JOURNAL OF VIROLOGY, Oct. 1991, p. 5190–5195
0022-538X/91/105190-06$02.00/0

Vol. 65, No. 10

Identification of a Signal Necessary for Initiation of Reverse Transcription of the Hepadnavirus Genome

CHRISTOPH SEEGER* AND JOHNNA MARAGOS

Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

Received 3 May 1991/Accepted 19 June 1991

Reverse transcription of the hepadnavirus genome initiates near the 3′ end of the RNA template and has previously been shown to depend on sequences flanking the initiation site for DNA synthesis (C. Seeger and J. Maragos, J. Virol. 64:16–23, 1990). DNA synthesis leads to the covalent attachment of a protein to the 5′ end of minus-strand DNA, and it is generally believed that this protein serves as the primer for reverse transcription. To examine priming in more detail, we have carried out a detailed genetic analysis of the nucleotide sequences at the origin of minus-strand DNA synthesis characterized in our earlier study. This mutational analysis has led to the identification of a short, four-nucleotide-long sequence as the signal for initiation of reverse transcription. This signal is a UUUC sequence motif flanking the position of the 5′ end of minus-strand DNA, which alone is not sufficient for DNA synthesis, indicating that positional effects are also important to specify the origin of DNA synthesis.

The DNA genomes of woodchuck hepatitis virus (WHV) and other hepadnaviruses replicate by reverse transcription of minus-strand DNA from an RNA intermediate, termed pregenomic RNA (5, 11, 21). Initiation of reverse transcription occurs close to the 3′ end of pregenomic RNA. DNA synthesis leads to the formation of a covalent link between protein and the 5′ end of minus-strand DNA (6). The protein, called terminal protein (TP), is encoded by the viral polymerase gene, which also encodes the viral reverse transcriptase, and it has been proposed that the TP actually functions as the primer for minus-strand DNA synthesis (2, 6). Support for this hypothesis is derived from the observations that short, nascent minus-strand DNA species of duck hepatitis B virus are linked to protein (12) and that the signals necessary for both initiation of DNA synthesis and the attachment of protein to the 5′ end of minus-strand DNA of WHV are contained in a short sequence motif spanning less than 20 nucleotides (20). Although the protein-primer model is attractive, since it would provide a rationale for the formation of the DNA-TP bond, the presence of protein at the 5′ end of minus-strand DNA can also be explained by other mechanisms. For example, protein could be attached to nascent minus-strand DNA via cleavage of a phosphodiester bond, similar to the mechanism proposed for the attachment of the poliovirus VPg protein to viral RNA (23). Clarification of this reaction in the hepadnavirus system will depend on the availability of an in vitro system allowing for the covalent attachment of deoxynucleoside monophosphate (dNMP) to the TP. This approach still remains elusive, since attempts to solubilize the polymerase from purified virions or to express the same product with enzymatic activity in *Escherichia coli* or yeast cells have so far been unsuccessful.

To better understand the reaction controlling priming of reverse transcription of minus-strand DNA in hepadnaviruses, we have performed a detailed genetic analysis of the nucleotide sequences important for minus-strand DNA synthesis of WHV. Specifically, we sought to genetically identify a minimal origin of minus-strand DNA synthesis on the pregenome of WHV and to test whether the polymerization of minus-strand DNA can occur independently of the reaction that links protein to DNA. Our results indicate that minus-strand DNA synthesis depends on a short UUUC sequence motif adjacent to the 5′ end of minus-strand DNA. Since this motif is repeated throughout the viral genome, this signal alone cannot control initiation of reverse transcription. We show, in fact, that the activity of the signal for DNA synthesis depends on its relative position on the RNA pregenome. Furthermore, our results suggest that the nucleotidyl-protein bond between DNA and the TP does not depend on a specific nucleotide on the RNA pregenome.

MATERIALS AND METHODS

Cell culture. Maintenance of HepG2 cells (1) and procedures for DNA transfection and purification of virion DNA from the culture medium of transfected cells were performed as described previously (19).

Molecular clones. The construction of pCMW82 has been described previously (19). Briefly, WHV clone pWHV81-2 was fused with pBC12/CMV/IL2 (4) to direct the synthesis of WHV pregenomic RNA from the cytomegalovirus immediate-early promoter on pBC12/CMV/IL2. Variants of pCM W82 were prepared by oligomer-directed site-specific mutagenesis as described by Taylor et al. (22). All mutations were verified by nucleotide sequence analysis. Construction of plasmids p82*dl*C and p82T3 is described in reference 20.

DNA oligomers. DNA oligomers for primer extension reactions were DNA oligomer CS3, mapping to positions 3229 to 3248 on the WHV genome, and DNA oligomer 2467, spanning positions 2467 to 2490.

Primer extension analysis. Primer extension reactions on DNA purified from viral particles were performed as described in reference 19.

Southern blot analysis. DNA samples were subjected to electrophoresis through agarose gels in 0.2 M NaOH and then transferred to nylon membranes (Amersham) and hybridized to ^{32}P-labeled WHV DNA in hybridization buffer (0.45 M $NaHPO_4$ [pH 7.2], 0.9 mM disodium EDTA, 6% sodium dodecyl sulfate, 9% formamide, 0.9 mg bovine serum albumin [Sigma] per ml) at 65°C. Films were exposed at

* Corresponding author.

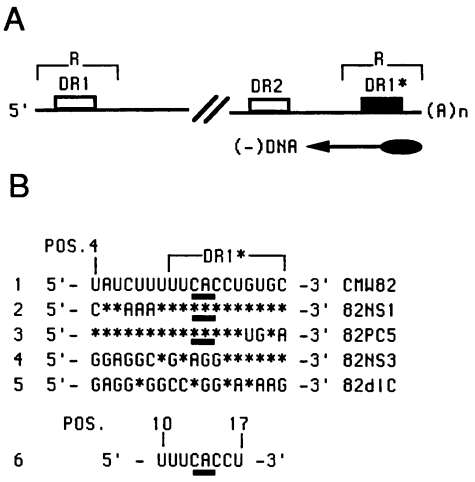

FIG. 1. Origin of minus-strand DNA synthesis. (A) Pregenomic RNA of WHV with direct repeats DR1 and DR2. The initiation site for minus-strand DNA synthesis is at DR1 close to the 3′ end of the RNA, marked DR1*. (B) Nucleotide sequence of pregenomic RNA of WHV from positions 4 to 21 surrounding the initiation site for minus-strand DNA synthesis. The horizontal bar indicates the nucleotides that correspond to the 5′ end(s) of minus-strand DNA (see text). Lines 2 and 3 show the nucleotide sequences of two WHV variants, p82NS1 and p82PC5, with a functional origin of DNA replication; lines 4 and 5 show the sequences of mutants p82NS3 and p82*dl*C, which are incompetent for minus-strand DNA synthesis beginning at DR1* (for details, see reference 20). Line 6 shows the nucleotide sequence pattern required for minus-strand DNA synthesis based on results obtained from the variants shown in lines 2 to 5 and from the analyses of additional variants described in reference 20.

−70°C to Kodak XAR-5 film with intensifying screens (DuPont).

Nomenclature. Numbering of the WHV2 genome was arranged to begin with the first ATG of the precore gene (position 1931 in reference 9).

RESULTS

Mutations at the origin of DNA synthesis do not block DNA synthesis. Synthesis of minus-strand DNA of WHV and other hepadnaviruses commences near the 3′ end of the RNA pregenome at direct repeat 1 (DR1), herein termed DR1* (Fig. 1A) (20). Deletion analysis of the region flanking DR1* showed that an RNA segment, spanning positions 10 to 17 on pregenomic RNA, is required for DNA synthesis (Fig. 1B). Based on the length of DNA products obtained with primer extension reactions on minus-strand DNA extracted from viral particles, the 5′ end of minus-strand DNA of WHV maps to position 13 or 14 on the RNA pregenome (Fig. 1B) (18). Reactions with virion DNA expressed in tissue culture cells consistently yielded a third DNA extension product, corresponding to 5′ ends of minus-strand DNA beginning at position 15 (Fig. 2C; compare lanes 8 and 9). Therefore, we anticipated that mutations between positions 10 and 17 would either abrogate minus-strand DNA synthesis or lead to the synthesis of minus strands with altered 5′ ends, or perhaps interfere with the attachment of protein to the 5′ end of the DNA.

To test this notion, we created six variants of the wild-type WHV expression vector pCMW82 with single base changes between nucleotides 13 and 16 (Fig. 2A). pCMW82 contains a cDNA equivalent of WHV pregenomic RNA fused to the cytomegalovirus immediate-early promoter-enhancer region, as described previously (19). The six derivatives of pCMW82 were assayed for virus production after transfection into HepG2 cells. Virus particles were concentrated from the supernatant of transfected cells; DNA was extracted and subjected to primer extension analysis for determination of the 5′ ends of minus-strand DNA. Primer extension analyses performed with virion DNA expressed with plasmids p82C14, p82G16, p82U15, and p82A15 yielded the same three DNA products, 105 to 107 nucleotides in length, as obtained from the extension reaction with virion DNA expressed with the wild-type plasmid, pCMW82 (Fig. 2B, lanes 2 to 6; Fig. 3B, lane 6). In contrast, reactions with virion DNA expressed with p82A13 and p82G13, in which the pyrimidine C residue at position 13 was substituted with the purines A and G, led to the synthesis of two additional shorter (103 and 104 nucleotides) extension products (Fig. 2C, lanes 5 and 6).

To test whether nucleotide changes at position 13 would interfere with the linkage of protein to minus-strand DNA, HepG2 cells were transfected with variants p82A13 and p82G13 and progeny virions were concentrated from the supernatant of the transfected cells. DNA was extracted from virions with and without incubation with proteinase K prior to phenol extraction. It is known that omission of proteinase K treatment prior to phenol extraction results in the loss from the aqueous phase of minus-strand DNA that is linked to protein (6). Purified virion DNA was subjected to electrophoresis through alkaline agarose, transferred to nitrocellulose, and hybridized with WHV-specific ^{32}P-labeled DNA. As shown in Fig. 2D, omission of proteinase K from the DNA purification procedure led to the loss of viral DNA species in all cases examined. This result indicated that the 5′ ends of minus-strand DNA synthesized with both mutants were covalently linked to protein.

To ensure that DR1* and not DR1, which is at the 5′ end of pregenomic RNA, directed minus-strand DNA synthesis, expression of virion DNA from p82T3, a plasmid carrying a one-nucleotide insertion between positions 9 and 10 at DR1*, was included as a control (Fig. 1B; Fig. 2B, lane 1) (20). This control also tested whether new DNA species were created by homologous recombination between the terminally redundant ends of the WHV sequences in pCMW82 that could have led to the synthesis of detectable levels of viral progeny. As expected from previous investigations, the major DNA products obtained in primer extension reactions with virion DNA synthesized with p82T3 were one nucleotide longer than those obtained with pCMW82 or any of the other constructs tested so far (Fig. 2B, lane 1). This result showed that under the described assay conditions, only minus strands originating from DR1* were detected.

In sum, our results so far demonstrated that synthesis of minus-strand DNA was not dependent on residues spanning positions 13 to 16 and suggested that the nature of the linkage between the TP to DNA was not particular for a specific nucleotide. The most surprising finding emerging from this analysis in conjunction with observations made previously (Fig. 1B) was that the primary nucleotide sequence requirement for minus-strand DNA synthesis is possibly limited to no more than the short stretch of uridylyl residues adjacent to the initiation sites for minus-strand DNA synthesis.

UUUC carries the signal for minus-strand DNA synthesis. The idea that a short stretch of uridylyl residues may be a part of the signal for reverse transcription emerged from the

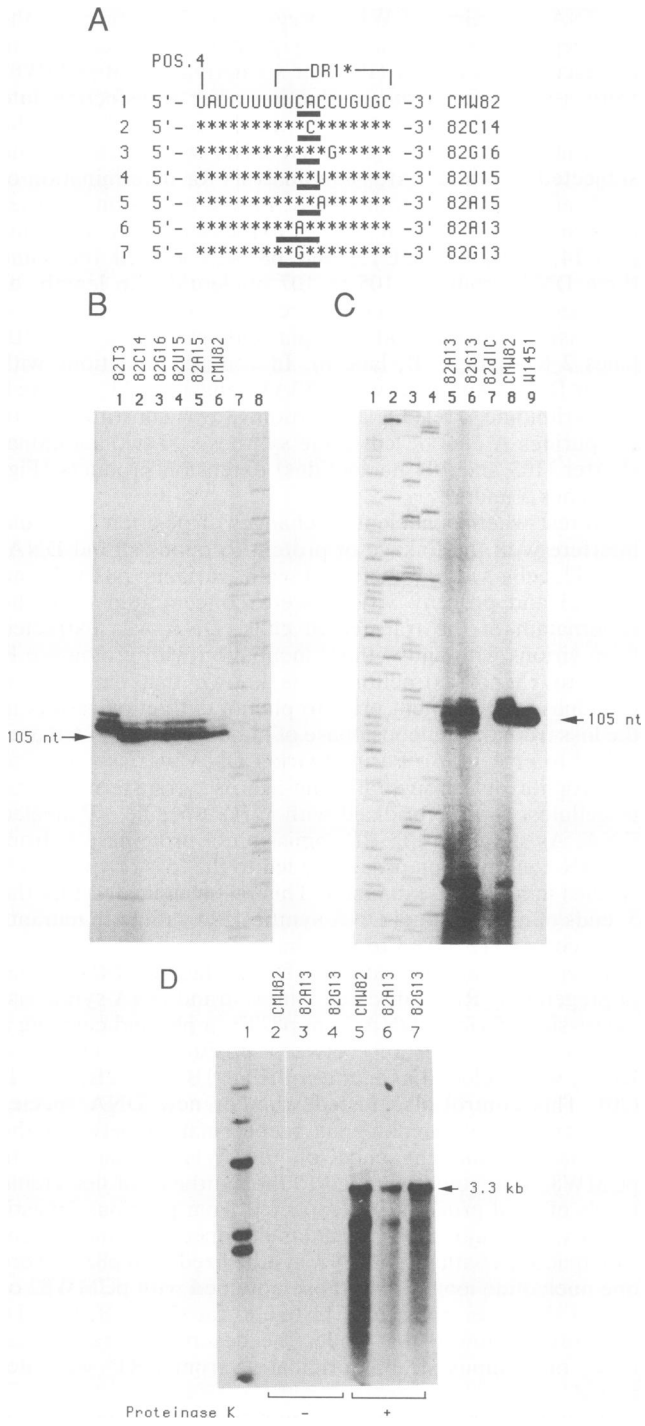

FIG. 2. Mutations that do not change the position of the 5′ end of minus-strand DNA. (A) Nucleotide sequences of WHV pregenomic RNA between positions 4 and 21 (line 1) and of six WHV variants with single base changes. Unchanged nucleotides are marked with asterisks; the 5′ ends of minus-strand DNA are marked by horizontal bars. (B) DNA products of primer extension reactions made on virion DNA purified from the supernatants of cells transfected with WHV variants p82T3, p82C14, p82G16, p82U15, and p82A15 and wild-type pCMW82 (lanes 1 to 6). DNA extension reactions were primed with a 5′-$^{32}PO_4$-labeled DNA oligomer, CS3 (positions 3229 to 3248 on the WHV genome). The size marker was the A and C tracks of a nucleotide sequence derived from pCMW82 primed with DNA oligomer CS2 (positions 3248 to 3229; lanes 7 and 8). (C) DNA products from reactions with virion DNA expressed with p82A13 and p82G13 (lanes 5 and 6). Lanes 7 to 9 show the DNA products obtained with virion DNA expressed with p82*dlC* and pCMW82 and with DNA purified from virus particles in the serum of a WHV-infected woodchuck. Lanes 1 to 4 show the nucleotide sequence ladder obtained with pCMW82 primed with DNA oligomer CS3. nt, nucleotides. (D) DNA extracted from virions expressed with pCMW82 (lanes 2 and 5), p82A13 (lanes 3 and 6), and p82G13 (lanes 4 and 7). DNA was separated by electrophoresis through 1.2% agarose under denaturing conditions, transferred to a membrane, and hybridized to $^{32}PO_4$-labeled WHV DNA. Virus particles were extracted with phenol with (lanes 5 to 7) or without (lanes 2 to 4) prior incubation with proteinase K. DNA fragments generated from lambda DNA incubated with the restriction endonuclease *Hin*dIII served as molecular weight standards (lane 1).

analysis of minus-strand DNA expressed with p82U13 (Fig. 3A, line 2). This construct contained, in addition to a C-to-T transition at position 13, three mutations at positions 16, 18, and 19, creating a UUCC motif on pregenomic RNA, similar to the sequence pattern at positions 10 to 13. When this construct was tested, these changes led to the synthesis of two additional DNA products, 110 and 111 nucleotides in length, corresponding to 5′ ends of minus-strand DNA initiating at positions 18 and 19, respectively (Fig. 3B, lane 6). To directly test whether UUUC was the signal for minus-strand DNA synthesis, we further modified p82U13 by changing the U residue at position 11 to C, hereby generating a UUUC motif beginning at position 8 (Fig. 3A, line 3). Transfection of p82CU into HepG2 cells led to the expression of virions with a predominant minus-strand DNA species originating at position 11 rather than at position 13, as observed with virion DNA expressed with the parental plasmid p82CU13 or the wild-type pCMW82 (compare Fig. 3C, lane 5, with Fig. 3B, lanes 4 and 6). The same result was obtained with a second variant, p82CA, in which the UUUC pattern was also moved by two nucleotides from position 10 to position 8 (Fig. 3A, line 4; Fig. 3B, lane 5).

Taken together, these results indicated that DNA synthesis depended on a sequence motif composed of a short, two- or three-nucleotide-long uridylyl track and a cytosyl residue located adjacent to the initiation site for reverse transcription of minus-strand DNA. Since pregenomic RNA contained additional copies of this sequence pattern, we speculated that DR1* was not the only site at which DNA synthesis could begin.

Synthesis of minus-strand DNA in the absence of DR1*. The RNA pregenome of WHV contains over 30 UUUC sequence motifs that, on the basis of our mutational analysis, could serve as potential initiation sites for minus-strand DNA synthesis (Fig. 4A). We had previously constructed a derivative of pCMW82, termed p82*dlC*, that lacked the UUUC pattern at DR1* and hence did not produce the expected 3.3-kb minus-strand DNA (Fig. 1B) (20). However, we noticed that the supernatants of cells transfected with p82*dlC* contained viral DNA products detectable with the help of the polymerase chain reaction (16). This observation was confirmed by Southern analysis of DNA extracted from the supernatant of HepG2 cells transfected with p82*dlC* (Fig. 4B). As expected, the virion DNA isolated from the supernatant of cells transfected with the wild-type construct pCMW82 contained 3.3-kb DNA species and shorter heterogeneous DNA forms that represented nascent DNA species predominantly of minus-strand DNA origin (Fig. 4B, lane 1). In the DNA sample purified from virion DNA expressed

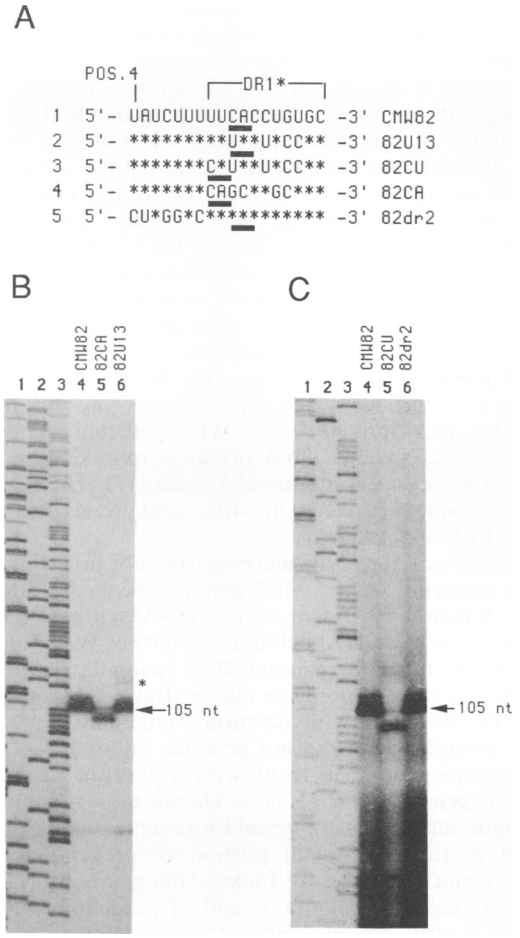

FIG. 3. Mutations affecting the position of minus-strand DNA. (A) Nucleotide sequences of pCMW82 (line 1) and variants p82U13, p82CU, p82CA, and 82dr2 (lines 2 to 5). Unchanged residues are depicted with asterisks; positions of the 5′ ends of minus-strand DNA are depicted with horizontal bars. (B and C) DNA products of the primer extension reactions performed with virion DNA expressed with p82CA and p82U13 (B; lanes 5 and 6) and p82CU and p82dr2 (C; lanes 5 and 6). Extension reactions on virion DNA expressed with pCMW82 are shown in lanes 4, and the C, G, and T tracks of a nucleotide sequence reaction with pCMW82 are shown in lanes 1 to 3. DNA oligomer CS3 (see Fig. 2B) served as a primer for extension reactions and for DNA sequencing. nt, nucleotides.

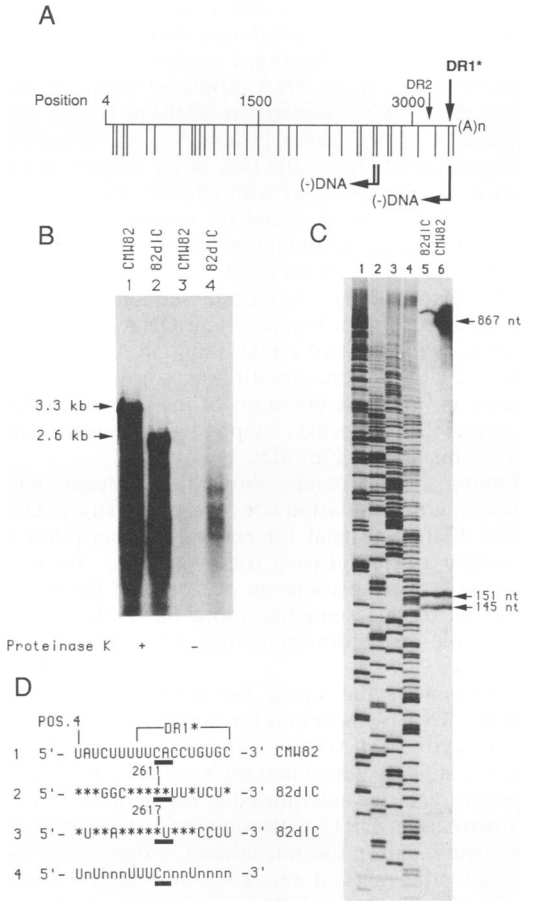

FIG. 4. Identification of additional sites for initiation of reverse transcription on the WHV pregenome. (A) Sequence of pregenomic RNA. Vertical bars below the line depict the presence of UUUC sequence motifs on pregenomic RNA of WHV. The initiation sites for minus-strand DNA at DR1* and at positions 2611 and 2617 are indicated (see text). (A)n, polyadenylate residues of pregenomic RNA. (B) Southern analysis of DNA extracted from virus particles in the supernatant of HepG2 cells transfected with pCMW82 (lanes 1 and 3) and p82*dl*C (lanes 2 and 4) (see legend to Fig. 2D). DNA was purified from viral particles with (lanes 1 and 2) and without (lanes 3 and 4) addition of proteinase K prior to extraction of DNA with phenol. Size markers were DNA fragments obtained after digestion of lambda DNA with the restriction endonuclease *Hin*dIII. (C) Primer extension analysis on virion DNA samples shown in lanes 1 and 2 of Fig. 4B (lanes 5 and 6). Extension reactions were primed with DNA oligomer 2467. The size marker was the A, C, G, and T tracks of a nucleotide sequence derived from pCMW82 primed with DNA oligomer 2467. nt, nucleotides. (D) Nucleotide sequence motifs at the initiation site for minus-strand DNA at DR1* (line 1) and at positions 2611 and 2617 (lines 2 and 3). Line 4 shows the nucleotides that are common to the sequence patterns shown in lines 1 to 3. Positions that vary among the three motifs are marked with the letter n. The horizontal bar shows the positions of the 5′ ends of minus-strand DNA.

with p82*dl*C, a ca. 2.6-kb-long DNA fragment and shorter DNA forms were present (lane 2). When proteinase K was omitted prior to extraction of viral DNA with phenol, the bulk of viral DNA was removed from the aqueous phase, suggesting that the 5′ ends of both the 3.3- and 2.6-kb DNA species were covalently attached to protein (Fig. 4B, lanes 3 and 4).

To identify the 5′ ends of the 2.6-kb minus-strand DNA species, we performed primer extension analysis on virion DNA isolated from the supernatant of cells transfected with p82*dl*C. With a DNA oligomer beginning at position 2467 on the WHV genome, two major extension products 145 and 151 nucleotides in length were obtained (Fig. 4C, lane 5). As observed in primer extension reactions for the identification of the natural 5′ ends of minus-strand DNA, two fainter bands were detected that were one and two nucleotides longer than the major DNA products. This result indicated that the 3′ ends of the observed DNA products corresponded to 5′ ends of minus-strand DNA and thus did not represent random pause sites for avian myeloblastosis virus reverse transcriptase on the minus-strand DNA template. The lengths of the two DNA products corresponded to minus-strand DNA species initiating at positions 2611 and 2617 on the WHV genome (Fig. 4D). Based on the position of the 5′

ends, the two minus-strand DNA species were 2,607 and 2,613 nucleotides long, in good agreement with the length of 2.6 kb estimated from Southern analysis (Fig. 4B). Extension reactions on virion DNA produced with the wild-type construct pCMW82 revealed, in addition to the expected DNA product of 867 bases in length, corresponding to 5′ ends originating at DR1*, the two DNA fragments obtained with virion DNA expressed with p82*dl*C. Thus, even in the presence of a functional signal for reverse transcription at DR1*, shorter minus strands were expressed from truncated initiation sites near position 2600.

A comparison of the nucleotide sequences flanking the natural initiation site of minus-strand DNA at DR1* and the initiation sites for the two 2.6-kb-long DNA species showed that the UUUC sequence motif was conserved among the three sites and that the positions of the 5′ ends of the three minus-strand DNA species mapped to the same site with respect to this signal (Fig. 4D).

In summary, these results showed that pregenomic RNA contained multiple initiation sites for minus-strand DNA and confirmed that the signal for reverse transcription is contained within a uridylyl tract followed by C. Since over 30 UUUC motifs were present on pregenomic RNA, the relative position of this sequence motif most likely played an important role in the determination of the 5′ end of minus-strand DNA.

The position of the signal for reverse transcription on pregenomic RNA is important for minus-strand DNA synthesis. To directly test whether the activity of the UUUC sequence motif was dependent on its relative position on the RNA pregenome, we substituted a fragment of seven nucleotides upstream of DR1* with sequences derived from DR2. Reverse transcription did not initiate at detectable levels at DR2 despite the marked sequence homology between the region flanking DR2 and DR1 (Fig. 3A, line 5; Fig. 4A). It was, however, conceivable that the absence of minus-strand DNA was due to the substitution of the first U residue of the UUUC motif by C. The resulting construct, p82dr2, was transfected into HepG2 cells, and virus particles were concentrated from the supernatant of the transfected cells. Following extraction of virion DNA, the 5′ ends of minus-strand DNA were determined by primer extension analysis. The extension reaction yielded the same DNA products as those obtained with virion DNA expressed from wild-type pCMW82 (Fig. 3C, lane 6). Thus, the DR2 sequence motif was activated following transposition to the DR1* region. This result directly confirmed the notion that in addition to nucleotide sequence composition, positional effects played a role for the selection of the initiation site for minus-strand DNA synthesis.

DISCUSSION

The purpose of this investigation was to identify the nucleotide sequences on the RNA pregenome of WHV that determine the initiation site for minus-strand DNA synthesis. Consideration of findings in the adenovirus system, in which a protein primes DNA synthesis (3, 7, 14, 15), and of our own investigations led us to the assumption that the nucleotide sequences flanking the 5′ end of minus-strand DNA were sufficient as signals for reverse transcription. However, our experiments revealed that the mechanism for priming DNA synthesis in hepadnaviruses is unique. It depends on a nucleotide sequence motif that is too short to independently govern the initiation reaction and therefore must rely on additional signals on the RNA pregenome. These could be provided by secondary structure forced upon RNA directly by nucleotide sequence composition or by the core protein encasing the RNA template.

The origin of hepadnavirus DNA synthesis requires signals from separate sequence elements on pregenomic RNA. From our results, we concluded that only selected sites bearing a short uridylyl track followed by a cytosyl residue can function as signals for reverse transcription. For example, while the sequence motifs near position 2600 provided signals for DNA synthesis, the five UUUC motifs between position 2700 and DR1* did not function as origins of DNA synthesis (Fig. 4A). In agreement with this notion were our previous results, showing that deletions removing the motif at DR1* abrogated minus-strand DNA synthesis from the 3′ end of pregenomic RNA (Fig. 1B). It is noteworthy that Southern or primer extension analyses on virion DNA isolated from virions in sera of WHV-infected woodchucks did not reveal specific DNA products representing minus-strand DNA species with novel 5′ ends (17). The reason for the discrepancy between in vitro- and in vivo-expressed virion DNA is not known.

An important question emerging from our investigations is which segments of the viral genome bear the additional signals required for minus-strand DNA synthesis. Results obtained with a series of deletion mutants of WHV indicated that a fragment of pregenomic RNA spanning positions 250 to 2200 was not required for minus-strand DNA synthesis. However, plasmids with deletions extending beyond this region towards DR1* did not produce minus DNA strands when complemented in *trans* with a plasmid carrying the wild-type genome of WHV (17). Therefore, it is conceivable that additional signals important for reverse transcription are located in the 3′-terminal portion of pregenomic RNA. Recent results reported by Junker-Niepmann et al. (8) suggest that sequences at the 5′ end of pregenomic RNA of hepatitis B virus provide signals for packaging of viral RNA and reverse transcriptase. It thus appears that at least three signals on pregenomic RNA are important for reverse transcription, two of which are located at the 5′ and 3′ ends of pregenomic RNA and one (or more) of which is located between position 2200 and DR1*. The role of the third signal may simply be to provide a structural scaffold for reverse transcription of minus-strand DNA.

How is the TP attached to the 5′ end of minus-strand DNA? Experiments presented in this report showed that mutations at or adjacent to the 5′ end(s) of minus-strand DNA do not abrogate DNA synthesis. Thus, it appears that the terminal protein of hepadnaviruses can undergo a covalent linkage with any of the four nucleotides. This observation contrasts with findings made in the adenovirus system, in which protein-mediated priming of DNA synthesis showed a strong specificity for the formation of protein-DNA linkage with dCMP (3, 7, 14). The potential of the TP to attach to different dNMP residues is, however, reminiscent of topoisomerases, for which the nucleotidyl-protein linkage can also occur with any of the four nucleotides (24).

Since primer extension analysis cannot confirm a colinear relationship between pregenomic RNA and minus-strand DNA of WHV, we cannot, a priori, exclude the possibility that formation of the protein-DNA linkage occurs by a template-independent mechanism. This model predicts that in a first step, dNMP is attached to the TP and that the 3′ hydroxyl group of this nucleotidyl residue is the primer for DNA synthesis. Hence, the specificity of the priming reaction would depend on the annealing of the dNMP residue with a particular nucleotide on pregenomic RNA, perhaps

similar to the mechanism described for RNA-primed transcription of influenza virus mRNA (13). However, expression of virion DNA with the mutants shown in Fig. 2A did not lead to the formation of 5′ ends that mapped to a particular nucleotide on pregenomic RNA, as would be expected if this model were correct. In addition, Lien et al. (10) demonstrated by Maxam and Gilbert nucleotide sequence analysis of virion DNA that the 5′ ends of minus strands of DHBV are colinear with the corresponding sequence on pregenomic RNA, indicating that the 5′ ends do not contain additional sequences not encoded by RNA.

With the help of genetic analyses, we have gained considerable insight into the structural organization of the hepadnavirus origin of minus-strand DNA synthesis. While considering primary nucleotide sequence a major factor for the determination of the signals necessary for DNA replication, it now appears that structural components of the viral replication machinery provide additional information necessary for DNA synthesis. As shown in this report for the initiation of minus-strand DNA synthesis, the mechanism of plus-strand DNA synthesis, which requires the transfer of an RNA primer from the 5′ end of the RNA genome to DR2 on minus-strand DNA, may similarly depend on the structural arrangement of the replication complex. It is conceivable that the activity of the hepadnaviral reverse transcriptase itself depends on conformational determinants imposed by the viral nucleocapsid.

ACKNOWLEDGMENTS

We thank Rich Katz, Bill Mason, and John Taylor for helpful comments and suggestions on the manuscript.

This work was supported by Public Health Service grants AI-24972 and AI-30544 from the National Institutes of Health.

REFERENCES

1. **Aden, D. P., A. Fogel, S. Plotkin, I. Damjanov, and B. Knowles.** 1979. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature (London) **282**:615–616.
2. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. **7**:4185–4192.
3. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58**:671–717.
4. **Cullen, B. R.** 1987. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:972–982.
5. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56**:651–693.
6. **Gerlich, W. H., and W. S. Robinson.** 1980. Hepatitis B virus contains protein attached to the 5′ terminus of its complete DNA strand. Cell **21**:801–809.
7. **Ikeda, J.-E., T. Enomoto, and J. Hurwitz.** 1982. Adenoviral protein-primed initiation of DNA chains in vitro. Proc. Natl. Acad. Sci. USA **79**:2442–2446.
8. **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.
9. **Kodama, K., N. Ogasawara, H. Yoshikawa, and S. Murakami.** 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutional relationship between hepadnaviruses. J. Virol. **56**:978–986.
10. **Lien, J., 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.
11. **Mason, W. S., J. M. Taylor, and R. Hull.** 1987. Retroid virus genome replication. Adv. Virus Res. **32**:35–96.
12. **Molnar-Kimber, K. L., J. W. Summers, J. M. Taylor, and W. S. Mason.** 1983. Protein covalently bound to minus-strand DNA intermediates of duck hepatitis B virus. J. Virol. **45**:165–172.
13. **Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug.** 1981. A unique cap(m^7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23**:847–858.
14. **Rawlins, D. R., P. J. Rosenfeld, R. J. Wides, M. D. Challberg, and T. J. Kelly.** 1984. Structure and function of adenovirus origin of replication. Cell **37**:309–319.
15. **Rekosh, D. M. K., W. C. Russell, A. J. D. Bellet, and A. J. Robinson.** 1977. Identification of a protein linked to the ends of adenovirus DNA. Cell **11**:283–295.
16. **Saiki, R. K., D. H. Gelfland, B. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
17. **Seeger, C.** Unpublished data.
18. **Seeger, C., D. Ganem, and H. E. Varmus.** 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science **232**:477–484.
19. **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.
20. **Seeger, C., and J. Maragos.** 1990. Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. J. Virol. **64**:16–23.
21. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
22. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13**:8765–8784.
23. **Tobin, G. J., D. C. Young, and J. B. Flanegan.** 1989. Self-catalyzed linkage of poliovirus terminal protein VPg to poliovirus RNA. Cell **59**:511–519.
24. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.