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pet, a Small Sequence Distal to the Pregenome Cap Site, Is Required for Expression of the Duck Hepatitis B Virus Pregenome

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We have found that transcription of the pregenome of an avian hepadnavirus, duck hepatitis B virus (DHBV), is dependent on the presence of a small element in the 5′ transcribed region of the pregenome-encoding sequence. This element, which we have named *pet* (positive effector of transcription), exerts its effect in *cis* in a position and orientation-dependent manner, suggesting that it may function as part of the nascent pregenome transcript. The requirement for *pet* depends on the presence in the transcription unit of a region of the DHBV genome located upstream of the envelope promoters, which specifically suppresses transcription of templates lacking *pet*. In the presence of this region, deletion of *pet* activates transcription from downstream promoters, suggesting that pregenome transcription complexes fail to reach the downstream promoters. In vitro transcription experiments support the model that *pet* is required for transcription elongation on the DHBV template. We speculate that *pet* is required to suppress transcription termination during the first passage of pregenome transcription complexes through a viral termination region on the circular viral DNA.

Hepadnaviruses are a family of DNA-containing viruses that includes human hepatitis B virus (HBV), woodchuck hepatitis virus (34), ground squirrel hepatitis virus (23), heron hepatitis virus (30), duck HBV (DHBV) (26), and goose HBV (29a). These viruses replicate primarily in the hepatocytes in vivo and establish chronic infection in the cells of their respective hosts. During the initiation of infection of hepatocytes, viral DNA is converted into a covalently closed circular DNA monomer of about 3 kb, which is found in the nucleus and serves as the template for transcription of viral RNAs (24, 25, 31, 33, 36). In the avian hepadnaviruses, two classes of mRNA (2) are transcribed from two distinct promoter regions in the covalently closed circular DNA (Fig. 1). The pregenome promoter is used for the production of a terminally redundant RNA (3.3 kb), the pregenome, that is polyadenylated approximately 270 nucleotides downstream of the promoter region during the second circuit of the genome by RNA polymerase II. The envelope mRNA promoters are located in a region 1,100 to 1,400 downstream of the pregenome promoter (7, 20). The two envelope mRNAs produced from these promoters are less than the genome in length (2.3 and 2.1 kb) and are polyadenylated at the same site as the pregenome transcript in a region that lies between the pregenome and envelope promoters. The pregenome transcript is translated to produce two proteins known to be required for viral DNA synthesis (the P protein and the capsid protein) and is also encapsidated and subsequently used as the template for production of the first viral DNA strand (the minus strand) by reverse transcription (16, 31).

In the present paper, we show that in the DHBV the production of the pregenome but not the envelope mRNAs is strongly dependent on a *cis*-acting element at the 5′ end of the RNA, which we have named *pet* (positive effector of transcription). We present evidence that *pet* is required to suppress premature termination of pregenome transcription in a large region between 500 and 1,200 nucleotides downstream of the pregenome initiation site during the first circuit of the genome by RNA polymerase II. We propose a model to explain how the circular template can be utilized for transcription without mutual interference of the two promoter regions.

MATERIALS AND METHODS

Plasmid construction. The various steps for construction of the different plasmids used in this study were performed by standard techniques. All incompatible restriction enzyme-generated ends were ligated after blunt-end formation with the Klenow fragment of DNA polymerase I and deoxyribonucleoside triphosphates either to remove protruding 3′ ends or to fill in recessed 3′ ends. The DHBV strain 16 sequences (22) in these constructs were derived from pSPDHBV5.1Ga12X, which contains two copies of the DHBV genome (15) cloned into the *Eco*RI site of pSP65. To eliminate transcription from the envelope promoters present in the upstream monomer in pSPDHBV5.1Ga12X, the DHBV sequence from *Eco*RI to *Bam*HI (nucleotide positions 1 and 1,658, respectively) was removed by cleavage and religation of the blunted ends. The derived plasmid was called pDV1.5wt (Fig. 2a). The plasmid pDV1.5wt.pA (Fig. 2b) was made by replacing sequences from *Msc*I (nucleotide 2372) to *Eco*RI (nucleotide 3021) in the 3′ DHBV monomer of pDV1.5wt with a 641-bp human growth hormone (hGH) polyadenylation signal-containing sequence excised from plasmid pOGH (Promega) (9) using the restriction enzymes *Pvu*II (nucleotide 2011) and *Eco*RI (nucleotide 2652).

All deletions were generated by removing selected sequences between appropriate restriction endonuclease sites (Fig. 3a; see also Fig. 6a). The construct pDV1.5inv.pA was produced by excision of a DHBV fragment from the 5′ *Afl*II (nucleotide 2526) to the 5′ *Eco*RV (nucleotide 2652) in pDV1.5wt.pA, blunting the *Afl*II ends, and recloning the same fragment in the opposite orientation.

To make *neo*-containing plasmids, a fragment (2.3-kb *Hin*dIII [nucleotide 5734] to *Bam*HI [nucleotide 3393]) which contained a bacterial neomycin resistance-encoding sequence and a simian virus 40 polyadenylation signal was excised from pRSVneo (11). In the constructs pDV1.5wt.neo, pDV1.5Δ6.neo, and pDV1.5ΔP.neo, this 2.3-kb fragment was fused with DHBV sequence at the *Eco*RI site (nucleotide 3021) at

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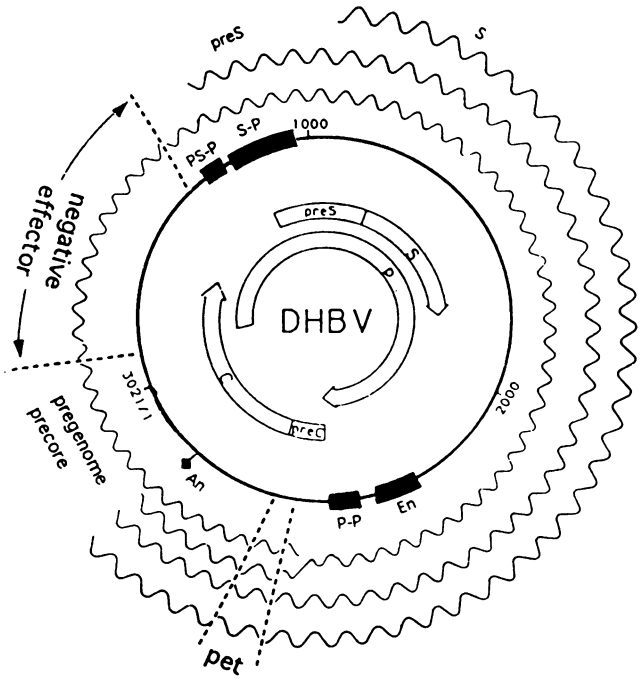

FIG. 1. Genetic and transcription map of the DHBV genome. The nucleotide numbering is according to Mandart et al. (22). Open reading frames are diagrammed in the center; RNA transcripts are on the outside. P-P, pregenome promoter; PS-S, preS promoter; S-P, S promoter; En, enhancer; An, polyadenylation site (data are from references 2, 7, 21, 22, and 37a).

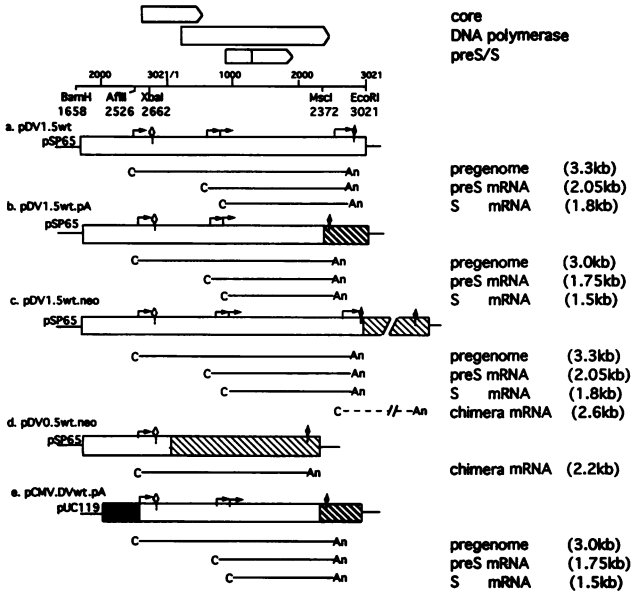

FIG. 2. Schematic representation of the plasmids used in this study and their RNA transcripts. The open reading frames are drawn at the top. The open boxes represent the sequences derived from the DHBV genome. The shaded boxes represented either the *neo*-encoding sequences, the hGH polyadenylation sequences, or the CMV immediate-early promoter-containing sequences. An, polyadenylation site; C, 5′ cap.

the 3′ end of the DHBV sequence (Fig. 2c). In the constructs pDV0.5wt.neo, pDV0.5Δ6.neo, and pDV0.5ΔP.neo, the *neo* fragment was fused with the DHBV sequence at the *Xba*I site (nucleotide 2662) within the 5′ half copy of the DHBV genome (Fig. 2d).

In all of the plasmids described above, the pregenome expression was driven by the authentic pregenome promoter. A second set of plasmids was derived from pUC119.CMV. DHBV (denoted by pCMV.DVwt in this study), in which the pregenome was transcribed from the cytomegalovirus (CMV) immediate-early promoter (33). The construct pCMV.DVwt. pA was generated by replacing a DHBV sequence from *Msc*I (nucleotide 2372) to *Eco*RI (nucleotide 3021) in pCMV.DVwt with a sequence containing the human growth hormone polyadenylation signal as described above (Fig. 2e). The deletion mutant pCMV.DVΔ6.pA was constructed by introducing a Δ6 deletion (Fig. 3a) into pCMV.DVwt.pA, and the deletion mutant pCMV.DVΔP.pA was made by deletion of the CMV promoter using restriction sites *Nco*I (nucleotide 445 in the CMV promoter region) and *Afl*II (nucleotide 2526 at the DHBV pregenome cap site).

To eliminate the potential influence of pregenome encapsidation on the stable levels of pregenome, a frameshift mutation was introduced into the core open reading frame at the *Eco*RV site (nucleotide 2652) in all of the constructs used to supply pregenome transcripts in this study. This frameshift mutation was obtained by deletion of 2 nucleotides at the *Eco*RV site as previously described (15).

Cells and transfection. DNA was transfected into the chicken hepatoma cell line LMH (5, 19) or HeLa cells by the calcium phosphate coprecipitation method described previously (32). Transfections were carried out with 10 μg of DNA per 60-mm-diameter tissue culture dish. Cotransfection was performed with 5 μg of each DNA.

RNA analysis. At 36 h posttransfection, RNA was isolated from the cell layer by the acid phenol-guanidine thiocyanate method (4). For Northern (RNA) blot analysis, RNA was glyoxylated, electrophoresed through a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labelled RNA specific for detection of plus strands, as described previously (36).

Primer extension analysis was performed essentially as described by Sambrook et al. (29). Briefly, a quarter of the RNA isolated from the transfected cells in one 60-mm tissue culture dish was mixed with 0.1 pmol of 5′-^{32}P-labeled oligonucleotide and denatured for 10 min at 85°C. The RNA-primer mixture was then annealed for 12 to 16 h at 40°C in a 30-μl hybridization solution containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% formamide. Nucleic acids were then precipitated with ethanol and dried for the primer extension analysis. The reverse transcription reaction was carried out in 25 μl of buffer supplied by the manufacturer with 10 U of avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42°C. After phenol extraction and ethanol precipitation, the reaction product was denatured and subjected to electrophoresis in 6% polyacrylamide gels containing 8 M urea.

In vitro transcription with HeLa nuclear extracts. Templates for in vitro runoff transcription were generated by cleavage of the three test plasmids pCMV.DVwt.pA, pCMV.DVΔ6.pA, and pCMV.DVΔP.pA at the *Eco*RV site in DHBV (nucleotide 720), approximately 1,200 bp downstream of the CMV promoter. HeLa cell nuclear extract in vitro transcription kits were purchased from Promega, and in vitro transcription reactions were performed according to the man-

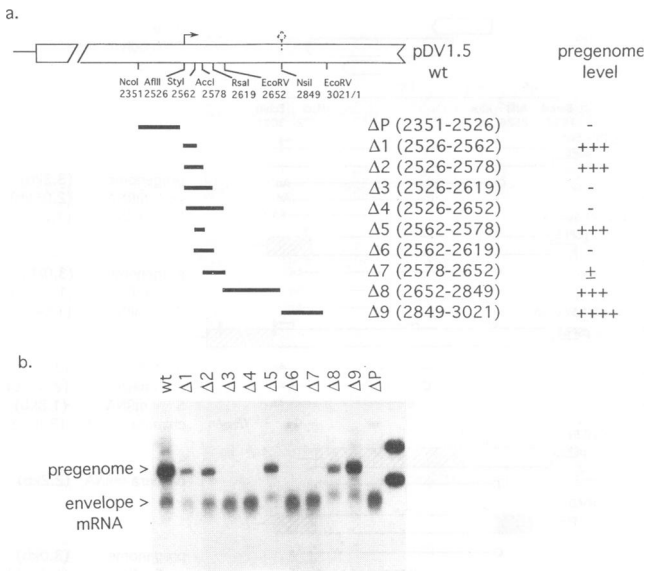

FIG. 3. Mapping the position of *pet*. (a) Summary of the various deletions in the 5′ pregenome-encoding region and their effects on pregenome levels. The numbers refer to the positions of the two restriction sites used to create each deletion. (b) Northern blots of viral RNA from LMH cells transfected with each mutant. RNA from 1/10 of a 60-mm plate was loaded in each lane. The lane at the right end contains 0.5 ng of glyoxylated DNA molecular size markers (4.6- and 3.0-kb fragment sizes are visible).

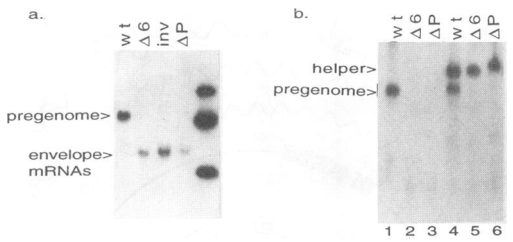

FIG. 4. Orientation and *cis* dependence of *pet* function. Northern blot analysis was used to determine the levels of virus-specific RNA. (a) Orientation dependence: RNAs from cells transfected with the wild-type (wt), Δ6, inv, and ΔP derivatives of pDV1.5wt.pA (Fig. 2b). A glyoxylated DNA molecular size marker (4.6, 3.0, and 1.4 kb) is shown in the right lane. (b) *cis* dependence: RNAs from cells transfected with the wt, Δ6, and ΔP derivatives of pDV1.5wt.pA (Fig. 2b) in the absence (left three lanes) or presence (right three lanes) of pDV1.5wt (Fig. 2a) as a helper. In this case, the helper genome was not core defective.

ufacturer's instructions, except that [^{32}P]UTP (800 Ci/mmol, 10 mCi/ml) rather than [^{32}P]GTP was used as the radiolabeled ribonucleotide. The reaction mixtures were incubated at 30°C for 1 h in 25 μl containing 400 μg of template per ml, 3 mM $MgCl_2$, 0.4 mM ATP, CTP, and GTP, 0.5 μM [^{32}P]UTP, and 8 U of nuclear extract. Template addition experiments showed that at 400 ng, the template was in excess (data not shown). When pulse-chase experiments were performed, the following modification was made. Following preincubation of the reaction mixtures (25 μl) at 30°C for 15 min in the presence of template and in the absence of ribonucleotides, 1 μl of pulse mixture containing 10 mM GTP, 10 mM ATP, 10 mM CTP, and 6.25 μM [^{32}P]UTP was added. After a further incubation at 30°C for 1, 3, or 5 min, the reactions were either terminated or were continued for another 15 min after the addition of 1 μl of 10 mM UTP. The reactions were stopped by the addition of 2 volumes of stop buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA [pH 7.5], 150 mM NaCl, 0.5% sodium dodecyl sulfate, 10 μg of yeast tRNA per ml). After phenol extraction and ethanol precipitation, the reaction products were denatured and subjected to electrophoresis in 5 or 6% polyacrylamide gels containing 8 M urea.

RESULTS

Mapping of the position of *pet*. We found that deletions within the DNA encoding the 5′ end of the pregenome caused a strong reduction in the levels of pregenome in transfected LMH cells. We constructed a series of terminal and overlapping deletion mutants to map the position of the element responsible for this effect and tested the levels of pregenome produced by these deletion mutants by Northern blot analysis (Fig. 3). In these experiments, we observed that deletions outside the region 2578 to 2652 did not affect pregenome levels, whereas deletions that extended into this region (Δ3, Δ4, Δ6, and Δ7) caused strong reductions in pregenome levels. We concluded that this region contained a positive regulatory element which we named *pet* (positive effector of transcription). In subsequent experiments, we used the Δ6 deletion (2562 to 2616) as the *pet*(−) template (i.e., the template lacking *pet*), since this was the smallest deletion that showed a maximal effect on pregenome levels, and the ΔP deletion, in which the pregenome promoter was deleted, for comparison with the wild-type *pet*(+) template.

***pet* activity is position- and orientation-dependent and is required in *cis*.** The preceding experiment demonstrated that the transcriptional enhancing activity is dependent on the position of *pet* in the pregenome transcription units. Deletion of the *pet*-encoding region from the 5′ end of the pregenome transcription inhibited transcription, even though a second copy of this sequence was present in the terminally redundant 3′ end of the pregenome-encoding sequence. Moreover, the activity of the 5′ copy of *pet* did not depend on the presence of the second copy, since the region encoding this copy could be substituted with human growth hormone sequences (as in experiments shown in Fig. 4) or specifically deleted (data not shown). Inversion of the *pet*-encoding sequence at the 5′ end of the genome destroyed its activity, as shown in Fig. 4a. We concluded that the activity of *pet* depended on its native orientation and location at the 5′ end of the pregenome transcription unit.

The experiment shown in Fig. 4b demonstrated that *pet* was required to be present in *cis* for its activity. A *pet*(−) construct that could be distinguished from the wild type by the size of its pregenome was cotransfected with a wild-type construct that could provide all of the viral proteins in *trans* to the *pet*(−) genome. Northern blot analysis showed, nevertheless, that the *pet*(−) mutation depressed the levels of the *pet*(−) pregenome and that these levels could not be restored by providing viral proteins in *trans*.

Deletion of *pet* relieves suppression of downstream promoters. We found that in all cases in which pregenome levels were reduced, the levels of the envelope transcripts were increased (Fig. 3b). This effect could be seen either when pregenome transcription was suppressed by deletion of the pregenome promoter (ΔP) or when *pet* was deleted (Δ6, for example). Suppression of transcription from downstream promoters by strong upstream promoters, referred to as promoter occlusion, is thought to be due to the passage of transcription complexes

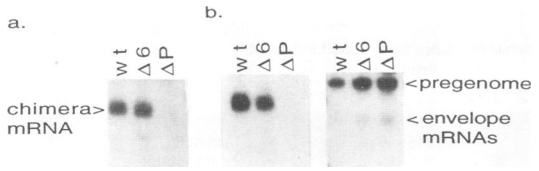

FIG. 5. The requirement for *pet* depends on *cis*-acting downstream sequences. Northern blot analysis for virus-specific or *neo*-specific RNA. (a) *pet* is not required when downstream DHBV sequences are absent. RNAs from cells transfected with the wild-type (wt), Δ6, and ΔP derivatives of pDV0.5wt.neo, hybridized with a *neo*-specific probe, are shown. (b) The downstream sequences act in *cis*. RNAs extracted from cells cotransfected with pDV1.5wt and either the wt, Δ6, or ΔP derivatives of pDV0.5wt.neo are shown. The right panel shows DHBV-specific RNA, and the left panel shows *neo*-specific RNA. Wild-type viral proteins do not suppress the Δ6 chimeric RNA.

through the affected promoter (8, 18). These observations suggested that deletion of *pet* caused a defect in transcription that relieved promoter occlusion, rather than a defect in pregenome RNA processing, transport, or stability, which would not be expected to relieve promoter occlusion.

Transcription can be restored to a *pet*(−) genome by deletion of downstream DHBV sequences. We have shown that *pet* was required in *cis* for transcription of the DHBV pregenome. Additional experiments suggested that the requirement for *pet* is to overcome a negative *cis*-acting element downstream of *pet* in the pregenome transcription unit. Initially, we constructed a reporter gene system to study the activity of *pet* in the absence of other viral elements. We fused the *neo* gene with a poly(A) signal to the pregenome-encoding sequence only 130 bp downstream of the pregenome cap site (pDV0.5wt.neo, shown in Fig. 2d) and tested the effect of the *pet*(−) mutation on the production of the chimeric reporter RNA. In this context, *pet* was not required to produce transcripts from the pregenome promoter (Fig. 5a). This result suggested that downstream viral sequences that had been removed from this construct were responsible for suppression of transcription from a *pet*(−) template. These downstream sequences did not supply an inhibitory viral protein, since cotransfection of these reporter constructs with a wild-type genome did not cause suppression of transcription from the *pet*(−) template (Fig. 5b). We concluded that the negative regulatory sequences acted in *cis* on the *pet*(−) genome.

We constructed a series of internal downstream deletions within the pregenome-encoding sequences to determine what regions could be deleted without relieving the requirement for *pet* (Fig. 6a). The requirement for *pet* was determined by primer extension analysis of the pregenome levels produced by each of the deleted templates in the presence or absence of *pet*. As shown in Fig. 6, only deletions that involved the region between nucleotides 1 and 720 (DHBV sequence) relieved the requirement for *pet*. Moreover, relieving the requirement for *pet* was not caused by activation of a different promoter or a shift in the transcription initiation site used by the pregenome promoter. We concluded that elements that could cause suppression of pregenome transcription were present in a region approximately 500 to 1,200 nucleotides downstream of the pregenome initiation site and that *pet* was required in *cis* to overcome the effects of this transcriptional suppression.

Effect of *pet* on transcription in vitro. The transcriptional defect due to the *pet*(−) mutation could be a defect in either transcription initiation or elongation through a region upstream of the envelope promoters. This region would include the elements that suppress pregenome transcription from a

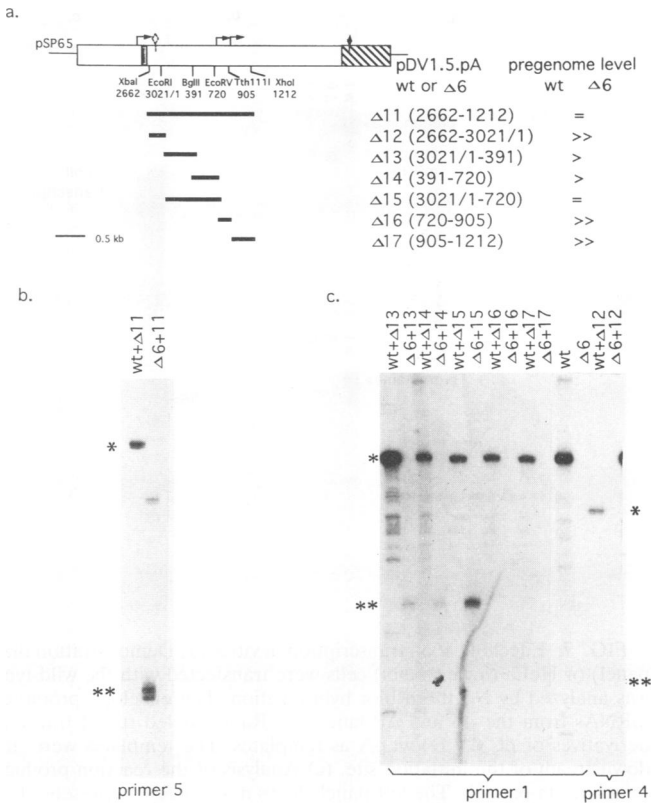

FIG. 6. Mapping of a region of transcriptional suppression located within the DHBV genome. (a) Location and effect of downstream deletions on dependence of pregenome transcription on *pet*. Deletions were introduced into pDV1.5wt as indicated. The relative pregenome levels of the wild-type (wt) and the Δ6 derivatives of each deletion mutant are summarized on the right. (b and c) Primer extension analysis of the relative pregenome levels of the wt and the Δ6 derivatives of each deletion mutant shown in panel a. Primer 1 (nucleotides 2816 to 2792) was used for most assays so that the relative levels of primer extension products could be directly compared. Primer 4 (nucleotides 126 to 102) and primer 5 (nucleotides 2662 to 2641) were used with deletion mutants in which the primer 1 binding site was deleted. The relevant primer extension products are indicated. *, *pet*(+) pregenomes; **, *pet*(−) pregenomes.

pet(−) template. We attempted to distinguish these two alternatives using an in vitro transcription system by comparing the characteristics of transcription from *pet*(+) and *pet*(−) templates. We found that pregenome expression was dependent on the presence of *pet* even when the DHBV promoter was substituted by the CMV immediate-early promoter. This dependence could be seen to occur both in LMH and in HeLa cells, as shown in Fig. 7a, by transfection of either cell line (LMH cells [upper panel] and HeLa cells [lower panel]) with *pet*(−) or *pet*(+) constructs driven by the CMV promoter (shown in Fig. 2e [pCMV.DV.wt.pA]). This property of *pet* allowed us to utilize HeLa cell nuclear extracts for in vitro transcription studies of the effect of *pet*.

In vitro transcription reactions were carried out by using each of these three plasmids cut at the DHBV *Eco*RV site (nucleotide 720), just downstream of the negative effector. Nuclear extracts were purchased from a commercial source. Of a total of six different extracts tested, three failed to detect any difference in utilization of the wild-type and *pet*(−) templates, while three extracts utilized the *pet*(−) template with greatly

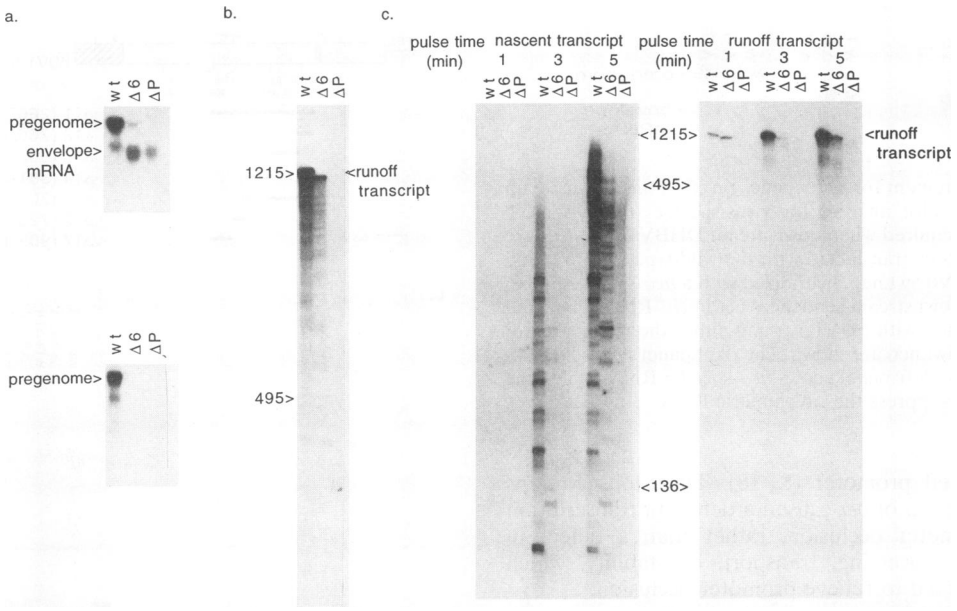

FIG. 7. Effect of *pet* on transcription in vitro. (a) Demonstration that the CMV promoter is *pet* dependent in LMH and HeLa cells. LMH (upper panel) or HeLa (lower panel) cells were transfected with the wild-type (wt), Δ6, or ΔP derivatives of pCMV.DVwt.pA (Fig. 2e), and total RNA was analyzed by Northern blot hybridization. The envelope promoters may not work in HeLa cells, as evidenced by the absence of envelope mRNAs from the Δ6 and ΔP lanes. (b) Radiolabeled runoff transcripts from 60-min in vitro transcription reactions using the wt, Δ6, and ΔP derivatives of pCMV.DVwt.pA as templates. The templates were linearized by cleavage at the *Eco*RV site at nucleotide 720, about 1,200 bp downstream of the initiation site. (c) Analysis of the reaction product of pulse-labeled in vitro transcription reactions using the wt, Δ6, and ΔP templates in panel b. The left panel shows nascent RNAs present at the end of a 1-, 3-, and 5-min pulse; the right panel shows runoff transcripts labeled in the same 1-, 3-, and 5-min pulse reactions after a 15-min chase with unlabeled UTP. The amount of label in the runoff transcripts is a specific measure of the total amount of incorporation into nascent transcripts during the indicated pulse times. Equal portions of the reaction mixture before and the after chasing were analyzed, and the autoradiogram in the left panel was exposed twice as long as that of the right panel.

decreased efficiency. Results from in vitro transcription experiments with one extract are shown in Fig. 7b and c. First, we measured the formation of runoff transcripts in a 1-h reaction. We observed that a strong reduction in the amount of runoff transcripts was produced by the deletion of *pet* (Fig. 7b). To examine the relative rates of transcription at different times following initiation, we carried out pulse-labeling reactions using the test templates. We monitored the incorporation of [^{32}P]UTP into specific nascent transcripts during the pulse period in two ways: (i) by measuring the amount of ^{32}P-labeled runoff transcript produced after an extensive cold chase and (ii) by determining the lengths of nascent strands produced during the pulse directly by gel electrophoresis. In a 1-min pulse, no nascent transcripts larger than 130 nucleotides (experimental limit of the assay) could be detected by direct examination of the pulsed product (Fig. 7c [left panel]); however, equal levels of radioactivity in the runoff transcripts were seen with the *pet*(+) and *pet*(−) templates (Fig. 7c [right panel]). These data indicated that the rates of incorporation of [^{32}P]UTP into the nascent transcripts on both templates during the 1-min pulse were the same, suggesting that both templates were utilized equally at the early stages of transcription, which included initiation and elongation through the beginning of the templates. In contrast, utilization of the *pet*(−) template resulted in incorporation of less label than the *pet*(+) template when the pulse period was extended to 3 or 5 min (Fig. 7c [right panel]). This result could be due to reduced elongation rates through the distal parts of the template during the pulse. In fact, longer nascent transcripts were produced from the *pet*(+) than from the *pet*(−) template (Fig. 7c [left panel]) at the ends of 3- and 5-min pulse periods. Overall, the transcriptional defect observed in the in vitro reactions with some nuclear extracts was consistent with the idea that the *pet*(−) mutation caused a transcriptional defect. In particular, elongation rates through distal regions of the *pet*(−) template were reduced compared with those of a *pet*(+) template.

Dominance of the pregenome promoter over the envelope promoters. An elongation defect produced by the *pet*(−) mutation was consistent with its coordinate effect of activating downstream promoters. To learn more about interference among the DHBV promoters, we tested whether both envelope promoters, the preS and S promoters, were activated by deletion of either *pet* (Δ6) or the pregenome promoter itself (ΔP). We used a primer extension assay to determine separately the levels of pregenome, preS, and S mRNAs in response to these deletions. The test plasmids used in this experiment were altered at the 3′ end of the pregenome-encoding sequences by substituting the terminally redundant region with a heterologous polyadenylation signal from the human growth hormone gene (for constructs, see Fig. 2 [pDV1.5wt.pA, pDV1.5Δ6.pA, and pDV1.5ΔP.pA]). This substitution simplified the interpretation of the primer extension analysis of the pregenome 5′ end. The primer extension assay, shown in Fig. 8a, confirmed that the *pet*(−) template generated only low levels of pregenome compared with those of wild-type *pet*(+) template after transfection and that the transcripts from both templates were initiated at the expected positions. In contrast, the levels of both the preS and the S mRNAs (clustered series of bands marked by asterisks in the middle and right panels of Fig. 8a) were both greatly increased

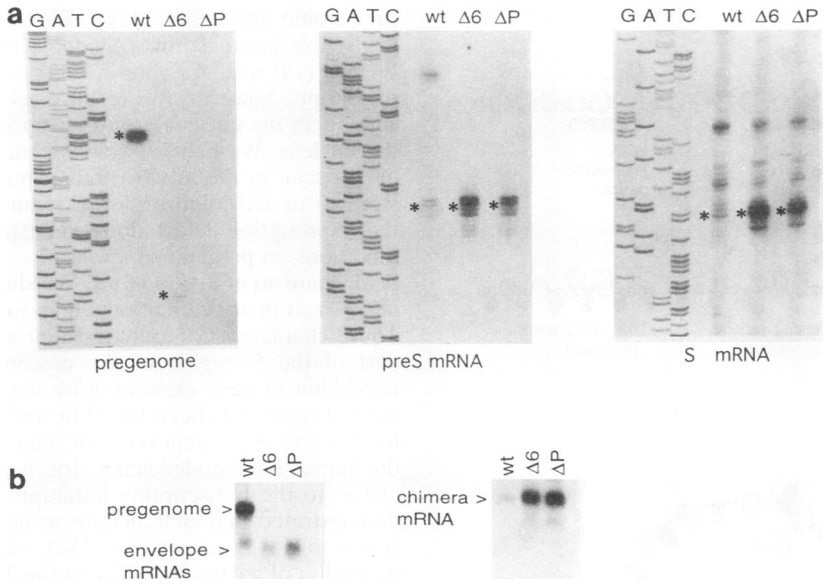

FIG. 8. Activation of downstream promoters by Δ6 or ΔP mutation. (a) RNA transcripts were assayed by primer extension on total RNAs extracted from cells transfected with the wild-type (wt), Δ6, and ΔP derivatives of pDV1.5wt.pA (Fig. 2b). Sequence ladders were derived from each primer used in the panel. Left panel, primer 1 (nucleotides 2816 to 2792) used to assay levels of both the wt and the Δ6 pregenomes; middle panel, primer 2 (nucleotides 931 to 906) used to assay preS mRNA levels; right panel, primer 3 (nucleotides 1184 to 1159) used to assay S mRNA. In this assay, the primer can be extended on either S, preS, or pregenome RNA. The strong pause above the cluster of S-specific bands is derived primarily from pregenome RNA in the wt lane and from preS mRNA in the Δ6 and ΔP lanes. (b) Northern blot analysis of activation of the downstream pregenome promoter. RNAs from cells transfected with the wt, Δ6, and ΔP derivatives of pDV1.5wt.neo (Fig. 2c). The left panel shows DHBV-specific RNAs from the upstream pregenome promoter and envelope promoters; the right panel shows *neo*-specific RNA from the downstream pregenome promoter.

in response to the *pet*(−) deletion (Δ6) or the pregenome promoter deletion (ΔP). Moreover, no shift in the start sites or relative enhancement of preS and S mRNA levels could be seen between the *pet*(−) and the ΔP template. Since both the preS and the S mRNAs were present at high levels in the *pet*(−)- and Δ6-transfected cells, we concluded that the preS promoter did not substantially suppress the S promoter, which is located about 200 nucleotides downstream.

We also investigated the behavior of the downstream copy of the pregenome promoter in response to the *pet*(−) and ΔP alterations of the template. In order specifically to detect the activity of the downstream pregenome promoter, we inserted a reporter sequence, *neo*, downstream of the promoter in each of the test plasmids (shown in Fig. 2 [pDV1.5wt.neo, pDV1.5Δ6.neo, and pDV1.5ΔP.neo]). Figure 8b shows that, although very little *neo*-specific RNA could be detected from the downstream promoter in the wild-type template, high levels of both the envelope transcripts (left panel) and the *neo*-containing transcripts (right panel) were seen in cells transfected with the *pet*(−) and ΔP constructs. We concluded that the upstream pregenome promoter suppressed the activity of the downstream pregenome promoter 3 kb away. In addition, in spite of the activation of the envelope promoters by the Δ6 and the ΔP mutations, transcription from these promoters did not suppress the downstream pregenome promoter. Thus, although the pregenome promoter was able to suppress the envelope promoters, the preS promoter did not suppress the S promoter, and these two promoters working together did not suppress the downstream pregenome promoter.

DISCUSSION

In this study, we have identified a *cis*-acting positive effector of transcription (*pet*) within the pregenome-encoding region from nucleotide +48 to +122 relative to the pregenome cap site. This downstream element appeared to be required for pregenome transcription only when certain additional DHBV sequences were present downstream in the transcriptional template.

Nature of the *pet*(−) defect. Two lines of evidence suggested that the effect of the *pet*(−) mutation was to decrease transcription of the pregenome. The first evidence that we encountered was the fact that *pet*(−) mutations, like the ΔP mutation, resulted in elevated levels of RNA transcripts from promoters located downstream of the pregenome promoter. This result is almost surely a consequence of relieving promoter occlusion by decreasing the number of pregenome transcription complexes that pass through the downstream promoters (7, 18). In the case of the ΔP mutation, the decrease in transcription complexes would have been due to an inhibition of the rate of transcriptional initiation of pregenomes. In the case of the *pet*(−) mutations, the decrease may have been caused by a blockage of transcription elongation at sites upstream of the affected promoters. The latter model, shown schematically in Fig. 9a, is consistent with our finding of a region of transcriptional suppression specific for *pet*(−) templates located just upstream of the envelope promoters. The effect of *pet* in the wild-type template may be to overcome blocks to transcriptional elongation through this region.

In vitro transcription experiments also support the model that *pet*(−) mutations caused defects in transcription. As in other studies (35), not all extracts tested showed the same activity. Differences between extracts were not due to experimental variation in the transcription reactions, since with any one extract the results were reproducible. We do not know the reason for variation in the properties of different extracts with respect to transcription of *pet*(−) templates. Differences could

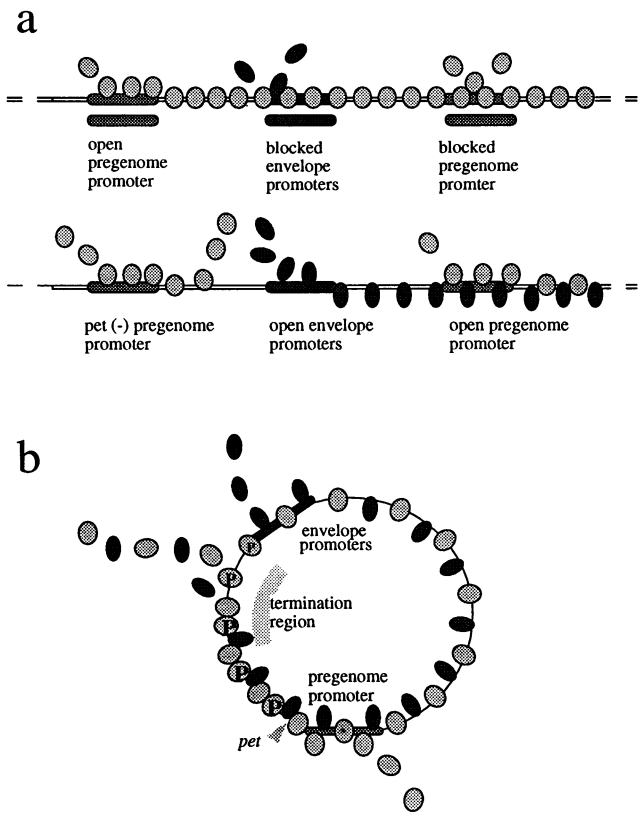

FIG. 9. Topology maps of transcription initiation and termination colinear and circular viral DNAs. (a) Transcription complexes on colinear *pet*(+) (top) or *pet*(−) (bottom) plasmid templates. Transcription complexes initiated at the upstream *pet*(+) pregenome promoter block assembly of transcription complexes at the envelope and downstream pregenome promoters. In the absence of *pet*, pregenome transcription complexes fail to travel through the putative termination region, allowing transcription from downstream promoters. (b) Transcription complexes on a circular viral template. The template immediately downstream of the pregenome promoter (bottom) is blocked by a combination of new and old pregenome transcription complexes as well as by envelope transcription complexes. Removal of old transcription complexes in the termination region (left) prevents suppression of the envelope promoters. New pregenome transcription complexes are not removed in the termination region because of the activity of *pet* (complexes marked with P).

have been due to variations in the amounts of a cellular factor that influenced the dependence of transcription elongation on *pet*. However, the fact that some extracts could distinguish between *pet*(−) and *pet*(+) templates suggests that *pet* has some function in transcription. In addition, pulse-labeling experiments indicated that the defect in transcription of the *pet*(−) template was evident only at longer pulse periods and resulted in an inhibition of nascent RNA elongation during the pulse relative to transcription on the *pet*(+) template. An effect of the *pet*(−) mutation on elongation of nascent strands is consistent with the model that *pet* is required to overcome blocks to transcription elongation through the region just upstream of the envelope promoters.

Other explanations for the effect of *pet*(−) mutations on pregenome levels cannot be ruled out. Hirsch and Ganem noted a similar reduction of pregenome levels caused by a deletion in the *pet*-encoding region and attributed this effect to pregenome instability (12a). We have shown that *pet*(−) transcripts produced by a Sindbis virus expression system for DHBV (14) are as stable in the cytoplasm as *pet*-containing transcripts; however, this result does not rule out a defect in stability in the nucleus or in transport of the pregenome from the nucleus. We believe, however, that the magnitude of the effects seen in the in vitro transcription experiments and the strength of activation of downstream promoters indicate that the transcription defect alone can explain the effect of *pet*(−) mutations on pregenome levels.

Mechanism of action of *pet*. We showed that *pet* exerted its effect in *cis* in an orientation- and position-dependent manner. These characteristics are consistent with *pet* functioning as a part of the 5′ region of the nascent RNA transcript. The regulation of gene expression by sequences within a 5′ transcribed region has been found in some viruses. The prototype for this type of element is the *tar* sequence, which is encoded in the human immunodeficiency virus type 1 long terminal repeat just 3′ to the transcription initiation site. Many studies have demonstrated that *tar* functions as an RNA component, consisting of a 59-nucleotide RNA stem-loop structure, and formation of a complex between this RNA stem-loop with the virus-encoded *tat* protein, along with a currently unidentified cellular cofactor(s), induces a dramatic increase in the level of mRNA synthesis from the adjacent long terminal repeat promoter element of human immunodeficiency virus type 1 (for details, see reference 12). Like *tar*, *pet* is located just 3′ to the transcription initiation site and could exert its effect through a folded structure on the nascent RNA shortly after initiation. However, should binding of a protein to *pet* be required for its activity, we do not believe such a protein to be virus-encoded, since mutations introduced into the open reading frames of capsid protein, P protein, preS protein, or S protein did not affect pregenome levels (data not shown). It is possible, therefore, that *pet* exerts its function by interacting with a cellular protein.

Possible role of *pet* in regulating transcription termination. Recognition of the dependence of pregenome transcription on the presence of *pet* allowed us to identify a second region of the genome that suppressed pregenome transcription specifically on a *pet*(−) template. We do not know the mechanism by which this region suppressed pregenome transcription, but we speculate that blocks to transcription elongation occurred within this region and that *pet* was required to overcome these blocks. In an infection, viral RNAs are transcribed from covalently closed circular viral DNAs. Active transcription termination events seem to be important for removing old transcribing complexes from a circular DNA template to allow room for the formation of new transcription complexes. Lying just distal to the viral polyadenylation site that is used in all transcripts, this second region would be in a position that might be expected to effect transcriptional termination. It has been shown with several genes that transcription termination is dependent on 3′-end processing of the nascent RNA (6, 10, 21, 38). It is also believed that transcription of sequences upstream of the pregenome cap site is required for utilization of the polyadenylation signal in hepadnaviruses (3, 27, 28). Thus, transcriptional termination should not occur during the first passage of pregenome transcription complexes through the putative termination region but only during the second passage, when 3′-end formation can occur. However, stalling of transcription complexes during their second passage through this region may contribute to 3′-end formation and subsequent termination, and such blocks to transcriptional elongation may also occur during their first passage through the region even in the absence of 3′-end formation. These blocks could limit the

rate at which pregenomes are synthesized, and pregenome transcription may require specific suppression of such transcription blocks during the first passage of the RNA polymerase through the template upstream of the envelope promoter region. We suggest that *pet*, positioned at the 5′ end of the nascent transcript, may be responsible for this initial suppression.

Lack of interference between pregenome and envelope promoters on the circular viral DNA. Our study demonstrated that transcription from the pregenome promoter could strongly suppress the activity of downstream envelope promoters (Fig. 8a). This suppression appears to be counterproductive for virus production, since envelope protein production was suppressed. However, the profound suppression of envelope mRNAs that we observed was probably an artificial consequence of the colinear rather than circular configuration of the pregenome-encoding sequence that we used for these studies. Our data showed that transcription from a DHBV pregenome promoter could suppress a second copy of that promoter 3 kb downstream (Fig. 8b). Therefore, in the circular viral DNA that is the natural transcriptional template, passage of transcription complexes through the pregenome promoter after one complete circuit through the genome would suppress new initiation events at that promoter. In this way, the pregenome promoter would be prevented from maximal utilization by self-regulation through promoter occlusion. If transcription complexes were specifically removed from the template during their second passage through a termination region upstream of the envelope promoters, suppression of transcription from the envelope promoters would be alleviated. Since use of the envelope promoters did not suppress a downstream pregenome promoter, interference with the pregenome promoter would not occur. This model is depicted schematically in Fig. 9b. We suggest that modulating differential utilization of termination signals by pregenome transcription complexes is the biological function of *pet*. This model provides an additional biological rationale for the presence of a termination region between the polyadenylation site and the envelope promoters.

Relationship of *pet* to the packaging signal ε. RNA sequences (ε) mapping close to *pet* have been proposed to form a stable stem-loop structure. This stem-loop is very well conserved among hepadnaviruses and plays an important role in viral RNA packaging (14, 17) and initiation of viral DNA synthesis (37). Our data demonstrate that *pet* and ε functions probably do not depend on the same RNA structure. First, we found that *pet* and ε functions could be segregated genetically by at least two mutants (Fig. 3a; Δ2 and Δ5) that express high levels of pregenome but are defective in packaging (2a). Therefore, some sequences that are required for packaging are not required for *pet* activity. These sequences, in fact, are responsible for almost all of the base pairing in the stem-loop structure that was proposed to exist in the DHBV pregenome (17), and this result excludes the possibility that *pet* function requires this particular stem-loop structure. In addition, some sequences required for *pet* activity are shared with the proposed stem-loop structure, implying that any putative RNA structure responsible for *pet* activity would require an alternate folding to that of the stem-loop, and therefore both *pet* and ε could not exist at the same time on a single RNA. Finally, the function of ε in RNA packaging and in DNA synthesis depends on the virus-encoded P protein (1, 13, 37), whereas no viral proteins are required for *pet* activity. The putative structural elements responsible for *pet* and ε could both function in *cis* on the same pregenome only if the *pet* structure were replaced by the ε structure after transcription and before packaging occurred.

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