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Hepatitis B Viral DNA Molecules Have Cohesive Ends

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Hepatitis B virus DNA made fully double stranded by a virion DNA polymerase reaction could be converted from circular to linear molecules by heating in 10 mM NaCl at 77°C or in 100 mM NaCl at 90°C for 15 min. Heat-generated linear hepatitis B virus DNA was reannealed to circular molecules by incubating in higher salt concentrations. The identity of the molecular forms was established by their electrophoretic mobility and appearance in electron micrographs. Recircularization was blocked by reacting linear molecules with nuclease S1 or avian myeloblastosis virus reverse transcriptase. These results suggest that the heated linear DNA had single-stranded ends with complementary nucleotide sequences. It also suggests that a discontinuity or nick is present in each strand of the circular DNA molecule after the single-stranded region is made double stranded by the virion DNA polymerase reaction. The difference in contour length by electron microscopy of circular and linear molecules spread under aqueous conditions suggested that the discontinuities in the two strands were about 270 base pairs apart. The amount of nucleotide incorporated into the ends of heat-generated linear hepatitis B virus DNA by reverse transcriptase suggested that the single-stranded ends were about 305 bases in length. This fully double-stranded linear DNA was cleaved with *Eco*RI or *Hpa*I restriction endonuclease. The sum of the two fragments generated by each totaled 3,510 base pairs, 310 base pairs greater than the contour length of circular hepatitis B virus DNA which represents a third estimate of the distance between the discontinuities in the two DNA strands of circular DNA. Restriction endonuclease cleavage also indicated that the ends of heated linear DNA which correspond to the discontinuities in the two strands of the circular DNA are at unique sites in the DNA with respect to the restriction sites.

The DNA of hepatitis B virus (HBV; also known as the Dane particle) has an unusual structure. It is a circular molecule with a length corresponding to double-stranded DNA of approximately 3,200 base pairs (bp) (2, 4, 6, 9). The DNA, however, is not completely double stranded. A single-stranded region is present in all molecules, and its length varies from 15 to 50% of the circular DNA length in different molecules (2, 4, 9). Thus, the DNA consists of a long strand (*a*) which is 3,200 nucleotides in length and a short strand (*b*) of variable length (1,700 to 2,800 nucleotides in different molecules). The single-stranded region is not randomly located in the DNA but occurs only within restriction fragments representing approximately 50% of the DNA (4). The virion contains a DNA polymerase (3, 7) which appears to use the 3′ end of the short strand as primer (6, 8) and closes the single-stranded gap to make fully double-stranded DNA molecules of uniform length (3,200 bp) (2, 4, 9). A discontinuity or nick appears to remain in the short strand after it is elongated by the DNA polymerase reaction. It has been suggested that the long strand (*a*) contains a nick or short gap on the basis of restriction endonuclease cleavage patterns of HBV DNA strands separated in alkali and copied with DNA polymerase I (9). However, there is not direct evidence concerning the structure of the long strand. Here we show that circular HBV DNA after closure of the single-stranded gap with virion DNA polymerase can be converted to a linear form by selected heating conditions and recircularized by annealing. We have shown that the ends of such heated linear DNA have unique locations with respect to restriction endonuclease cleavage sites in the DNA. These results indicate that a discontinuity or nick exists at a unique site in the long strand (*a*) of HBV DNA and the nick remaining in strand *b* after the virion DNA polymerase reaction also occurs at a unique site.

MATERIALS AND METHODS

Preparation of HBV (Dane particles). Dane particle-rich plasma was obtained from patient donor I-263 (hepatitis B surface antigen subtype adw_2), who

had a high level of serum Dane particle DNA polymerase activity. This material was clarified by centrifugation at 10,000 rpm for 10 min. Dane particles were pelleted by centrifugation in a Spinco 21 rotor for 6 h at 15°C. The resulting pellets were resuspended in 10 ml of 0.01 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA (TNE)–0.1% 2-mercaptoethanol (ME)–1 mg of bovine serum albumin per ml, pH 7.5, to make a P_1 preparation. To further purify Dane particles, the P_1 material was pelleted through 30% sucrose in an SW40 rotor for 4 h at 15°C. The pellet (P_2) was suspended in 200 μl of TNEME and sonicated for 2 min.

Endogenous HBV DNA polymerase reaction. The following reaction conditions were established to completely convert the single-stranded region of HBV DNA molecules to double-stranded DNA. Two hundred microliters of P_2 was added to 100 μl of a mixture containing 1 mM dATP, 1 mM dTTP, 0.15 mM dCTP, 0.15 mM dGTP, 1.5 μM [α-^{32}P]dCTP, 1.5 μM [α-^{32}P]dGTP (Amersham-Searle, both 350 Ci/mmol), 75 mM $MgCl_2$, 0.2 M Tris-hydrochloride, 0.24 M NH_4Cl, 1.5% Nonidet P-40, and 0.3% 2-mercaptoethanol (pH 7.5) at 37°C. After 1 h the reaction was adjusted to 0.3 mM dCTP and 0.3 mM dGTP. At 4 h the reaction was terminated by the addition of 30 μl of 0.5 M EDTA.

Isolation and purification of HBV DNA. Proteinase K and sodium dodecyl sulfate were added to the DNA polymerase reaction mixture to give final concentrations of 1 mg/ml and 1%, respectively. The solution was incubated at 56°C for 2 h. The reaction mixture was extracted three times with phenol equilibrated with TNEME. One-tenth volume of 2 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol were added to precipitate the DNA. After 2 h at −20°C, the ethanol precipitate was pelleted in a Microfuge for 5 min and resuspended in 10 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA.

Reverse transcriptase reactions. In some experiments avian myeloblastosis virus reverse transcriptase (supplied by J. Beard) was used to convert any single-stranded regions remaining in the DNA after the virion DNA polymerase reaction to double-stranded DNA. The reaction mixture containing HBV DNA (1 to 100 ng), 2.5 mM dCTP, 2.5 mM dGTP, 0.5 mM [^{3}H]dATP and [^{3}H]dTTP (15.1 and 17.1 Ci/mmol, respectively), 50 mM Tris-hydrochloride (pH 8.3), 40 mM KCl, 6 mM MgCl, 0.1 mg of bovine serum albumin per ml, and 2 to 5 U of enzyme was incubated at 37°C for 2 h. A similar reaction was used to convert the single-stranded ends of DNA made linear by heating to double-stranded DNA. In this reaction, nonradioactive dATP was replaced with [α-^{32}P]dATP (400 Ci/mmol), and dTTP, dCTP, and dGTP were present at 2.5 mM each. After the reaction, the DNA was extracted with phenol and precipitated with ethanol as described above.

Endonuclease digestion. Endonuclease S1 digestion of DNA (1 to 100 ng) was carried out with 50, 100, or 150 U of enzyme (Miles Laboratories) in 45 μl of 30 mM sodium acetate–1 mM zinc acetate–50 mM NaCl (pH 4.6) at 37°C for 45 min. DNA was digested with *Eco*RI and *Hpa*I restriction endonucleases (0.5 U) in appropriate buffers at 37°C for 20 min. Reactions were stopped by the addition of EDTA.

DNA electron microscopy. HBV DNAs made completely double stranded by the virion DNA polymerase reaction were spread by an aqueous technique (1) before and after conversion to the linear form by heating. Phage PM2 form II DNA molecules were used as an internal standard for length measurements. Molecules were traced and measured with a digitizer attached to a Hewlett-Packard programmable calculator.

Gel electrophoresis. Horizontal 0.7% agarose slab gel electrophoresis was run at 35 to 40 V for 16 to 19 h at 4°C. Electrophoresis in vertical composite gels composed of 0.5% agarose and 2% polyacrylamide was carried out at 180 V for 2 to 2.5 h at room temperature. Electrophoresis buffer for both was 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA. Restriction DNA fragments of known size generated from phage lambda and plasmid PBR322 DNA were used as size markers in gel electrophoresis.

RESULTS

Conversion of circular HBV DNA to a linear form by heating and recircularization by annealing. To test the integrity of the long (*a*) strand of HBV DNA, a DNA preparation made fully double stranded and radiolabeled by the virion DNA polymerase and extracted as described in Materials and Methods was subjected to heating under a variety of conditions; its structure was analyzed by gel electrophoresis. Figure 1 shows an autoradiogram from such an experiment. Figure 1, track 1, shows the gel position of the double-stranded circular ^{32}P-labeled DNA as extracted from Dane particles. Track 2 shows that DNA incubated in 0.10 M NaCl–0.01 M Tris-hydrochloride–0.001 M EDTA (pH 7.5) at 90°C for 15 min was converted quantitatively to a form which migrated in the position expected for linear DNA. This conversion was not observed when DNA in 0.10 M salt was incubated at lower temperatures. Experiments not shown demonstrated that DNA converted to a linear form with *Eco*RI restriction endonuclease, which cleaves the DNA at one site (A. Siddiqui, F. R. Sattler, and W. S. Robinson, Proc. Natl. Acad. Sci. U.S.A., in press), or with nuclease S1 had electrophoretic mobilities like the heated DNA in track 2 (Fig. 1). As described later, examination of the heated DNA by electron microscopy confirmed its linear conformation. At lower ionic strength, the conversion was found to occur at a lower temperature. Tracks 3 to 11 (Fig. 1) show the effects of increasing temperature of incubation on DNA in 0.01 M NaCl–0.01 M Tris-hydrochloride–0.001 M EDTA (pH 7.5). The circular to linear transition occurred between 73°C (track 7) and 77°C (track 8) under these salt conditions. At 83°C (track 10) and above (track 11) the DNA in 0.01 M salt was completely denatured and migrated as single-stranded DNA. DNA in 0.10 M salt was not completely

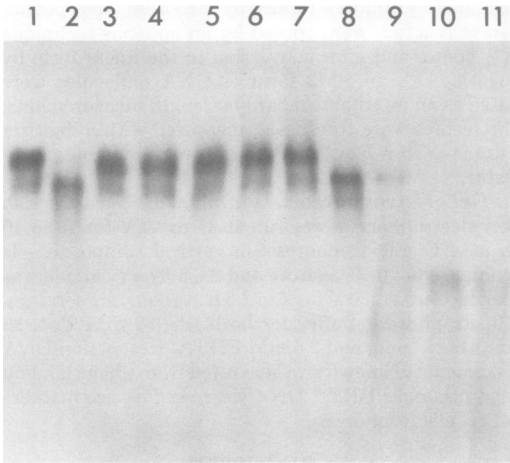

FIG. 1. *Autoradiogram of HBV ^{32}P-labeled DNA before and after heating and electrophoresis in an agarose gel. HBV DNA was made completely double stranded by the virion DNA polymerase in the presence of ^{32}P-labeled deoxynucleoside triphosphates. Aliquots were incubated for 15 min in 0.10 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA at room temperature (track 1) and 90°C (track 2), and in 0.01 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA at 60°C (track 3), 63°C (track 4), 67°C (track 5), 70°C (track 6), 73°C (track 7), 77°C (track 8), 80°C (track 9), 83°C (track 10), and 87°C (track 11). Each was rapidly cooled in ice water after heating and analyzed by gel electrophoresis.*

denatured until the temperature was increased to 94°C.

When HBV DNA made linear by heating was incubated in 0.1 M NaCl–0.01 M Tris-hydrochloride–0.001 M EDTA (pH 7.5) at 60°C for 2 h, it was quantitatively converted back to the circular form (Fig. 2). In 0.4 M NaCl, complete recircularization was observed in 1 h at 60°C.

The finding that circular HBV DNA can be converted completely to a linear form under selected heating conditions indicates that the long strand (*a*) as well as the short strand (*b*) must contain a nick or gap. The rapid recircularization under appropriate salt and temperature conditions is consistent with the presence of single-stranded cohesive ends on the linear form.

Electron microscopic appearance and contour length of HBV DNA before and after heating. The HBV DNA preparation used in the experiment shown in Fig. 2 was spread with circular PM2 phage DNA and examined by electron microscopy before heating when its electrophoretic mobility was that of circular DNA (Fig. 2, track 1) and after heating when it behaved in electrophoresis like linear DNA (Fig. 2, track 2). Almost exclusively circular HBV DNA molecules were observed by electron microscopy before heating and almost exclusively linear forms were observed after heating.

The contour lengths of 66 circular HBV DNA molecules were measured using the PM2 form II DNA molecules (considered to have a length of 3.33 μm) (2) on the same grid as an internal-length standard, and the results are shown in Fig. 3. The mean contour length of the circular HBV DNA molecules was 1.04 μm, with a standard error of the mean of 0.01 μm. A contour length of 1.04 μm corresponds to a length of approximately 3,200 bp for double-stranded DNA. This was calculated using a figure of 3,074 bp per μm of contour length based on the published countour length (10) and sequence length (5) of simian virus 40 DNA. The mean contour length of 49 heated linear HBV DNA molecules (shown in Fig. 5) measured in the same way was 0.95 μm, with a standard error of the mean of 0.01 μm. The mean contour length difference between the circular and heated linear HBV DNA was thus 0.09 μm or 8.4% of the length of

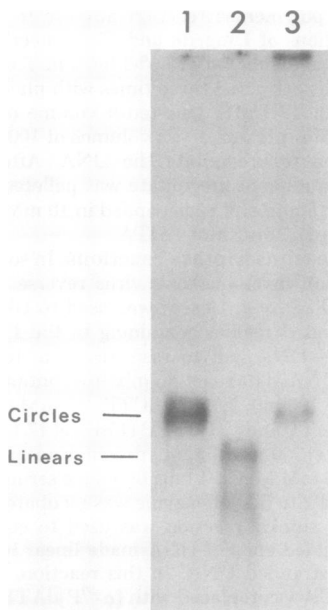

FIG. 2. *Autoradiogram of HBV ^{32}P-labeled DNA after heating and after heating followed by incubation in high salt. Aliquots of HBV ^{32}P-labeled DNA prepared as described in the legend to Fig. 1 were incubated for 15 min in 0.01 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA at room temperature (track 1) and 77°C (track 2) before agarose gel electrophoresis. A portion of the DNA after incubation at 77°C was adjusted to 0.10 M NaCl and incubated for 2 h at 60°C before gel electrophoresis (track 3).*

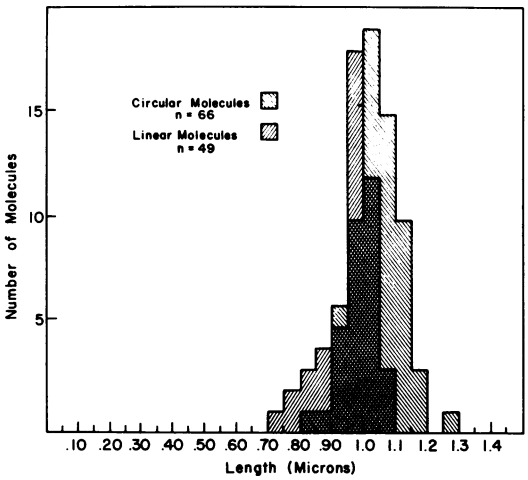

FIG. 3. *Histogram of HBV DNA contour lengths. Electron micrographs of HBV DNA were obtained before and after heating the DNA under the conditions described in the legend to Fig. 2 and spreading with form II PM2 DNA as described in the text. The electron micrographs were projected, and molecular forms were traced with a digitizer attached to a programmable calculator. The HBV DNA molecules before heating were almost exclusively in a circular form, and the contour lengths of 66 molecules were determined with reference to the contour length of circular PM2 DNA molecules. The HBV DNA molecules were almost exclusively linear in the preparation after heating, and the contour length of 49 molecules was determined in the same way. The specific lengths were entered into banks of 0.05 width and the histogram printouts are superimposed in the figure.*

the circular form. This corresponds to a DNA length difference of approximately 270 bp. The difference was highly significant ($P < 10^{-7}$) when tested by the Wilcoxon rank sum analysis. The finding that the heated linear form was shorter than circular HBV DNA is consistent with denaturation of a 270-bp region of the DNA by heating and the formation of single-stranded cohesive ends approximately 270 nucleotides in length, which would not be apparent in electron micrographs of DNA spread by the aqueous technique used here.

Nuclease S1 and reverse transcriptase reactions block recircularization of heated linear HBV DNA. Removal of single-stranded cohesive ends by digestion with the single-strand-specific endonuclease S1 or conversion of the single-stranded ends to double-stranded DNA with reverse transcriptase would be expected to prevent recircularization of heated linear HBV DNA, as illustrated in Fig. 4. The results of the experiments shown in Fig. 5 and 6 demonstrated that modification of heated linear DNA with these enzymes did prevent recircularization. After heated linear HBV DNA was incubated with three different concentrations of nuclease S1, it failed to recircularize in 0.1 M NaCl at 60°C for 2 h (Fig. 5, tracks 8, 9, and 10), unlike the same heated linear DNA not incubated with nuclease S1 (Fig. 5, track 4). Similarly, after serving as a template in a reverse transcriptase reaction, heated linear HBV DNA failed to recircularize (Fig. 6).

Locations of the ends of heated linear HBV DNA with respect to restriction endonuclease cleavage sites. To localize the ends of heated linear HBV DNA, a preparation of circular HBV DNA with the single-stranded region converted to double-stranded DNA with the virion DNA polymerase before isolation, as described in Materials and Methods, was made linear by heating. The single-stranded ends of this DNA were made double stranded and radiolabeled by reverse transcriptase in the presence of ^{32}P-labeled deoxynucleoside triphosphates. When DNA made linear by heating was treated with nuclease S1 and compared by gel electrophoresis with the same heated DNA after

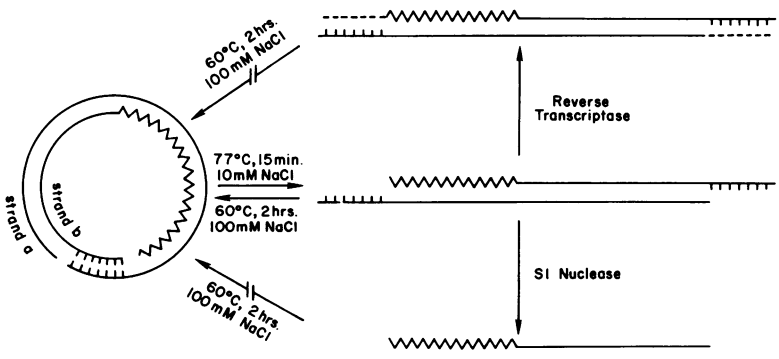

FIG. 4. *Model of interconversion of HBV DNA molecular forms by heating, reannealing, and reaction with nuclease S1 or reverse transcriptase.* ∿∿∿ *represents DNA synthesized by the virion DNA polymerase, and - - - represents DNA synthesized by a reverse transcriptase reaction with heated linear DNA.*

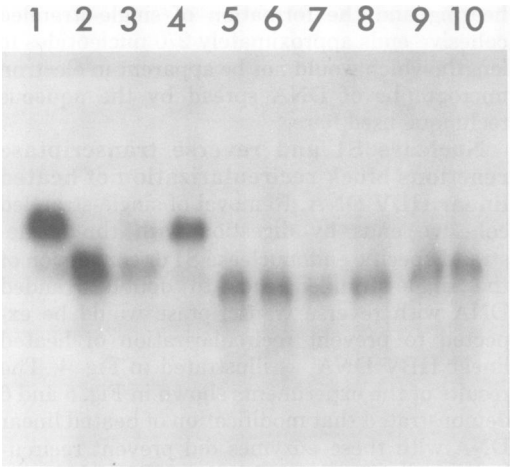

FIG. 5. *Autoradiogram of HBV ^{32}P-labeled DNA made linear by heating and incubated with nuclease S1. Agarose gel electrophoresis of HBV ^{32}P-labeled DNA prepared as described in the legend to Fig. 1 was carried out before (track 1) and after (track 2) heating as described in the legend to Fig. 2. Aliquots of the heated DNA were incubated for 2 h at 60°C in 0.10 M NaCl (track 4) or for 45 min at 37°C in S1 buffer (track 3), or with S1 buffer and 50 (track 5), 100 (track 6), or 150 (track 7) U of nuclease S1 before agarose gel electrophoresis. Portions of each DNA sample incubated with nuclease S1 were made up to 0.10 M with NaCl and further incubated at 60°C for 2 h before gel electrophoresis (tracks 8 to 10).*

a reaction with reverse transcriptase, a difference in electrophoretic mobility corresponding to a length difference of approximately 400 bp was observed (Fig. 7). This elongation by reverse transcriptase is consistent with conversion of single-stranded ends to double-stranded DNA by this reaction. Aliquots of the linear DNA made fully double stranded and end labeled with reverse transcriptase were digested with restriction endonuclease *Eco*RI and with *Hpa*I, both of which cleave circular HBV DNA of hepatitis B surface antigen subtype adw$_2$ one time (Siddiqui et al., in press). Figure 8 shows that *Eco*RI digestion of the linear DNA yielded ^{32}P-labeled DNA fragments of approximately 1,860 and 1,650 bp (track 3), and *Hpa*I produced fragments of approximately 2,620 and 880 bp (track 4). *Hpa*I is known to cleave HBV (adw$_2$) DNA approximately 980 bp in a clockwise direction from the unique *Eco*RI site in DNA oriented as shown in Fig. 9 (8a; Siddiqui et al., in press). The sum of that distance (980 bp) and the 880-bp fragment generated by *Hpa*I digestion of the linear DNA is equal to the size of the 1,860-bp fragment generated by *Eco*RI. This suggests that the ends of heated linear DNA and thus the nick in the long strand (*a*) and the nick remaining in the short strand (*b*) after complete closure of the single-stranded region in circular DNA can be localized with respect to the unique *Eco*RI and *Hpa*I cleavage sites, as shown in Fig. 9.

The two fragments generated by *Eco*RI and *Hpa*I in the experiment shown in Fig. 8 are clearly unevenly radiolabeled. This is thought to be due to a failure to completely close the single-stranded gap with the virion DNA polymerase before DNA isolation in that experiment. Because of this, the single-stranded ends after heating would have been of uneven length and thus the amount of radioactive DNA synthesis with reverse transcriptase would be different at the two ends. Consistent with this is the fact that the more heavily labeled fragment in each case (1,650-bp *Eco*RI fragment and 2,620-bp *Hpa*I fragment) includes the DNA region containing

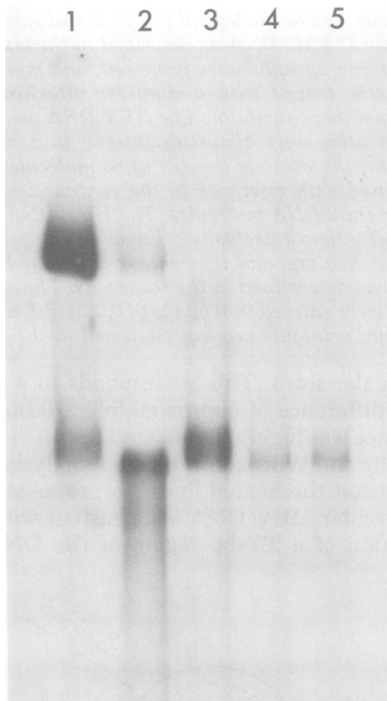

FIG. 6. *Autoradiogram of HBV ^{32}P-labeled DNA made linear by heating and used as template in a reverse transcriptase reaction. Composite gel electrophoresis of HBV ^{32}P-labeled DNA was carried out before (track 1) and after (track 2) treatment with 150 U of nuclease S1 as described in the text. Aliquots of the heated linear DNA were incubated in a reverse transcriptase reaction as described in the text and analyzed by composite gel electrophoresis before (track 4) and after further incubation at 60°C for 2 h in 0.10 M NaCl (track 5). The position of ^{32}P-labeled HBV DNA after digestion of the circular form with restriction endonuclease EcoRI is shown in track 3.*

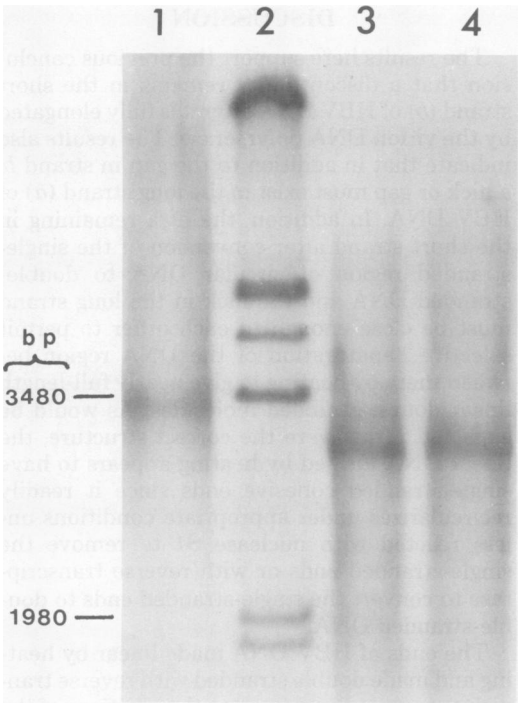

FIG. 7. *Comparison of HBV DNA made linear by heating and treated with avian myeloblastosis virus reverse transcriptase with linear DNA treated with nuclease S1. HBV heated linear DNA made fully double stranded and radiolabeled with reverse transcriptase (track 1) as described in the legend to Fig. 6 and heated linear DNA treated with 100 U of nuclease S1 (track 3) as described in the legend to Fig. 5 were analyzed by agarose gel electrophoresis and autoradiography. An aliquot of the nuclease S1-treated linear DNA was incubated under conditions known to produce recircularization (track 4) as described in the legend to Fig. 5. Track 2 shows the position of fragments of ^{32}P-labeled DNA of lambda phage after digestion with HindIII and EcoRI restriction endonucleases.*

the single-stranded gap and in other experiments when the single-stranded gap was completely closed with reverse transcriptase after DNA isolation and before heating, the two ends were in fact evenly radiolabeled after heating (data not shown).

The sum of the sizes of the two DNA fragments generated by either *Eco*RI or *Hpa*I is approximately 3,510 bp. This estimated length for heated linear DNA after the single-stranded ends has been made double stranded is approximately 310 bp longer than the length estimated above for circular HBV DNA in electron micrographs, suggesting that the length of each of the cohesive ends may be 310 nucleotides; i.e., the distance between the nick in the long strand (*a*) and the nick remaining in the short strand (*b*)

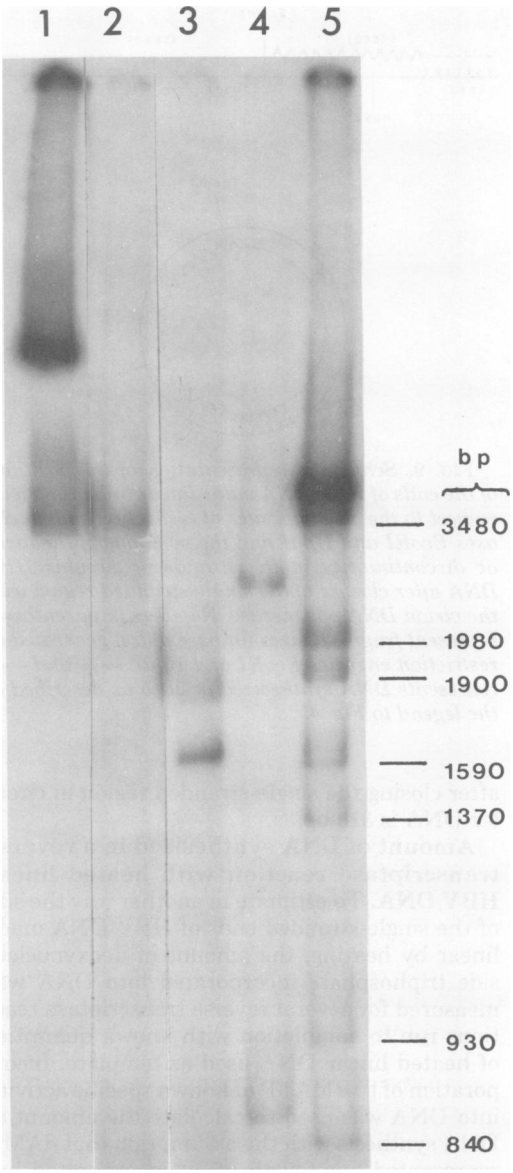

FIG. 8. *Autoradiogram of HBV ^{32}P-labeled DNA made linear by heating, made fully double stranded, and radiolabeled with reverse transcriptase, and digested with restriction endonucleases EcoRI and HpaI. HBV DNA with the single-stranded region made completely double stranded by the virion DNA polymerase was made linear by heating and incubated in a reverse transcriptase reaction containing ^{32}P-labeled deoxynucleoside triphosphates to make the ends fully double stranded and radioactive. Electrophoresis was carried out in composite gels before (track 2) and after digestion with EcoRI (track 3) or HpaI (track 4). Track 5 shows the position of fragments of ^{32}P-labeled DNA of lambda phage after digestion with HindIII and EcoRI restriction endonucleases. Track 1 shows the position of circular HBV ^{32}P-labeled DNA made fully double stranded and radiolabeled with the virion DNA polymerase.*

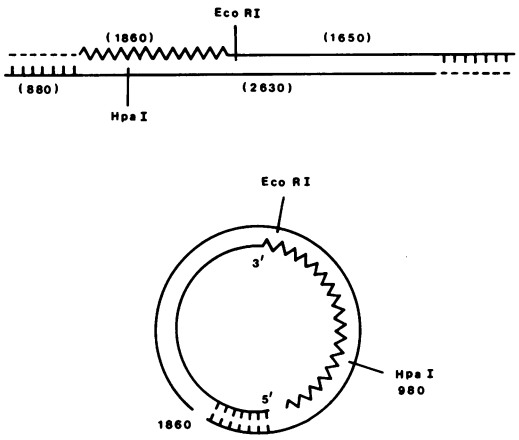

FIG. 9. *Schematic representation of the positions of the ends of HBV DNA made linear by heating with respect to the cleavage sites of restriction endonucleases EcoRI and HpaI and the positions of the nicks or discontinuities in the strands of circular HBV DNA after closure of the single-stranded region with the virion DNA polymerase. Numbers in parentheses represent fragment sizes (in base pairs) generated by restriction enzymes EcoRI and HpaI.* ∿∿∿ *and - - - represents DNA synthesized in vitro as described in the legend to Fig. 4.*

after closing the single-stranded region in circular DNA is 310 bp.

Amount of DNA synthesized in a reverse transcriptase reaction with heated linear HBV DNA. To estimate in another way the size of the single-stranded ends of HBV DNA made linear by heating, the amount of deoxynucleoside triphosphate incorporated into DNA was measured for several reverse transcriptase reactions run to completion with known quantities of heated linear DNA used as template. Incorporation of [^{32}P]dATP of known specific activity into DNA was used to calculate the amount of DNA synthesis with the assumption that dAMP represented one-fourth of all incorporated nucleotide. These experiments indicated that approximately 610 nucleotides had been added to each heated linear DNA molecule at the completion of the reaction, or 305 nucleotides per end for equal incorporation at the two ends. This estimate of the length of the single-stranded ends is in good agreement with the above size estimates by electron microscopic contour length difference between circular and heated linear molecules (270 bp) and by the difference in estimated circle length in electron micrographs and the length of heated linear DNA with single-stranded ends made double stranded as estimated by gel electrophoresis (310 bp).

DISCUSSION

The results here support the previous conclusion that a discontinuity remains in the short strand (*b*) of HBV DNA after it is fully elongated by the virion DNA polymerase. The results also indicate that in addition to the gap in strand *b*, a nick or gap must exist in the long strand (*a*) of HBV DNA. In addition, the nick remaining in the short strand after conversion of the single-stranded region of circular DNA to double-stranded DNA and the nick in the long strand must be close enough to each other to permit selective denaturation of the DNA region between them by heating to give nearly full-length linear double-stranded molecules. As would be expected if this were the correct structure, the linear DNA formed by heating appears to have single-stranded cohesive ends since it readily recircularizes under appropriate conditions unless reacted with nuclease S1 to remove the single-stranded ends or with reverse transcriptase to convert the single-stranded ends to double-stranded DNA.

The ends of HBV DNA made linear by heating and made double stranded with reverse transcriptase must correspond to the positions of the two nicks in the DNA (one in strand *a* and one remaining in strand *b* after closure of the single-stranded gap). Cleavage of such linear DNA with restriction endonucleases *Eco*RI and *Hpa*I has led to the conclusion that the site of the nick in each strand is unique and has permitted localization of the nicks relative to the restriction sites in this DNA as illustrated in Fig. 9.

It was shown previously that DNA synthesis is initiated during the virion DNA polymerase reaction in multiple restriction fragments of HBV DNA (4). Since there appears to be only one single-stranded region in each circular DNA molecule (4, 8), DNA synthesis must be initiated at different sites in different molecules. Thus, the 3′ end of the short strand (*b*) is considered to vary in location in different molecules. The finding here that the location of the nick remaining in strand *b* after its complete elongation by the virion DNA polymerase is a unique one indicates that the 5′ end of strand *b* must occur at this unique site. Thus, the variable length of the single stranded region must be determined by the variable location of the 3′ end and the fixed position of the 5′ end of strand *b*.

The exact distance between the two nicks in circular DNA, a distance which should correspond to the length of the single-stranded ends of heated linear DNA, is not certain from the experiments here because of the uncertainty in the measurements of DNA length by electron

microscopy and gel electrophoresis. However, the estimates of 270 bp, which is the difference in contour length of circular and heated linear molecules, 310 bp, which is the difference in contour length of circular molecules and the estimated length by gel electrophoresis of heated linear DNA made completely double stranded with reverse transcriptase, and 305 bp, which is the amount of DNA synthesized when the single-stranded ends of heated linear DNA are made completely double stranded with reverse transcriptase, are remarkably close and appear to provide a reasonable order of magnitude for the distance between the nicks in the two strands of this DNA.

DNA with a single-stranded region and a virion DNA polymerase which closes the single-stranded gap have not been found in other viruses. The role these features play in virus infection and replication is not clear and may not be clarified easily since at this time no cell culture system exists to study virus replication.

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