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Structure of Hepatitis B Dane Particle DNA Before and After the Dane Particle DNA Polymerase Reaction

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DNA isolated from the hepatitis B antigen form known as the Dane particle was examined by electron microscopy before and after the endogenous Dane particle DNA polymerase reaction. The most frequently occurring form was an untwisted circular double-stranded DNA molecule approximately 1 μm in length. Less frequently occurring forms included circular DNA of approximately unit length and having one or more small single-stranded regions, similar circular molecules with one or more tails either shorter or longer than 1 μm in length, and very small circular molecules with tails. There was no increase in frequency or length of tails after a DNA polymerase reaction, suggesting that tails were not formed during this reaction. The mean length of circular molecules increased by 23% when DNA was spread in formamide compared with aqueous spreading, suggesting that single-stranded regions are present in most of the molecules. The mean length of circular molecules obtained from aqueous spreading increased by 27% after a Dane particle DNA polymerase reaction. This indicates that single-stranded regions were converted to double-stranded DNA during the reaction.

The Dane particle (3), one of the particulate forms of hepatitis B surface antigen (HB_sAg) in the blood of patients infected with hepatitis B virus, has been shown to contain a small circular DNA molecule (9) and DNA polymerase activity (5, 10). The Dane particle is the only viral antigen form known to contain nucleic acid, its ultrastructure is consistent with that of a virus, the antigen on its surface (HB_sAg) is thought to react with virus-neutralizing antibody (6, 7), and it has a unique internal core antigen (HB_cAg) (1). Although these features suggest that the Dane particle may be the complete form of hepatitis B virus, there is no direct evidence showing that it is infectious.

The structure of the DNA from Dane particles and the mechanism of the DNA polymerase reaction are not completely understood. The results of Robinson et al. (9) suggested that the open appearance of the circular molecules after aqueous spreading was due to either a nicked circular or low-superhelix density, closed circular DNA conformation, and the coincident sedimentation of new DNA made radioactive in a Dane particle DNA polymerase reaction with the circular molecules suggested that the circular DNA was the template for the enzyme. Overby et al. (8) reported finding linear tails attached to circular molecules in Dane particle DNA preparations and interpreted these to be "replicative forms" synthesized during the DNA polymerase reaction. The tail lengths and number of molecules with tails were stated to increase after the reaction, although the changes were not quantitated. It was concluded that the circular DNA was being replicated by the "rolling-circle" mechanism during the DNA polymerase reaction. A different conclusion about the Dane particle DNA polymerase reaction was reached by Summers et al. (12) after they examined the DNA fragments produced by the restriction endonuclease *Hae* III after Dane particle DNA had served as a template for DNA synthesis. It was concluded that the circular DNA contained a single-stranded region that was made double stranded during the Dane particle DNA polymerase reaction.

To further understand the structure of Dane particle DNA and the mechanism of the DNA polymerase reaction, we have studied the frequency of different molecular forms of DNA from Dane particles and the length distribution of circular molecules before and after the DNA polymerase reaction.

MATERIALS AND METHODS

Preparation of Dane particles. Dane particle-rich plasma was obtained from donor no. 390, and a P_1 fraction containing Dane particles was prepared by centrifugation for 4 h at 4°C in a Spinco 21 rotor at

21,000 rpm as previously described (11). The resulting pellet was suspended in TNE-ME-BSA (0.01 M Tris-hydrochloride [pH 7.5], 0.15 NaCl, 0.001 M EDTA, 1% mercaptoethanol, and 1 mg of bovine serum albumin per ml) to yield P_1.

DNA polymerase reaction and preparation of DNA. P_1 preparations were incubated either in the DNA polymerase reaction mixture as previously described (5) or in a reaction mixture without deoxyribonucleoside triphosphates (control). After 4 h of incubation at 37°C, the reaction mixture was layered over a discontinuous 10, 20, and 30% sucrose gradient containing TNE and centrifuged at 50,000 rpm in a Spinco SW65 rotor at 4°C. The pellet containing Dane particle cores was dissolved in 100 μl of TE-SDS (0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA, and 1% sodium dodecyl sulfate), Pronase was added to give a final concentration of 1 mg/ml, the solution was incubated for 1 h at 37°C, and the DNA was extracted twice with phenol equilibrated with TNE-ME. The aqueous phase was layered over a 5 to 20% sucrose gradient containing TNE, and the DNA was pelleted to the bottom of a polyallomer tube in a Spinco SW65 rotor at 50,000 rpm for 18 h at 4°C.

DNA electron microscopy. DNA was spread by the aqueous (9) or formamide techniques (J. Ferguson and R.W. Davis, *in* J. K. Koehler, ed., *Advanced Techniques in Biological Electron Microscopy*, vol. 2, in press). The spreading solution contained 40% formamide, and the hypophase contained 10% formamide.

Length measurements of DNA molecules. Molecules were traced with a Science Accessories Corp. Graf/Pen attached to a Tektronix 31 programable calculator. Phage PM-2 form II DNA molecules were used as an internal standard for all length measurements, and the lengths of other molecules on an electron microscope grid were calculated based on their length relative to PM-2 DNA, which was considered to have a length of 3.33 μm.

RESULTS

Dane particle DNA forms and their frequency before and after a DNA polymerase reaction. Figure 1 shows several molecular forms found in significant numbers in Dane particle DNA preparations. Previous experiments (9) have shown that no molecules were observed on grids after treatment of similar preparations with pancreatic DNase I, indicating that all of the molecules were DNA. Table 1 shows the frequency with which these forms were observed in preparations before and after a Dane particle DNA polymerase reaction. The most abundant molecular form, both before and after a reaction, was the untwisted circle measuring about 1 μm in length and having the appearance of double-stranded DNA (Fig. 1A). A much smaller number of circular molecules of the same length had linear tails shorter than 1 μm attached to the circular DNA (Fig. 1B–E). There appeared to be a lower frequency of these molecules after a DNA polymerase reaction than in DNA preparations from Dane particles that had not been incubated in complete DNA polymerase reaction mixture. Even fewer circular molecules with linear tails longer than 1 μm were found, and the frequency did not change significantly after the reaction. Even rarer molecules appeared to have two or more tails originating from the same or different sites on the circular DNA (Fig. 1J). A few molecules appeared to have single-stranded regions or gaps within the circle (Fig. 1F–H). The number of molecules having single-stranded regions decreased after the reaction. Since the length of these gap regions is very short, the inherent error in assigning the end of a duplex region and the start of a single-stranded region represents a significant proportion of the gap size. It is, therefore, not possible to accurately measure the length of these regions by the formamide-mounting technique alone. The other DNA form seen in significant numbers in all Dane particle DNA preparations was the very small circle having linear tails of variable length (Fig. 1I). The frequency of this form was not different before and after the DNA polymerase reaction.

Difference in electron microscopic length of circular DNA molecules when spread by aqueous and formamide techniques, and before and after the Dane particle DNA polymerase reaction. If single-stranded regions of sufficient length are present in the circular DNA from Dane particles, the electron microscopic length of the molecules when spread in formamide, which extends the single-stranded DNA, should be greater than the length after spreading in a nondenaturing buffer (aqueous spreading) in which single-stranded DNA is not extended. The results in Table 2 show that the mean length of circular molecules from Dane particles not subjected to a DNA polymerase reaction increased by 23% when spread in formamide compared with aqueous spreading, which is consistent with the presence of single-stranded regions in the DNA. The mean length difference for molecules spread by the two techniques was only 12% for a DNA preparation from Dane particles after a DNA polymerase reaction. The length of Dane particle DNA molecules in each experiment was calculated using the known length of PM-2 DNA molecules on the same grid as an internal length standard.

If the single-stranded regions of the circular Dane particle DNA molecules became double stranded during the Dane particle DNA polymerase reaction, the apparent length of the molecules on electron microscope grids after

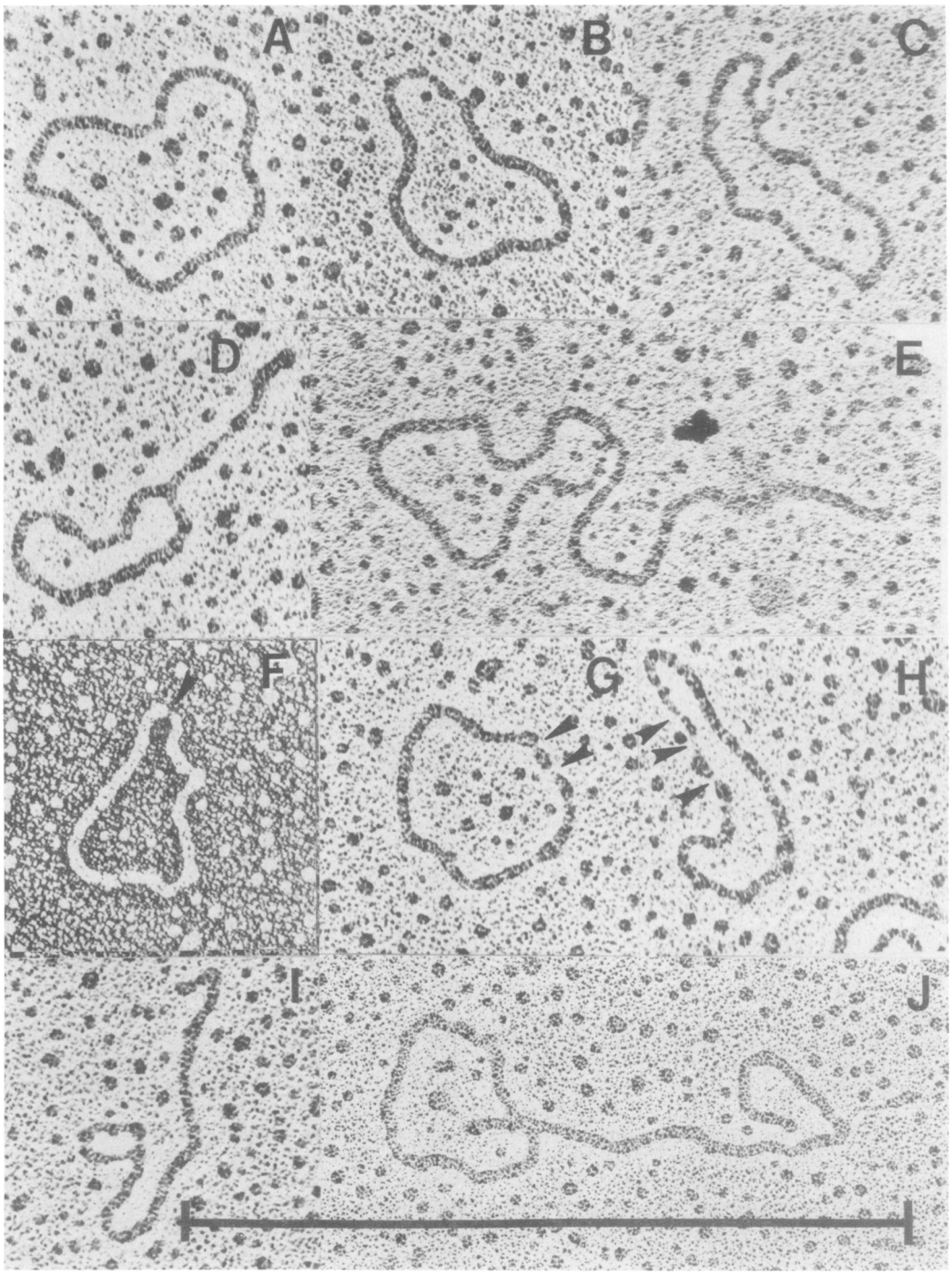

FIG. 1. *Electron micrographs of Dane particle DNA forms. DNA was extracted from Dane particles not subjected to DNA polymerase reaction conditions and spread by the formamide technique. (A) A 1-μm-length plain circle; (B–E) circles with one tail; (F) circles with one small gap, (G) two gaps, and (H) multiple gaps; (I) a small circle with a tail; and (J) a circle with two tails emanating from the same site on the circle contour are representative of the population scored by four independent investigators. The black arrows in F–H denote the position of gap regions. The solid bar at the bottom represents 1 μm on the electron microscope grid.*

TABLE 1. *Frequency of Dane particle DNA forms*

Molecular form	No DNA polymerase reaction (control)		After DNA polymerase reaction	
	No. of molecules (n)	Frequency (f)[a] (%)	No. of molecules (n)	Frequency (f)[a] (%)
Plain circle	510	81 ± 3	959	93 ± 1.6
Circle with tail <1 μm	44	7 ± 2	23	2.2 ± 1
Circle with tail >1 μm	7	1.1 ± 0.8	0	0
Circle with 1 gap	46	7.3 ± 2	12	1.2 ± 0.7
Circle with 2 or more gaps	5	<1	2	<0.5
Small circle with tail	6	<1	30	2.9 ± 1
Circle with 2 tails	12	1.9 ± 1	13	1.3 ± 0.7
Total molecules	633		1,029	

[a] f_{actual} (95% confidence limit) = $f_{observed} \times (1 \pm \alpha)$, where $\alpha = 1.96 \times \sqrt{(1-f_{observed})/nf_{observed}}$.

TABLE 2. *Mean length of circular DNA molecules from Dane particles before and after a DNA polymerase reaction*[a]

When measured	Length after aqueous spreading (μm)	Length after formamide spreading (μm)	Difference in length after aqueous and formamide spreading (%)
Before reaction	0.683 ± 0.135,[b] (n = 181)	0.882 ± 0.129, (n = 163)	23
After reaction	0.936 ± 0.111, (n = 169)	1.060 ± 0.081, (n = 218)	12
Difference in length before and after reaction (%)	27	17	

[a] Dane particle DNA polymerase reactions, DNA extractions, and length measurements were carried out as described in Materials and Methods. The lengths of 30 to 60 PM-2 DNA molecules on each grid were measured as length standards. Variations of PM-2 lengths between groups were such that they would minimize the observed differences found between the hepatitis B virus molecules.

[b] Mean ± standard error.

aqueous spreading should be greater for DNA after a reaction than before. The results in Table 2 show that after a DNA polymerase reaction the mean measured length of circular DNA molecules after aqueous spreading increased by 27%, suggesting that the DNA polymerase reaction converted single-stranded regions of the circular molecules to double-stranded DNA. The difference in mean length for DNA before and after a DNA polymerase reaction was 17% when the molecules were spread in formamide. This is due to the fact that single-stranded regions are shorter than the corresponding duplex DNA length in formamide spreadings (J. Ferguson and R. W. Davis, *in* J. K. Koehler, ed., *Advanced Techniques in Biological Electron Microscopy*, vol. 2, in press). A significant reduction in the mean length of single-stranded regions during the Dane particle DNA polymerase reaction probably accounts for the smaller difference in length of postreaction molecules between aqueous and formamide spreading (12%) than the difference for prereaction DNA molecules spread by the two techniques (23%). A Student's t test was applied to the data to test the significance of the differences. The P value was less than 0.005, with a degree of freedom of 2.0 or greater for the differences shown in Table 2.

The distribution of lengths for circular molecules examined under the four conditions described in Table 2 is shown in Fig. 2. The results suggest that not only does the apparent length of the circular molecules increase after a Dane particle DNA polymerase reaction, but the population of molecules becomes more homogeneous with respect to length.

DISCUSSION

Our findings suggest that the circular DNA molecules isolated from Dane particles contain one or more single-stranded regions that are extended to give a longer apparent length by electron microscopy when the DNA is spread in formamide compared with the length after aqueous spreading. The increase in mean length of the circular molecules spread by the aqueous technique after the Dane particle DNA polymerase reaction compared with DNA before the reaction is consistent with the conver-

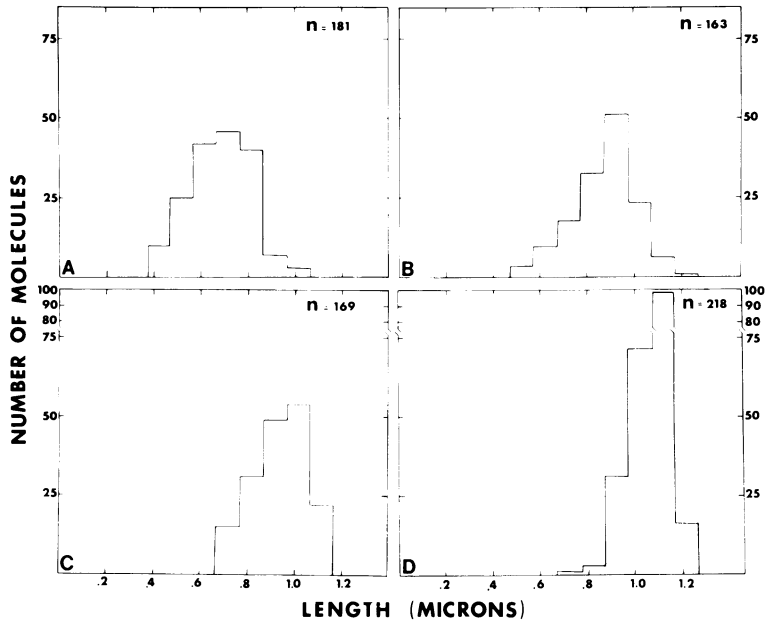

FIG. 2. *Histogram of Dane particle circular DNA lengths. DNA was extracted from Dane particles not subjected to a DNA polymerase reaction and spread by the aqueous (A) and formamide (B) techniques. Dane particles from the same preparation were incubated in a complete DNA polymerase reaction mixture for 4 h at 37°C, and the DNA was extracted and spread by the aqueous (C) and formamide (D) techniques. The method for the Dane particle DNA polymerase reaction, DNA extraction, DNA spreading and DNA length measurements are described in Materials and Methods.*

sion of single-stranded regions to double-stranded DNA during the reaction. These findings are in agreement with the conclusions of Summers et al. (12) on the structure of Dane particle DNA and the action of the endogenous DNA polymerase. Their evidence for a single-stranded region was the observation that avian myeloblastosis virus DNA polymerase, which contains no exonuclease activity and thus cannot introduce radioactive nucleotides into preexisting double-stranded DNA, successfully used the Dane particle DNA as a primer for DNA synthesis, presumably using single-stranded regions in the DNA as a template. Because the endogenous DNA polymerase activity in Dane particles was said to introduce radioactive nucleotides into the same restriction endonuclease *Hae* III-generated DNA fragments as did the avian myeloblastosis virus DNA polymerase, it was concluded that the endogenous Dane particle enzyme used the same single-stranded regions as template. The published data, however, show radioactivity in almost all *Hae* III fragments after a reaction with either polymerase, suggesting that both enzymes introduce radioactive nucleotides into all parts of the circular molecule and not into specific regions. The latter result is consistent with other mechanisms for the endogenous DNA polymerase reaction as well as the possibility that single-stranded gaps in the circular molecules are filled in during the reaction. Our finding of an increase in double-stranded DNA length after the reaction strongly supports the possibility that single-stranded gaps are filled during the reaction.

Although our results indicate that the circular molecules from Dane particles contain single-stranded regions, the number of such regions per molecule and the exact size of the single-stranded part of the molecule are not given by the experiments here. The finding of a few molecules with more than one visible single-stranded region (Fig. 1G and H; Table 1) suggests that at least some molecules exist in this form. Single-stranded regions less than about 150 nucleotides in length would not be visible by the methods used here, so that such small regions undoubtedly could exist in all of the molecules. The finding that almost all DNA fragments after *Hae* III digestion are radioactive after a Dane particle DNA polymerase reaction with radioactive nucleotides suggests that in Dane particle DNA preparations single-stranded regions exist in all parts of the circular molecules (12; T. Landers, H. Greenberg, and W. S. Robinson, unpublished data). The fact that the mean length of the circular mole-

cules increased by about one-fourth both during a DNA polymerase reaction and after spreading the DNA in formamide compared with aqueous spreading provides an approximation of the mean size of the single-stranded region. However, this is probably a minimum estimate, because the in vitro DNA polymerase reaction in the experiments here may not have closed the entire single-stranded gap, as suggested by the additional increase in mean length when postreaction DNA was spread in formamide compared with aqueous spreading. The more heterogeneous length distribution for the prereaction circular molecules spread by the aqueous technique compared with the length distribution after the reaction (Fig. 2) suggests that the single-stranded region may not be the same length in all molecules. The increase in mean circle length and the more homogeneous length distribution after the DNA polymerase reaction suggest that most of the DNA molecules, rather than a small subpopulation in the preparation, participate in the DNA polymerase reaction. From their data Summers et al. (12) estimated that 16 to 58% of the length of different molecules was single stranded. Lutwick and Robinson (7a) studied the reassociation of DNA made radioactive in a Dane particle DNA polymerase reaction and found that the complexity of the newly synthesized DNA corresponded to one-fourth to one-half of the amount of DNA in a molecule the size of the circular Dane particle DNA. Thus, the estimates of the length of the single-stranded DNA region by different methods show reasonable agreement.

The results here indicate that the genetic coding capacity of the circular DNA from Dane particles is greater than thought from previous studies of DNA length determined by electron microscopy, which analyzed only the double-stranded portion of the molecule and suggested a size around 1.6×10^6 daltons (9). The mean length of postreaction molecules spread in formamide in this study (1.060 μm) would correspond to 2.08×10^6 daltons or 3,150 nucleotide pairs for double-stranded DNA. This is probably a minimum estimate of the size for the reasons given above. In theory, a double-stranded DNA of this size could code for 126,000 daltons of protein. This size is smaller than the DNA of other DNA viruses and is not enough DNA to code for the number of protein bands shown to be present in HB_sAg preparations by gel electrophoresis (2, 4, 13). It is not known, however, how much protein is virus coded, and it has not been shown that the DNA described here represents the complete complement of viral DNA.

The significance of the unit length of the small circular molecules with tails is unclear, but they represent less than 10% of the molecules in any DNA preparation studied by us. Overby et al. (8) suggested that they were forms of replicating DNA formed during the endogenous Dane particle DNA polymerase reaction. We have found no increase in the frequency of circular molecules with tails or in length of tails in DNA extracted from Dane particles after a Dane polymerase reaction compared with unreacted DNA (Table 1). It is not known whether the small decrease in frequency of molecules with tails is significant. Thus, our findings do not support the concept that circular molecules with tails are formed during the Dane particle DNA polymerase reaction or that tail lengths increase during the reaction, as expected if DNA replication by the rolling-circle mechanism was occurring during the Dane particle enzyme reaction as suggested by Overby et al. (8). Our findings indicate that the reaction involves synthesis of the complementary strand at single-stranded regions within the circular molecules. The biological utility of single-stranded gaps in a viral DNA that are closed by a virion enzyme is not apparent. These properties have not been described for other viruses.

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