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Mutations in the ε Sequences of Human Hepatitis B Virus Affect both RNA Encapsidation and Reverse Transcription

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Hepadnaviruses replicate by reverse transcription of an RNA intermediate within subviral core particles in the cytoplasm of infected hepatocytes. Recognition of the ε encapsidation signal located on the 5′ end of the pregenomic RNA by the viral polymerase occurs early in core particle assembly. The ε sequences contain a set of nested inverted repeats which form a stable stem-loop structure shown to play a role in RNA packaging and recently implicated as the site of initiation of minus-strand DNA synthesis. We have introduced a variety of site-directed mutations into the ε sequences of human hepatitis B virus to study their effects on viral replication in transfected HuH7 cells. We have identified two classes of mutations: those which adversely affect packaging and those which package RNA but adversely affect DNA synthesis. Analysis of these mutants has allowed us to identify separate features of the ε *cis*-acting signal which function in the processes of RNA packaging and reverse transcription.

Human hepatitis B virus (HBV) is a member of the hepadnavirus family of small enveloped animal viruses all characterized by a pronounced liver tropism and narrow species specificity. Hepadnaviral replication is carried out by reverse transcription of an RNA intermediate within subviral core particles assembled in infected hepatocytes (21, 23). Core particle assembly involves the interactions of the structural proteins, core (C) and polymerase (P), with the pregenomic RNA (pgRNA). pgRNA serves as the template for reverse transcription, as well as for translation of the C and P proteins. The incorporation of P protein and pgRNA into assembling core particles is mutually dependent (3), and the argument for the central role of the P protein in RNA packaging has been supported by several genetic studies (2, 8, 9, 14, 26). Although C protein domains involved in self-assembly and RNA packaging have been defined (13, 22, 28), the interactions of P and pgRNA with C protein have not been further elucidated.

In HBV, the *cis*-acting signal for encapsidation, termed ε, has been defined by a region of 85 nucleotides (nt) near the 5′ end of pgRNA which is sufficient to direct packaging of foreign RNA sequences into viral core particles (16). Deletion analyses of the related duck hepatitis B virus (DHBV) have identified a similar *cis*-acting packaging domain at the 5′ end of pgRNA but showed that additional sequences near the middle of pgRNA are required for RNA encapsidation (5, 15). The 5′ packaging regions of both HBV and DHBV contain several nested inverted repeats with the capacity to form a bulged stem-loop structure (Fig. 1) (16). Structural probing analyses with single strand- and double strand-specific nucleases have confirmed the presence of this folded structure, including the base-paired regions shown in Fig. 1, in packaged HBV RNA (17, 24). Furthermore, genetic studies using a heterologous RNA packaging system have shown that major disruptions of this structure in HBV interfere with encapsidation (17, 24). The ε sequences are located within the terminal redundancy on pgRNA and are present at both the 5′ and 3′ ends of the molecule. Mutations in the 3′ homologous sequences have no effect on RNA packaging (15), and it is not clear what distinguishes the 5′ sequences as the signal for encapsidation. Moreover, some debate continues about the possible requirement in HBV packaging for sequences adjacent to the ε stem-loop (3, 10, 16, 24).

Our current understanding of reverse transcription in hepadnaviruses is far from complete. Initiation of DNA replication occurs through a novel mechanism in which the polymerase itself acts as a primer for the minus strand by forming a covalent linkage with the first nucleotide of the DNA (34, 36). The 5′ end of the minus strand has been mapped to a location within an 11- to 12-nt direct repeat sequence present at both the 5′ and 3′ ends of the terminally redundant pgRNA, termed DR1 (20, 29, 38). Mutational analyses of woodchuck hepatitis virus (WHV) have identified the 3′ copy of DR1 as the apparent site of minus-strand initiation (31). However, studies on DHBV P protein expressed in vitro have suggested that the priming of minus-strand DNA occurs on template sequences within the bulge region of the ε stem-loop, producing a short DNA oligomer covalently bound to P. Following initiation, the nascent DNA is apparently dissociated from its template in ε and translocated to complementary sequences in the 3′ DR1 copy (35, 37). Mutational analysis of the DHBV stem-loop has shown a strong correlation between the sequence requirements for in vivo RNA packaging and in vitro DNA priming (25) and has suggested that both of these processes depend upon interactions between the P protein and the RNA structure. Although it has been presumed that replication in HBV proceeds via a similar priming mechanism, this question has not been directly addressed for HBV.

We have introduced a variety of site-directed mutations into the ε sequences of HBV to investigate the functional requirements of the region for viral replication. The phenotypes of the viruses carrying these mutations fell into two classes: those which affected pgRNA encapsidation and those which affected DNA replication. The range of mutations which adversely affected RNA packaging suggests that this process is extremely sensitive to changes, not only in secondary structure but also in primary sequences of ε. The second class of mutations did not affect packaging but resulted in a severe defect in DNA synthesis, thus providing the first evidence that the ε sequences in

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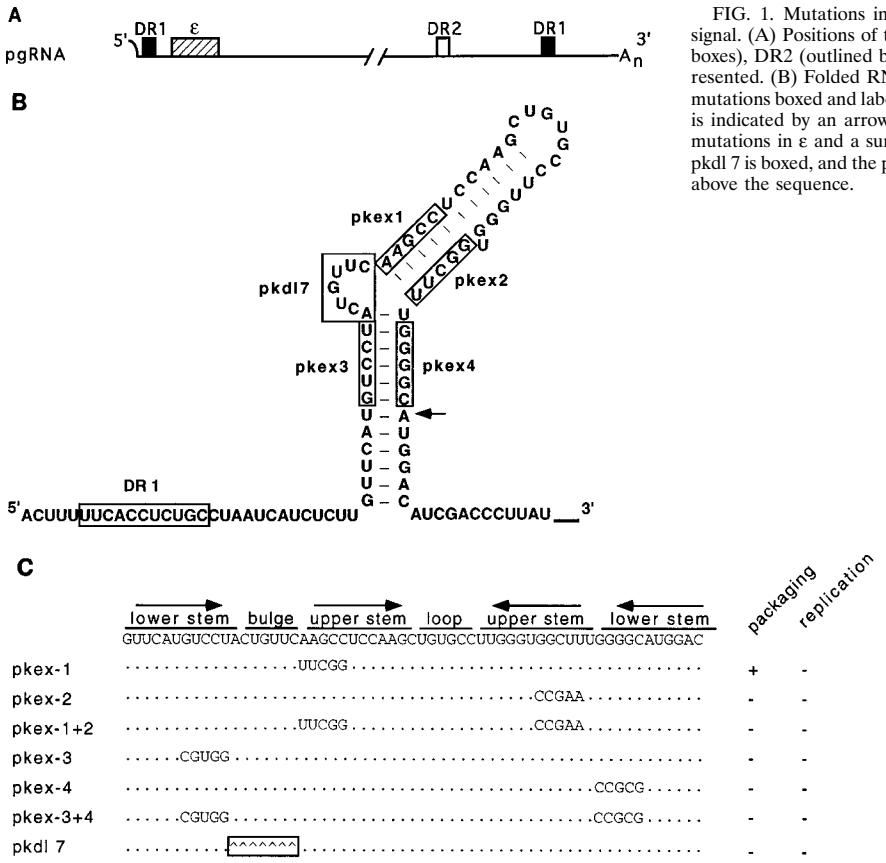

FIG. 1. Mutations in the stem-loop structure of the HBV ε encapsidation signal. (A) Positions of the *cis*-acting signals on pgRNA. DR1 sequences (solid boxes), DR2 (outlined box), and the ε packaging signal (hatched box) are represented. (B) Folded RNA structure of the ε sequence, with the regions of the mutations boxed and labelled. The start site for translation of the C gene product is indicated by an arrow. (C) Nucleotide sequences and positions of the stem mutations in ε and a summary of the mutant phenotypes. The 7-nt deletion in pkdl 7 is boxed, and the positions of the inverted repeats are shown by the arrows above the sequence.

HBV play a critical role in reverse transcription. Analysis of these mutants has additionally allowed us to identify separate features of the ε *cis*-acting signal which function in the processes of RNA packaging and DNA synthesis.

MATERIALS AND METHODS

Cloned DNAs and DNA constructions. Plasmid pTHBV, which contains two head-to-tail copies of the HBV genome, subtype ayw, was a gift from G. Acs (11). pCMV-1 was a gift from C. Seeger and contains a hybrid genome consisting of the *Fsp*I (nt 1804)-to-*Bst*XI (nt 2829) fragment of HBV contiguous with the *Bst*XI (nt 1022)-to-*Sph*I (nt 3302) portion of WHV under the control of the cytomegalovirus (CMV) immediate-early promoter. pBluescript (KS−) and M13mp18 phage were obtained from Stratagene. All HBV sequences in the DNA constructions were derived from pTHBV, and nucleotide positions were numbered beginning with the unique *Eco*RI site as position 1.

Cloning manipulations were carried out by standard methods (27). pBShbv NNB contains an overlength HBV genome consisting of an *Nco*I (nt 1370)-digested full-length HBV genome and the *Nco*I (nt 1370)-to-*Bgl*II (nt 1986) fragment of HBV cloned into pBluescript (KS−). pCMVhbv was constructed by replacing the *Bst*XI (nt 2829)-to-*Hin*dIII WHV sequences of pCMV-1 with the *Bst*XI (nt 2829)-to-*Sac*I fragment of pBShbvNNB containing the corresponding HBV sequences. The core-minus HBV variant, pBShbvNNB C−, was constructed from pBShbvNNB by linearization at the *Bgl*II site (nt 1986), blunting of the ends with the Klenow fragment of DNA polymerase 1, and religation; these steps create a frameshift in the core open reading frame, leading to premature termination at codon 46. pBSCMVNBs contains the 735-nt *Nco*I-to-*Bsp*EI fragment of pCMVhbv cloned into the polylinker of pBluescript (KS−). M13CMVhbv was made by placing the *Nco*I fragment of pCMVhbv, which carries a portion of the CMV promoter sequences and the 5′ end of the HBV genome, into an *Nco*I linker in the *Sma*I site of M13mp18. Mutations in M13CMVhbv were created by standard techniques of oligonucleotide-mediated site-directed mutagenesis (18).

Cell culture. HuH7 human hepatoma cells were cultured in minimal essential medium (GIBCO Laboratories) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimal essential medium nonessential amino acids, and 10% fetal bovine serum. For each transfection, 10 μg of plasmid DNA was introduced into HuH7 cells in a 10-cm-diameter dish by the calcium phosphate procedure described by Chen and Okayama (7), with reagents from Stratagene. Mock transfection mixtures contained calcium phosphate precipitates with no added DNA.

Isolation of core particles. Four to five days after transfection, cells were lysed in 6 ml of 1% Nonidet P-40–50 mM Tris (pH 7.4)–50 mM NaCl–200 mM KCl–1 mM EDTA–0.01% 2-mercaptoethanol. The lysates were cleared of cellular debris by centrifugation in an SW-41 rotor at 10,000 rpm for 40 min and were pelleted over a 4-ml cushion of 25% sucrose, 20 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA at 25,000 rpm for 4 h. The pellets were resuspended in 1% Nonidet P-40 and 50 mM Tris (pH 7.4).

Endogenous polymerase assay. Core particles obtained from one-half dish of transfected cells were used for each experiment. The reaction conditions for the endogenous polymerase assay were based on a published procedure (6). Labelled DNA products were separated on a 1% agarose gel and were viewed by autoradiography.

Isolation of total RNA. Total RNA was prepared from transfected cells by a standard guanidinium thiocyanate lysis procedure (1). To eliminate possible plasmid DNA contamination, resuspended RNAs were digested with 10 U of RNase-free DNase I in 5 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 50 μg of acetylated bovine serum albumin per ml at 37°C for 15 min, phenol-chloroform extracted, and ethanol precipitated. Each sample was digested with DNase I nuclease twice, and the quality of the RNAs was assessed on formaldehyde-agarose gels.

Isolation of encapsidated (core) RNA. Four to five days after transfection, two dishes of transfected cells were lysed and pooled and core particles were harvested by centrifugation as described above. The pellets were resuspended in 400 μl of 25 mM Tris (pH 7.4)–0.5% Nonidet P-40–8 mM $CaCl_2$–10 mM $MgCl_2$ before treatment with 24 U of S7 micrococcal nuclease and 10 U of DNase I for 30 min at 37°C. Sodium dodecyl sulfate, EDTA, and proteinase K were added to final concentrations of 1%, 20 mM, and 200 μg/ml, respectively, and the incubation continued for 2 h at 37°C, followed by phenol and chloroform extractions and ethanol precipitation. The DNase I digestions were repeated once.

In vitro synthesis of RNA probes. pBSCMVNBs DNA was purified on cesium chloride gradients and linearized with *Hin*dIII to provide templates for in vitro transcription by T7 RNA polymerase. High-specific-activity radiolabelled RNA probes were synthesized according to a published protocol (4). The integrity of RNA probes was assessed on denaturing polyacrylamide gels.

RNase protection analysis. RNase protections were performed according to a published procedure (1). Briefly, samples of total RNA (30 μg) or core RNA (from the equivalent of one dish of transfected cells) were annealed with 3×10^5 cpm of ^{32}P-labelled probe at 45°C overnight. RNase digestions were carried out with a mixture of RNase A and RNase T_1 (Ambion Corp.) at final concentrations of 2.5 μg/ml and 50 U/ml, respectively. The digested products were separated on a 6% denaturing polyacrylamide gel for analysis by autoradiography. Undigested labelled probe was included as a size marker.

RESULTS

Analysis of mutations which disrupt the stem-loop structure of ε. To examine the *cis*-acting elements in the HBV ε encapsidation signal, a series of mutations was made within the conserved stem-loop (Fig. 1). In mutants pkex-1 and pkex-2, five consecutive nucleotides were altered on the left and right sides, respectively, of the upper stem. Each mutation alone abolishes base pairing in the upper stem, while the double mutation pkex-1+2 restores the formation of the stem in the presence of the altered sequences. Similarly, the 5-nt exchanges in the pkex-3 and pkex-4 mutations disrupt base pairing in the lower stem which is restored in the double mutation pkex-3+4 (Fig. 1). In addition to these sequence exchanges, a 7-nt deletion of the bulge sequences, pkdl 7, was made (Fig. 1). Each mutation was transferred into the 5′ copy of the ε sequences of pCMVhbv, which allows expression of a replication-competent HBV genome under the control of the CMV immediate-early promoter. DNA sequencing of the final plasmid constructions confirmed the presence of the mutations.

Mutant viral genomes were introduced into HuH7 cells by calcium phosphate-mediated transfections; 4 days later, viral cores were isolated from the transfected cell lysates and were assayed for DNA replication in endogenous polymerase reactions. As shown in Fig. 2 in the lanes marked HBV, wild-type genomes yielded the expected relaxed circular and linear products in these assays. In contrast, no detectable replicative intermediates were produced by the mutants. The slight smearing in the lanes marked pkex-1+2 and pkdl 7 was not consistently present. These results suggest that an early step in viral replication has been interrupted in the mutants.

To ensure that the observed defects were not simply the result of impaired C protein synthesis, mutant genomes were tested for their abilities to complement a core-minus defect in cotransfection experiments. As expected, the core-minus genome pBShbvNNB C− was unable to replicate. However, in cotransfections of pBShbvNNB C− with each of the mutants, the levels of labelled DNA products were similar to those of the wild type, indicating no impairment of C protein synthesis.

Primary sequences within ε are required for RNA encapsidation. The inabilities of the mutants to produce viral replicative intermediates could be due to a defect in RNA packaging or DNA synthesis. The efficiency of RNA packaging was determined in RNase protection assays using radiolabelled antisense RNA probes prepared by in vitro transcription of pBSCMVNBs linearized with *Hin*dIII (Fig. 3A). Total RNAs were prepared from cells transfected with the mutant genomes by lysis in guanidinium thiocyanate, and encapsidated RNAs were prepared from core particles harvested by centrifugation. The isolated RNAs were incubated with labelled probe under annealing conditions prior to digestion with RNase T_1 and RNase A. The 735-nt antisense probe which includes CMV promoter sequences was predicted to protect a 527-nt portion of the 5′ end of pgRNA. As shown in Fig. 3, a protected species of the expected size was present in total RNA preparations from each of the mutants. The protected fragments in the mutants were slightly smaller than the wild-type fragments as a result of the presence of mismatched bases at the position of each mutation.

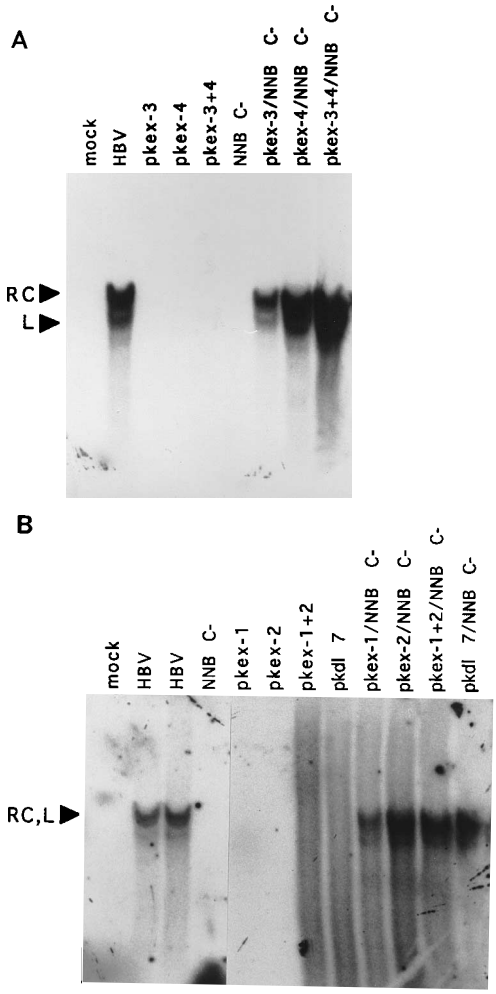

FIG. 2. Viral replication of stem mutants. Viral genomes carrying the mutant sequences indicated above the lanes were transfected into HuH7 cells. Core particles were harvested from cell lysates after 4 days and were assayed in endogenous polymerase reactions. (A and B) Results with mutants in the lower stem (A) and mutants in the upper stem and bulge (B). pBShbvNNB C− (NNB C−) contains a frameshift in the C open reading frame that terminates prematurely at codon 46. RC, relaxed circular DNA; L, linear duplex DNA; mock, mock transfection. The reaction products are indicated by arrowheads.

In the lower-stem mutants, neither single mutants pkex-3 and pkex-4 nor double mutant pkex-3+4 showed evidence of encapsidated RNA, indicating that base pairing in the lower stem was not sufficient for RNA packaging (Fig. 3B). Therefore, at least some of the primary sequences which have been altered in these mutants are necessary for the recognition of ε and encapsidation of pgRNA. In the analysis of the mutations in the upper stem, the results were somewhat different (Fig. 3C). Although RNA was not found in core particles of pkex-2 and pkex-1+2, RNA encapsidation in pkex-1 was unaffected. We conclude that base pairing in the lower portion of the upper stem is not required for encapsidation. Rather, some or all of the primary sequences altered in pkex-2 are evidently required for RNA packaging in HBV. Furthermore, the defect observed in the endogenous polymerase analysis of pkex-1 suggests that some later stage of viral replication requires either specific sequences on the left side of the upper stem or

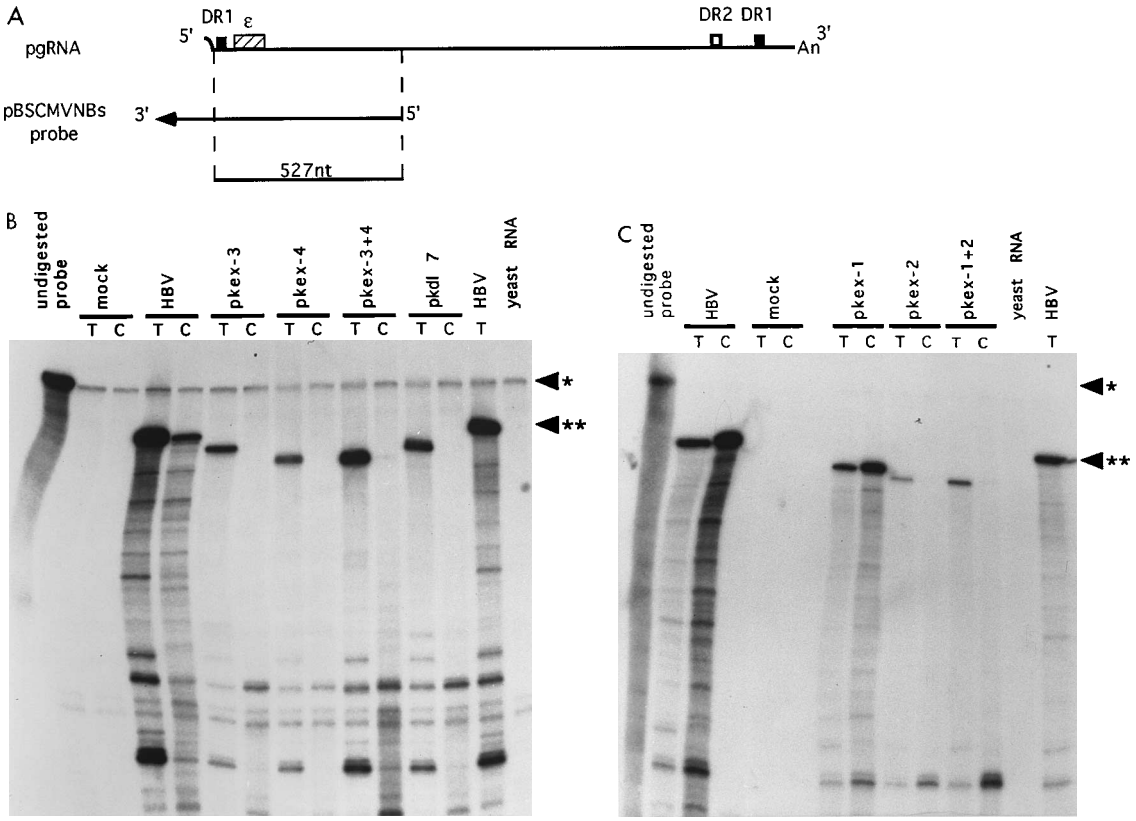

FIG. 3. RNase protection analysis of stem mutants. RNAs were prepared from total transfected cell lysates or from core particles isolated from transfected cell lysates. (A) Diagram of pgRNA showing the position of the probe. The protected species of the wild-type RNA is indicated by the line at the bottom. The symbols used are as described in the legend for Fig. 1. (B) Analysis of lower-stem and bulge mutants and (C) analysis of upper stem mutants. T, total cellular RNA; C, encapsidated RNA; *, position of the 735-nt undigested RNA probe; **, position of the wild-type 527-nt protected species. mock, mock transfection.

the presence of the stem. In addition to mutations in the stem portions of ε, we analyzed a mutant missing 7 nt in the bulge region. RNase protection analysis of this mutant, pkdl 7, revealed complete abrogation of RNA packaging (Fig. 3B). Thus, the presence of the unpaired bulge sequences is essential for packaging.

Point mutations within the unpaired bulge sequences abrogate DNA replication but not RNA encapsidation. Recent studies of DHBV have indicated that minus-strand initiation occurs within the bulge sequences of ε (35, 37). The short oligomer of DNA which is formed is then translocated to complementary sequences in the 3′ DR1. In the minus-strand DNA of HBV, the first 3 nt attached to the primer are 3′-AAG-5′ (38). This short sequence is complementary to the 3′ portion of the ε bulge, 5′-CUGUUC-3′, which could be used as a template for priming. To examine the role of the ε bulge sequences in viral replication, a set of these ε-bulge mutations was made, by altering one or two bases in the upper portion of the bulge (Fig. 4A). Each mutation was cloned into the 5′ ε sequences of pCMVhbv for analysis. Endogenous polymerase reactions of core particles from cells transfected with the mutants revealed severe replication defects (Fig. 4B and C). In ε-A, the level of DNA synthesis was reduced to less than 10% of the wild-type level, with a small amount of linear duplex DNA and no relaxed circles produced. The predominant species of DNA synthesized was represented by a small band which may correspond to the single-strand DNA form. Although the overall reduction in DNA synthesis might have been predicted, the effect on plus-strand synthesis evidenced by the absence of relaxed circles was surprising. Similarly, the ε-G mutant produced only linear and single-strand DNA forms, in barely detectable amounts. DNA synthesis in ε-AG was completely undetectable.

The bulge mutants were next examined for their abilities to package viral RNA. None of these mutants displayed a reduction in pgRNA encapsidation in RNase protection analyses (Fig. 4D). The results indicate that these altered ε sequences, while having no effect on RNA packaging, nonetheless play a critical role in reverse transcription. By analogy to the DHBV model, the phenotypes of these mutations can be explained as a defect in translocation of the primer from ε to DR1 and differences among the mutants may reflect a low tolerance for the mismatched bases in ε-A and ε-G.

To test the requirement for homology between ε and DR1 in viral replication, the same sequence alterations made in ε-A and ε-G were placed into the 3′ DR1 of pCMVhbv. Viral cores produced by genomes with the single mutations DR1-A and DR1-G or double mutations ε/DR1-A and ε/DR1-G were assayed for endogenous polymerase activity (Fig. 4C). Surprisingly, mutants DR1-A and DR1-G were more severely affected than ε-A and ε-G and produced no labelled DNA. If the ε-bulge mutants were defective in translocation of a nascent minus strand to noncomplementary wild-type DR1 sequences, then we expected to see a similar phenotype in the reciprocal situation in which wild-type ε sequences were translocated to a mutant DR1. Moreover, double mutants ε/DR1-A and ε/DR1-G showed no evidence of viral replication, which indicates that the presence of complementary sequences in the

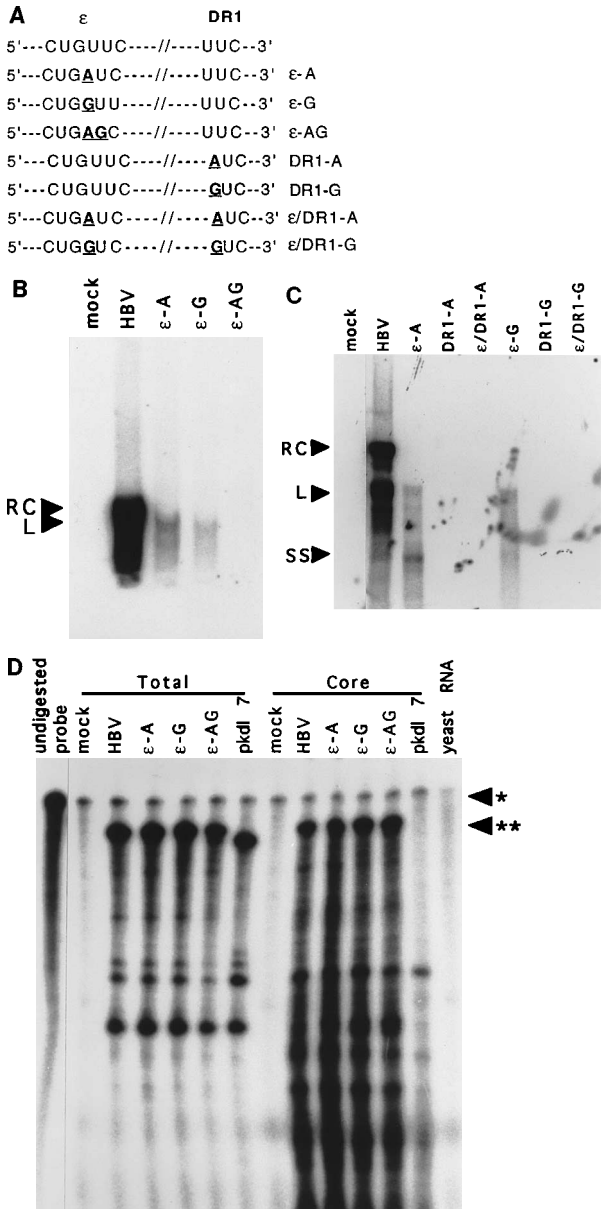

FIG. 4. Analysis of the ε bulge and DR1 mutations. (A) Sequences of the ε and DR1 point mutations. The point mutations are indicated by underlined letters in boldface type. (B and C) Endogenous polymerase assays of intracellular core particles isolated from HuH7 cells transfected with the ε (panel B) and ε and DR1 (panel C) mutant genomes indicated above the lanes. RC, relaxed circular DNA; L, linear duplex DNA; SS, single-strand minus-strand DNA. (D) RNase protections of total and encapsidated (core) RNAs from cells transfected with the mutant genomes. *, position of the 735-nt undigested probe; **, size of the 527-nt protected species (see Fig. 3). mock, mock transfection.

ε-primed minus strand and the 3′ DR1 is not sufficient to allow DNA replication. On the basis of these results, it is clear that specific sequences in the ε bulge and in the 3′ DR1 are critical for the process of minus-strand synthesis.

DISCUSSION

A stem-loop structure at the 5′ end of pgRNA has been shown to play a critical role in RNA encapsidation in both HBV and DHBV (17, 24, 25) and to be involved in minus-strand DNA synthesis in DHBV (35, 37). The presence of this structure in packaged HBV RNA has been confirmed in previously reported structural probing analyses (17, 24). To assess the essential features of this folded structure, we have introduced a variety of mutations into this region of the HBV genome, termed ε, and have analyzed their effects on viral replication. Two classes of mutant phenotypes were distinguished: those which were unable to package pgRNA and those which packaged RNA but were defective in DNA synthesis. The implications of these findings are discussed below.

Analysis of the mutations in the upper and lower stems of the ε stem-loop and of the 7-nt deletion mutant suggests that the process of pgRNA encapsidation is extremely sensitive to changes in both primary sequences and potential secondary structure in this region. Disruption of the lower stem in pkex-3 and pkex-4 completely abrogated RNA packaging, consistent with the results of similar mutations reported by Pollack and Ganem (24). However, they reported that a compensatory double mutation in the lower stem restored RNA encapsidation to nearly wild-type levels, while our compensatory double mutant, pkex-3+4, was not packaged. The discrepancy in these results may be accounted for by the differences in the two sets of mutants. The mutations of Pollack and Ganem were 4-base substitutions, whereas ours were 5-base substitutions; and the specific sequence alterations made were not the same in both experiments. Either the additional bases altered in our mutants are essential for packaging or else the sequences which we have introduced more drastically interfere with recognition of ε than the mutations of Pollack and Ganem. We conclude that, in addition to lower-stem base pairing, primary sequences in this region contribute significantly to the *cis*-acting signal in RNA packaging.

Neither of the mutants carrying altered sequences on the right side of the upper stem, pkex-2 and pkex-1+2, was able to package RNA, but pkex-1 pgRNA was encapsidated with wild-type efficiency. On the basis of these results, it is apparent that base pairing in the lower portion of the upper stem is not necessary but primary sequences in the right upper stem are essential for RNA encapsidation. Thus, although the sequences altered in pkex-1 and pkex-2 are normally paired (17, 24), base pairing in this region of the RNA is not required for packaging. Similar findings were reported by Pollack and Ganem (24), who also showed that efficient packaging requires base pairing in the upper portion of the upper stem adjacent to the loop. The phenotypes of pkex-1, pkex-2, and pkex-1+2 have allowed us additionally to identify separate features of the *cis*-acting signal in the processes of RNA packaging and DNA synthesis. Although upper stem formation and the 5 nt encompassed by pkex-1 are completely expendable in packaging, at least some of these features are essential for reverse transcription.

The replication defect in pkex-1 provides the first evidence that the ε encapsidation signal in HBV plays a critical role in reverse transcription. This observation is consistent with a mechanism of replication similar to that of DHBV, in which the minus strand initiates within the bulge sequences of the ε stem-loop and translocates to DR1 (35, 37). If priming occurs at ε and the template for minus-strand initiation is provided by the unpaired nucleotides immediately adjacent to the upper stem, it is likely that base pairing in this region may be important for positioning of the polymerase at the appropriate site. It is equally conceivable that the specific sequences altered in pkex-1 are required for reverse transcription. Our results do not completely rule out the more exotic possibility that minus-strand priming in HBV occurs at the 3′ DR1 and that sequences of ε are necessary for positioning the polymerase at

the appropriate initiation site, for example, through RNA-RNA interactions bringing together the two ends of pgRNA or through altering the structure of P and allowing it to recognize a *cis*-acting signal at the 3′ end of pgRNA.

Additional evidence of ε-mediated minus-strand priming in HBV was provided by examination of bulge mutants ε-A, ε-G, and ε-AG. Our results with pkdl 7 and with these bulge mutants show that, consistent with previous reports, although the presence of the unpaired bases is required for RNA encapsidation, specific sequences are not critical for this function (17, 24). Moreover, the severe replication defects in the ε-bulge mutants point to an essential role for these sequences in DNA synthesis. A previous report (17) described two mutants in the ε bulge of HBV which were completely replication competent, albeit at reduced efficiencies. In the ε packaging signal of HBV, the three nucleotides in the bulge which are complementary to the 5′ end of minus-strand DNA are 5′-UUC-3′. The mutations described by Knaus and Nassal (17) were all conservative pyrimidine-to-pyrimidine changes at these positions and may not disrupt pairing of the 3′ end of the primer DNA after translocation. Our mutations were all purine substitutions, and the mismatched base pairing which would result from translocation of our mutant sequences to DR1 may not be as well tolerated by the polymerase.

While the level of overall DNA synthesis was profoundly reduced, an interesting aspect of the ε-A and ε-G phenotypes was the selective loss of relaxed circular DNA. This result suggests that plus-strand DNA synthesis was adversely affected in the mutants. The plus-strand primer is derived from a short oligoribonucleotide located at the 5′ end of pgRNA which is cleaved by RNase H and translocated to the DR2 position on minus-strand DNA (19, 30, 33). Relaxed circles are then formed by elongation of the plus strand through a short terminal redundancy on the minus strand which enables plus-strand jumping. One possible explanation for these results is that the minus-strand DNAs produced by ε-A and ε-G do not contain the terminal redundancies required for plus-strand jumping. Such an effect could result from the translocation of a nascent minus strand from the ε region to an inappropriate position on the RNA, or from the impairment of signals responsible for positioning of the polymerase correctly at the 3′ DR1. Deletion analyses of WHV and DHBV have shown that, in the absence of 3′ DR1 sequences, minus-strand DNA synthesis from a variety of aberrant positions located at the 3′ end of the pgRNA can still occur (12, 32). Alternatively, it is possible that our mutations have in some way interfered with translocation of the plus-strand primer to DR2.

The phenotypes of the ε bulge and DR1 mutants are consistent with a model of reverse transcription in which the template for the initiation of minus-strand synthesis is provided by sequences in the 5′ ε bulge region (35, 37). According to this model, the defects in both the ε and DR1 mutants likely reflect problems in translocation of the primer resulting from noncomplementarity between the sequences of the ε-primed DNA and the DR1 site on pgRNA. The inabilities of double mutants ε/DR1-A and ε/DR1-G to produce replicative intermediates indicate that complementarity between ε and DR1 is not sufficient to allow DNA synthesis. Viewed in the context of the ε-mediated priming model, these results and the differences between the ε and DR1 mutants imply that the process of primer translocation depends upon the presence of specific sequences in these two regions of the pgRNA. Analyses of the effects of similar ε/DR1 compensatory double mutations on DHBV replication in vivo show significantly reduced efficiencies of DNA synthesis relative to that of the wild type (35, 37). One possible explanation is that sequences in either DR1 or ε serve an additional function as a recognition signal necessary for translocation of the primer. It is interesting that, in contrast to the results of studies of WHV and DHBV (12, 32, 35, 37), HBV genomes carrying single mutations in the 3′ DR1 sequences were not able to synthesize DNA. This observation suggests that the signals required for reverse transcription to proceed may be more stringent in HBV than in other hepadnaviral variants.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. Wiley-Interscience, New York.
2. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64:**5324–5332.
3. **Bartenschlager, R., and H. Schaller.** 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. **11:**3413–3420.
4. **Berkowitz, R. D., J. Luban, and S. P. Goff.** 1993. Specific binding of human immunodeficiency virus type 1 *gag* polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. J. Virol. **67:**7190–7200.
5. **Calvert, J., and J. Summers.** 1994. Two regions of an avian hepadnavirus RNA pregenome are required in *cis* for encapsidation. J. Virol. **68:**2084–2090.
6. **Chang, C., K. Jeng, C. Hu, S. J. Lo, T. Su, L.-P. Ting, C.-K. Chou, S. Han, E. Pfaff, J. Salfeld, and H. Schaller.** 1987. Production of hepatitis B virus *in vitro* by transient expression of cloned HBV DNA in a hepatoma cell line. EMBO J. **6:**675–680.
7. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
8. **Chen, Y., W. S. Robinson, and P. Marion.** 1992. Naturally occurring point mutation in the C terminus of the polymerase gene prevents duck hepatitis B virus RNA packaging. J. Virol. **66:**1282–1287.
9. **Chen, Y., W. S. Robinson, and P. L. Marion.** 1994. Selected mutations of the duck hepatitis B virus P gene RNase H domain affect both RNA packaging and priming of minus-strand DNA synthesis. J. Virol. **68:**5232–5238.
10. **Chiang, P.-W., K.-S. Jeng, C.-P. Hu, and C. Chang.** 1992. Characterization of a *cis* element required for packaging and replication of the human hepatitis B virus. Virology **186:**701–711.
11. **Christman, J. K., M. Gerber, P. M. Price, C. Flordellis, J. Edelman, and G. Acs.** 1982. Amplification of expression of hepatitis B surface antigen in 3T3 cells co-transfected with a dominant-acting gene and cloned viral DNA. Proc. Natl. Acad. Sci. USA **79:**1815–1819.
12. **Condreay, L. D., T.-T. Wu, C. E. Aldrich, M. A. Delaney, J. Summers, C. Seeger, and W. S. Mason.** 1992. Replication of DHBV genomes with mutations at the sites of initiation of minus- and plus-strand DNA synthesis. Virology **188:**208–216.
13. **Hatton, T., S. Zhou, and D. N. Standring.** 1992. RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their roles in viral replication. J. Virol. **66:**5232–5241.
14. **Hirsch, R. C., J. E. Lavine, L. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature (London) **344:**552–555.
15. **Hirsch, R. C., D. D. Loeb, J. R. Pollack, and D. Ganem.** 1991. *cis*-acting sequences required for encapsidation of duck hepatitis B virus pregenomic RNA. J. Virol. **65:**3309–3316.
16. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9:**3389–3396.
17. **Knaus, T., and M. Nassal.** 1993. The encapsidation signal on the hepatitis B virus RNA pregenome forms a stem-loop structure that is critical for its function. Nucleic Acids Res. **21:**3967–3975.
18. **Kunkel, T. A., J. D. Roberts, and R. A. Zabour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**376–382.
19. **Lien, J.-M., C. E. Aldrich, and W. S. Mason.** 1986. Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand

DNA synthesis. J. Virol. **57:**229–236.
20. **Lien, J.-M., D. J. Petcu, C. E. Aldrich, and W. S. Mason.** 1987. Initiation and termination of duck hepatitis B virus DNA synthesis during virus maturation. J. Virol. **61:**3832–3840.
21. **Loeb, D. D., and D. Ganem.** 1993. Reverse transcription pathway of the hepatitis B viruses, p. 329–355. *In* A. M. Skalka and S. P. Goff (ed.), Reverse transcriptase. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Nassal, M.** 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. J. Virol. **66:**4107–4116.
23. **Nassal, M., and H. Schaller.** 1993. Hepatitis B virus replication. Trends Microbiol. **1:**221–228.
24. **Pollack, J. R., and D. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
25. **Pollack, J. R., and D. Ganem.** 1994. Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. J. Virol. **68:**5579–5587.
26. **Roychoudhury, S., A. F. Faruqi, and C. Shih.** 1991. Pregenomic RNA encapsidation analysis of eleven missense and nonsense polymerase mutants of human hepatitis B virus. J. Virol. **65:**3617–3624.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Schlicht, H.-J., R. Bartenschlager, and H. Schaller.** 1989. The duck hepatitis B virus core protein contains a highly phosphorylated C terminus that is essential for replication but not for RNA packaging. J. Virol. **63:**2995–3000.
29. **Seeger, C., D. Ganem, and H. E. Varmus.** 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science **232:**477–484.
30. **Seeger, C., and J. Maragos.** 1989. Molecular analysis of the function of direct repeats and a polypurine tract for plus-strand DNA priming in woodchuck hepatitis virus. J. Virol. **63:**1907–1915.
31. **Seeger, C., and J. Maragos.** 1990. Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. J. Virol. **64:**16–23.
32. **Seeger, C., and J. Maragos.** 1991. Identification of a signal necessary for initiation of reverse transcription of the hepadnavirus genome. J. Virol. **65:**5190–5195.
33. **Staprans, S., D. D. Loeb, and D. Ganem.** 1991. Mutations affecting hepadnavirus plus-strand DNA synthesis dissociate primer cleavage from translocation and reveal the origin of linear viral DNA. J. Virol. **65:**1255–1262.
34. **Tavis, J. E., and Ganem, D.** 1993. Expression of functional hepatitis B virus polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription. Proc. Natl. Acad. Sci. USA **90:**4107–4111.
35. **Tavis, J. E., S. Perri, and D. Ganem.** 1994. Hepadnavirus reverse transcription initiates within the stem-loop of the RNA packaging signal and employs a novel strand transfer. J. Virol. **68:**3536–3543.
36. **Wang, G.-H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell **71:**1–20.
37. **Wang, G.-H., and C. Seeger.** 1993. Novel mechanism for reverse transcription in hepatitis B viruses. J. Virol. **67:**6507–6512.
38. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Bucher, R. Sprengel, R. Cattaneo, and H. Schaller.** 1987. Replication strategy of human hepatitis B virus. J. Virol. **61:**904–911.