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Restriction endonuclease cleavage map and location of unique features of the DNA of hepatitis B virus, subtype adw_2

[DNA structure/surface antigen subtypes of hepatitis B virus (HBsAg subtypes)]

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ABSTRACT **DNA of hepatitis B virus (HBV) of hepatitis B surface antigen (HBsAg) subtype adw_2 made fully double stranded by the virion DNA polymerase and radiolabeled either by the virion DNA polymerase reaction or by nick-translation with ^{32}P-labeled deoxynucleoside triphosphates was used to establish a map of restriction endonuclease cleavage sites by the method of double and triple enzyme digestion and to determine the relative positions of several unique physical features of this DNA. The five restriction sites for the enzyme *Hin*cII, the two sites each for *Bam*HI, *Ava* I, and *Bgl* II, and the single sites for *Eco*RI, *Pst* I, *Hpa* I, and *Taq* I were positioned relative to each other. Within this map, the single-stranded region in HBV DNA has been localized and the locations of nicks in each strand (*a* and *b*) have been determined with respect to restriction sites on the circular map. Comparison of restriction endonuclease cleavage patterns of DNAs of HBV of HBsAg subtypes adw_2, ayw_3, and adrq+ revealed consistent differences among subtypes and occasional differences within subtypes.**

Hepatitis B virus (HBV) has several interesting features. The virion (also known as the Dane particle) has a complex structure (1, 2) with hepatitis B surface antigen (HBsAg) on its outer lipid-containing envelope (1, 2) and the distinct hepatitis B core antigen (HBcAg) on its internal core or nucleocapsid (2). Substantial evidence indicates that the HBsAg is specified by the viral genome (3). A group-specific determinant *a* found in all HBsAg preparations and two pairs of subtype determinants (*d*, *y* and *w*, *r*), which are for the most part mutually exclusive and thus behave as alleles, have been described (4, 5). Antigenic heterogeneity of the *w* determinant and additional determinants such as *q* and *x* or *g* have been found (6). Virus strains of the eight HBsAg subtypes ayw_1, ayw_2, ayw_3, ayw_4, ayr, adw_2, adw_4, and adr as well as more complex and less common subtypes such as adyw and adywr have been identified (7). Both group-specific and type-specific HBsAg determinants appear to reside in the major 22,000-dalton polypeptide, which can be isolated from purified HBsAg particles (8).

Hepatitis B virions contain a circular DNA molecule (9) that is partially double stranded. The single-stranded portion varies in length from approximately 15–50% of the circle length in different molecules (10–12). Thus the DNA consists of a long strand, *a*, of constant length (3200 nucleotides) and a short strand, *b*, which varies in length from approximately 1700 to 2800 nucleotides in different molecules. A DNA polymerase activity in the virion (13, 14) closes the single-stranded gap in each molecule to make fully double-stranded circular DNA with a uniform length of approximately 3200 base pairs (bp) (10–12). DNA synthesis is initiated for this reaction at the 3′ end of the short strand, which occurs at different sites within a specific region (50%) of the DNA in different molecules (12, 15). There is also evidence that a nick exists in the long strand (*a*) (10, 16) and that the 5′ end of the short strand (*b*) occurs at a fixed site (16). The circular DNA can be converted to a linear form with single-stranded cohesive ends by selectively denaturing the region between the 5′ end of the short strand and the nick in the long strand by heating under appropriate conditions (16).

We have constructed a map of restriction endonuclease cleavage sites of HBV DNA (HBsAg subtype adw_2) and have localized certain physical features of the DNA within this map, including the single-stranded region, the 5′ end of the short strand, and the nick in the long strand. We have also found systematic differences in the restriction endonuclease cleavage patterns for DNA of HBV of HBsAg subtypes adw_2, ayw_3, and adrq+.

MATERIALS AND METHODS

Plasma rich in Dane particles was obtained by plasmaphoresis from patients persistently infected with HBV (HBsAg carriers). HBsAg subtyping was kindly performed by A. M. Courouce-Pauty. The detailed methods of Dane particle preparation and DNA extraction have been described (9). The single-stranded region of HBV DNA was always made double-stranded by an extensive virion DNA polymerase reaction in the presence of high deoxynucleoside triphosphate (dNTP) concentrations as described (12) before DNA extraction. Dane particle DNA was radiolabeled either during the endogenous DNA polymerase reaction, in which case radioactive nucleotides are incorporated into only approximately 50% of the molecule (12), or by nick-translation (17), which results in uniform radiolabeling. ^{32}P-labeled dNTPs with a specific activity of 350 Ci/mmol (1 Ci = 3.7×10^{10} becquerels) were purchased from Amersham-Searle. *Escherichia coli* DNA polymerase I was a gift of Arthur Kornberg.

Restriction endonucleases were used in standard reaction conditions suggested by New England BioLabs, from which the enzymes were purchased. For monitoring completeness of digestion, unlabeled DNA with known restriction pattern such as pBR322 plasmid or PM2 phage DNA was included in the reaction. Polyacrylamide gel electrophoresis and autoradiography were carried out as described (12). DNA fragments for subsequent restriction analysis were excised from gels and recovered by electroelution (18).

HBV DNA made fully double-stranded by the endogenous DNA polymerase reaction was heated at 77°C for 15 min in 10 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA to convert the circular molecules to linear forms and then immediately chilled (16). Avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase) (provided by J. Beard, Life

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; bp, base pair(s).

Science, St. Petersburg, FL) was used to make the single-stranded ends double stranded as described (19).

RESULTS

Circular HBV 390 DNA (adw_2) made fully double stranded by the virion DNA polymerase reaction in the presence of ^{32}P-labeled dNTPs was cleaved into a unit-length linear form by *Eco*RI, *Taq* I, *Hae* II, *Pst* I, and *Hpa* I (not shown) restriction endonucleases, indicating the existence of a single cleavage site in this DNA for each of the enzymes. *Ava* I, *Bam* HI, and *Bgl* II restriction endonucleases cleaved the molecule into at least two fragments, although only one of the fragments was radiolabeled by the endogenous DNA polymerase reaction in each case. HBV 390 DNA uniformly labeled with ^{32}P by nick-translation was digested with *Hin*cII endonuclease and a gel electrophoresis pattern of DNA fragments similar to that previously reported by Landers *et al.* (12) was obtained (Fig. 1, tracks 1 and 6). The four visible bands consisted of five DNA fragments, designated here as *Hin*cII-A (980 bp), *Hin*cII-B (766 bp), *Hin*cII-B′ (728 bp), *Hin*cII-C (546 bp), and *Hin*cII-D (314 bp), as will be subsequently shown.

In order to localize the cleavage sites of the enzymes that cleave HBV 390 DNA once or twice within the five *Hin*cII fragments, uniformly labeled DNA was first digested with *Hin*cII and then a second enzyme, and the digests were analyzed by gel electrophoresis (Fig. 1). Digestion with *Hin*cII and either *Hae* II (Fig. 1, track 9) or *Bam*HI (Fig. 1, track 3) resulted in a reduction in intensity of the *Hin*cII second band and the appearance of two smaller fragments, the sum of whose sizes equalled the size of the second band. Extensive experiments indicated that this was not due to incomplete digestion of the second band but to the presence of two DNA fragments in it.

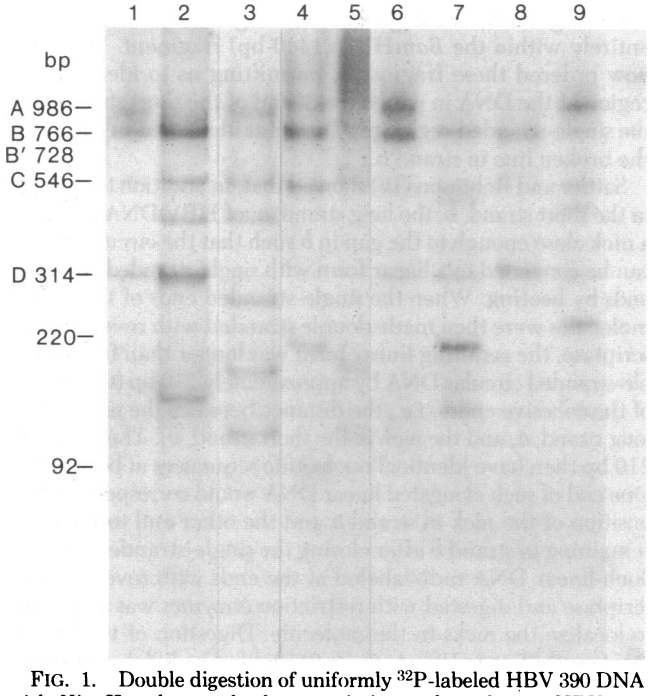

FIG. 1. Double digestion of uniformly ^{32}P-labeled HBV 390 DNA with *Hin*cII and several other restriction endonucleases. HBV 390 DNA made completely double stranded by the virion DNA polymerase was radiolabeled by nick-translation using ^{32}P-labeled dNTPs. Before electrophoresis in 3–7% polyacrylamide gels and autoradiography, aliquots of the [^{32}P]DNA were incubated with: track 1, *Hin*cII alone; track 2, *Hin*cII and *Bgl* II; track 3, *Hin*cII and *Bam*HI; track 4, *Hin*cII and *Eco*RI; track 5, *Hin*cII and *Pst* I; track 6, *Hin*cII alone; track 7, *Hin*cII and *Ava* I; track 8, *Hin*cII and *Taq* I; and track 9, *Hin*cII and *Hae* II.

The two fragments are designated B and B′ and the fragment cleaved by *Bam*HI is defined as B′. The two fragments could be detected as a double in some gels with the best resolution and sizes of 766 and 728 bp have been assigned to B and B′, respectively.

Digestion of *Eco*RI/*Hpa* I Fragments with *Hin*cII. Digestion of HBV 390 DNA uniformly labeled by nick-translation with the single cutters *Eco*RI and *Hpa* I together yielded a 2320-bp fragment (*Eco*RI/*Hpa* I-A) and a 980-bp fragment (*Eco*RI/*Hpa* I-B) (data not shown). Each DNA fragment was extracted from a gel, digested with *Hin*cII, and analyzed by polyacrylamide gel electrophoresis. The results of digestion of *Eco*RI/*Hpa* I-A with *Hin*cII are shown in Fig. 2, track 4. *Hin*cII-A, -B or -B′ (or both), and -C and a DNA fragment of approximately 92 bp can be observed. The second band was shown to contain only *Hin*cII-B′ by its cleavage with *Bam*HI (data not shown). Digestion of *Eco*RI/*Hpa* I-B with *Hin*cII yielded B or B′ or both and a DNA fragment of approximately 220 bp (Fig. 2, track 5). Resistance of the first band to *Bam*HI (data not shown) indicated that it contained only the *Hin*cII-B fragment and not -B′. Thus *Hin*cII-B and -B′ are separated by digestion of HBV 390 DNA with *Eco*RI and *Hpa* I. The sum of the sizes of the 92-bp DNA fragment from *Eco*RI/*Hpa* I-A and the 220-bp DNA fragment from *Eco*RI/*Hpa* I-B is approximately the size of *Hin*cII-D, which does not appear in *Hin*cII digests of either *Eco*RI/*Hpa* I fragment. This is consistent with the findings that *Hin*cII-D is cleaved by *Eco*RI into fragments of approximately 92 and 220 bp (Fig. 1, track 4). Although, as described above, we have shown that *Hpa* I cleaves HBV 390 DNA at a single site, *Hpa* I was found not to cleave any *Hin*cII fragment of this DNA in a separate experiment. *Hpa* I and *Hin*cII recognize overlapping sequences (20), and one of the *Hin*cII cleavage sites is also the *Hpa* I cleavage site. Because *Eco*RI/*Hpa* I-B consists of only a 220-bp portion of the *Hin*cII-D plus the complete *Hin*cII-B fragment, *Hin*cII-B must be adjacent to *Hin*cII-D in HBV 390 DNA. In addition, *Hpa* I must cleave HBV 390 DNA at the end of the *Hin*cII-B fragment not adjacent to *Hin*cII-D.

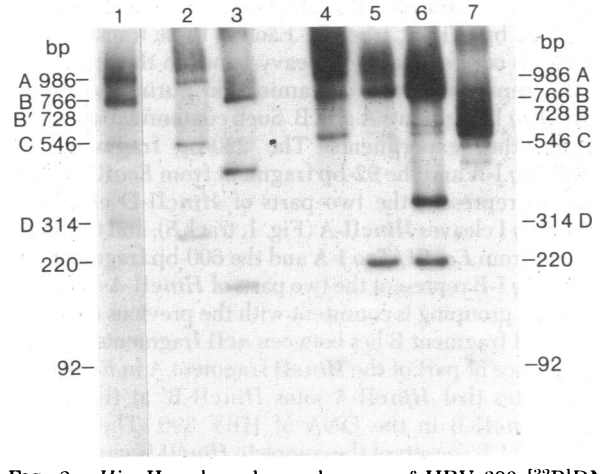

FIG. 2. *Hin*cII endonuclease cleavage of HBV 390 [^{32}P]DNA fragments generated by double enzyme digestion. Aliquots of HBV 390 [^{32}P]DNA prepared as described for Fig. 1 were digested with *Eco*RI plus *Bam*HI, *Eco*RI plus *Hpa* I, and *Eco*RI plus *Taq* I, and the two fragments A and B resulting from each digestion were separated by agarose gel electrophoresis and recovered as described in the text for digestion with *Hin*cII. After digestion with *Hin*cII, HBV 390 [^{32}P]DNA (track 1), *Eco*RI/*Bam*HI fragments A (1860 bp) (track 2) and B (1464 bp) (track 3), *Eco*RI/*Hpa* I fragments A (2320 bp) (track 4) and B (980 bp) (track 5), and *Eco*RI/*Taq* I fragments A (2054 bp) (track 6) and B (1255 bp) (track 7) were analyzed by gel electrophoresis and autoradiography as described for Fig. 1.

Digestion of *Eco*RI/*Bam*HI Fragments with *Hin*cII. Digestion of HBV 390 DNA with *Eco*RI and *Bam*HI together yielded a 1860-bp fragment (*Eco*RI/*Bam*HI-A) and a 1400-bp fragment (*Eco*RI/*Bam*HI-B) (data not shown). Recovery of each fragment and digestion with *Hin*cII produced the findings shown in Fig. 2. Digestion of *Eco*RI/*Bam*HI-A with *Hin*cII yielded *Hin*cII-A, -C, and fragments of approximately 270 bp and 92 bp (Fig. 2, track 2). Digestion of *Eco*RI/*Bam*HI-B with *Hin*cII yielded *Hin*cII-B (B′ is cleaved by *Bam*HI) and DNA fragments of approximately 454 bp and 170 bp (Fig. 2, track 3). As shown above, *Eco*RI cleaves *Hin*cII-D into fragments of 220 and 92 bp and *Bam*HI cleaves both *Hin*cII-B′ and -D. This suggests that the 92-bp fragments arising from *Eco*RI/*Bam*HI-A are one portion of *Hin*cII-D cleaved by *Eco*RI. The 170-bp fragment arising from *Eco*RI/*Bam*HI-B must represent the remainder of the *Hin*II-D fragment after cleavage with *Bam*HI, which has been shown in other experiments to cleave *Hin*cII-D at a site approximately 50 bp from the *Eco*RI site. The 50-bp fragment would have run off the bottom of the gel under the electrophoresis conditions used. The sum of the sizes of the 270-bp fragment from *Eco*RI/*Bam*HI-A and the 454-bp fragment from *Eco*RI/*Bam*HI-B closely approximates the size of *Hin*cII-B′ (a fragment shown above to be cleaved by *Bam*HI). Thus a portion of *Hin*cII-B′ is present in *Eco*RI/*Bam*HI-A and the remainder in *Eco*RI/*Bam*HI-B. *Eco*RI/*Bam*HI-B thus consists of *Hin*cII-B, part of -B′, and part of -D. Because *Hin*cII-D was shown above to be adjacent to *Hin*cII-B, *Hin*cII-B′ must be adjacent to the other end of *Hin*cII-B in HBV 390 DNA. It is also clear from the composition of *Eco*RI/*Bam*HI-A that the *Hin*cII fragments A and C must be adjacent in HBV 390 DNA.

Digestion of *Eco*RI/*Taq*I Fragments with *Hin*cII. Digestion of HBV 390 DNA with *Eco*RI and *Taq* I together yielded a 2054-bp fragment (*Eco*RI/*Taq* I-A) and a 1255-bp fragment (*Eco*RI/*Taq* I-B) (data not shown). Recovery of each fragment and digestion with *Hin*cII gave the findings shown in Fig. 2. Digestion of *Eco*RI/*Taq* I-A with *Hin*cII yielded *Hin*cII-B or -B′ or both and DNA fragments of approximately 340 and 220 bp (Fig. 2, track 6). Digestion of *Eco*RI/*Taq* I-B with *Hin*cII yielded *Hin*cII-C and fragments of approximately 600 and 92 bp (Fig. 2, track 7). Each of these tracks contains faint bands corresponding to heavy bands in the other track, and this represents cross contamination during isolation of *Eco*RI/*Taq* I fragments A and B. Such contamination was not seen in other experiments. The 220-bp fragment from *Eco*RI/*Taq* I-A and the 92-bp fragment from *Eco*RI/*Taq* I-B appear to represent the two parts of *Hin*cII-D cleaved by *Eco*RI. *Taq* I cleaves *Hin*cII-A (Fig. 1, track 8), and the 340-bp fragment from *Eco*RI/*Taq* I-A and the 600-bp fragment from *Eco*RI/*Taq* I-B represent the two parts of *Hin*cII-A cleaved by *Taq* I. This grouping is consistent with the previous conclusion that *Hin*cII fragment B lies between *n*cII fragments D and B′. The presence of part of the *Hin*cII fragment A in *Eco*RI/*Taq* I-A indicates that *Hin*cII-A joins *Hin*cII-B′ at the end not joining *Hin*cII-B in the DNA of HBV 390. The fact that *Eco*RI/*Taq* I-B consists of the complete *Hin*cII fragment C and parts of *Hin*cII fragments A and D indicates that *Hin*cII-C lies between *Hin*cII fragments D and A in HBV 390 DNA.

This experiment completes the order of all *Hin*cII fragments in HBV 390 DNA and the order is illustrated in Fig. 3. The experiments we have done also permit localization of the cleavage sites of *Eco*RI, *Pst* I, *Hpa* I, *Taq* I, *Bam*HI, and *Bgl* II relative to the *Hin*cII cleavage sites, and these are also illustrated in Fig. 3.

Location of the Unique Physical Features of HBV DNA. In a pulse-chase reaction, Landers *et al.* (12) showed that DNA

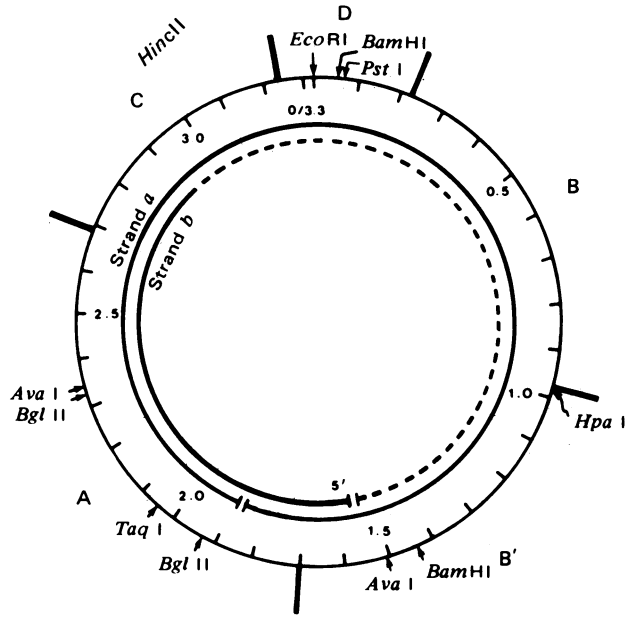

FIG. 3. Physical map of HBV 390 DNA. The outer circle represents a scale of DNA length in kilobase pairs and the positions established for cleavage sites of the restriction endonuclease are indicated. The inner circles represent the long (*a*) and short (*b*) strands of the DNA oriented with respect to the restriction sites indicated in the outer scale. The broken line in strand *b* represents the region within which the 3′ end of strand *b* may occur in different molecules and the corresponding region in strand *a* is that which may be single stranded in different molecules.

synthesis occurred in *Hin*cII fragments B, B′, C, and D during the virion DNA polymerase reaction, indicating that DNA synthesis is initiated and the single-stranded region exists within these fragments. The single-stranded region is included almost entirely within the *Bam*HI-B (1440-bp) fragment. We have now ordered these fragments, permitting us to identify the region of the DNA in which the 3′ end of the short strand and the single-stranded region may occur as illustrated in Fig. 3 by the broken line in strand *b*.

Sattler and Robinson (16) showed that, in addition to the gap in the short strand, *b*, the long strand, *a*, of HBV DNA contains a nick close enough to the gap in *b* such that the circular DNA can be converted to a linear form with single-stranded cohesive ends by heating. When the single-stranded ends of the linear molecules were then made double stranded with reverse transcriptase, the resulting linear form was longer than fully double-stranded circular DNA by approximately 210 bp (the length of the cohesive ends—i.e., the distance between the nick in the long strand, *a*, and the nick in the short strand, *b*). The terminal 210 bp then have identical nucleotide sequences at both ends. One end of such elongated linear DNA would correspond to the position of the nick in strand *a* and the other end to the nick remaining in strand *b* after closing the single-stranded region. Such linear DNA radiolabeled at the ends with reverse transcriptase and digested with restriction enzymes was analyzed to localize the nicks in the molecule. Digestion of the linear DNA with *Taq* I (Fig. 4, track 2) yielded radiolabeled fragments of approximately 3100 and 430 bp; *Pst* I digestion (track 3) yielded ^{32}P-labeled fragments of approximately 1810 and 1710 bp; and *Hpa* I (track 5) produced ^{32}P-labeled fragments of approximately 2580 and 890 bp. Digestion with both *Pst* I and *Hpa* I (track 4) resulted in ^{32}P-labeled fragments of approximately 1810 and 890 bp. These results indicate that the nick in strand *a* is approximately 1810 bp clockwise from the *Pst* I site or approximately 890 *bp* clockwise from the *Hpa* I site

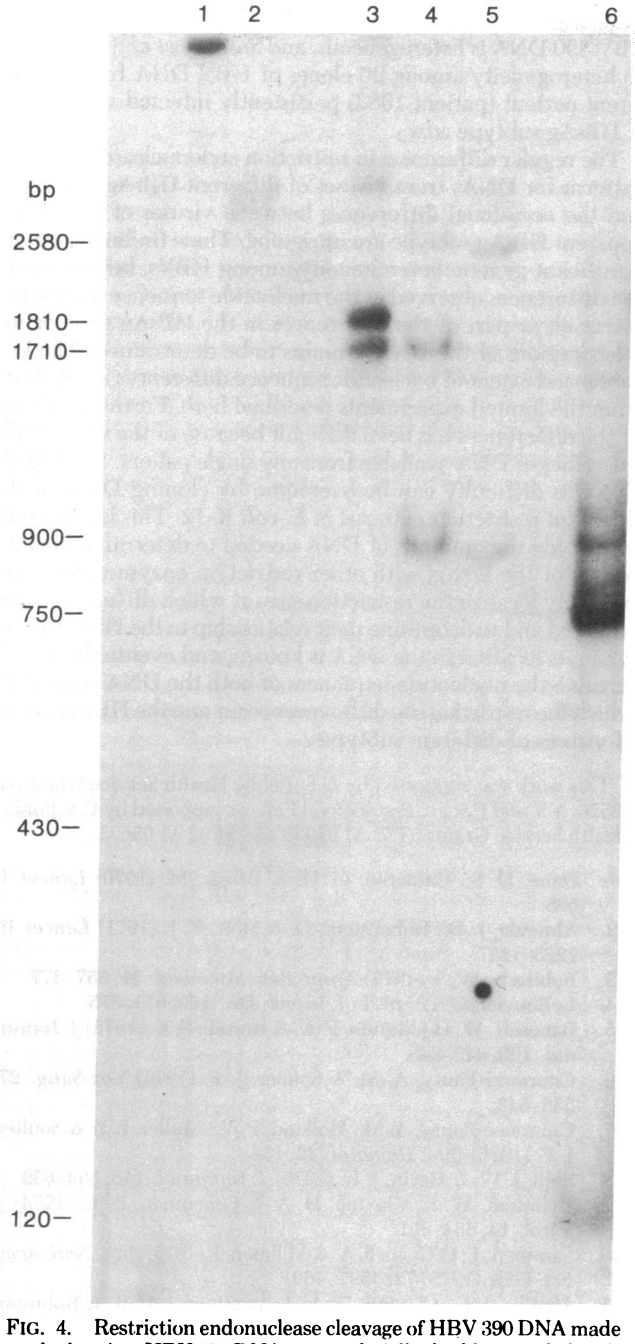

FIG. 4. Restriction endonuclease cleavage of HBV 390 DNA made linear by heating. HBV 390 DNA was made fully double stranded with the virion DNA polymerase and converted to linear DNA by heating, and the single-stranded ends were converted to double-stranded DNA and radiolabeled with reverse transcriptase in the presence of ^{32}P-labeled dNTPs. The DNA was then analyzed by agarose gel electrophoresis and autoradiography after incubation with: track 1, no enzyme; track 2, *Taq* I; track 3, *Pst* I; track 4, *Pst* I plus *Hpa* I; track 5, *Hpa* I; and track 6, *Hin*cII.

in DNA oriented as in Fig. 3. The nick remaining in strand *b* after the single-stranded region is made double stranded is approximately 1650 bp clockwise from the *Pst* I site and approximately 680 bp clockwise from the *Hpa* I site. These locations are near the *Hin*cII cleavage site between *Hin*cII fragments A and B′, which can be calculated from the earlier experiments to be approximately 1714 bp clockwise from the *Eco*RI site or 1664 bp from the *Pst* I site in HBV 390 DNA as shown in Fig. 3. Digestion of the linear DNA with *Hin*cII (Fig. 4, track 6) produced ^{32}P-labeled fragments of approximately 980 bp, 750 bp, and a doublet near 100 bp. The 980-bp fragment appears to represent *Hin*cII-A and the 750-bp fragment, *Hin*cII-B′. The 100-bp doublet is interpreted to consist of fragments of near equal length cleaved from each of the ends, which are redundant in this elongated linear DNA. That suggests that the nick in the long strand is approximately 100 bp clockwise from the cleavage site between *Hin*cII fragments A and B′ and the nick remaining in the short strand after the single-stranded gap has been closed is approximately 100 bp counterclockwise from the cleavage site between *Hin*cII fragments A and B′ in DNA oriented as in Fig. 3. These features are illustrated in Fig. 3.

***Hin*cII Cleavage Patterns of HBV DNAs of Different HBsAg Subtypes.** Fig. 5 shows ethidium bromide-stained DNA fragments resulting from *Hin*cII digestion and gel electrophoresis of HBV DNA from seven different patients. The HBsAg subtype of the virus whose DNA is shown in tracks 1 through 4 was adw_2, that in 5 and 6 was ayw_3, and that in 7 was adrq+. The patterns of the four adw_2 DNAs in Fig. 5 generally correspond to the pattern shown above for HBV 390 DNA. The DNAs of the ayw_3 viruses appear to lack the *Hin*cII-A and -D fragments found in adw_2, and a large fragment of approximately 1280 bp, which is near the sum of the sizes of *Hin*cII-A and -D, is present. The second band in track 6 and the third band in tracks 6 and 7 also appear to differ slightly in position from those of the *Hin*cII-B and -C fragments, respectively, of adw_2. The DNA of the adrq+ virus appears to lack the *Hin*cII-C and -D fragments found in adw_2 and an additional fragment of approximately 860 bp (equal to the sum of the sizes of *Hin*cII-C and -D of adw_2) is present. This suggests that the *Hin*cII cleavage site between fragments C and D in DNA of adw_2 virus is not present in the DNA of this adr virus. Striking differences between subtypes were also observed when the same DNAs were digested with *Hae* III and *Hin*fI restriction endonucleases. In addition to differences in restriction endonuclease cleavage patterns that appear to correlate with HBsAg subtype differences among viruses, we have observed DNAs of the same subtype (e.g., adw_2 and ayw_3) that have restriction patterns different from the "typical" patterns shown in Fig. 5. These we have designated DNAs with "atypical" restriction patterns. We have examined DNAs of 16 adw_2 virus strains of

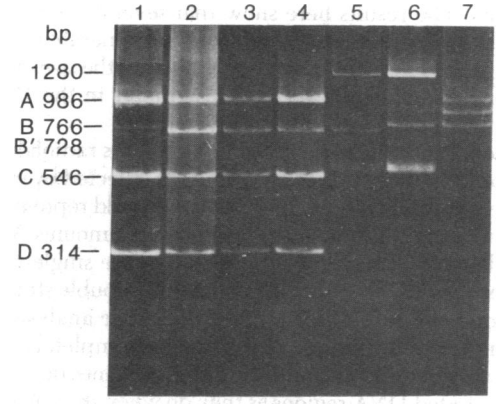

FIG. 5. *Hin*cII endonuclease cleavage pattern of DNA from HBV of different HBsAg subtypes. Each DNA was made fully double stranded with the virion DNA polymerase. DNAs from HBV of HBsAg subtype adw_2 obtained from patients I-53 (track 1), I-117 (track 2), I-108 (track 3), and I-162 (track 4); subtype ayw_3 from patients I-35 (track 5) and 1042 (track 6), and subtype adrq+ from patient 808 (track 7) were then digested with *Hin*cII and analyzed by electrophoresis in a 3–7% polyacrylamide gradient gel. The DNA fragments were stained with ethidium bromide, illuminated with a UV lamp, and photographed.

which 13 (patient numbers 22, 390, 984, 985, 1083, I-6, I-53, I-80, I-108, I-117, I-162, and I-263) had typical and 3 (1018, 1035, and I-191) had atypical restriction endonuclease cleavage patterns. Three ayw_3 HBV strains revealed two (975 and 1042) that were indistinguishable and one (I-35) with a slightly different restriction pattern. Two adrq+ HBV strains (808 and 1047) had DNAs that were similar.

DISCUSSION

Here we present a map of restriction endonuclease cleavage sites in HBV DNA of HBsAg subtype adw_2. We have determined the location of: the 3′ end of the short strand, which is variable; the single-stranded region, which is bounded on one side by the 3′ end of the short strand and which is made variable in length by the variable location of the 3′ end; and the unique sites of the nick in the short strand remaining after closure of the single-stranded region and the nick in the long strand with respect to restriction sites. This has permitted more precise determination of the topography of the special physical features of this DNA. The actual role that these features and the DNA polymerase in the virion play during virus replication is not clear, but it seems likely they are involved in DNA replication or integration.

HBV DNA of the same subtype (adw_2) was recently cloned and a map of certain restriction endonuclease cleavage sites was determined by the 5′-end labeling method (21). The order and locations of restriction sites investigated in both studies are the same and confirm that the entire unaltered HBV DNA was cloned.

The sum of the *Hin*cII fragments of fully double-stranded HBV 390 DNA was 3340 bp when carefully measured with respect to pBR322 plasmid DNA restriction fragments of known size. This figure is in agreement with estimates of the size of intact fully double-stranded HBV DNA molecules measured in electron micrographs (11) and by electrophoretic mobility (12). Landers *et al.* (12) previously suggested that HBV 390 DNA molecules might be heterogeneous in base sequence, because the sum of the sizes of *Hae* III and *Hin*cII restriction fragments appeared to exceed the size of intact DNA molecules. Landers *et al.* (12) suggested that both the *Hae* III and the *Hin*cII-A bands (the largest bands generated by each enzyme and that contain DNA fragments that substantially overlap) appeared as doublets and thus may each contain two DNA fragments. The results here show that several restriction enzymes cleave *Hin*cII-A once or twice and generate fragments the sum of whose sizes is that of one fragment the size of A. This makes the presence of two DNA fragments in the *Hin*cII-A band of HBV 390 DNA extremely unlikely.

Landers *et al.* (12) also observed fragments radiolabeled by the virion DNA polymerase that were not detected by ethidium bromide staining and suggested that these could represent DNA fragments present in less than stoichiometric amounts. We have found that such fragments appear when the single-stranded region of the DNA is not made completely double stranded by the virion enzyme before restriction enzyme analysis. Under such conditions the DNA appears to be incompletely cleaved, presumably because certain restriction enzymes do not cleave single-stranded DNA regions as they do when the same regions are double stranded. When HBV DNA contains large single-stranded regions, fragments larger than the *Hae* III or *Hin*cII-A fragments may be observed by ethidium bromide staining and autoradiography when DNA is cleaved by these enzymes. These findings emphasize the importance of obtaining complete closure of the single-stranded region by the virion DNA polymerase or with reverse transcriptase before carrying out the kind of studies described here. Our data do not suggest that HBV 390 DNA is heterogeneous, and Sninsky *et al.* (21) found no heterogeneity among 20 clones of HBV DNA from a different patient (patient 1083) persistently infected with HBV of HBsAg subtype adw_2.

The regular differences in restriction endonuclease cleavage patterns for DNAs from viruses of different HBsAg subtypes and the occasional differences between viruses of the same apparent HBsAg subtype are intriguing. These findings suggest significant genetic heterogeneity among HBVs, but whether the differences observed at the nucleotide sequence level represent all or part of the differences in the HBsAg gene or in other regions of the DNA remains to be determined. The location and extent of nucleotide sequence differences is not clear from the limited experiments described here. Further analysis of the differences has been difficult because of the very small quantities of DNA available from any single patient. It is hoped that this difficulty can be overcome by cloning DNAs with different restriction patterns in *E. coli* K-12. This is expected to provide the amounts of DNA needed to determine the behavior of the DNAs with other restriction enzymes, to more precisely localize the restriction sites at which differences are observed and to determine their relationship to the HBsAg gene when its location in the DNA is known, and eventually to determine the nucleotide sequences of both the DNA regions at which the restriction site differences occur and the HBsAg genes of viruses of different subtypes.

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