Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 3997–4001, July 1982
Biochemistry

Asymmetric replication of duck hepatitis B virus DNA in liver cells: Free minus-strand DNA

(strand-specific probes)

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Communicated by Baruch S. Blumberg, April 2, 1982

ABSTRACT **In order to study the replication of the DNA genome of duck hepatitis B virus, an avian virus related to human hepatitis B virus, we have characterized viral DNAs present in the livers of viremic ducks by agarose gel electrophoresis and the Southern blot procedure. In addition to relaxed circular DNA similar to virion DNA, livers contained a heterogeneous population of rapidly migrating species. The conformation of the rapidly migrating species was markedly sensitive to salt, suggesting that these species were largely single stranded. The largest major rapidly migrating species was shown to have an electrophoretic mobility that was insensitive to preheating of the DNA to 100°C and was similar to that of denatured virus DNA 3 kilobases long, suggesting that this DNA was a single-stranded copy of the entire virus genome. Hybridization with strand-specific probes demonstrated that this 3-kilobase species, as well as more rapidly migrating DNAs, were predominantly minus strands.**

Virions of hepatitis B virus (HBV) contain a 3.2-kilobase (kb) circular, partially double-stranded DNA (1, 2). One strand of this virion DNA is intact except for a discontinuity at a specific location. The other strand is heterogeneous in length, varying from 50% to 100% of full size (3, 4). The 5′ terminus of this incomplete strand is located at a unique site *ca.* 300 base pairs (bp) from the 5′ end of the complete strand (5), creating a cohesive overlap to maintain the circular conformation of virion DNA; the 3′ end location varies (3). This unusual DNA structure and the presence of an endogenous, virion-associated, DNA polymerase capable of filling in the single-stranded region by elongation from the 3′ end of the incomplete strand are characteristic of a family of HBV-like viruses which currently includes woodchuck hepatitis virus (6), ground squirrel hepatitis virus (7), and duck hepatitis B virus (DHBV) (8).

The presence of a single-stranded region in many or all of the virion DNAs and of a virion DNA polymerase capable of filling in this region raises the possibility of a novel mechanism for viral DNA synthesis. Studies of the virion DNA alone have not revealed this mechanism. In order to investigate this mechanism, we have analyzed viral DNAs present in the livers of Pekin ducks infected with DHBV because liver is a site at which a major amount of viral DNA synthesis occurs. A recent study of viral DNAs present in HBV-infected livers as detected by agarose gel electrophoresis and the Southern blot method (9) had revealed virion-sized DNAs as well as more rapidly migrating species (10). The large amount of these latter DNAs suggested that they were derived from intermediates in the synthesis of virion DNA, but their structure was not determined.

In this report, we present data suggesting that these rapidly migrating DNAs in livers infected with HBV-like viruses are mostly single-stranded, the largest being unit sized but with a substantial amount being shorter than unit sized, and that they correspond to the complete strand of the virion DNA. Single-stranded DNA corresponding to the incomplete strand has not been observed, consistent with the idea that the two strands are synthesized by separate pathways. In a separate study (11), we have observed that the template for the single-stranded DNA is RNA—i.e., that one step of viral DNA synthesis occurs by reverse transcription.

MATERIALS AND METHODS

One-day-old Pekin ducks and embryonated Pekin duck eggs were obtained from commercial suppliers. Approximately 10–20% of the ducklings hatched from these sources are viremic (DHBV), as a consequence of vertical transmission through the egg (ref. 8; A. O'Connell and T. London, personal communication). In some cases, eggs that had been incubated for 14–17 days were injected intravenously with 10 μl of serum from a viremic duckling because this procedure appeared to produce an increased incidence of viremia in newly hatched ducklings (unpublished data). Viremic ducklings were identified by testing for viral DNA in the serum with a hybridization spot test. Briefly, 3 μl of serum was mixed with 3 μl of 1 M NaCl/0.1 M NaOH, incubated 10 min at room temperature, and spotted on a nitrocellulose filter. Alternatively, 5 μl of serum was spotted directly onto a dry filter which was then floated for 20 min on 1 M NaCl/0.1 M NaOH. The alkali was neutralized by soaking the filter in 1 M NaCl/0.1 M Tris·HCl, pH 7.4, and rinsing with 0.3 M NaCl/0.03 M sodium citrate. The filter was then hybridized with a probe to viral DNA, as described below. The studies described herein were carried out on ducks up to 7 weeks old. [α-^{32}P]TTP (800 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. Electrophoresis-grade agarose, low-melting point agarose, large-fragment DNA polymerase I, and the bacteriophage M13mp7 (12) cloning kit were from Bethesda Research Laboratories. Nitrocellulose sheets (BA85) (0.45-μm pore size) were obtained from Schleicher & Schuell. *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. Nuclease S1 of *Aspergillus oryzae* was purchased from Miles.

Cloning of the DHBV Genome. The DHBV genome (length, 3,000 bp) contains a single *Eco*RI restriction endonuclease site. The virion DNA was filled in via the endogenous reaction, purified, cleaved with *Eco*RI, and ligated with the *Eco*RI site of the bacteriophage λ vector, Charon 16A (13). This DNA was packaged *in vitro* (14), and DHBV DNA-containing plaques were selected according to published procedures (15). The hybridization probe for this selection was the radiolabeled product of the endogenous reaction of DHBV (8). The locations of sev-

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Abbreviations: HBV, hepatitis B virus; DHBV, duck hepatitis B virus; kb, kilobase(s); bp, base pair(s).

eral restriction enzyme sites on the DHBV insert are shown in Fig. 1.

Subcloning of the entire insert from Charon 16A into the *Eco*RI site of the single-stranded bacteriophage M13mp7 and preparation of M13 bacteriophage DNA were carried out as recommended by the manufacturer (Bethesda Research Laboratories).

Extraction of Total Liver DNA. Ducks were sacrificed by injection with Nembutal and exsanguination, and livers were removed and stored at −80°C. To extract DNA, ≈0.2 g was homogenized with a Dounce homogenizer (A pestle) in 5 ml of ice-cold 10 mM Tris·HCl/10 mM EDTA, pH 7.4, followed by addition of 5 ml of 0.2 M NaCl/0.02 M Tris·HCl/2 mM EDTA, pH 7.4, containing 4 mg Pronase per ml and 0.2% $NaDodSO_4$ and incubation for 2 hr at 37°C. The nucleic acids were deproteinized and precipitated, as described (8), and resuspended in 5 ml of 5 mM Tris·HCl/1 mM EDTA, pH 7.4. Pancreatic RNase was added to a concentration of 100 μg/ml, and the mixture was incubated for 1 hr at 37°C. After addition of Pronase to 0.5 mg/ml and $NaDodSO_4$ to 0.5% and incubation for another 1 hr at 37°C, NaCl was added to 0.15 M and deproteinization and precipitation were performed, as above. The DNA was resuspended in 1 mM Tris·HCl/1 mM EDTA, pH 7.4, and stored at 4°C.

Analysis of DHBV DNA Sequences by the Southern Blot Procedure. Electrophoresis at 0.8 V/cm was carried out in horizontal slab gels prepared with electrophoresis grade agarose (Bethesda Research Laboratories) containing 0.04 M Tris·HCl, 0.02 M sodium acetate, and 1 mM EDTA (pH 7.2). After electrophoresis, DNA was denatured and transferred to nitrocellulose sheets by the method of Southern (9) as modified by Wahl *et al.* (16). Hybridization for 16–24 hr at 42°C was carried out by a published procedure (16), except that bovine DNA was substituted for salmon sperm DNA.

^{32}P-Labeled probe was prepared from 300 ng of denatured Charon 16A DNA (carrying the DHBV insert) in a 20-μl reaction mixture containing 0.1 M Tris·HCl (pH 7), 100 μg of oligonucleotide primers per ml, 20 mM $MgCl_2$, dATP, dGTP, and dCTP, each at 20 μM, 5 μM [^{32}P]TTP, and 1 unit of DNA polymerase I (17). Incubation for 5–10 min at 37°C resulted in >50% incorporation of the [^{32}P]TTP. Four volumes of 10 mM Tris·HCl, pH 7.4/10 mM EDTA containing wheat germ ribosomal RNA at 312 μg/ml and 0.125% $NaDodSO_4$ was then added, followed by 0.1 vol of 2 M sodium acetate and 2 vol of 100% ethanol. The precipitate was collected, washed, resuspended in 100 μl of H_2O, denatured at 100°C, and added to 10 ml of hybridization solution (16).

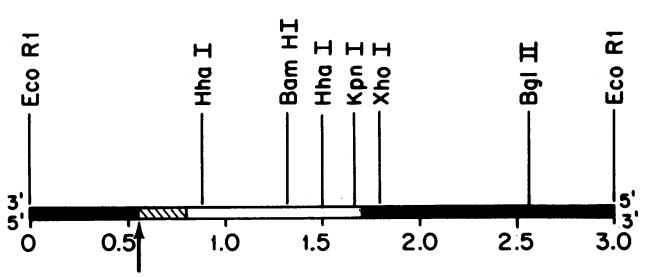

FIG. 1. Location of restriction endonuclease sites on the entire DHBV genome cloned into the *Eco*RI site of Charon 16A. Distances are in kbp. Some viruses have a second *Bam*HI site approximately 0.1 kb to the right of the *Bam*HI site shown in the figure. The arrow indicates the tentative location of the nick in the complete strand of virion DNA. The tentative placement of this nick relative to selected cleavage sites was determined as described (8). Hatched area, cohesive overlap between the 5′ termini of the complete and incomplete strands; open area, approximate extent of the single-stranded region present in some molecules, placed by assuming a cohesive overlap and single-stranded region analogous to HBV (3–5).

Strand-specific probes were prepared from single-stranded M13mp7 virion DNAs carrying the DHBV insert. Because M13 phage DNA is exclusively plus strand, only one strand of insert DNA is present in the virus, the polarity of the insert depending upon the orientation achieved during ligation into the M13 replicative form DNA. A reaction mixture containing 3 ng of M13mp7 phage DNA (no insert) per μl, 50 mM NaCl, 7 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 3.8 mM dithiothreitol, 39 μg of oligonucleotide primers per ml, dATP, dGTP, and dCTP at 20 μM each, 3.8 μM [^{32}P]TTP, and 0.018 unit of large-fragment DNA polymerase I per μl was incubated 20–30 min at 37°C, resulting in 15% or greater incorporation of [^{32}P]TTP. The nucleic acids were collected, as above, resuspended in H_2O, and heat denatured. The product derived from 40 ng of template was then hybridized (2 hr at 68°C) to 40 ng of M13mp7 phage DNA carrying the DHBV insert in 36 μl of 0.75 m NaCl/0.075 M sodium citrate, pH 7.0, to produce a probe in which the label was associated with double-stranded M13 sequences and the insert sequences remained single-stranded. This hybridized DNA was diluted into 10 ml of hybridization solution (16).

After filter hybridization, nitrocellulose sheets were subjected to autoradiography at −80°C with a Du Pont Cronex Lightning Plus intensifying screen and Kodak XAR-5 film.

Preparative Gel Electrophoresis. DNA from infected liver was subjected to horizontal slab gel electrophoresis in 1% agarose gels prepared with low-melting-point agarose containing the buffer components described above and 0.5 μg of ethidium bromide per ml. The location of double-stranded marker DNAs was visualized with UV light; the relative position of DHBV DNA species was determined in a preliminary experiment using the procedure of Southern (9) as described above. In subsequent experiments, the appropriate region of the gel, as defined by the location of marker DNAs, was cut out, melted by incubation for 20 min at 68°C, cooled to 37°C, and extracted two times with 0.5 vol of phenol (equilibrated with 50 mM Tris·HCl at pH 8) that had been warmed to 37°C. Then 0.1 vol of 2 M sodium acetate and 2 vol of 100% ethanol were added to precipitate the nucleic acids, which were collected by centrifugation for 30 min at 30,000 rpm in the Beckman SW 50.1 rotor.

RESULTS

Rapidly Migrating DHBV-Specific DNAs in Infected Liver Are Largely Single-Stranded. DHBV infects cells of the liver and pancreas of Pekin ducks. This is reflected by the presence of various viral DNA species, some of which are likely to be intermediates in the formation of virion DNA. Comparison of the viral DNAs in virions, liver, and pancreas by agarose gel electrophoresis is shown in Fig. 2. Under the conditions of electrophoresis, double-stranded DNA the size of the DHBV genome (3 kb) migrated slower when in a relaxed circular conformation than when in a linear conformation; the supercoiled form migrated faster than the linear form (refs. 8 and 18; unpublished data). Lane A shows virion DNA that consists primarily of 3-kb double-stranded relaxed circular DNA (8, 19). It should be noted that, with DHBV, the bulk of virion DNA migrates as essentially fully double-stranded DNA, with only a minor amount containing the large, single-stranded regions that allow for extensive radioactive labeling in the endogenous polymerase reaction (8, 19). The small amount of a slightly more rapidly migrating DNA, in lane A, is probably a 3-kb double-stranded linear molecule. In addition to these two species, liver (lane B) and pancreas (lane C) contained a number of more rapidly migrating species not readily detectable in virions. One of these,

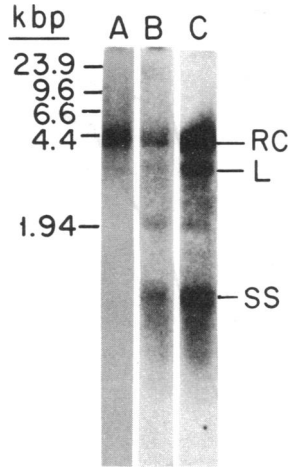

FIG. 2. Autoradiogram of DHBV DNAs in virions, liver, and pancreas from a 10-day-old viremic duck. DNAs were subjected to electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose, and detected by hybridization with ^{32}P-labeled Charon 16A–DHBV probe. Lanes: A, virion DNA (from particles present in 20 μl of serum); B, 0.55 μg of liver DNA; C, 5 μg of pancreas DNA. Hybridization was not obtained to DNA from tissues of uninfected ducks (not shown). The size of linear duplex marker DNAs is indicated. RC, double-stranded relaxed circular DNA, 3 kbp; L, double-stranded linear DNA, 3 kbp; SS, see text.

migrating adjacent to the 1.94-kbp marker, is probably the supercoiled closed circular DNA of 3 kbp which we have found to be enriched in DNA extracts from liver nuclei (data not shown). The amount of this supercoiled structure in Fig. 2 may be an underestimate of the *in vivo* abundance because a single nick would convert this species to a relaxed circular molecule with a slower migration. In any case, this report focuses on the discrete species marked "SS" as well as those species migrating more rapidly than SS and appearing as a smear of hybridization in Fig. 2.

Preliminary experiments revealed that the electrophoretic mobility of species SS did not vary with the agarose gel concentration in a manner expected for the known conformations of double-stranded DNA (18), suggesting that this species might be single-stranded. To test this possibility, we compared the sedimentation rates of DHBV DNA species in the liver in sucrose gradients containing two different concentrations of salt, inasmuch as the conformation of single-stranded DNA is markedly sensitive to salt concentration compared to double-stranded DNA (20). The sedimentation of the 3-kb double-stranded relaxed circular DNA was relatively unaffected by NaCl concentration whereas SS and the collection of more rapidly migrating species sedimented much faster at the higher salt concentration (Fig. 3*B*) than at the lower (Fig. 3*A*), suggesting that these species were extensively single-stranded.

In order to obtain further information on the single-strandedness and length of SS, we separated this species from other DHBV DNAs by a combination of sucrose gradient sedimentation, as in Fig. 3*A*, and preparative gel electrophoresis. The electrophoretic mobility of this species was then proven not to be altered by prior heating to 100°C, suggesting that it was single-stranded. In addition, its mobility was shown to be similar to that of 3-kb single-stranded DNA prepared by denaturing the *Eco*RI insert of a Charon 16A clone of the entire DHBV genome (Fig. 4), implying that SS was a single-stranded copy of the viral genome.

The results described in Figs. 3 and 4 suggested that the entire collection of rapidly migrating DHBV DNAs present in

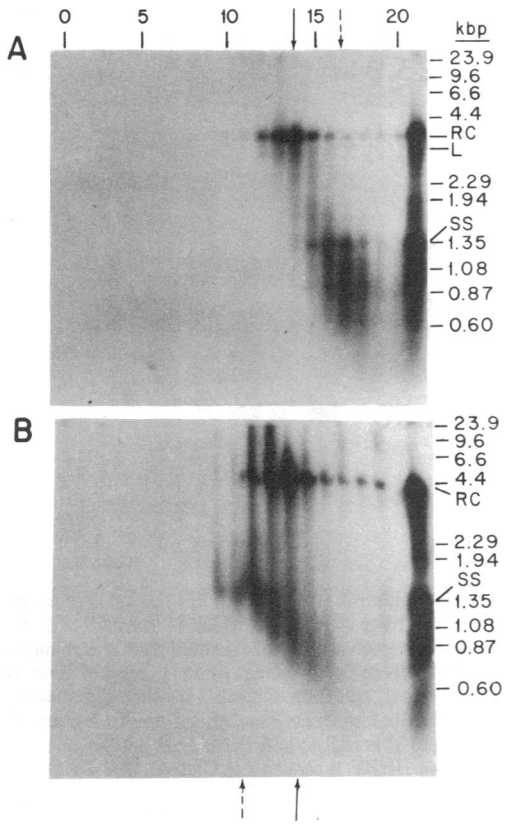

FIG. 3. Sedimentation analyses of DHBV-specific DNAs present in the liver of a 15-day-old viremic duck. Aliquots (10 μg) of liver DNA in 40 μl of 1 mM Tris·HCl/1 mM EDTA, pH 7.4, were sedimented into 3.8 ml of 5–20% (wt/vol) sucrose gradients containing either 1 mM Tris·HCl/1 mM EDTA at pH 7.4 (*A*) or 1 M NaCl/10 mM Tris·HCl; pH 7.4/1 mM EDTA (*B*) for 100 min at 55,000 rpm in an SW 56 rotor at 20°C. The gradients were collected from the bottom as 5-drop fractions and equal volumes of each fraction were subjected to electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with a Charon 16A–DHBV probe. As controls, 2-ng aliquots of purified 3-kb DHBV insert from a Charon 16A clone were sedimented in parallel gradients, either before or after heat denaturation (3 min at 100°C), subjected to electrophoresis, transferred to nitrocellulose, and detected by hybridization. The peak fractions of double-stranded (↓) and single-stranded (↓) cloned DNA are marked. The direction of sedimentation was from right to left. Lane at far right contained total liver DNA (1 μg). L, location of linear 3-kbp DNA.

infected liver were predominately single-stranded molecules of varying length, with a maximum length equal to that of the viral genome (3 kb). Consideration of the structure of virion DNA raised the idea that the single-stranded molecules in the liver might correspond to the complete strand of the virion DNA. This possibility was tested by hybridizing Southern blots of liver DNAs with strand-specific probes.

Clones of the Plus and Minus Strands of DHBV DNA. Strand-specific probes were prepared by cloning the entire DHBV genome into the single-stranded bacteriophage vector M13mp7. The strand specificity of two such clones (8 and 409) was tested as shown in Fig. 5. The incomplete strand of DHBV DNA was radiolabeled in the endogenous reaction, denatured, and then allowed to hybridize in the presence of the cloned DNAs. Clone 409 phage DNA accelerated this hybridization and therefore, is complementary to the product of the endogenous DNA polymerase reaction of DHBV; clone 8 phage DNA competed with hybridization of the radiolabeled DNA and therefore is homologous to this product. In previous studies

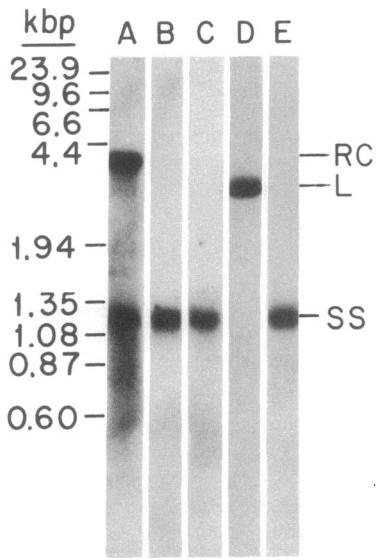

FIG. 4. Electrophoretic mobility of the major rapidly migrating viral DNA in the liver of a viremic duck is insensitive to heating to 100°C. Viral DNA species SS was separated from the remaining viral species in a total liver DNA extract (lane A, 1 μg of liver DNA), as described in the text, and then subjected to electrophoresis on a 1.5% agarose gel either before (lane B) or after (lane C) heating for 3 min at 100°C in 1 mM Tris·HCl/1 mM EDTA, pH 7.4. As a control, the 3-kb linear duplex DHBV insert in the Charon 16A–DHBV clone was subjected to electrophoresis either before (lane D) or after denaturation (lane E) by heating for 3 min at 100°C. The DNAs were transferred to nitrocellulose and detected by hybridization with a Charon 16A–DHBV probe.

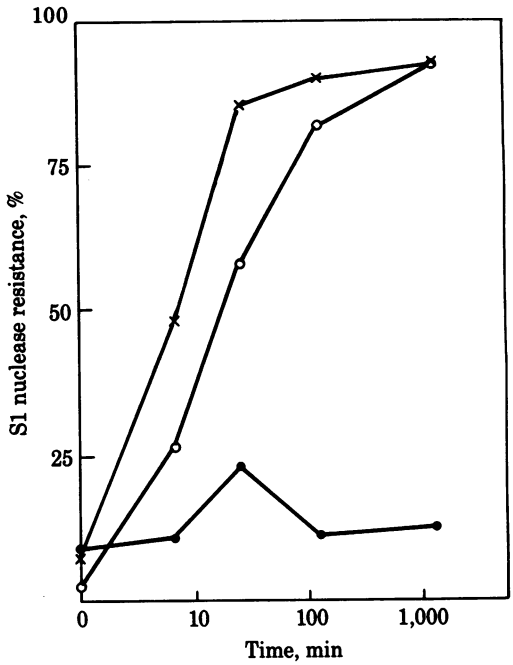

FIG. 5. Strand specificity of M13–DHBV clones as determined by hybridization with the endogenous polymerase product of DHBV. DHBV [^{32}P]DNA was prepared in the endogenous reaction of DHBV virions, as described (8). This DNA (10,000 cpm) was mixed with 0.1 μg of either M13mp7 (○), M13–DHBV clone 8 (●), or M13–DHBV clone 409 (×) phage DNA, heated 3 min at 100°C, quick chilled, and then annealed at 68°C in a final volume of 100 μl containing 0.6 M NaCl, 0.04 M Tris·HCl (pH 7.4), 2 mM EDTA, and 1 mg/of yeast tRNA per ml. Aliquots (15 μl) were withdrawn at the indicated times and diluted to 60 μl, and 25 μl portions were tested for resistance to nuclease S1 digestion. Digestions were for 1 hr at 53°C by addition of 25 μl of [^{32}P]DNA to 200 μl of 0.3 M NaCl/0.03 M sodium acetate, pH 4.5/3 mM $ZnSO_4$ containing 40 μg of denatured bovine DNA per ml, either with or without 1,000 units of S1 nuclease per ml. After this incubation, acid-precipitable DNA was collected on glass fiber filters and radioactivity was determined in Liquifluor-toluene scintillation fluid (New England Nuclear) in a Beckman scintillation counter. The percentage S1 resistance was normalized to the S1 resistance of the nondenatured ^{32}P-labeled DHBV DNA (62.5%).

(ref. 19; unpublished data) we found that the major polyadenylylated RNAs found in DHBV-infected liver are complementary to the complete strand of virion DNA, indicating that, by definition, this is the minus strand and that the incomplete strand is the plus strand of DHBV. Thus, M13 clone 8 phage DNA carries the plus strand of DHBV, and clone 409, carries the minus strand.

Single-Stranded DHBV-Specific DNA Sequences in the Liver Are Minus Strands. The liver and SS DNA preparations described in Fig. 4 were subjected to agarose gel electrophoresis and transferred to nitrocellulose, and DHBV sequences were detected by hybridization with plus- or minus-strand-specific probes (Fig. 6). The SS DNA species, as well as the more rapidly migrating DHBV DNAs, were not detected by a probe that recognizes the plus strand of DHBV but were detected by the probe complementary to the minus strand of DHBV. These rapidly migrating species therefore appear to be predominately minus strands, with little or no associated plus strand. The two faint bands just above SS, marked with asterisks, may represent minus-strand DNA with small amounts of growing plus-strand DNA.

In order to extend these observations, liver and pancreas DNAs from an additional three viremic ducks were subjected to Southern analysis and hybridization with strand-specific probes. The results were similar to those shown in Fig. 6. The rapidly migrating DHBV DNAs were not detected by a probe that recognizes the plus strand but were detected by a probe that recognizes the minus strand of DHBV (data not shown).

DISCUSSION

A major portion of the viral DNA isolated from infected tissues behaves as a collection of single-stranded minus-strand species with lengths shorter than or equal to the length of the DHBV genome (3 kb). This conclusion is supported by four observations: (*i*) marked sensitivity of conformation (sedimentation rate) to ionic strength; (*ii*) hybridization with plus-strand, but not minus-strand, viral DNA; (*iii*) comigration of the major, discrete species with single-stranded viral DNA with a length of 3 kb; and (*iv*) insensitivity of the electrophoretic mobility of the major species to incubation at 100°C. Because both the 3-kb minus-strand DNA (SS) from liver and the minus-strand of DHBV DNA appear to possess a covalently bound protein (21) that presumably is analogous to that found at the 5′ terminus of the minus strand of HBV DNA (22), it seems likely that these two DNA molecules are identical. Also, if the minus-strand DNA in liver is converted to virion DNA by initiation and elongation of plus-strand DNA from a single origin near the 5′ terminus of the minus-strand DNA (5), then "free" minus-strand DNAs in the liver, including those <3 kb long, might actually have a small amount of associated plus-strand DNA corresponding in extent to the cohesive overlap observed in virion DNA of HBV-like viruses. More data are needed to resolve these questions.

Although the isolation of 3-kb minus strands from infected tissues was not totally unexpected, the presence of free minus strands smaller than 3 kb was surprising. These are unlikely to

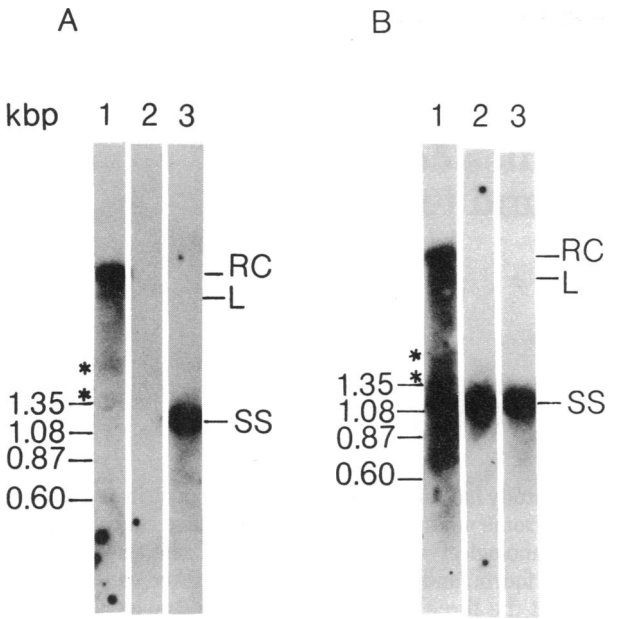

FIG. 6. Rapidly migrating viral DNAs in infected liver are mostly minus strand. The total liver DNA (lane 1, 1 μg) SS DNA (lane 2), and cloned, denatured DHBV DNA (lane 3) described in Fig. 4 were subjected to electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with a probe that will detect plus strands (M13–DHBV clone 409) (*A*) or a probe that will detect minus strands (M13–DHBV clone 8) (*B*).

arise from random cleavage of the 3-kb DNA because in all the samples we have examined there appeared to be at least two relatively discrete classes of size <3 kb, in addition to species of random lengths (e.g., Fig. 3). At least two alternatives remain: either these species smaller than 3 kb represent full-sized minus strands of defective viruses with deletions of viral sequences, or they are nascent chains that are released during the DNA extraction. In the latter instance, the discrete subgenomic species could reflect points at which chain elongation slows or stops as a result of specific structural impediments, resulting in the accumulation and subsequent release, during DNA extraction, of particular size categories of nascent strands. This possibility is at least consistent with other data showing that the template for the minus strand is RNA (11). Thus, nascent DNA attached to a template would have been released by the RNase digestion used during the DNA isolation procedure. Infected duck livers contain a polyadenylylated viral RNA of approximately genome length that might serve as the template for the synthesis of the minus-strand DNAs (19).

We are grateful to Laura Coates and Daniel Gzesh for excellent technical assistance. This work was supported by U.S. Public Health Service Grants AI-15166, CA-26012, CA-22651, CA-06927, and RR-05539, by American Cancer Society Grant MV-7E, and by an appropriation from the Commonwealth of Pennsylvania.

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