Protein Covalently Bound to Minus-Strand DNA Intermediates of Duck Hepatitis B Virus

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Analysis of duck hepatitis B viral DNA by gel electrophoresis, Southern blotting, and binding to benzoylated naphthoylated DEAE-cellulose showed that a protein is bound to the minus-strand virion DNA as well as to the full-length single strand, minus-strand species, and minus-strand DNA intermediates isolated from replicating complexes present in infected duck liver. By utilizing a modified dideoxynucleotidyl sequencing method, it was shown that the protein is covalently bound to the smallest detectable growing strands (ca. 30 bases) and that minus-strand synthesis begins at a unique site. These results support the notion that the protein may function as a primer for synthesis of the minus-strand DNA.

A protein has been shown to be covalently bound to the DNA and RNA genomes of several distinct families of viruses, including Picornaviridae, Caliciviridae, Adenoviridae, Comoviridae, and Luteoviridae (23). Since the protein is covalently bound to the 5' end, several groups of investigators have suggested that the protein may act as the primer for DNA and RNA synthesis. Recent studies of the mechanism of replication of poliovirus (14, 15), *Bacillus subtilis* phage ϕ 29 (13), and particularly adenoviruses (3, 8) support the "primer" hypothesis. Gerlich and Robinson (6) have demonstrated that hepatitis B virus (HBV) also contains a protein covalently linked to its genome. In contrast to the other double-stranded DNA viruses, such as adenovirus and ϕ 29, the protein appears to be linked only to the minus strand. This observation raises the question of whether this bound protein may also act as primer for minus-strand synthesis, whether the protein serves as a packaging signal for the genome analogous to the ϕ 29 protein (1), or whether the protein exhibits other functions. To investigate this question, we are studying the properties of the genome of the duck hepatitis B virus (DHBV), which is similar in molecular structure to HBV (12).

DHBV has a small circular genome which consists of two duplexed DNA strands: the minus strand is $\approx$ 3 kilobases and the plus strand varies in length up to unit size. The mechanism of replication has recently been elucidated (22). Briefly, after infection, transcription of RNA and translation of proteins occurs; DNA polymerase and a genome-sized RNA are assembled into an immature core structure. The polymerase synthesizes minus-strand DNA from the

RNA template, utilizing a reverse transcriptase-like activity. After completion of minus-strand DNA synthesis, plus-strand DNA replication begins. This mechanism explains the recent finding of significant quantities of single-stranded full-length minus-strand DNA in infected liver (11).

The possibility that the protein which is covalently bound to the minus-strand DNA of HBV may act as a primer for minus-strand synthesis has been addressed in the analogous DHBV system by determining (i) whether a protein is covalently bound to the DNA and (ii) the length of the DNA intermediate on which the bound protein is first observed. In this report, we demonstrate that a protein is covalently bound to the minus strand of virion DNA, to the full-length single-stranded minus-strand species found in infected liver, and to minus-strand DNA intermediates which are isolated from replicating complexes present in infected liver. By utilizing a modified dideoxynucleotidyl sequencing technique (18), we show that the smallest fragment we can detect (<30 bases) contains a covalently bound protein and that the 5' end of the minus-strand DNA appears to be unique.

MATERIALS AND METHODS

Ducks. One-day-old ducklings were purchased from a commercial supplier. Ducklings were bled by cardiac puncture, and sera were assayed for the presence of DHBV DNA by dot hybridization, probing with a [32 P]dTTP-labeled DHBV DNA which was prepared from a clone containing DHBV in a lambda Charon 16A vector (11). Six- to fourteen-day-old infected ducklings were used.

Virus. One- to 4-milliliter serum samples were layered onto 10 to 20% sucrose gradients containing 0.15

M NaCl and 20 mM Tris-hydrochloride (pH 7.4). The virus was pelleted by centrifugation at 24,000 rpm for 16 h in an SW40 rotor. Endogenous polymerase reactions were performed as described previously (12). As noted in the text, some endogenous polymerase reactions were performed in the absence of radiolabeled nucleotides solely to complete the synthesis of the plus strand.

Isolation of protein-DNA complex and protease K treatment. The endogenously labeled virions were dissociated by heating at 60°C in 2% sodium dodecyl sulfate (SDS)-2% β -mercaptoethanol (β -ME) for 20 min. The protein-DNA complex was layered onto a 5 to 20% sucrose gradient consisting of 10 mM Tris-hydrochloride (pH 7.4), 10 mM EDTA, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 50,000 rpm for 4.3 h in an SW60 rotor, 0.1-ml fractions were collected and assayed for the presence of DHBV DNA by electrophoresis into an SDS-containing 0.7% agarose gel, blotting to a nitrocellulose filter by the Southern technique (20), hybridizing with a [32 P]dTTP-labeled DHBV-specific probe, and autoradiography.

To extract protein-bound DNA from infected liver tissue, 0.1 g of liver was homogenized in 1.5 ml of urea-SDS buffer (8 M urea, 0.1% SDS, 10 mM Tris-hydrochloride [pH 7.4], 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) and incubated for 1 h at 22°C. After gentle sonication to reduce viscosity, the sample was layered onto a 9- by 0.5-cm column of Sepharose 4B equilibrated with urea-SDS buffer. One milliliter of the relevant fractions as determined by optical density at 254 nm was layered onto a 9- by 0.5-cm column of Sepharose 4B equilibrated with TE + SDS buffer (10 mM Tris-hydrochloride [pH 7.4], 10 mM EDTA, 0.1% SDS) to remove the urea.

Replicating core complexes were extracted from infected liver and were endogenously labeled at 37°C for 30 min in the presence of 100 μ g of actinomycin D per ml, 50 mM NaCl, 20 mM Tris-hydrochloride (pH 7.4), 7 mM MgSO_4 , 0.1% β -ME, 13 pmol of [32 P]dTTP, and 50 μ M dATP, dCTP, and dGTP, as described previously (22). Cores were found to be dissociated by a variety of procedures, including (i) heating in 2% β -ME-2% SDS at 60°C for 20 min, (ii) incubation at 37°C for 30 min in 0.1% SDS-20 mM EDTA-10 mM dithiothreitol, and (iii) incubation at 37°C for 30 min in 0.1% SDS-20 mM EDTA. Disruption was complete, as measured by autoradiography of the samples after electrophoresis into an SDS-containing 1.5% agarose gel. Procedures (i) and (iii) were used as indicated in figure legends.

Where noted in the text, samples were digested with 0.5 mg of protease K (Sigma Chemical Co.; self-digested for 2 h at 37°C) per ml in buffer containing 0.1% SDS for 1 h at 37°C. Where indicated, NaCl was added to a final concentration of 0.1 M, and the sample was deproteinized in the presence of 10 μ g of tRNA carrier by extraction with an equal volume of equilibrated (50 mM Trizma base [pH 8.0]) phenol-chloroform (1:1). Samples were concentrated as noted by ethanol precipitation in 0.3 M NaAc performed in siliconized plastic tubes.

It should be noted that the protein-DNA complex readily absorbed onto plastic and particularly glass in the absence of 0.1% SDS. Siliconized pipettes and tubes were used whenever possible. Resuspension of

the complex after concentration by ethanol precipitation was increased to 50 to 90% recovery by heating the sample at 68°C for 5 min in 10 to 20 mM EDTA-0.1% SDS. Treatment with protease K eliminated these problems. Samples were stored at -80°C and were used within 2 to 3 weeks.

Chromatography on BND-cellulose. The protein-DNA complex was separated from the SDS by filtration through a 9- by 0.5-cm column of Sepharose 2B or Sephadex G-50 equilibrated with TE (10 mM Tris-hydrochloride [pH 7.4], 10 mM EDTA) and recovered in the excluded volume as detected by Cerenkov radiation. NaCl was added to a final concentration of 0.3 M, and chromatography with benzoylated naphthoylated DEAE (BND)-cellulose was performed as originally described by Sedat et al. (19) with modifications by Stillman and Bellett (21). Briefly, the DNA sample was passed through the column (1 by 0.5 cm) three times to ensure binding and then washed three times (1.0 ml each time) with 0.3 M NaCl-TE. Double-stranded DNA was eluted in 1 M NaCl-TE (0.45 ml five times) followed by elution with 1 M NaCl-2% caffeine in TE to collect single-strand-containing DNA (5 to 10 fractions of 0.45 ml). After background levels of Cerenkov radiation were obtained, elution of protein-bound DNA with warmed (37°C) 1 M urea-1% SDS in TE was begun.

Gel electrophoresis, transfer, hybridization, and autoradiography. Gel electrophoresis was performed in electrophoresis buffer (40 mM Tris-hydroxyacetate [pH 7.5], 20 mM sodium acetate, 1 mM EDTA) or in modified SDS-containing electrophoresis buffer (40 mM Tris-hydroxyacetate [pH 7.5], 5 mM sodium acetate, 1 mM EDTA, 0.1% SDS) (2). The type and percentage gel are given in the figure legends. The gels were either dried or blotted with a nitrocellulose filter by the Southern technique (20) in the presence of 20 $\times$ SSC (1 $\times$ SSC: 0.15 M NaCl plus 0.015 M sodium citrate). Where applicable, slices of agarose gel were electroeluted in threefold diluted SDS-containing electrophoresis buffer at 20 V for 16 h at 20°C. Molecular weight markers were *Hind*III-digested phage λ DNA and *Hae*III-digested phage ϕ X174 RF DNA.

Hybridization of the nitrocellulose filter was performed with either [32 P]dTTP-labeled DHBV DNA cloned in lambda vector charon 16A (11) or [32 P]dTTP-labeled cDNA of M13mp7 which was annealed to minus- or plus-strand clones of DHBV in M13mp7 (11). α - 32 P-deoxynucleotides at a specific activity of 670 to 800 Ci/mmol were purchased from New England Nuclear Corp., Boston, Mass. The nitrocellulose filter was washed, air dried, and autoradiographed at -80°C with Kodak XAR-film and an intensifying screen.

Dideoxynucleotidyl sequencing. The method of Sanger et al. (18) was modified in two ways. First, the buffer was changed to that used for the endogenous polymerase reaction in the DHBV cores. Second, to shorten the length of the labeled products, the concentration of dideoxynucleotides was increased fourfold relative to the Sanger protocol.

Cores were incubated at 37°C for 30 min in four 100- μ l reactions containing 13 pmol each of [32 P]dTTP, [32 P]dATP, [32 P]dCTP, and [32 P]dGTP, 20 mM Tris-hydrochloride (pH 7.4), 7 mM MgSO_4 , 50 mM NaCl, 0.1% β -ME, 100 μ g of actinomycin D per ml, and 2.6 nmol of dideoxynucleotides. All four cold deoxynu-

cleotides (0.5 nmol) were then added, and the samples were incubated an additional 15 min to minimize any premature truncation. One-half of each sample was treated with 0.5 mg of protease K per ml–0.1% SDS–20 mM EDTA, deproteinized with phenol-chloroform, and ethanol precipitated. The other half was dissociated with 0.1% SDS–20 mM EDTA at 37°C for 30 min and concentrated by ethanol precipitation. The samples were resuspended in 2 μ l of 6 M urea–40 mM NaOH–0.1% SDS–0.4 mM EDTA–0.03% xylene cyanol–0.03% bromophenol blue, denatured at 100°C for 3 min, and loaded onto an 8% polyacrylamide gel containing 0.1% SDS, 8 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA. Gels were electrophoresed in buffer containing 0.1% SDS at 2,000 V (50°C) and autoradiographed.

RESULTS

Protein-bound virion DNA. The electrophoretic mobility of virion DNA was found to be sensitive to protease digestion, as shown in Fig. 1. Virion DNA was labeled with [32 P]dTTP in an endogenous reaction (12), and the DNA was isolated in the absence of protease digestion, as described in Materials and Methods. Briefly, the radiolabeled virions were dissociated by heating at 60°C in 2% SDS–2% β -ME and separated from the majority of free proteins by sedimentation into a 5 to 20% sucrose gradient containing TE and SDS. The labeled DNA-complex was recovered from the gradient, and a portion was treated with protease K. Both the treated and untreated samples were electrophoresed on a 0.7% agarose gel. As shown in Fig. 1, the protease K-treated sample (+) migrated more rapidly into the gel than the untreated sample (–).

Sedat et al. (19) have shown that fully double-stranded DNA bound to BND-cellulose can be eluted with 1 M NaCl, but single-strand-containing DNA requires 1 M NaCl containing 2% caffeine. In addition, Stillman and Bellett (21) have shown that protein-bound DNA is eluted only with 1 M urea–1% SDS. This technique was used to compare the effect of protease K treatment on endogenously 32 P-labeled virion DNA. The putative protein-DNA complex from virions was isolated as described in Materials and Methods, and a sample was layered onto a Sepharose 2B column equilibrated with TE to remove unbound SDS. The sample was collected in the excluded volume, NaCl was added to a final concentration of 0.3 M, and the sample was chromatographed on BND-cellulose as described previously (21). As shown in Fig. 2A, the majority (>90%) of the virion DNA which was isolated in the absence of protease digestion was eluted only with urea-SDS buffer, supporting the notion that the virion DNA is bound to protein (hereafter referred to as protein-DNA complex). In contrast, protease K-treated DNA (Fig. 2B) was eluted in 1 M NaCl–2% caffeine,

implying that it was partially single stranded, as expected.

Protein bound to minus-strand virion DNA. Since in the virion only the incomplete strand, i.e., the plus strand, can incorporate nucleotides in the endogenous reaction (12), denaturation of the endogenously labeled DNA before binding it to BND-cellulose would enable us to determine whether the protein is bound to the plus strand. The protein-DNA complex was denatured by heating at 90°C for 15 min in TE + SDS, quick chilled (15 s), and layered onto a Sephadex G-50 column equilibrated with TE. It was collected in the excluded volume and chromatographed on BND-cellulose. As shown in Fig. 3, >90% of the radioactivity eluted in the caffeine-containing buffer, suggesting that the plus-strand DNA is not protein bound.

To test the inference that the minus strand must be protein bound, virion DNA, after an endogenous reaction with only cold nucleotides, was isolated in the absence of protease digestion. After heat denaturation (90°C for 15 min), half the sample was protease K treated, and the samples were electrophoresed in duplicate on a single SDS-containing 1.5% agarose gel. A Southern transfer was performed, and each half

FIG. 1. Agarose gel electrophoresis of endogenously labeled virion DNA in the absence or presence of protease K digestion. Virion DNA was endogenously labeled with [32 P]dTTP and isolated in the absence of protease digestion (see the text). A sample containing ca. 500 trichloroacetic acid-precipitable cpm which was treated with protease K (+) and an untreated sample (–) were electrophoresed into a 0.7% agarose gel. The gel was dried and autoradiographed. Kbp, Kilobase pairs.

FIG. 2. BND-cellulose chromatography of virion protein-DNA complex. Virion DNA was endogenously labeled and isolated in the absence of protease digestion (see the text). A portion of the sample was digested with protease K and deproteinized. Both treated and untreated samples were layered onto Sepharose 2B columns and collected in the excluded volume. NaCl was added to a final concentration of 0.3 M, and chromatography was performed as described by Stillman and Bellett (21). (A) Chromatography of virion DNA isolated in the absence of protease digestion. (B) Chromatography of protease K-treated sample.

of the blot was hybridized to a strand-specific probe. Figure 4 shows that protease K treatment of the minus strand increased its mobility in the gel (lane 3 versus lane 2), whereas the same treatment had no effect on the migration of the plus strand (lane 6 versus lane 5). These data strongly suggest that the protein detected in the assays employed is bound only to the minus strand.

Protein bound to replicating minus-strand intermediates. The question arises as to at what stage the protein becomes linked to the minus strand. Three approaches have been used to investigate whether the protein is bound to nascent minus-strand DNA intermediates. First, Mason et al. (11) have shown that isolation of infected liver DNA resulted in a continuous spectrum of DHBV intermediates, including not only distinct species of nicked circular DNA, linear double-stranded DNA, and covalently closed circular DNA, but also a significant amount of full-length single-stranded DNA spe-

cies which are of minus polarity (11) (Fig. 5, lanes 1 and 2). To determine whether these species are covalently bound to a protein, infected liver DNA was isolated by using SDS and urea to dissociate noncovalently bound proteins (see Materials and Methods). The DNA eluted in the void volume from the Sepharose 4B column was analyzed before or after pronase digestion by electrophoresis into a 0.7% agarose gel (Fig. 5A) and a 1.5% agarose gel (Fig. 5B). In each fraction, full-length double-stranded DNA (DS) and full-length, single-stranded DNA (SS) species migrated more rapidly into the gel after pretreatment with pronase (Fig. 5, lanes 1 and 2 versus lanes 3 and 4). In contrast, pretreatment with pronase failed to alter the mobility of a species migrating in the position expected of covalently closed circular DNA (Fig. 5B) or of marker DNAs added to the samples (data not shown). These results suggested that the protein is covalently bound before or upon completion of minus-strand synthesis.

Recently, Summers and Mason (22) isolated replicating complexes from infected liver that carry out synthesis of minus-strand DNA within immature or unpackaged viral cores. We have investigated at what stage the protein-DNA complex is formed by determining the smallest size of nascent minus strand that is linked to protein. Nascent minus strands were labeled with [32 P]dTTP in an endogenous DNA polymerase reaction in the presence of 100 μ g of actinomycin D per ml, which inhibits plus-strand DNA synthesis (22). DNA was extracted by heating at 60°C in 2% β -ME-2% SDS. The sample was electrophoresed into an SDS-con-

FIG. 3. BND-cellulose chromatography of heat-denatured, endogenously 32 P-labeled virion protein-DNA complex. 32 P-labeled virion protein-DNA complex was denatured at 90°C for 15 min in the presence of TE + SDS. The sample was layered onto a Sephadex G-50 column in TE and collected in the excluded volume. NaCl was added to a final concentration of 0.3 M, and chromatography on BND-cellulose was performed as described by Stillman and Bellett (21).

FIG. 4. Agarose gel electrophoresis of heat-denatured protein-DNA complex. A sample of virion protein-DNA complex was treated with protease K. Treated and untreated samples were heated at 90°C for 15 min in the presence of TE + SDS and loaded in duplicate onto an SDS-containing 1.5% agarose gel. A Southern transfer was performed, and the nitrocellulose filter was hybridized to DHBV minus-strand-specific (lanes 1 to 3) and plus-strand-specific (lanes 4 to 6) M13mp7-derived probes (11). Lanes 1 and 4, protein-DNA complex; lanes 2 and 5, heat-denatured protein-DNA complex; lanes 3 and 6, protease K-treated heat-denatured DNA. kbp, Kilobase pairs.

taining 1.5% agarose gel. Regions of the gel corresponding to various sizes of nascent minus strands were electroeluted in SDS-containing buffer overnight. One-half of each sample was treated with protease K, and the samples were electrophoresed into an SDS-containing 5% polyacrylamide gel. As shown in Fig. 6, the protease K-treated samples migrated more rapidly into the gel than untreated samples in all size classes examined. These data show that the protein is linked to the minus strand early in its synthesis.

To determine the length of the shortest protein-bound growing minus strand, we carried out an endogenous DNA polymerase reaction on cores in the presence of dideoxynucleotides (18) and actinomycin D. If the 5' end of the minus strand occurs at a unique site, we would expect (i) to obtain a sequencing ladder from the blocked reactions and (ii) that the position of this

ladder in the gel would be altered by protease treatment. As shown in Fig. 7A, lanes 1 to 4, protease K-treated samples did generate a sequencing ladder, showing that minus-strand synthesis begins at a unique site. Lanes 5 to 8 (Fig. 7A) showed that the sample which was not protease K treated did not migrate readily into the gel. Although the data are consistent with the notion that any fragment detectable in the protease K-treated samples had been bound to a protein, one could also argue that all short nascent DNA strands were actually not covalently bound to a protein, but had instead become trapped in the extraneous proteins during the concentration step. To test the latter possibility, an equal mixture of deproteinized and untreated samples was concentrated and electrophoresed into a sequencing gel. As shown in Fig. 7B, the presence of protein did decrease the recovery of the protease K-treated sample to 10 to 20%. However, its presence did not alter the electrophoretic mobility of the protease K-treated sample. These data suggest that a protein had been covalently bound to all minus-strand DNA intermediates readily detectable in the deproteinized sample. Comparison with marker DNAs indicat-

FIG. 5. Agarose gel electrophoresis of liver DNA isolated in the absence of protease digestion. Liver DNA was isolated in the absence of protease digestion (see the text), half of each fraction was digested with 0.5 mg of pronase per ml at 37°C for 30 min, and the samples were electrophoresed into a 0.7% (A) and a 1.5% (B) agarose gel. A Southern transfer was performed, and the nitrocellulose blot was hybridized with a ³²P-labeled DHBV DNA cloned into the lambda vector, charon 16A. SS, Full-length single-stranded minus-strand species; DS, double-stranded DHBV; CCC, the position expected for covalently closed circular DNA. Lanes 1 and 2 are pronase-treated samples of the untreated samples in lanes 3 and 4, respectively.

FIG. 6. Polyacrylamide gel electrophoresis of ^{32}P -labeled minus-strand DNA intermediates. Replicating cores were labeled in the presence of 100 μg of actinomycin D per ml and dissociated by heating at 60°C for 20 min in 2% β -ME–2% SDS. The sample was loaded onto an SDS-containing 1.5% agarose slab gel and electrophoresed at 3.2 V/cm for 6 h, and every third slice was electroeluted in SDS-containing buffer at 20 V overnight. The samples were concentrated by ethanol precipitation, and one-half of each sample was treated with protease K. The samples were then electrophoresed into an SDS-containing 5% polyacrylamide gel. The gel was dried and autoradiographed for 16 h (A) or 96 h (B). Lanes 1, 3, 5, and 7 are protease K-treated samples of the untreated samples in lanes 2, 4, 6, and 8, respectively. bp, Base pairs.

ed that the shortest detectable fragment in the protease-treated samples is ca. 30 bases from the 5' end of the minus strand, the smallest strand readily labeled in the endogenous core reaction (data not shown).

DISCUSSION

Duck hepatitis B virion DNA contains a protein covalently bound to the minus strand, as shown by protease K-induced changes in electrophoretic mobility and chromatography on BND-cellulose. The protein could not be dissociated from the DNA by (i) gel electrophoresis in the presence of 0.1% SDS (Fig. 1), (ii) denaturation of the DNA strands by heating at 90°C for 15 min in 0.1% SDS (Fig. 4), or (iii) alkali treatment in 100 mM NaOH at 37°C for 1 h (data not shown). The failure of alkali to hydrolyze the

protein-DNA bond implies that, in contrast to adenovirus (16), the linkage is not a phosphodiester bond between the initial nucleotide and serine or threonine. Other linkages which have been observed include a phosphodiester bond to tyrosine and phosphoamide bonds to lysine (7, 17). Gerlich and Robinson (6) have demonstrated that HBV also exhibits a protein covalently bound to the complete strand, and recently, Ganem et al. (5) found an analogous protein bound to the complete strand of ground squirrel hepatitis virus. These findings suggest that the protein may play a prominent role in the mechanism of replication of this class of viruses.

The mechanism of replication of HBV-like viruses (22) involves the packaging of a "pregenomic" RNA into an immature viral core. The polymerase synthesizes the minus-strand DNA

FIG. 7. Modified dideoxynucleotidyl sequencing of minus strand from replicating cores of infected liver. (A) Cores were endogenously labeled in the presence of 2.6 nmol of dideoxynucleotides and 100 μg of actinomycin D per ml (see the text). Lanes 1 to 4 were treated with protease K and extracted with phenol-chloroform. Lanes 5 to 8 were dissociated by heating at 37°C for 30 min in 0.1% SDS–20 mM EDTA. Lanes 1 and 5, ddATP; lanes 2 and 6, ddGTP; lanes 3 and 7, ddCTP; lanes 4 and 8, ddTTP. (B) Cores were endogenously labeled in the presence of 2.6 nmol of ddGTP. Lane 1, protease K-treated phenol-chloroform-extracted DNA; lane 2, ethanol concentration of equal mixture of deproteinized sample and protein-bound DNA; lane 3, protein-bound DNA.

from the RNA template with a reverse transcriptase-like activity. Minus-strand replication initiates at a unique site, as shown by the sequencing ladder generated by minus-strand synthesis in replicating cores (Fig. 7A). The elongation of the minus strand is complete before the synthesis of the plus strand begins (22). Since initiation of DNA synthesis requires the presence of a primer (9), a possible mechanism by which a protein could become covalently attached to the minus strand would be by acting as a primer for minus-strand DNA synthesis. A priming function for DNA or RNA synthesis has been attributed to the 5' terminal proteins found in poliovirus (14, 15), ϕ 29 (13), and particularly adenovirus (3, 8). If the DHBV protein is to function as a primer, then two predictions must be met. First, the protein must be bound at the 5' end of the strand. By using a modified sequencing reaction, we were able to show that protein is bound to nascent minus strands of greater than 30 nucleotides (Fig. 7). In addition, Gerlich and Robinson (6) have been unable to readily phosphorylate the 5' ends of HBV virion DNA, implying that the protein is bound at least near the 5' end.

Second, one would predict that the protein-DNA linkage occurs at the beginning of replication; i.e., all minus-strand DNA intermediates, regardless of length, must exhibit a covalently bound protein. Minus-strand intermediates isolated from replicating cores exhibited a greater electrophoretic mobility after protease K treatment (Fig. 6), implying the existence of a covalently bound protein in all size classes examined. Furthermore, the minus-strand DNA intermediates truncated by dideoxynucleotides migrated very slowly into a sequencing gel unless the samples were first protease K treated (Fig. 7A), presumably because the sequencing gel conditions are unfavorable for electrophoresis of proteins (10). DNA intermediates lacking a covalently bound protein would have migrated into the gel according to length, as demonstrated by the inability of the untreated sample to alter the migration of a protease K-treated and deproteinized sample (Fig. 7B). In contrast, protease K treatment of samples resulted in the appearance in the gel of a sequence ladder typical of initiation of DNA synthesis at a specific site (Fig. 7A). Subsequent experiments have shown that the shortest readily detectable fragment contains <30 bases, showing that the linkage process occurs as an early step in the synthesis of minus-strand DNA. Thus, these data are consistent with the hypothesis that a protein may serve as the primer for minus-strand DNA synthesis.

Other functions for proteins covalently bound to viral genomes have been proposed. In ϕ 29 replication, the covalently bound protein has been shown to be required for packaging of the

viral genome (1). In contrast to the polio RNA genome, the polio mRNA is not linked to a covalently bound protein, most likely resulting from posttranscriptional cleavage (4). These findings suggest that the polio protein may also play a role in the packaging process. The assembly of DHBV cores must involve mechanisms for packaging not only the pregenome RNA, but also the DNA polymerase, as well as the putative protein primer. Because minus-strand DNA synthesis initiates at a unique site on the RNA template, one can readily envision the need for a protein primer or a DNA polymerase-primer complex to recognize and bind to that site on the pregenome. This recognition may be an initial step in assembly of immature cores. Further studies are under way to investigate this possibility.

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