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Genome of hepatitis B virus: Restriction enzyme cleavage and structure of DNA extracted from Dane particles

(*in vitro* DNA synthesis/endogenous DNA nucleotidyltransferase/endonuclease R·*Hae*III)

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ABSTRACT **DNA extracted from Dane particles has been characterized by gel electrophoresis and restriction enzyme cleavage with endonuclease R·*Hae*III (from *Hemophilus aegyptius*). Dane particle DNA is proposed to be a double-stranded circular DNA approximately 3600 nucleotides in length containing a single-stranded gap of 600–2100 nucleotides. The endogenous DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) reaction appears to repair this single-stranded gap.**

The Dane particle, a 42 nm, virus-like body seen in the blood of patients with active and chronic hepatitis, is thought to be the viral agent of hepatitis B (1). Kaplan *et al.* (2) demonstrated the presence of a primed DNA polymerase activity (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) in the cores of Dane particles. Such cores were shown by Robinson *et al.* (3) to contain a small double-stranded circular DNA molecule which acted as the substrate for the DNA polymerase. We have investigated the structure of unreacted DNA from Dane particles by *in vitro* radiolabeling of the extracted DNA and specific cleavage of the radioactive DNA with a restriction enzyme from *Hemophilus aegyptius* (4). The structure is related to the role of the endogenous DNA polymerase.

MATERIALS AND METHODS

Enzymes. *Escherichia coli* DNA polymerase I and avian myeloblastosis virus (AMV) DNA polymerase were generous gifts of Dr. Lawrence Loeb (The Institute for Cancer Research, Philadelphia). Endonuclease R·*Hae*III was purified from *H. aegyptius* ATCC 11116 according to Middleton *et al.* (4) except that a streptomycin sulfate precipitation was substituted for the Bio-Gel chromatography step.

Dane Particles. Human plasma enriched in Dane particles by sedimentation twice through 20% sucrose into a 65% sucrose cushion was kindly provided by Dr. William Robinson, Stanford University.

Extraction of DNA. DNA was extracted from Dane particle preparations by digestion with 500 μg/ml of Pronase for 2 hr at 37° in the presence of 0.5% sodium dodecyl sulfate or Sarkosyl, 0.1 M NaCl, 10 mM Tris·HCl (pH 8), and 10 mM EDTA. The digested preparation was then extracted at room temperature with an equal volume of phenol equilibrated with 0.5 M Tris·HCl (pH 8) and precipitated with ethanol.

Endo R·*Hae*III Digestions and Gel Electrophoresis. DNA was digested with endo R·*Hae*III and subjected to slab gel electrophoresis as previously described (6). [^{32}P]DNA was located on the gels by autoradiography after drying.

Abbreviations: AMV, avian myeloblastosis virus; Py, polyoma virus.

RESULTS

Since only small amounts of Dane particle DNA were available we labeled the DNA by *in vitro* replication. Extracted DNA was denatured in 0.2 N NaOH and the complementary strand was synthesized at 15° using nucleoside [^{32}P]triphosphates and *E. coli* DNA polymerase I. Oligonucleotides derived from calf thymus DNA provided the primers for DNA synthesis (5, 6). The fully double-stranded product consisted of a series of newly synthesized strands 50–500 nucleotides in length duplexed with the unlabeled template strand. *In vitro* synthesized DNA was completely susceptible to cleavage with restriction enzymes (Summers, manuscript in preparation).

DNA was extracted from 0.2 ml of a Dane particle preparation which contained 10^{10}–10^{11} Dane particles per ml as estimated by electron microscopy, denatured, and replicated *in vitro* using [α-^{32}P]dTTP as described in Table 1. The radioactive product was sedimented through a neutral sucrose gradient and appeared as a single peak of about 13–14 S (Fig. 1). This S value corresponds to a molecular weight for linear double-stranded DNA of 1.8 to 2.3 × 10^6 (8).

Aliquots of each fraction of the neutral sucrose gradient were digested with endo R·*Hae*III and the fragments were separated on a 5% polyacrylamide gel (Fig. 1). The sizes of the *Hae*III fragments indicated in Fig. 1 as nucleotide pairs were calculated from the migration positions relative to those of the polyoma *Hae*III fragments run on the same gel (6, 9).

Undigested aliquots of fractions 14–20 of the sucrose gradient in Fig. 1 were subjected to electrophoresis through a 2% polyacrylamide–0.5% agarose gel (Fig. 2). *In vitro* replicated DNA from Dane particles can be resolved into two

Table 1. *In vitro* replication of denatured Dane particle DNA

Minutes reaction	Total pmol of dTMP incorporated
5	7.0
10	11.7
20	20.9
40	20.3

Dane particle DNA, precipitated with ethanol, was dissolved in 5 μl of H_2O and 2 μl of 1 M NaOH was added to denature the DNA. After 5 min at 4°, the sample was adjusted to pH 7.5 with 30 μl of 0.33 M Tris·HCl, pH 7. Seventy μl of H_2O containing 0.7 μmol of $MgCl_2$, 1 nmol each dATP, dCTP, and dGTP, 0.5 nmol of [α-^{32}P]-dTTP (10 Ci/mmole), 2 μg of oligonucleotide primers, and 0.5 μg of DNA polymerase I were added and the reaction was incubated at 15°. Aliquots were removed and assayed for incorporation of radioactive precursor into acid-insoluble material.

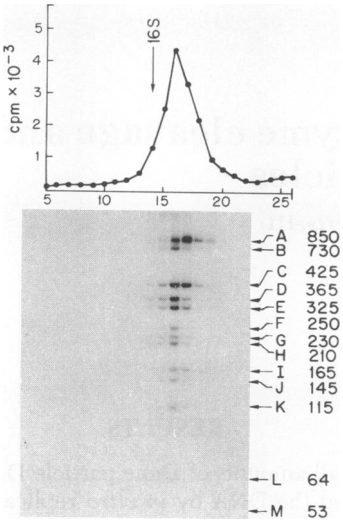

FIG. 1. Neutral sucrose sedimentation and cleavage patterns of DNA from Dane particles. After 60 min incubation, the reaction in Table 1 was adjusted to 0.5% with sodium dodecyl sulfate and 10 mM with EDTA and layered on a 3.8 ml of 5–20% sucrose gradient containing 150 mM NaCl, and 50 mM Tris–HOAc, pH 6.0, and centrifuged at 55,000 rpm in a Beckman SW56 rotor, at 20° for 150 min. A sedimentation marker of polyoma linear DNA (16 S) was centrifuged in a separate gradient. Fractions were collected from the bottom of the tube and 5 μl aliquots were assayed for acid-precipitable radioactivity. Aliquots (20 μl) of fractions 1–20 were digested with endo R·*Hae*III and layered on a 5% polyacrylamide slab gel. The gel was run at 100 V for 3 hr at room temperature, dried, and developed by autoradiography. Fragments L and M, although not visible in the photograph, are seen on the autoradiogram.

components. A discrete band accounting for most of the radioactivity migrates at a position of linear DNA of 3600 nucleotide pairs. This DNA component peaks in fraction 16 in the gradient which corresponds to 13.5 S, or approximately 3400 nucleotide pairs. Since circular DNA of this S value would migrate much slower in the gel (10) we conclude that the *in vitro* replicated DNA is linear. A second component

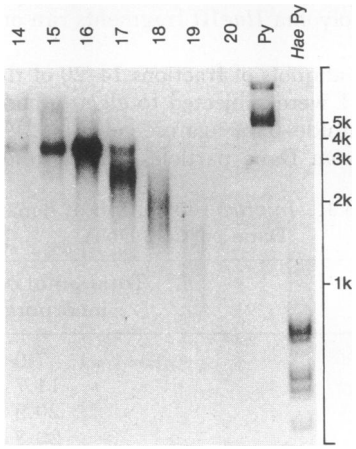

FIG. 2. Migration of undigested DNA from Dane particles, replicated *in vitro*. Aliquots of fractions 14–20 from the sucrose gradient in Fig. 1. were layered on a 2% polyacrylamide–0.5% agarose slab gel. Polyoma double-stranded circular and linear DNA (Py) labeled *in vitro* as well as polyoma *Hae*III fragments (*Hae*Py) were layered on the same gel as molecular weight markers. The gel was run at 50 V for 7 hr at room temperature, dried, and developed by autoradiography. Molecular weight calibration of the gel in nucleotide pairs is shown at the right (5k = 5000 pairs, etc.)

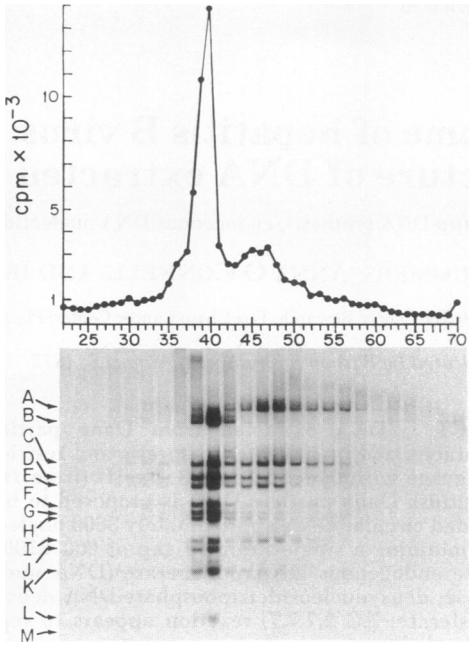

FIG. 3. Cleavage patterns of *in vitro* replicated Dane particle DNA components of various size. [^{32}P]DNA from Dane particles was prepared as described in Table 1. A portion of this DNA was applied in a volume of 5 μl to a 1% agarose gel (1.5 × 110 mm) poured in a 100 μl capillary pipette. The gel was run at 50 V for 4 hr at room temperature. The gel was removed and sliced into 1/16 inch (1.6 mm) sections and the radioactivity (Cerenkov) was counted. Radioactive fractions were eluted from the gel slices and digested simultaneously with 100 μl of *Hae*III digestion buffer containing a 1:10 dilution of the enzyme stock. After 24 hr at 37° carrier RNA (10 μg) and Sarkosyl (1%) were added to each digestion and the nucleic acids were precipitated with 2.5 volumes of 95% ethanol. The precipitated digests were subjected to electrophoresis through a 5% polyacrylamide slab gel and the gel was developed by autoradiography. Some additional fragments due to incomplete digestion of the DNA are seen in gel fractions 36 and 42.

of the *in vitro* replicated DNA is heterogeneous in size and migrates at positions ranging from less than 1500 nucleotide pairs (fractions 19 and 20) to about 3600 nucleotide pairs. A small amount of radioactivity migrates at positions which correspond to more than 3600 nucleotide pairs (fractions 14 and 15).

A second preparation of DNA was denatured and replicated *in vitro* and fractionated by electrophoresis through a 1% agarose tube gel. The DNA in each region of the gel was then digested with endo R·*Hae*III and the *Hae*III cleavage pattern was determined. The radioactive profile of the agarose gel and the *Hae*III fragments from fractions of the gel are shown in Fig. 3. Striking differences in the relative abundance of certain fragments are seen in Fig. 3 between the 3600 nucleotide component (fractions 38–40) and the smaller component (fractions 42–70). Fragment *Hae*III-A is virtually absent in the 3600 nucleotide component. The sum of all the fragments found in this discrete-sized component adds up to 3132 nucleotide pairs, which accounts for 87% of the 3600 nucleotide pairs estimated from the migration of this species on the gel in Fig. 2.

In contrast to the 3600 nucleotide species, the class of shorter molecules migrating between fractions 44 and 48 contains fragment *Hae*III-A, but is depleted in *Hae*III-B, F, G, H, J, and L, relative to the other fragments. Still smaller molecules migrating between fractions 50 and 70 contain reduced amounts of fragments D, E, and K relative to frag-

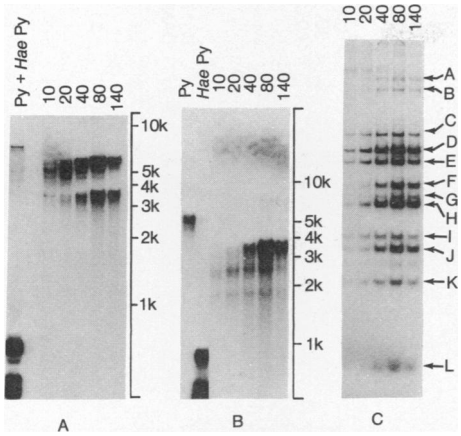

FIG. 4. (A) Migration of undigested Dane particle DNA replicated *in vitro* without prior denaturation. Carrier RNA (15 μg) and Sarkosyl (0.5%) were added to the aliquots of the reaction in Table 2, and the nucleic acids were precipitated with 2.5 volumes of 95% ethanol. The ethanol precipitates were dissolved in 20 μl of H_2O. Five microliters of each aliquot was applied to a 2% polyacrylamide–0.5% agarose slab gel. Polyoma circular and linear [^{32}P]DNA and ^{32}P-labeled polyoma *Hae*III fragments were applied to the same gel as molecular weight markers for calibrating the gel as shown on the right. The gel was run at 100 V for 3 hr and developed by autoradiography. (B) Migration of the single strands of undigested Dane particle DNA replicated *in vitro* without prior denaturation. Five microliters of the aliquots and markers in (A) were adjusted to 0.2 M with NaOH and applied to the 2% acrylamide–0.5% agarose slab gel in (A) (7). (C) Distribution of radioactivity among the *Hae*III fragments of Dane particle DNA replicated *in vitro* without prior denaturation. Five microliters of the aliquots in (A) was digested with endo R-*Hae*III and applied to a 5% polyacrylamide gel. Electrophoresis was for 3 hr at 100 V. Radioactivity in the gel was detected by autoradiography.

ments A, C, I, and M, which are the most abundant. The smaller components, therefore, seem to be derived from a restricted region of the total genome of 3600 nucleotide pairs. The small amount of slower migrating species (fractions 26–34) contains all of the *Hae*III fragments identified in Fig. 1. These molecules, therefore, do not contain additional DNA sequences but are probably partial duplications of the 3600 nucleotide genome.

The two major classes of molecule, which are present in approximately equimolar amounts in total digests, arise from the two strands of a partially double-stranded DNA molecule, as shown in Fig. 5. The following experiment demonstrates this.

Dane particle DNA was extracted from the same Dane particle preparation used in Table 1. The DNA was replicated without prior denaturation using AMV DNA polymerase and [α-^{32}P]dATP as the radioactive precursor. Since AMV DNA polymerase contains no exonuclease activity and cannot act on double-stranded DNA at nicks (11), there is no possibility of introducing radioactivity into double-stranded regions of the DNA. Elongation of 3'-OH ends using a single-stranded DNA template is possible, however. As can be seen in Table 2, the amount of incorporation occurring under these conditions indicates the presence of substantial single-stranded regions in the undenatured DNA.

Portions of the DNA synthesized during the course of the reaction were applied before and after denaturation to a 2% polyacrylamide–0.5% agarose gel to determine how the size of the intact product and the labeled single strands (Fig. 4A and B) changed during the course of the reaction. A third portion was digested with endo R-*Hae*III and the fragments

Table 2. *In vitro* replication of native Dane particle DNA

Minutes incubation	Total pmol of dAMP incorporated
10	0.58
20	1.04
40	2.08
80	2.86
140	3.05

DNA extracted from 50 μl of a Dane particle preparation was replicated without prior denaturation in a reaction mixture containing 20 mM Tris·HCl (pH 7.4), 0.35 μmol of $MgCl_2$, 0.5 nmol each dCTP, dGTP, dTTP, and 0.38 nmol of [α-^{32}P]dATP (46 Ci/mmol), 0.25 μmol of dithiothreitol, and 1 μg AMV polymerase in a volume of 50 μl. Aliquots (10 μl) were removed at the indicated times and added to 50 μl of 10 mM EDTA, pH 7.0, to stop the reaction. Samples (5 μl) of the diluted aliquots were assayed for incorporation of radioactive precursor into acid-insoluble material.

were separated on a 5% polyacrylamide gel to determine what regions of the genome acted as template (Fig. 4C).

The radioactive product at the end of the reaction with the AMV polymerase sedimented around 15 S in neutral sucrose (data not shown). However, the native DNA migrating through the gel (Fig. 4A) separated into two main components, typical of the behavior of linear and circular double-stranded DNA of the same molecular weight (10). The faster form migrated like double-stranded linear DNA 3600 nucleotides in length. The slower migrating species, accounting for about 70% of the radioactivity (the autoradiogram has been over-exposed to show the distribution of radioactivity at early times), is probably a circular form 3600 nucleotides in length. Both forms of DNA migrated more rapidly at early times during the reaction, indicating that substantial single-stranded regions were being repaired.

Fig. 4B shows the distribution of radioactivity in the single strands of DNA after various times of reaction with the AMV polymerase. The radioactive product at the end of the reaction with the AMV polymerase was about 3600 nucleotides in length. At early times, however, radioactivity appeared only in molecules less than 3600 nucleotides in length, indicating that elongation of the shorter strands occurred during the course of the reaction.

Fig. 4C shows the distribution of radioactivity among the *Hae*III fragments. It is clear that some regions of the genome were much more heavily labeled than others, with fragments D, E, F, H, J, and L containing the most radioactivity. These fragments, along with B and G, are precisely those which are deleted from the shorter molecules in Fig. 3. Those present in the greatest proportion in the shorter molecules, A, C, I, and M, contained little radioactivity after replication with the AMV polymerase and thus were not found in single-stranded regions in the undenatured DNA.

Thus, the short DNA strand in the Dane particle DNA can be elongated on a single-stranded template which contains regions of the genome found primarily in the 3600 nucleotide strand. The 3600 nucleotide strand as well as the shorter strand are linear, yet the product is primarily a double-stranded circular DNA. These findings can be adequately explained by the proposed structure illustrated in Fig. 5.

This structure suggests a mechanism for the endogenous DNA polymerase reaction described by Kaplan *et al.* (2), that is, elongation of the short strand and consequent repair of the single-stranded gap in the Dane particle DNA. Incorporation of radioactivity in the endogenous DNA polymerase reaction would therefore result in labeling of only the

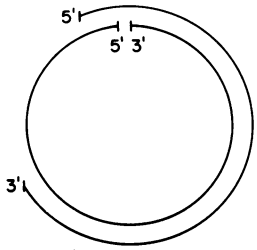

FIG. 5. Proposed structure of the DNA extracted from Dane particles.

single-stranded regions of the DNA, i.e., those regions that were labeled in the reaction with the AMV polymerase. That this is the case is shown in Fig. 6A. The distribution of radioactivity among the *Hae*III fragments of DNA labeled in an endogenous reaction after 30 min (50% maximum incorporation) and 3 hr is virtually identical to that observed during the course of the reaction with the AMV polymerase. Also, as shown in Fig. 6B, the product at the end of the reaction is primarily a slower migrating, probably circular form, with a minor linear component of 3600 nucleotide pairs.

DISCUSSION

The DNA extracted from Dane particles is a single molecular species with a complexity of about 3600 nucleotides. This is not very different from the genome complexity of small DNA viruses, i.e., parvoviruses and papovaviruses, although it is distinguishably smaller. Our evidence supports the findings of Robinson *et al.* (3) and of Overby *et al.* (12) that the majority of the DNA extracted from Dane particles is circular. Although, to our knowledge, no one has previously reported the existence of circular DNA with single-stranded gaps in Dane particles, many of the gaps are less than 20% of the genome length. Single-stranded regions may have contributed to the wide range of molecular lengths measured by Robinson *et al.*, since length measurements on single-stranded DNA may depend strongly on ionic conditions. Gapped circular DNA occurs naturally in eukaryotes through an asynchrony in the replication of the different strands of circular DNA, as in mitochondrial DNA replication (13). The relevance of the gapped circular DNA of Dane particles to its mode of replication is not known, however.

Several questions remain concerning the proposed structure of the DNA and the distribution of *Hae*III sites between the short strand and the 3600 nucleotide strand. (*i*) Since the short strand is postulated to cover a discontinuity in the 3600 nucleotide strand, it seems probable that the discontinuity lies opposite the *Hae*III-A fragment. This would result in the loss of the *Hae*III-A fragment from the 3600 nucleotide strand and the appearance of two new fragments, possibly *Hae*III-B and a smaller fragment. This model will have to be tested by establishing the physical order of the fragments in both strands. (*ii*) A higher molecular weight strand which contains both *Hae*III-A and -B is evident in Fig. 3. We do not have a satisfactory explanation for the occurrence of this strand. (*iii*) A minority of the DNA labeled in the endogenous reaction and in the reaction with the AMV polymerase appears to be linear. Such molecules could result from denaturation of the segment of double stranded DNA between the 5′ end of the short strand and the 5′ end of the 3600 nucleotide strand.

The endogenous DNA polymerase reaction apparently carries out the same synthetic reaction that the AMV polymerase carries out on intact Dane particle DNA. This suggests that the structure of the extracted DNA is the same as that in the Dane core and is not an artifact of the isolation. The endogenous reaction appears to repair the single-stranded gap in the double-stranded circular DNA. The biological significance, if any, of this reaction is not known. Only clinical specimens of these particles are available and it is not known how infectious these specimens are. It is possible that the vast majority of particles found in the serum of humans are defective virions for one reason or another. However, the inclusion of a primed DNA polymerase activity in Dane particles is indicative of a role in repair of the gapped molecules.

Although it is possible that the Dane particle DNA may code for the virion DNA polymerase, the specificity observed in the endogenous reaction appears to reside entirely in the template DNA and not in the DNA polymerase.

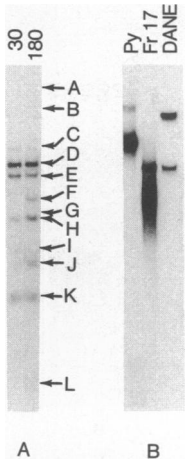

FIG. 6. (A) Distribution of radioactivity among the *Hae*III fragments of Dane particle DNA labeled by the endogenous DNA polymerase reaction. Twenty microliters of the Dane particle preparation was incubated with NP-40 (0.4%) and 30 mM dithiothreitol for 30 min at room temperature. The following additions were made in a final volume of 200 μl: 10 μmol of Tris·HCl (pH 8.1), 4 μmol of $MgCl_2$, 20 nmol of EDTA, 10 μmol of KCl, 30 nmol each dATP, dCTP, and dGTP, 4 nmol of [α-^{32}P]dTTP (8 Ci/mmol). After 30 min at 37°, 0.35 μmol of unlabeled dTTP was added to one half (100 μl) of the reaction and the incubation continued for an additional 2.5 hr. The reactions were stopped by adjusting to 10 mM with EDTA and 0.5% with Sarkosyl. Both reactions were digested with 500 μg/ml of Pronase for 2 hr at 37°. The DNA was sedimented on a neutral sucrose gradient (not shown) as in Fig. 1 and the sharp 15S peak was pooled. A portion was digested with endo R·*Hae*III, applied to a 5% polyacrylamide slab gel, and subjected to electrophoresis at 100 V for 3 hr. The radioactivity in the gel was detected by autoradiography. (B) The product from a 3-hr endogenous reaction in (A) was applied to a 2% polyacrylamide–0.5% agarose slab gel. Polyoma linear and circular [^{32}P]DNA and Dane particle [^{32}P]DNA replicated *in vitro* (fraction 17, Fig. 1) were applied to the same gel as molecular weight markers. The gel was developed by autoradiography.

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