65 binding and selectively block synthesis of plus-strand DNA, without affecting RNA packaging or minus-strand synthesis. The DBS sequence, which is located downstream of the pgRNA polyadenylation site, overlaps the core (C) protein coding region. All mutations introduced into this site severely affected viral replication. However, these effects were shown to result from dominant negative effects of mutant core polypeptides rather than from *cis*-acting effects on RNA recognition. Thus, the 5′ UBS but not DBS sites play important *cis*-acting roles in HBV DNA replication; however, the involvement of p65 in these roles remains a matter for investigation.

The hepatitis B virus (HBV) belongs to the family of hepadnaviruses (hepatotropic DNA viruses), agents that produce persistent infection of liver cells and cause liver disease in a variety of hosts (reviewed in reference 13). Like all hepadnavirus particles, HBV particles are composed of an enveloped nucleocapsid containing a 3.2-kb partially duplex circular DNA which is covalently linked to the viral polymerase (2). The DNA molecule is generated through reverse transcription of an RNA intermediate called pregenomic RNA (pgRNA) (33). The pgRNA is packaged by the viral polymerase into core particles. Following encapsidation, reverse transcription occurs, and cores bearing the mature DNA genome then undergo envelopment and release (38).

pgRNA performs two essential functions in the life cycle of the hepadnaviruses: (i) it serves as the template for reverse transcription (33) and (ii) it is the mRNA encoding the polymerase (P) and the core (C) protein (6, 7, 29). To ensure the synthesis of a full-length DNA molecule, the pgRNA has a terminal redundancy (4, 11, 12). This redundancy contains information essential for several processes in addition to reverse transcription, including polyadenylation, packaging, and translation (Fig. 1B). Moreover, each copy of the terminal repeat plays a distinct role in these processes. For example, the 5′ copy of this sequence is essential for viral RNA packaging (15–17, 24) and translation (5), while polyadenylation and reverse transcription require sequences in and adjacent to both terminal redundancies (9, 27, 31, 34, 37).

Hepadnavirus reverse transcription follows a complex pathway. The present view of this process, schematized in Fig. 1A, is supported by a substantial body of genetic and biochemical evidence. The viral polymerase promotes packaging of the pgRNA and initiation of the minus-strand synthesis by binding to the 5′ stem-loop structure (15–17, 24, 25). Following RNA binding, DNA synthesis is initiated, using P protein as a primer in a reaction templated by the bulge of the stem-loop, thereby producing a polymerase-linked oligonucleotide. By virtue of its complementarity with so-called DR1 sequences in the terminal repeat, this short oligonucleotide is translocated to the copy of DR1 contained within the 3′ redundancy (30, 34, 37), whereupon the minus strand can be further elongated. During elongation, the pgRNA is degraded by the RNase H activity of the viral polymerase (20). When the polymerase reaches the 5′ end of the RNA template, the terminal 15 to 18 nucleotides (nt) of the template are protected from the RNase H activity (19). This short RNA is then translocated to the complementary DR2 sequence near the 5′ end of the minus strand, where it is used as the primer for the synthesis of plus-strand DNA (32). Elongation proceeds to the 5′ end of the minus-strand template; to continue plus-strand synthesis, the polymerase must transfer to the 3′ end of the minus strand, again via a homology-dependent mechanism that results in circularization of the viral DNA.

Since the terminal redundancy of the pgRNA plays essential roles in the HBV viral life cycle and since genetic evidence suggested the involvement of host factors in some of these processes (25, 27), we previously looked for host factors able to recognize RNA sequences within and adjacent to the terminal redundancy (23). One factor of nuclear origin, p65, was found able to specifically bind multiple sites on the pgRNA in vitro. One site, UBS (upstream binding site), lies within the terminal redundancy and is thus present twice in pgRNA; the other site, DBS (downstream binding site), lies just downstream of the polyadenylation cleavage site so that only one copy of DBS is present in the mature pgRNA (Fig. 1B). Although both binding sites overlap with regions important for polyadenylation processes (27), we earlier showed that p65 binding to these sites is not required for efficient polyadenylation of the pgRNA.

In the present study, the possible roles of UBS and DBS in the HBV life cycle are further examined. To this end, mutations able to abolish p65-pgRNA interactions in vitro were

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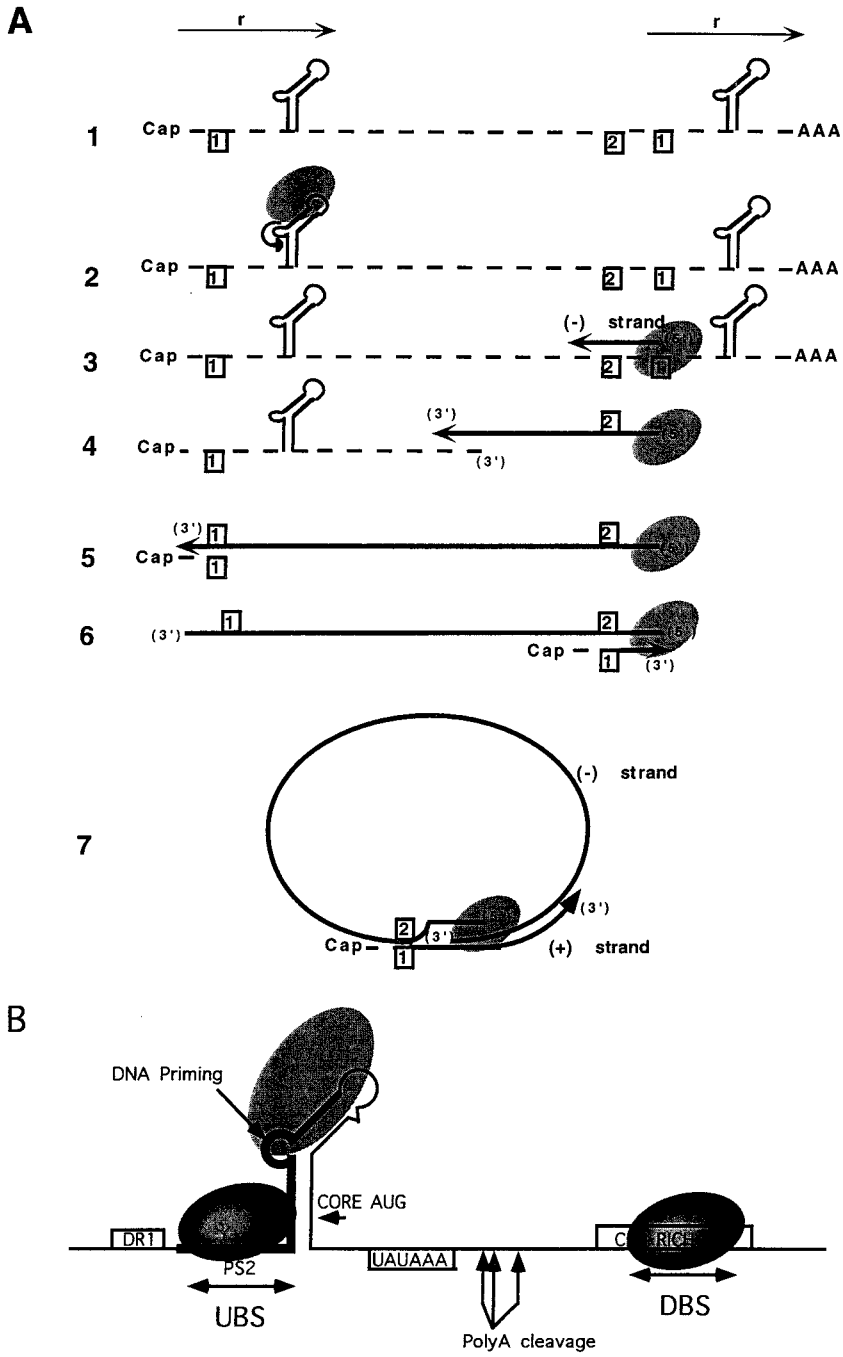

FIG. 1. Hepadnavirus reverse transcription pathway. (A) Scheme of the widely accepted model for hepadnavirus DNA synthesis process (see text for more details). r, terminal repeat. (B) Schematic representation of the functional elements present within and adjacent to the terminal redundancy and known to play essential roles in the hepadnavirus life cycle (for a recent review, see reference 13). The locations of the initial binding of the viral polymerase to the pgRNA and of the p65 binding sites are noted with respect to all of the functional elements. Black bars, PS2 polyadenylation-enhancing element; DR1, extent of direct repeat sequence involved in DNA strand transfers; UAUAAA, HBV polyadenylation signal; arrows, poly(A) addition sites; box, U-rich region required for accurate and efficient poly(A) addition.

introduced into the intact viral genome, and the effects of the lesions on the production of progeny genomes were analyzed. Our results indicate that one copy of UBS is essential for plus-strand synthesis, while the DBS sites appear to play no *cis*-acting role in viral replication.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs. *Taq*I polymerase was purchased from Perkin-Elmer; *Pfu*I polymerase was purchased from Stratagene. All enzymes and kits were used according to the manufacturers' instructions.

Plasmids and site-directed mutagenesis. In all constructs, the numbering of the sequence and the restriction sites correspond to those for HBV adw2 (36). All DBS mutations (D1, D2, D3, and D4), all UBS mutations (U1, U2, and U3), and the stop codon in core (SC) were introduced with the megaprimer method for in vitro mutagenesis by using two parallel templates as previously described (35). Briefly, this approach requires only one specific primer to introduce the mutation and two vector primers to generate and amplify the desired mutated fragment. The HBV *Hpa*I-*Bst*EII fragment was cloned into pSELECT (Promega) to generate the first template (pSEL[H-B]). The second template used

was obtained by cloning the HBV *Eco*RI-*Apa*I fragment into pBS-SK(−) (Stratagene), generating pSK/HBV[E-A]. In the mutagenic oligonucleotides, the mutations (see Fig. 3A and 6A for sequences of the mutations introduced) were surrounded by sequence complementary to HBV that were 25 nt long on each side. In the first round of amplification, the mutagenic oligonucleotides were used as 5′ primers and an oligonucleotide complementary to the vector SP6 promoter was used as the 3′ primer. The PCR-amplified fragment was then gel purified and used as the megaprimer on the second template, pSK/HBV[E-A]. After 10 cycles with only template and megaprimer, an oligonucleotide complementary to the HBV sequence surrounding the *Eco*RV site (800 bp 5′ of UBS and 900 bp 5′ of DBS) was added as the 5′ primer and the SP6 oligonucleotide was added as the 3′ primer. The product of the second PCR amplification was purified by using QIAQUICK (Quiagen), digested with *Nco*I and *Apa*I, and swapped with the wild-type *Nco*I-*Apa*I fragment of pGEM-HBV[E-A], which was obtained by cloning the HBV *Eco*RI-*Apa*I fragment into pGEM7Z(+) (Promega). The mutations were screened by sequencing, and the chosen mutant clones were sequenced between *Nco*I and *Apa*I sites to exclude the presence of any additional mutations. For the combined stop core mutations with DBS2 mutation (SC-D2), the template used for the first round of PCR was pSEL HBV/D2[H-B], obtained by cloning the *Hpa*I-*Bst*EII fragment from D2-5′ (see below).

The 1.5-mer HBV constructs containing the mutations in the 5′ copy of either UBS or DBS (U1-5′, U2-5′, U3-5′, D1-5′, D2-5′, D3-5′, and D4-5′) were obtained by first deleting from the mutant pGEM-HBV[E-A] the fragment between *Sma*I in the pGEM polylinker and *Eco*RV in the HBV. Then, an *Apa*I HBV monomer, derived from the 1.5-mer HBV env⁻ construct (3), was added to this intermediate.

The 1.5-mer HBV constructs containing the mutations in the 3′ copy of either UBS or DBS (U1-3′, U2-3′, U3-3′, D1-3′, D3-3′, and D4-3′) were obtained by swapping the wild-type (wt) *Hin*dIII-*Xba*I fragment of 1.5-mer HBV env⁻ with the same fragment from mutant pGEM-HBV[E-A]. For D2-3′, a similar swap was done by using the *Sac*I-*Xba*I restriction sites since the mutation created a new *Hin*dIII site.

The 1.5-mer mutant constructs D1-5′/YMHD, D2-5′/YMHD, D3-5′/YMHD, D4-5′/YMHD, U1-5′/YMHD, D1-3′/YMHD, D2-3′/YMHD, D3-3′/YMHD, D4-3′/YMHD, and U1-3′/YMHD were obtained by combining the YMHD mutation (14, 25, 26) from 1.5-mer HBV/YMHD with either the UBS or the DBS mutations. The 3′ constructs were obtained by swapping the *Nco*I-*Nco*I fragment of the D-3′ or U-3′ mutants with the same fragment from 1.5-mer HBV/YMHD. The mutations in the 5′ copy of DBS were combined by swapping the *Apa*I-*Apa*I fragment of the 5′ DBS mutants with the same fragment from 1.5-mer HBV/YMHD. For the U1-5′ mutant, the fragment swapped was *Hin*dIII-*Eco*RI.

To provide wt core in *trans* in some transfection experiments, pHBV-C2 was used (42). Briefly, this construct allows expression from a cytomegalovirus promoter of the pgRNA devoid of the stem-loop and with an insertion mutation in the viral polymerase. The only expected translated product is the core protein. Two DBS mutations (D2 and D3) were also transferred into this construct, generating pHBV-CD2 and pHBV-CD3.

Cell cultures, transfections, and RNA and DNA analyses. HepG2 human hepatoma cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal calf serum. Transfections were performed by calcium phosphate precipitation as previously described (8). In each transfection, we used 7.5 mg of HBV construct DNA along with 3 mg of plasmid pXGH5 for the growth hormone assay (HGH-TGES Transient Gene Expression kit; Nichols Institute) used to determine the transfection efficiency. When wild-type core was provide in *trans*, in addition to the DNA mentioned above, we added 7.5 μg of pHBV-C2.

The total RNA was prepared by using RNazol (Tel-Test Incorporated), and part of the total RNA was used to poly(A) select the mRNA with Oligotex (Qiagen).

The intracellular core particles and nucleic acid were extracted as previously described (32), with the only modifications that micrococcal nuclease (0.6 U/ml; Sigma) was used in place of RNase A and the final nucleic acid precipitation was carried out in the presence of glycogen (Boehringer Mannheim) as a carrier.

Primer extension analysis was carried out as previously described (32). The oligonucleotide used as a primer of the 5′ end of the plus strand is complementary to nt 1804 to 1816 of the HBV plus strand.

Southern and Northern blotting analysis were performed by standard procedures (28). The *Apa*I-*Apa*I HBV fragment from 1.5-mer HBV env⁻ was labeled by random priming (Rediprime; Amersham) and used for all hybridization experiments. The total or the poly(A)-selected RNA was also hybridized as a standard to the *GAPDH* cDNA labeled as described above.

RESULTS

Experimental design. The UBS and DBS sequences were previously identified as binding sites on HBV pgRNA for a host nuclear factor, p65 (23). To analyze the roles of UBS and DBS in the viral life cycle, we introduced mutations able to abolish the p65-pgRNA interaction in vitro (23) into a standard overlength HBV construct, called 1.5-mer HBV (Fig. 2A) (3). With this construct, all viral transcripts are transcribed from their natural HBV promoters and the resulting pgRNA is terminally redundant. By using this construct, it is possible to introduce mutations specifically at either terminal repeat, thereby allowing separate assessment of the role of each copy of the repeated sequences.

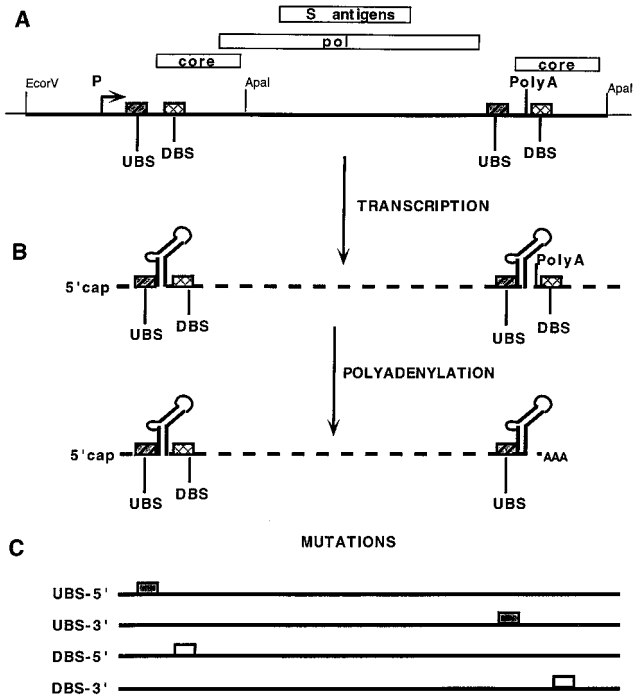

FIG. 2. Experimental strategy. (A) 1.5-mer HBV construct and locations of the HBV ORFs, of the p65 binding sites (UBS and DBS), and of the poly(A) cleavage site (PolyA). Thin line, vector DNA; thick line, HBV DNA; P, pgRNA promoter. (B) pgRNA (dotted line) transcribed from the 1.5-mer HBV construct in its pre-mRNA form and as a mature polyadenylated RNA. The locations of the stem-loops and of the p65 binding sites are shown with respect to the polyadenylation site. (C) Depiction of mutated copy of either UBS or DBS in the different constructs.

Since the UBS sequence is present at both ends of the mature pgRNA and the DBS sequence is present at both ends in the nuclear pre-mRNA (Fig. 2B), we decided to introduce mutations in each of these sites, at either the 5′ or the 3′ repeat (Fig. 2C). (Mutations in the 5′ copy of UBS are referred to as U-5′ lesions, while those in the 3′ copy are designated U-3′ lesions; a similar convention was followed for the DBS mutations.) The 1.5-mer HBV mutants were used to transfect HepG2 cells. In each transfection experiment, half of the transfected cells were used to extract total or polyadenylated mRNA. The other half was used as a source of intracellular core particles, from which we extracted the HBV nucleic acids. The level of viral DNA synthesis was determined by Southern blotting analysis of the nucleic acids extracted from the core particles. For some mutants, the pgRNA packaging efficiency was determined by Northern blotting analysis of the RNA contained in the core particles.

Mutations in UBS block plus-strand DNA synthesis. We previously determined that within the 23-nt binding region of UBS, the central 10 bp are critical, since mutations in this core (e.g., U2 and U3 [Fig. 3]) severely inhibited binding of p65 (23). Mutations U2 and U3 were introduced into either the 5′ or the 3′ copy of UBS in the 1.5-mer HBV construct, and the

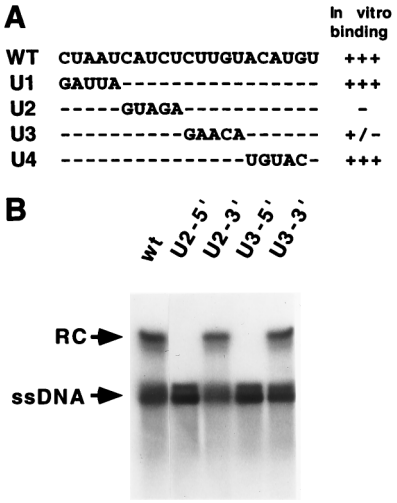

FIG. 3. Effects of mutations in the central core of the UBS sequence on HBV DNA synthesis. (A) Sequence of the minimal region defining UBS and depiction of the nucleotide sequence changes introduced in each mutant RNA. At the right is schematic scoring of the UV cross-linking results of the binding assay: +++, 25 to 100% of wt binding; −, less than 5% of wt binding; +/−, 5 to 10% of wt binding (23). (B) Southern analysis of the nucleic acids extracted from the intracellular core. The wt lane shows the migration pattern of DNA extracted from the wt core particles. RC, relaxed circular DNA; ssDNA, HBV minus-strand single-stranded DNA.

mutant genomes were transfected into HepG2 cells; the DNA contained within the mutant viral progeny was then examined. As shown in Fig. 3B, these mutations, when present in the 5′ copy of UBS, profoundly altered viral DNA synthesis. The fastest-migrating product represents the single-stranded DNA which results from minus-strand synthesis. The slower-migrating product represents the relaxed circular DNA, which is the DNA present in the mature virions. The single-stranded DNA accumulated to similar levels in the wt and all mutant viruses. However, the accumulation of the relaxed circular DNA was completely inhibited when the mutations were present in the 5′ copy of UBS. This result suggests that the 5′ copy of UBS is involved in HBV DNA maturation. By contrast, the same mutations in the 3′ copy of UBS had no effect on the HBV DNA synthesis.

To determine if the phenotype of 5′ UBS mutations is correlated with p65 binding, we sought to examine the effects of adjacent lesions in the viral genome. Because the region immediately 3′ to this copy of UBS is known to be essential for RNA encapsidation, lesions there cannot be inspected for defects in plus-strand synthesis. Accordingly, we concentrated on lesions upstream of U2 and U3. The adjacent mutation U1, which does not affect the binding of p65 to the pgRNA in vitro (23), was chosen for examination. As shown in Fig. 4A, this mutation displayed a phenotype different from those of the other two mutations: it almost completely abolished DNA synthesis when present in the 5′ copy of UBS.

Since the U1 mutation affected both plus- and minus-strand DNA synthesis, the possible effects of this mutation on RNA encapsidation were analyzed. To facilitate the comparison between the pgRNA packaging levels of virus containing either the wt or mutant U1, we introduced the mutation into the 1.5-mer HBV/YMHD background. In this construct, the viral polymerase carries a mutation, YMHD, which ablates the DNA synthesis function in the polymerase but leaves unaltered its ability to bind and package the HBV pgRNA (14, 25, 26). Introduction of the U1 mutation into the 5′ copy of UBS in 1.5-mer HBV/YMHD, U1-5′/YMHD, decreased the efficiency of pgRNA encapsidation, while the same mutation in the 3′ copy of UBS, U1-3′/YMHD, appeared not to affect encapsidation significantly (Fig. 4B). For both mutants, the poly(A)-selected pgRNA accumulated at normal levels (Fig. 4C).

Taken together, these data indicate that the sequences comprising central core of the 5′ copy of UBS are required for viral replication and establish a correlation between p65 binding to this region and successful plus-strand synthesis. The flanking sequences appear to be involved in a separate process, RNA packaging, which precludes mutational analysis of any additional contributions that they might make to plus-strand synthesis.

5′ UBS mutations block the priming reaction for plus-strand synthesis. The inhibition of accumulation of relaxed circular DNA showed with the UBS2 and UBS3 mutants could be caused by a block of either (i) the completion of minus-strand DNA synthesis through the region responsible for the generation of the plus-strand primer; (ii) the generation or translocation of the plus-strand RNA primer from the 3′ to the 5′ end of the minus strand (Fig. 1A, lines 5 and 6), (iii) the priming event after translocation (Fig. 1A, line 6), or (iv) the circularization event (Fig. 1A, line 7).

Some mutations previously generated in duck hepatitis B virus specifically block the translocation of the RNA primer without affecting the priming reaction; as a result, a full-length double-stranded linear DNA molecule was generated (19, 32). To understand if the mutations U2 and U3 had the same effect on viral DNA synthesis, we compared the electrophoretic mobilities of the viral DNA products with those of cloned linear monomers of viral DNA. No DNA species with the mobility of duplex linear DNA was observed in this analysis (data not shown).

Alternatively, the U2 and U3 mutations in the 5′ copy of UBS might specifically block the circularization event that must occur for the DNA maturation (Fig. 1A, construct 7). Mapping the 5′ end of the putative plus strand allows determination of whether the block occurs at this step. For this purpose, we used a primer complementary to nt 1804 to 1816, a region of the plus strand downstream of DR2, the site to which the RNA primer is transposed (Fig. 5A). If translocation and priming occurred, and the viral polymerase reached the 5′ end of the minus strand, elongation from the 1804–1816 primer should generate a 210-bp fragment. A fragment of the same size is, of course, also expected with the wt viral DNA. But as Fig. 5B shows, the primer extension failed to generate a signal for both U2-5′ and U3-5′ mutants, while as expected, the 210-bp fragment was generated in the wt DNA.

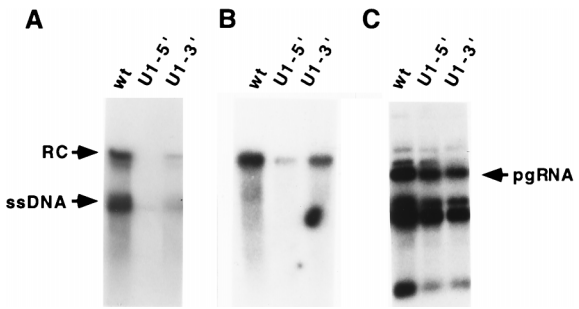

FIG. 4. Effects of mutations in the 5′ portion of UBS. (A) Southern analysis of the nucleic acid extracted from the viral particles. RC, relaxed circular DNA; ssDNA, HBV minus-strand single-stranded DNA. (B) Northern analysis of the encapsidated pgRNA. (C) Northern analysis of the poly(A)-selected RNA extracted from the same transfected cells as for panel B.

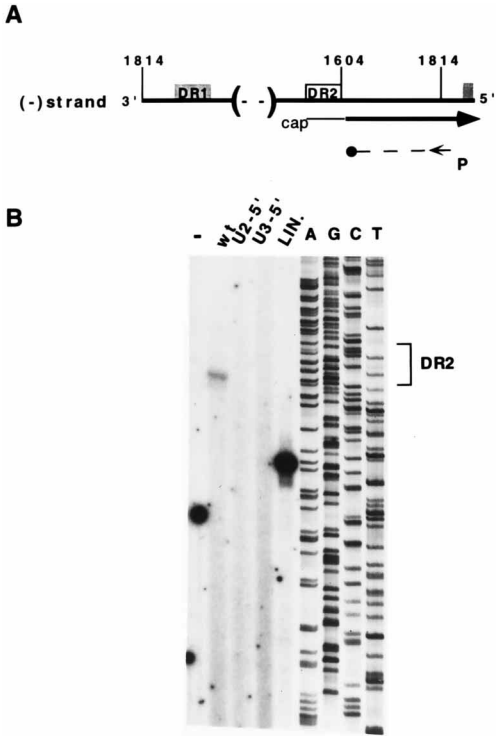

FIG. 5. Primer extension on the viral plus-strand DNA. (A) Schematic depiction of the primer extension. The primer (P) hybridization site is shown with respect to some landmarks on the minus strand and the localization of the plus strand. The large parentheses indicate that the drawing is not to scale; the thick line represents a single filament of DNA; the thin line represents RNA; ● is the termination site of the primer extension, since the samples were pretreated with NaOH at 95°C to destroy the RNA primer (32). (B) Primer extension data for the indicated mutants.

This result, taken together with the lack of duplex linear DNA molecules, indicates that mutations in UBS that ablate p65 binding block the priming of the plus strand of the HBV DNA. Either plus-strand RNA primers fail to be generated (e.g., due to impaired minus-strand DNA synthesis through this region or to impaired cleavage of the primer) or those which are generated fail to prime plus-strand DNA synthesis either in situ or following transposition to DR2.

DBS mutations inhibit HBV DNA synthesis. Like UBS, DBS is 22 nt long and is pyrimidine rich; unlike UBS, most of the 22-nt sequence is required for efficient p65-pgRNA interactions. Mutations in DBS were engineered into the 5′ or 3′ copies of DBS in the HBV 1.5-mer vectors as described above. Note that DBS overlaps with the core open reading frame (ORF) and the mutations that we introduced to abolish p65 binding in vitro (summarized in Fig. 6A) change the amino acid sequence of core between residues 20 and 25 (Fig. 6A). Thus, any phenotype observed in vivo with a mutant 5′ copy of DBS, which is in the known functional copy of the core gene in the 1.5-mer HBV env$^-$ construct, could be also due to lesions in the core protein. For this reason, for 5′ DBS mutations in the intact viral genome, we provided in *trans* a wt source of core protein (3′ DBS mutations were examined without complementing C protein). As shown in Fig. 6B, HBV DNA synthesis was severely inhibited by mutations in either copy of DBS, even when C protein was supplied in *trans.*

Since synthesis of both minus and plus strands was inhibited in these mutants, we looked at the effects of these mutations on the pgRNA packaging efficiency. As we did for the U1-5′ mutant, we subcloned the DBS mutations into the HBV mutant YMHD to facilitate the analysis of RNA packaging in the wt and DBS mutant constructs. Northern analysis of the total (not shown) and poly(A)-selected (Fig. 6D) mRNA revealed that none of the mutations at either copy of DBS affected the levels of any viral transcript. However, most of the mutations in the 5′ copy of DBS completely inhibited the packaging of the pgRNA (Fig. 6C). In contrast, no significant effect on the RNA packaging was observed when the same mutations were introduced in the 3′ copy of DBS (Fig. 6C).

The inhibition of viral DNA synthesis in the DBS mutants is caused by defective core protein. Because DBS lies outside of the region that we and others have previously shown to be involved in RNA packaging, we suspected that the inhibition of packaging by the mutations in the 5′ copy of DBS was due to the mutations introduced into the core protein. As this inhibition was only weakly relieved by wt core protein in *trans* (data not shown), then if the observed phenotype is caused by mutated core proteins, such mutants must represent dominant negatives. To explore this possibility, we conducted the experiment shown in Fig. 6E. A construct with a stop mutation in the 5′ copy of the core gene (SC-5′) was cotransfected with variable ratios of two plasmid expression vectors encoding wt C proteins and C proteins bearing either D2 or D3 mutations. The encapsidated progeny viral DNA was assayed by Southern blot hybridization. As shown Fig. 6E, increasing levels of mutant C proteins strongly disrupted the replication of a wt genome, confirming that lesions in residues 20 to 25 of C protein generate potent dominant negatives.

In contrast to the behavior of mutations in the 5′ copy of DBS, mutations in the 3′ copy display a severe effect on DNA synthesis without apparently affecting pgRNA encapsidation (Fig. 6C). This result was quite surprising because the 3′ copy of DBS is not present in the mature polyadenylated pgRNA which is in the nucleocapsids. The discovery that C proteins bearing lesions in residues 20 to 25 are potent dominant negatives suggested a possible interpretation: contrary to expectation, the 3′ copy of the core gene of the 1.5-mer HBV construct is expressed at low levels. If this is so, then some of the effects of 3′ DBS mutations might again be attributable to defective C proteins.

We therefore conducted several studies to examine the possibility that the 3′ copy of ORF C is expressed from the 1.5-mer HBV construct. First, we tested the ability of the HBV construct with a stop codon in the 5′ copy of the core gene (SC-5′) to produce core particles with mature DNA molecules. As shown in Fig. 7A, a small amount of encapsidated viral DNA is indeed produced by this mutant.

This result suggests that either the C-gene stop codon is a leaky mutation or low levels of expression occur from the downstream copy of core gene. To distinguish between the two alternatives, the same C-gene stop codon was introduced into the 3′ copy of either the wt genome or the mutant DBS2-3′, generating SC-3′ or SC/D2-3′, respectively. The levels of encapsidated viral DNA produced in HepG2 cells transfected by these two constructs were compared to those generated by the wt genome and the mutant D2-3′. As shown in Fig. 7B, the introduction of the stop codon completely abolished the phenotype of the mutation in the 3′ copy of DBS. This result confirms that a small amount of mutated core is indeed expressed from the downstream gene and is responsible for the observed phenotype of 3′ DBS mutations. The reason that 3′ DBS lesions appear to affect DNA synthesis while 5′ DBS lesions affect RNA packaging appears to be due to the relative levels of expression of the dominant negative C protein. At the high levels expressed from the 5′ C gene, C assembly is pre-

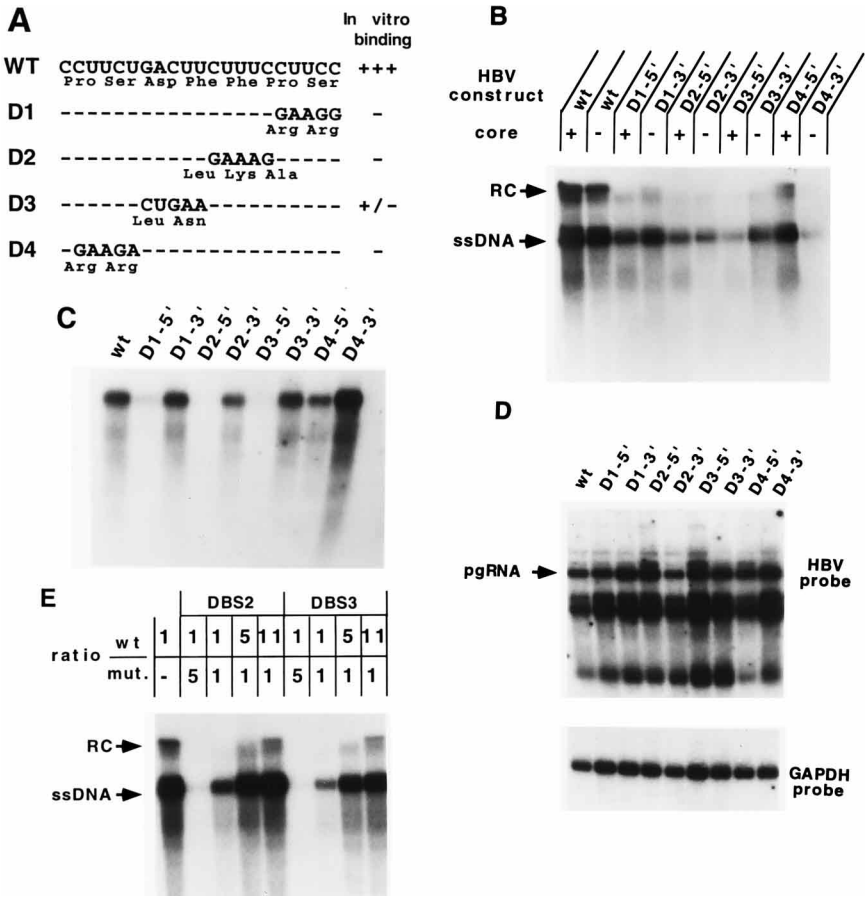

FIG. 6. Effects of mutations in the DBS sequence. (A) Sequence of the minimal region defining DBS, depiction of the nucleotide sequence changes introduced in each mutant RNA, and consequent changes in the core amino acid sequence. At the right is schematic scoring of the UV cross-linking results of the binding assay as in Fig. 3A (23). (B) Southern analysis of the nucleic acid extracted from the viral particles. Wild-type core was expressed by the cotransfecting pHBV-C2 plasmid when the mutations were in the 5′ copy of the core gene. RC, relaxed circular DNA; ssDNA, HBV minus-strand single-stranded DNA. (C) Northern analysis of the encapsidated pgRNA. (D) Northern analysis of the poly(A)-selected RNA extracted from the same transfected cells as for panel C. (E) Southern analysis of a three-plasmid cotransfection experiment. Each transfection was done with 7.5 μg of SC-5′ plus 7.5 μg of a combination of pHBV-C2 (expressing wt C) and either pHBV-CD2 or pHBV-CD3 (expressing mutated C). The molar ratio of pHBV-C2 and plasmid expressing the mutated core (mut.) is indicated above each lane.

sumably severely disrupted, leading to an absence of functional capsids, while at the lower levels expressed from the 3′ position, aberrant capsids which are competent to package RNA but which interfere with proper reverse transcription can be assembled. Consistent with this view is the experiment of Fig. 6E; as shown there, low ratios of mutant to wt C proteins have defects primarily in plus strand in DNA synthesis, while higher levels progressively abolish all DNA synthesis, as would be expected from a packaging defect.

We do not know the identity of the mRNA from which this 3′ C gene is expressed. Presumably, the viral C promoter at the 3′ end of the genome is used, and some transcripts escape polyadenylation within ORF C to express the full coding region. Either these RNAs are present at such a low level that they escape detection by Northern blotting or they comigrate with authentic viral transcripts.

DISCUSSION

In this work, we have examined the potential roles of the host nuclear factor p65 in the replication of HBV. Our approach was to mutationally ablate the known sites of p65-RNA interaction and examine the resulting mutant genomes for the ability to undergo RNA encapsidation and DNA synthesis. Our results indicate that lesions in the different p65 binding sites defined by our earlier in vitro studies result in substantially different phenotypes in vivo and that not all of these phenotypes are the result of *cis*-acting effects.

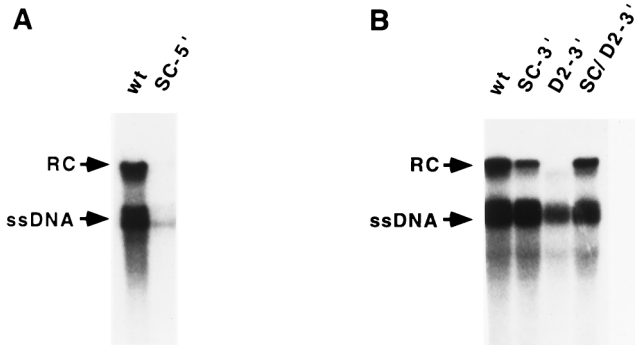

FIG. 7. Effects of a stop codon in the core gene in the 1.5-mer HBV constructs. (A) Southern analysis of the viral particle DNA. (B) Southern analysis of viral particles DNA. wt, 1.5-mer HBV env$^-$; SC-5′, stop codon in the 5′ copy of core; SC-3′ in the 3′ copy of the core; D2-3′, DBS2 mutation in the 3′ copy of core; SC/D2-3′, stop codon and DBS2 mutations in the 3′ copy of the core. RC, relaxed circular DNA; ssDNA, HBV minus-strand single-stranded DNA.

The only one of the several potential p65 interaction sites in pgRNA that is documented to be essential in *cis* is the 5′ copy of UBS. Lesions here appear to block plus-strand DNA synthesis, and detailed examination of relevant mutations suggests that the block is at the level of the generation or elongation of the initial RNA primer. The phenotype of the UBS-5′ mutations is novel. Although mutations 3′ to the primer cleavage site have been shown to influence plus-strand DNA synthesis (32), in all prior cases the result was increased in situ priming, at the expense of primer translocation. The present mutations are the first *cis*-acting lesions of which we are aware in which production of all forms of duplex viral DNA is inhibited.

Is this phenotype due to impaired binding of p65? Our data do not answer this question with certainty. On the one hand, a good correlation is observed between impairment of p65 binding and impaired plus-strand priming (though only a limited number of mutations could be examined for this phenotype, since nucleotides immediately flanking the central core of UBS appear to play roles in earlier steps of replication). This correlation favors the involvement of p65 in this process; for example, p65 might be involved in optimally positioning the RNase H activity on the template or in stabilizing the newly made primer for elongation. On the other hand, the 5′ UBS site that generates this phenotype appears to be absent from the related ground squirrel hepatitis virus genome (23); given that the replication pathways of HBV and ground squirrel hepatitis virus proceed through identical intermediates, it seems a likely presumption that these intermediates are generated by similar mechanisms. If this is so, it would argue against a role for p65 in this process and suggest instead that the phenotype is due to alterations in the RNA structure that impair either distal minus-strand synthesis or primer generation. Clearly, further detailed investigation of this issue is required. Given our present results, it is unlikely that further mutational studies will shed more light on this matter; rather, identification and cloning of p65 may be required to provide the next important clue.

Lesions in DBS have strong phenotypes with respect to viral replication, but in no case do these appear to be due to *cis*-acting effects. Rather, the effects of these mutations appear to result from their translation into mutant C proteins that display potent dominant negative phenotypes. At low concentrations these proteins generate mutant capsids in which DNA synthesis (notably plus-strand synthesis) is disrupted, despite apparently normal levels of RNA encapsidation. This result further affirms that capsid structure influences viral DNA replication, as initially indicated by studies of Yu and Summers (39), who identified C-protein mutants that were assembly competent but also displayed defects in plus-strand synthesis. As the ratios of mutant to wt C proteins rise, the effects on viral replication become more profound, progressively inhibiting the synthesis of both DNA strands and RNA encapsidation. It is likely that at high concentrations of mutant C proteins, capsid assembly is completely disrupted, though we cannot exclude that apparently normal core particles are formed but are specifically unable to encapsidate RNA. Further biophysical studies of these mutant C proteins will be required to pinpoint the exact nature of the defect in core assembly at the different mutant/wt ratios. Recent progress in defining the structure of the viral capsid (2a) as well as the structure of the C protein dimer assembly unit (10a) should inform such studies.

Our results also provide an important cautionary note regarding the use of overlength HBV constructs in the study of viral replication. It is generally assumed that in such constructs the 3′ C gene cannot be expressed, as the polyadenylation signal for pgRNA is located within this gene. However, our studies clearly document that low levels of expression from this coding region do indeed occur. Potent dominant negative mutations can be generated adventitiously by mutagenesis of presumed *cis*-acting elements in this region, and their level of expression suffices to generate major phenotypes on viral replication. Future mutational studies of DNA sequences in this region should be conducted in the context of null mutations in the 3′ ORF C.

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