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Mutations Affecting Hepadnavirus Plus-Strand DNA Synthesis Dissociate Primer Cleavage from Translocation and Reveal the Origin of Linear Viral DNA

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Hepadnaviruses replicate their circular DNA genomes via reverse transcription of an RNA intermediate. The initial product of reverse transcription, minus-strand DNA, contains two copies of a short direct repeat (DR) sequence, termed DR1 and DR2. Plus-strand DNA synthesis initiates at DR2 on minus-strand DNA, using as a primer a short, DR1-containing oligoribonucleotide derived by cleavage and translocation from the 5′ end of pregenomic RNA. To clarify the sequence requirements for plus-strand primer cleavage and translocation, we have constructed mutants of the duck hepatitis B virus bearing base changes in or around the DR1 sequence in the primer. A point mutation at the terminal nucleotide of DR1 has a striking phenotype: normal levels of duplex viral DNA are produced, but nearly all of the DNA is linear rather than circular. Mapping of the 5′ end of plus-strand DNA reveals that primer cleavage occurs with normal efficiency and accuracy, but the primer is not translocated to DR2; rather, it is extended in situ to generate duplex linear DNA. Other mutations just 3′ to DR1 similarly affect primer translocation, although with differing efficiencies. Linear DNA found in wild-type virus preparations has the same fine structure as the mutant linears described above. These results indicate that (i) plus-strand primer cleavage and translocation are distinct steps that can be dissociated by mutation, (ii) lesions in sequences not included in the primer can severely inhibit primer translocation, and (iii) elongation of such untranslocated primers is responsible for the variable quantities of linear DNA that are found in all hepadnaviral stocks.

Persistent infection with hepatitis B viruses (hepadnaviruses) frequently leads to chronic hepatitis and cirrhosis and is strongly associated with the development of hepatocellular carcinoma (9). The molecular mechanisms of viral persistence are now starting to be understood. Genomic persistence is mediated by the maintenance of a nuclear pool of closed circular viral DNAs that serve as templates for viral transcription; this pool is maintained by an unusual genomic replication strategy involving reverse transcription of an RNA intermediate (27, 29). While formally analogous to retroviral replication, the hepadnaviral reverse transcription pathway differs mechanistically from its retroviral counterpart at most of its important steps (14, 23, 31). Given the central role of this pathway in viral persistence, a detailed understanding of each of these steps is important to a full understanding of hepadnaviral biology. In this paper, we present an analysis of the events involved in one of these steps, the priming of plus-strand DNA synthesis, using the duck hepatitis B virus (DHBV) as an experimental system.

DHBV virion DNA is a relaxed circular species of 3.0 kb whose 5′ ends map to the region of a short direct repeat (DR) of 12 nucleotides (nt) present twice in the mature genome (14, 18). These repeats are designated DR1 and DR2. Pregenomic RNA, the template for reverse transcription, is transcribed from the genome by host RNA polymerase II. It is a terminally redundant RNA (3) that contains two copies of the viral DR1 sequence but only a single copy of DR2 (Fig. 1). This RNA is packaged into a viral core particle together with the reverse transcriptase (7, 11, 27). After encapsidation, viral minus-strand synthesis is initiated at the 3′ copy of DR1 (25), presumably by employing the viral P protein as a primer (1, 2). Elongation of this chain generates the full-length minus strand, which is actually terminally redundant by several nucleotides. During minus-strand elongation, the RNA of the resulting RNA-DNA hybrid is degraded by the RNase H activity of the P gene product (20, 27).

Viral plus-strand DNA contains at its 5′ end a capped oligoribonucleotide of 16 to 18 nt. Both biochemical and genetic experiments indicate that this RNA is derived from the 5′ end of pregenomic RNA and contains a copy of DR1 sequences (14, 23). This RNA is believed to be the primer for plus-strand synthesis. Since the RNA-DNA joints in plus strands are located near the boundary of DR2, this RNA must be cleaved near the 3′ end of DR1 and then translocated and annealed to the homologous DR2 sequences for priming to occur (Fig. 1). In this paper, we have examined some of the sequence requirements for the generation of a functional plus-strand primer. Our results indicate that the cleavage and translocation reactions can be dissociated by mutation and that RNAs that have been cleaved but not translocated can also function as primers. Elongation of such untranslocated primers generates unit-length linear DNA, a form of viral DNA that has been found in all viral stocks but whose origin has heretofore been unexplained.

MATERIALS AND METHODS

Plasmid constructions. All cloned mutated DHBV genomes were generated by oligomer-directed site-specific mutagenesis of subgenomic DNA fragments, followed by DNA sequence analysis (to exclude possible secondary mutations) and reconstruction of unit-length monomeric and dimeric genomes. Plasmid constructions were performed

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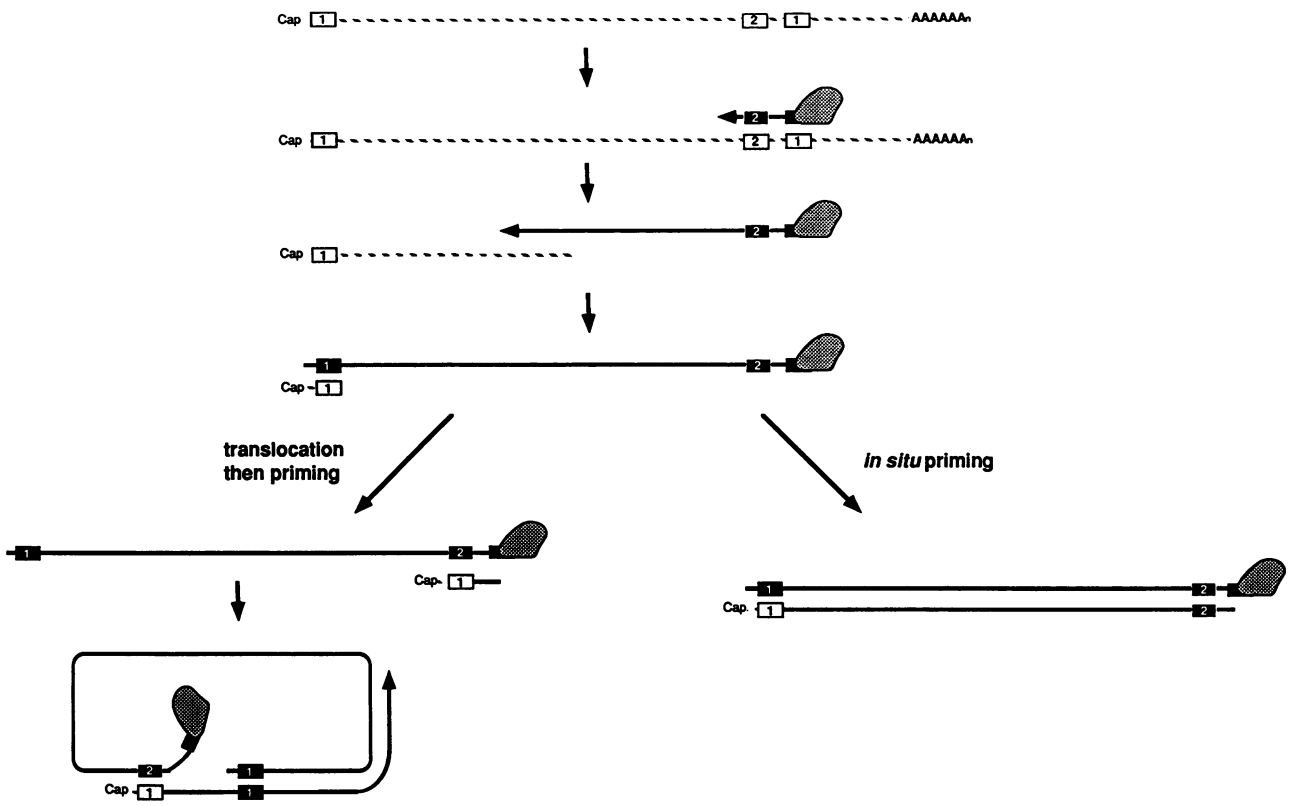

FIG. 1. The hepadnaviral replication pathway. Dashed line (row 1) indicates the terminally redundant pregenomic RNA, bearing two copies of DR1 and one copy of DR2 sequences (boxes). Minus-strand initiation (row 2) occurs in the region of the 3′ DR1 element (25) and is thought to be primed by P protein (shown as a stippled blob). During elongation of the minus strand (dark line), genomic RNA in nascent DNA-RNA hybrids is degraded by the RNase H activity (20) of P protein (row 3). After minus-strand synthesis, cleavage of the 5′ end of genomic RNA occurs at DR1, generating a short, capped RNA (row 4) that can be translocated to the DR2 region of minus-strand DNA for subsequent elongation (row 5, left) or can remain in situ and be elongated (row 5, right). If translocated and elongated, circularization of the genome can occur through a plus-strand transfer presumably mediated by the short terminal redundancy of minus-strand DNA (row 6).

with the European strain of DHBV (26). The wild-type plasmid used in these studies is a tandem dimer of the European strain of DHBV that was previously described (10, 13). Mutants DR1-12, DR2-12, DR1-13, DR1-*Pvu*, and DR1-*Xho* (Fig. 2) were obtained by mutagenesis of a 0.32-kb *Nco*I-*Xba*I (nt 2351 to 2666) subfragment of the DHBV genome, using either the method of Zoller and Smith (32) for mutant DR1-12 or the method of Kunkel et al. (12) for all other mutants. Mutant DRS-12 (Fig. 2) was derived by mutagenesis of the 0.32-kb *Nco*I-*Xba*I subfragment of mutant DR1-12 by using the method of Kunkel et al. (12).

Cell culture and transfections. LMH cells, a chicken hepatoma cell line (5), were grown in a 1:1 Dulbecco minimal essential medium-Ham nutrient mixture F12 supplemented with 10% fetal calf serum (GIBCO Laboratories) and passaged twice weekly at a 1:3 dilution. Transfections of cloned dimeric genomes were performed by the calcium phosphate coprecipitation method as described previously (10).

Isolation of viral nucleic acid. At 3 days posttransfection, cytoplasmic cores were prepared by polyethelene glycol precipitation (13) as follows. Cells from a 100-mm plate were lysed in 1 ml of 10 mM Tris (pH 7.5)-1 mM EDTA-50 mM NaCl-8% sucrose-0.25% Nonidet P-40. Nuclei were removed by centrifugation for 3 min in an Eppendorf microcentrifuge at 4°C, and the cytoplasmic extract was adjusted to 6 mM $MgCl_2$ and digested with DNase 1 (50 μg/ml) and RNase A (20 μg/ml) for 30 min at 37°C. Cores were precipitated by adding 330 μl of 26% polyethylene glycol-1.4 M NaCl-25 mM EDTA, incubating at 4°C for 30 min, and pelleting in an Eppendorf microcentrifuge for 4 min. Cores resuspended in 100 μl of 10 mM Tris (pH 7.5)-6 mM $MgCl_2$ were then redigested with DNase I for an additional 15 min at 37°C, followed by the addition of 5 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 500 μg of proteinase K (Boehringer-Mannheim) per ml and incubation for 1 h at 37°C. Core nucleic acid was extracted twice with equal volumes of phenol and $CHCl_3$ (1:1) and then once with 1 volume of $CHCl_3$. The aqueous phase was then precipitated with 2 volumes of ethanol after the addition of 10 to 20 μg of carrier yeast tRNA. The ethanol precipitate was washed with 70% ethanol, dried, and suspended in 50 μl of TE (10 mM Tris [pH 7.5]-1 mM EDTA).

Serum virion DNA was isolated as follows. DHBV-positive duck serum was clarified by low-speed centrifugation. A 4-ml volume of serum was then placed on 1 ml of a 15% sucrose solution in 20 mM Tris (pH 7.5)-150 mM NaCl. The virus was pelleted by ultracentrifugation in a Beckman SW50.1 rotor for 3 h at 47 krpm at 4°C. The resultant pellet was resuspended in 100 μl of core reaction buffer (20 mM Tris [pH 7.5]–50 mM NaCl–10 mM β-mercaptoethanol–1 mM EDTA–1% Triton X-100) overnight at 4°C. The next day, $MgCl_2$ was added to a concentration of 6 mM and DNase I was added to a final concentration of 75 μg/ml, and this mixture was incubated at 37°C for 1 h, followed by the

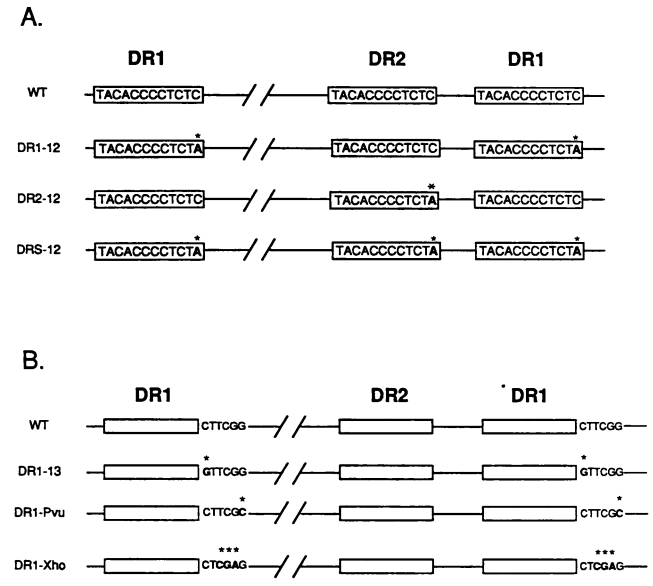

FIG. 2. Structure of the mutants affecting DR sequences or surrounding regions. (A) DR1 mutants. Wild-type (WT) DHBV pregenomic RNA is schematized at the top by a line, with the nucleotide sequences of DR1 and DR2 shown in boxes. Mutants DR1-12, DR2-12, and DRS-12 are each depicted below the wild type as they would appear in pregenomic RNA; the mutated nucleotide is indicated by boldface type and an asterisk. (B) Mutants 3′ to DR1. At top is shown wild-type (WT) DHBV pregenomic RNA, with DRs indicated as blank boxes and relevant sequences downstream of DR1 indicated. Below the wild type are depicted the mutants DR1-13, DR1-*Pvu*, and DR1-*Xho* as they would appear in pregenomic RNA, with the mutated nucleotides indicated by boldface type and asterisks.

addition of 7.5 mM EDTA, 1% SDS, and 500 μg of proteinase K (Boehringer-Mannheim) per ml and incubation for 1 h at 37°C. Phenol extraction and ethanol precipitation were performed as described above for core DNA isolation.

Southern blot analysis. DNA electrophoresis and transfer to Hybond-N (Amersham) were carried out by using standard methods (15). Prehybridization, hybridization, and washing of the filters were carried out by the methods of Church and Gilbert (4). Radiolabeled DHBV probe was prepared by using random oligonucleotide primers to prime synthesis from a gel-purified monomer of cloned DHBV DNA (21).

Primer extension analysis. For primer extension analysis, core nucleic acid was subjected to alkaline hydrolysis in 200 mM NaOH for 5 min at 95°C, which was terminated by the addition of Tris hydrochloride to a final concentration of 333 mM (24). DNA was precipitated with 2 volumes of ethanol. Pellets were washed in 70% ethanol, dried, and resuspended in 10 μl of TE for further analysis. These conditions represent limit digestion conditions: doubling both the concentration and time of hydrolysis did not change the pattern of primer extension products.

A synthetic 24-base oligonucleotide (complementary to positions 2622 to 2599 of DHBV-3) was used as the primer for primer extension analysis of the 5′ end of plus-strand DNA. 5′ End-labeling of the oligonucleotide primer with [γ-^{32}P]ATP was carried out as described by McGraw (17). Primer extension analysis was performed as described by Seeger and Maragos (24) with several modifications. A 1- to 2-ng amount of core DNA plus 400 pg of end-labeled primer in a final volume of 7 μl was incubated at 95°C for 2 min. Samples were chilled immediately on ice. This mixture was supplemented with dATP, dCTP, dGTP, and TTP (final concentration, 100 μM), 50 mM Tris (pH 8), 6 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol, and 17 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America Inc.). The final volume of the reaction mixture was 10 μl. The reaction mixture was incubated at 42°C for 1 h. Samples were then ethanol precipitated by using 2.5 M ammonium acetate as the salt, washed with 75% ethanol, vacuum dried, and then resuspended in 6 μl of formamide-dye solution. Samples were incubated at 80°C for 10 min prior to electrophoresis on a 6% polyacrylamide-8 M urea-25% formamide sequencing gel. After electrophoresis, the gel was fixed and dried; autoradiography was then performed at −70°C with an intensifying screen for 12 h to 10 days. The 5′ ends of plus DNA were determined by comparison with a sequencing ladder of cloned DHBV-3 DNA generated by priming with the same end-labeled oligonucleotide used in the primer extension analysis. Formamide was used in the gel to resolve a sequence compression that occurs at the 3′ end of DR1.

To verify that under these conditions all DNA strands were maximally extended, we carried out a control extension by using the same primer on cloned DHBV DNA cleaved at nt 2489 with *Taq*I. This generated a single extension product of the length predicted by the viral DNA sequence (not shown). To further demonstrate that the ends we visualized were not artifacts of primer extension, we also examined alkali-treated virion DNA after cleavage at the *Acc*I site in precore and 3′ end-labeling with Klenow polymerase and ^{32}P-labeled deoxynucleoside triphosphates. Sizing of the labeled products on a sequencing gel revealed three bands corresponding to molecules with the same three 5′ termini we had earlier mapped by primer extension (14a).

RESULTS

DR mutations. To examine the sequence requirements for viral plus-strand primer generation, we constructed the mutants depicted in Fig. 2. In mutant DR1-12, the 3′ nucleotide of DR1 has been changed from C to A. In mutant DR2-12, the same lesion is present only in DR2, while in mutant DRS-12, both DR1 and DR2 harbor this mutation. In all cases, the mutations were introduced into subgenomic DNA fragments; after verifying the identity of the mutations by DNA sequencing, the lesions were recombined in vitro back into unit-length and then dimeric DHBV genomes. (Details of the mutagenesis and cloning steps are summarized in Materials and Methods.) The replication of each of these mutants was then assayed by transfection into permissive LMH hepatoma cells (5) followed by Southern blotting of viral DNA within purified cytoplasmic core particles.

When wild-type DHBV DNA was assayed in this way, the expected pattern of progeny DNA forms was observed (Fig. 3): fully duplex relaxed circular DNA, unit-length linear duplex DNA, and unit-length and shorter single strands of minus-strand polarity (lane 1). Analysis of mutant DR1-12 revealed a strikingly different phenotype. Although viral minus-strand synthesis was largely undisturbed and the total amount of viral DNA synthesis was nearly normal, most of the duplex DNA was found as linear rather than relaxed circular DNA (lane 2). This phenotype was found only when the lesion was present in DR1. When the mutation was present in DR2 alone (mutant DR2-12, lane 3), the pattern of progeny DNA was similar to that of wild type. Interestingly, restoration of full DR complementarity by constructing the

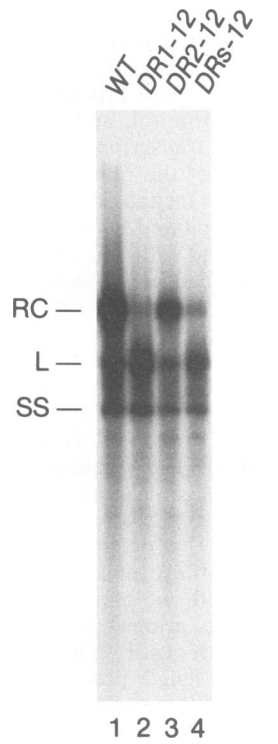

FIG. 3. Encapsidated viral DNA forms produced by wild-type or DR-mutant genomes. LMH cells were transfected with cloned dimers of wild-type DHBV DNA (lane 1) or viral DNA containing either the DR1-12 (lane 2), DR2-12 (lane 3), or DRS-12 (lane 4) mutations. Three days posttransfection, cytoplasmic viral core particles were prepared as described in Materials and Methods and their DNA was purified by proteinase K digestion and phenol-chloroform extraction. DNA isolated from equal quantities of transfected cells was electrophoresed through 1.25% agarose gels, transferred to nylon membranes, and hybridized to [^{32}P]DHBV DNA; filters were then washed and autoradiographed as described in Materials and Methods. Shown at left are the positions of the relaxed circular (RC), linear (L), and single-stranded (SS) DNA forms of DHBV.

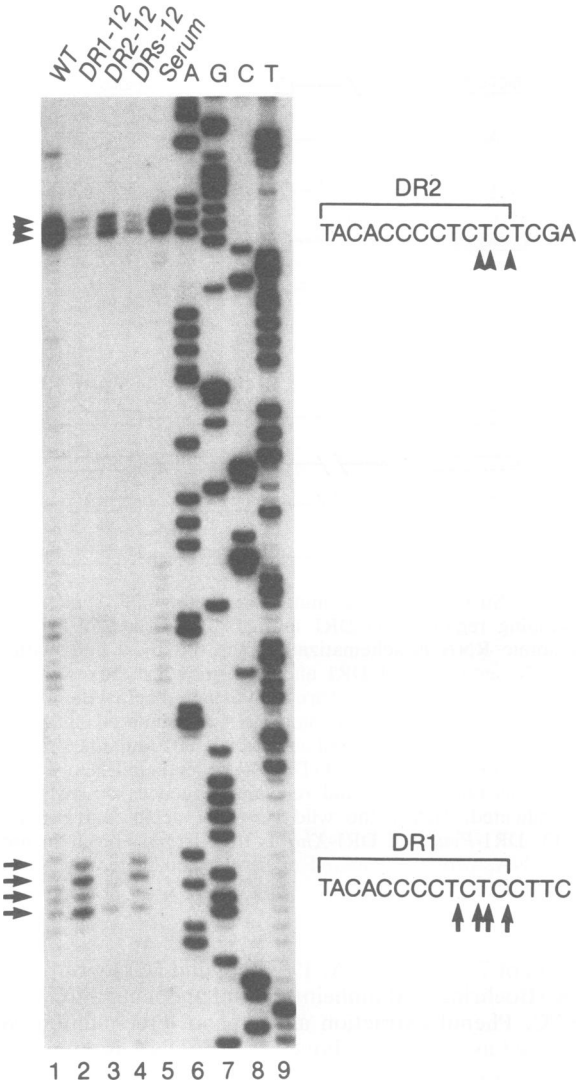

FIG. 4. Mapping of the 5′ ends of wild-type or DR-mutant plus-strand DHBV DNA. Cytoplasmic core particles were prepared from LMH cells transfected with wild-type DHBV DNA (lane 1) or with DR1-12 (lane 2), DR2-12 (lane 3), or DRS-12 (lane 4) DNAs. DNA was extracted from these preparations as described in the legend to Fig. 3, alkali treated to remove the plus-strand RNA primer, and then annealed to an excess of the 5′ end-labeled oligonucleotide primer complementary to plus-strand DNA spanning nt 2622 to 2599 in the preC region of DHBV (26). After primer extension with deoxynucleoside triphosphates and avian myeloblastosis virus polymerase, the extension products were examined by electrophoresis through 6% polyacrylamide-8 M urea-25% formamide gels and autoradiography. Lane 5 shows similar analysis of DNA extracted from DHBV virions derived from serum of a viremic duck. Lanes 6 to 9 show a DNA sequencing ladder generated from cloned DHBV DNA template by using the same primer as for primer extension. Shown at right is a schematic diagram illustrating the positions of the RNA-DNA junctions (denoted with arrows) in DHBV plus-strand DNA in conventional priming (top) or in situ priming (bottom).

double mutant DRS-12 did not correct the phenotype, but again resulted in the disproportionate production of linear DNA (lane 4).

On the basis of these observations, we speculated that the linear DNA might have arisen by the generation of abnormal RNA primers that could not be translocated. If such RNAs could be elongated from their native position, duplex linear DNA would result (Fig. 1). We shall refer to this as in situ priming. To test this idea, we employed a primer-extension method to map the 5′ end of the plus-strand DNA produced by wild-type and mutant genomes. In plus strands initiated in the conventional way (Fig. 1), the RNA-DNA junction should map to the region of DR2, while in those arising from in situ priming it should map to DR1 (Fig. 4A). Accordingly, progeny DNA was extracted from cytoplasmic core particles, alkali treated to remove the RNA primer, and annealed to a 5′ end-labeled oligonucleotide complementary to a region of plus-strand DNA beginning 53 nt 3′ to the last nucleotide of DR1. After primer extension with deoxynucleoside triphosphates and reverse transcriptase, the labeled extension product was electrophoresed on a 6% polyacrylamide-8 M urea gel alongside a DNA sequencing ladder generated by using the same primer (Fig. 4). We consistently observed that the 5′ ends of the wild-type plus-strand DNA map to a triad of sites within the 3′ three nucleotides of DR2; two of these sites correspond exactly to the 5′ ends previously identified by Lien et al. (14). In contrast, in mutant

DR1-12, 90% of the plus-strand 5′ ends map to four sites within DR1 (lane 2). This confirms that, as suspected, the majority of plus strands in this mutant arose via in situ priming. A small proportion of the plus strands in DR1-12 did map to DR2, commensurate with the small amount of relaxed circular DNA evident in the Southern blot analysis of Fig. 3. These proportions suggest that virtually all of the 5′ ends in this sample have been detected. By using alkaline gel electrophoresis and Southern blotting with probes specific for plus strands, we have detected no 5′ ends 3′ to this region, i.e., ones that might have resulted from aberrant translocation of the primer to downstream sites or from alternate initiations at downstream sites as has been described for woodchuck hepatitis virus (24) (data not shown).

Intriguingly, the fact that three of the four RNA-DNA joints in DR1-12 map to the wild-type triad of sites within the DR indicates that the cleavage reaction involved in primer generation was largely undisturbed by the mutation. Thus, the inability to translocate the primer was not due to infidelity of the cleavage reaction. Clearly, the cleavage and translocation reactions have somewhat different sequence requirements. (Further evidence for this will be presented below.) The RNA product of the most 3′ cleavage would be expected to bear the mutant base at its 3′ end; this lesion might account for its failure to be translocated. However, the other cleavages should produce RNA primers of wild-type sequence, two of which are identical in length to wild-type primers, yet these too are poorly translocated. In addition, of those primers that were translocated to DR2, there appears to be no enrichment in favor of the wild-type junctions. How can we account for this? One interpretation could be that primary RNA cleavage actually occurs only after the 3′ nucleotide of DR1; after translocation to DR2, some "nibbling" of the primer may occur prior to elongation to generate the other RNA-DNA junctions detected in the final product. Alternatively, cleavage might have occurred at all four sites prior to translocation, with the apparently normal primers failing to be transferred because the adjacent mutation in DR1 impairs translocation. In the following section, we present evidence that lesions outside the primer can affect translocation, which is consistent with the latter model.

Mutations 3′ to DR1. To further explore the sequence requirements for primer generation, we constructed additional mutations in the region of DR1. In particular, we wanted to see if any component of the cleavage or translocation machinery recognized elements outside of DR1. Accordingly, we constructed mutant DR1-13, in which the first nucleotide 3′ to DR1 has been altered, as well as mutants DR1-*Pvu* and DR1-*Xho*, in which single or multiple base changes have been engineered into the region 3′ to DR1 (Fig. 2). Each mutation was then built back into a dimer-length DHBV genome; mutant genomes were transfected into permissive hepatoma cells and assayed for encapsidated progeny DNA by Southern blotting as before (Fig. 5). Similar phenotypes resulted in all cases. Each of these lesions resembled that of DR1-12 in that they produced normal levels of free minus-strand DNA and abnormal ratios of linear to circular duplex DNA. In DR1-13 and DR1-*Xho*, however, the defects were less extreme than in DR1-12; typically, only 50 to 70% of the total duplex DNA in these mutants was linear.

The 5′ ends of the plus-strand DNAs of each of these mutants were then mapped by using the primer-extension assay described above. In each case, the extended products revealed evidence for both conventional and in situ priming

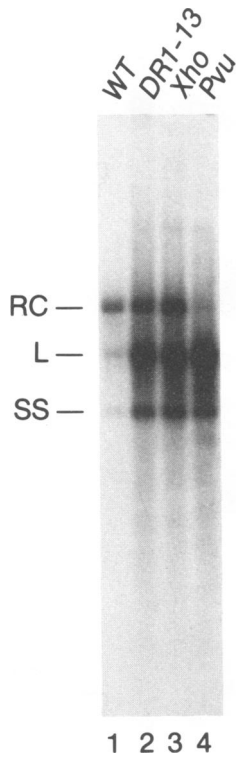

FIG. 5. Viral DNA forms in cells transfected with mutants bearing lesions flanking DR1. LMH cells were transfected with cloned DNA from wild-type DHBV (lane 1), mutant DR1-13 (lane 2), mutant *Xho* (lane 3), or mutant *Pvu* (lane 4); at day 3 posttransfection, cytoplasmic cores were purified and viral DNA was extracted as described in the legend to Fig. 3. Viral DNAs were electrophoresed through a 1.25% agarose gel, transferred to a nylon membrane, and hybridized with a [^{32}P]DHBV DNA probe homologous to the entire DHBV genome. Shown at left are the positions of the relaxed circular (RC), linear (L) and single-stranded (SS) viral DNA forms.

(Fig. 6). The ratios of 5′ ends in DR2 to those in DR1 were consistent with the ratios of relaxed circular DNA to linear DNA detected by Southern blotting of the encapsidated DNAs (Fig. 5). In all cases, the sites of three of the four RNA cleavages within the DR were identical in both conventionally and in situ-primed molecules, although the relative proportions of the individual cleavage products varied modestly (but reproducibly) from mutant to mutant.

The phenotypes of these mutations are instructive because they further illustrate the differing sequence requirements of primer cleavage and translocation. From the total level of plus-strand DNA and from the positions of the RNA-DNA junctions, we can infer that no significant impairment of the cleavage mechanism took place. However, the presence of increased levels of in situ priming indicates that the lesions substantially impaired translocation, despite the fact that the cleaved RNA primers themselves were all of wild-type sequence and fully homologous to DR2. The implications of this for the translocation mechanism will be considered further in the Discussion.

Linear DNA in wild-type infection. The discovery that linear DNAs in the above mutants arose from in situ priming led us to examine the possibility that such aberrant priming events might occur at a lower frequency during wild-type DHBV infection. Many authors have noted the presence of

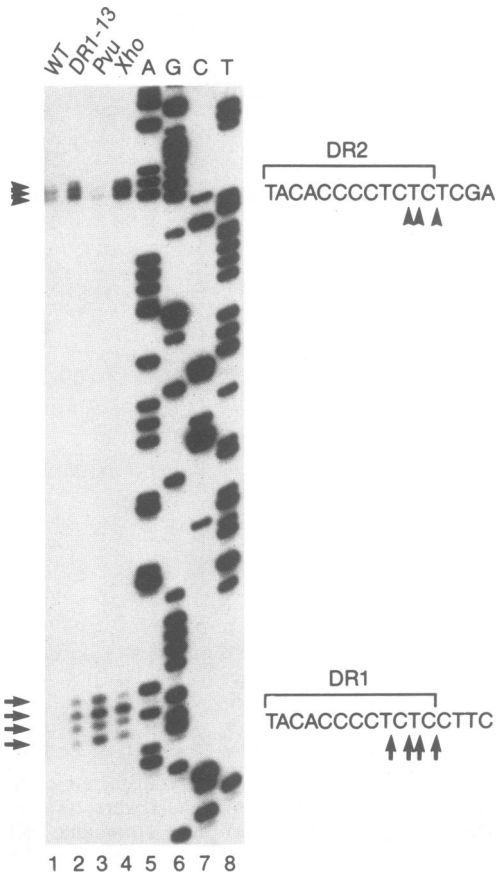

FIG. 6. Mapping the 5′ ends of plus-strand DNA from mutants with lesions flanking DR1. Viral DNA was extracted from cytoplasmic cores purified from LMH cells transfected with wild-type DHBV DNA (lane 1) or with DNA from mutants DR1-13 (lane 2), DR1-*Pvu* (lane 3), or DR1-*Xho* (lane 4). After alkali treatment to remove linked RNA, the 5′ ends of plus-strand DNA were identified by primer extension exactly as described in the legend to Fig. 4. Lanes 5 to 8 show a sequencing ladder generated from wild-type DHBV DNA template by using the same primer as for primer extension. Shown at right is a schematic diagram of the positions of the RNA-DNA junctions (arrows) in conventionally primed plus strands (top) and in situ-primed molecules (bottom).

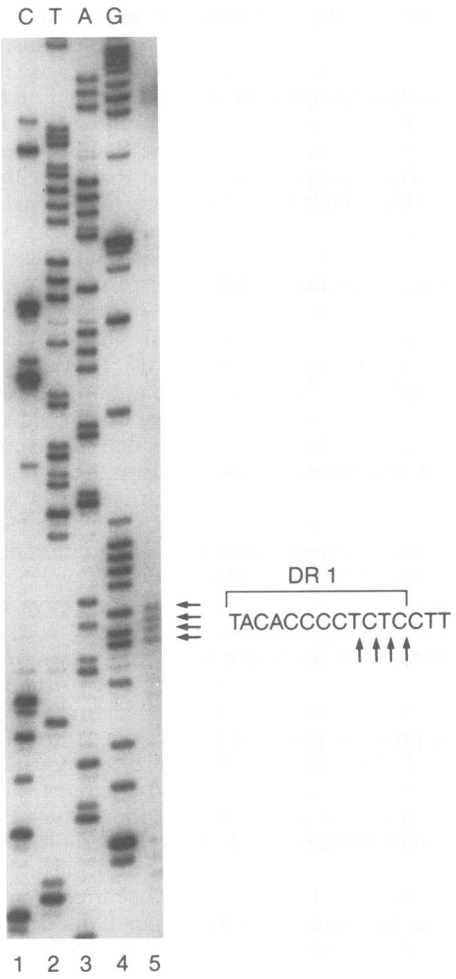

FIG. 7. Mapping the 5′ ends of linear DHBV DNA from wild-type infection. From three 100-mm dishes of LMH cells transfected with wild-type DHBV DNA, cytoplasmic cores were prepared and their DNA was extracted and fractionated on a 1.25% low-melting-point agarose gel in Tris-borate buffer. After visualization of bands with ethidium bromide, the species corresponding to 3.0-kb linear DNA was excised; after melting the agarose at 65°C, DNA was recovered by phenol extraction, alkali treated, and examined by primer extension (lane 5) as described in the legend to Fig. 6. Lanes 1 to 4 show a sequencing ladder generated from wild-type cloned DHBV DNA by using the same primer as for primer extension. Shown at right is a schematic diagram of the positions of the RNA-DNA junctions indicated by arrows.

duplex linear DNA in human and animal hepadnaviruses (8, 16, 28). Typically such molecules, which can represent up to several percent of the total virion DNA, have been ascribed to denaturation of the 5′ cohesive termini during DNA preparation. However, the conditions of pH or temperature required to melt such regions (up to 250 nt for the mammalian viruses) are not achieved during extract preparation (22). Since the termini of these linears have never been precisely mapped, the origin of linear DNA has remained largely unexplained.

To see if wild-type linear DNAs arise through in situ priming, we examined viral DNA from LMH cells transfected with wild-type DHBV DNA. Although the amounts of linear DHBV DNA in these stocks is small, as judged by Southern blotting (cf. Fig. 3 and 4), we were able to prepare sufficient quantities for analysis by pooling cytoplasmic cores purified from multiple transfected plates. After DNA extraction and electrophoresis in 1.25% agarose gels, linear DNA was visualized by staining with ethidium bromide and excised from the gel. Purified linears were then alkali treated, and their termini were mapped in the primer extension assay. Figure 7 (lane 5) shows that the termini map to the cleavage sites in DR1 predicted by the in situ priming model. (Small quantities of molecules whose termini map to DR2 likely represent contaminating incomplete relaxed circular DNA molecules).

DISCUSSION

We have examined the sequence requirements for the generation of the RNA primer for DHBV plus-strand DNA synthesis by using mutant viral genomes with lesions in and around DR1. Our results indicate that the sequence requirements for primer cleavage are differentiable from those for

primer translocation. As best we can judge, none of our mutations importantly affects the efficiency or specificity of primer cleavage, with the proviso that only RNA primers competent for elongation are scored in our assay. All of the mutations, however, affect the efficiency of primer translocation. This defect was initially discovered in mutant DR1-12, in which the lesion is within DR1. However, from the fact that lesions outside DR1 also impair primer translocation (sometimes quite drastically, as in mutant DR1-*Pvu*), we infer that some feature of the surrounding region, either primary sequence or secondary structure, is also being sensed, directly or indirectly, by the translocation machinery.

How might regions outside the primer be involved in primer translocation? One possibility is that factors bound to these regions might facilitate pairing of the region surrounding DR1 to the region of minus-strand DNA surrounding DR2 prior to the cleavage reaction. After annealing of the DRs, unpaired RNAs could be resected and the fully base-paired RNA primer could then be extended. However, the existence of in situ priming argues against this model: if such pairing were an obligatory precursor to cleavage, then cleavage could not occur without translocation. Also, the failure of DRS-12 to correct the defect of DR1-12 or to shift the RNA-DNA joint in plus-strand DNA argues against a model in which primers are paired prior to cleavage. (A similar conclusion has also been recently drawn for woodchuck hepatitis virus DNA synthesis [24].) All in all, it seems more likely that translocation follows cleavage and that proteins involved in translocation are bound to the genome at least in part through interactions with the 3′ end of DR1 and downstream sequences. Such binding may well occur prior to cleavage. We are presently constructing additional mutations in this area to further define the extent of the relevant region and to try to gain insight into the nature of the recognition reaction. However, it seems likely that a detailed understanding of the biochemistry of primer translocation will require an in vitro system in which this reaction can be faithfully reproduced.

The finding that mutant DR2-12 (Fig. 2) can prime plus-strand synthesis normally (Fig. 3 and 4) reveals another interesting feature of the priming reaction. In this mutant, priming occurs despite a one-base mismatch at the 3′ end of the primer, and mapping of the 5′ end of plus-strand DNA indicates that nucleotides could be added to the mispaired 3′ OH group with normal efficiency (Fig. 4). This suggests that conventional Watson-Crick base pairing is not the sole factor in recognition and extension of the translocated RNA primer.

Analysis of linear DNA generated by wild-type DHBV replication indicates that, as in the mutants, linear viral genomes arise via in situ priming (Fig. 7). This observation resolves a longstanding puzzle regarding the origin of this DNA species and indicates that the translocation machinery has a detectable error rate even in the absence of *cis*-acting mutations. This in situ priming resembles in many respects the normal mechanism of retroviral plus-strand priming. In retroviruses, the plus-strand primer is generated by the RNase H activity of the *pol* gene product (30). This degrades most of the genomic RNA following minus-strand synthesis but spares a short purine-rich RNA segment base paired just upstream of the U3 region; this then serves as the primer without any translocation step (30). On the basis of this analogy, it is attractive to speculate that the RNase H activity of the P protein is likewise responsible for plus-strand primer cleavage in hepadnaviruses, but there is as yet no direct evidence for this.

Is is not clear whether linear DNA serves any particular role in the viral life cycle. We do know that mutant DR1-12 is not infectious for ducks: after intrahepatic injection of cloned viral DNA, none of eight recipients displayed viral replicative intermediates in the liver (26a). Thus, linear DNA cannot replace circular DNA altogether, a result that is expected given that such molecules could not serve as transcriptional templates for pregenomic RNA. (They are in fact more analogous to cDNA copies of genomic RNA.) However, it is noteworthy that in linear DNA arising in this fashion, full or partial copies of DR1 are present near both termini. Since free DNA ends are often recombinogenic (6), and since the integrated viral genomes found in hepatocellular carcinoma specimens often contain a DR sequence near the host-virus junction (19), linear DNAs could in principle be precursors to such integrants. If assays for hepadnaviral integration can be developed, mutants like DR1-12 may allow experimental tests of this possibility.

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