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DNA Synthesized in the Hepatitis B Dane Particle DNA Polymerase Reaction

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Radioactive DNA was prepared in extensive (4 h) Dane particle DNA polymerase reactions. In different experiments the amount of new DNA, determined by the amount of nucleotide incorporation into an acid-insoluble form, was between 29 and 45% of the total circular DNA isolated from Dane particle preparations after the reaction. DNA reassociation kinetics were used to determine the complexity of the newly synthesized DNA. In different experiments $C_0t_{1/2}$ values, corresponding to between 625 and 1,250 nucleotide pairs, were obtained for the radioactive Dane particle DNA. These results suggest that a unique region (or regions), corresponding to approximately one-fourth to one-half of the circular Dane particle DNA template, was copied one time during the reaction. DNA and RNA extracted from hepatitis B virus-infected liver but not from uninfected liver accelerated the rate of reassociation of radioactive DNA from Dane particles. These Dane particle DNA base sequences were found in alkali-stable, rapidly sedimenting DNA from infected liver as well as in DNA sedimenting at a rate similar to the DNA extracted from Dane particles. These findings are consistent with Dane particle DNA being hepatitis B virus DNA that is integrated into high-molecular-weight cellular DNA and transcribed into RNA in infected liver.

Previous work from this laboratory (10, 18, 19) has shown that the 42-nm Dane particle (5), one of the particulate forms of hepatitis B surface antigen (HB_sAg) in the blood of patients infected with hepatitis B virus (HBV), contains a small, circular double-stranded DNA and DNA polymerase activity that uses the circular DNA as a template. The DNA molecules were shown to have an average length of 0.78 μm and the appearance of open or nicked circular molecules (18). A double-stranded DNA of that length would consist of approximately 2,450 nucleotide pairs. Although there is no direct evidence that the Dane particle is infectious HBV, its ultrastructure is consistent with that of a virus (5) and it is the only HB_sAg form known to contain nucleic acid. The Dane particle contains two unique antigens, an internal core antigen (1) and HB_sAg which is thought to react with virus-neutralizing antibody (11, 12) and for which there are virus-specified subtypes (13, 14). It is not known whether Dane particle DNA is unique DNA as expected for viral DNA or heterogeneous DNA as might be expected if random host DNA were incorporated into Dane particles.

Since the endogenous DNA polymerase reaction uses the Dane particle DNA as a template (18), this reaction can be used to make radioactive DNA to investigate the complexity of the newly synthesized Dane particle DNA and to look for Dane particle DNA base sequences in the DNA and RNA of HBV-infected and uninfected liver.

MATERIALS AND METHODS

Materials. [^{3}H]dCTP (20 Ci/mmol), [^{3}H]dGTP (20 Ci/mmol), α-[^{32}P]dCTP (136 Ci/mmol), α-[^{32}P]dGTP (136 Ci/mmol), α-[^{32}P]dATP (136 Ci/mmol), and α-[^{32}P]TTP (136 Ci/mmol) were purchased from New England Nuclear Corp. Nonidet P-40 was a gift of the Shell Oil Co. Simian virus 40 (SV40) ^{3}H-labeled DNA was a gift of Paul Berg. ϕX174 ^{3}H-labeled DNA replicative form (rf) was a gift of John Scott. Salmon sperm DNA and calf thymus DNA were purchased from the Sigma Chemical Co. PM-2 virus was grown and purified as described by Espejo et al. (7), and some cultures were incubated with $^{32}PO_4$ to prepare PM-2 ^{32}P-labeled DNA. Optical grade cesium chloride was purchased from Kowecki Berylco Industries.

Liver tissue. Liver tissue of four patients with chronic HBV infection and chronic active hepatitis (no. 354, 353, 356, 357) and hepatoma tissue from one chronic HB_sAg carrier (no. 353) were obtained from Myron Tong, John Wesley Hospital, Los Angeles, Calif. Hepatoma tissue from HB_sAg-negative patients (FT-74-879, FT-75-386) was obtained from the Office of Program Resources and Logistics, National Cancer Institute. Liver tissue obtained from 10 un-

infected autopsy patients (no. 10, 86, 89 to 96) was provided by the Department of Pathology, Stanford University School of Medicine.

DNA and RNA extraction from tissue. Liver tissue was homogenized in a Waring blender in 5 volumes of 0.05 M Tris-hydrochloride, pH 7.5, 0.15 M NaCl, and 0.005 M EDTA. The homogenate was made 1% with sodium dodecyl sulfate and 1 mg of Pronase per ml and incubated overnight at 37°C. After three phenol extractions, 2 volumes of ethanol were added to precipitate the DNA and RNA. The precipitate was redissolved in buffer containing 0.001 M Tris-hydrochloride, pH 7.5, and 0.01 M EDTA, and the volume was adjusted to give an absorbance at 260 nm (A_{260}) of around 40. Solid CsCl (25 g) was added to 20 ml of the nucleic acid solution for centrifugation in a Spinco 42 Ti rotor at 35,000 rpm for 36 h at 20°C. The band of DNA in the middle of the tube and the pellet of RNA on the bottom were recovered separately and dialyzed against 0.01 M Tris-hydrochloride, pH 7.5, and 0.01 M EDTA.

Dane particle preparations. Plasma from chronic HB_sAg human carriers with high concentrations of Dane particles in their blood was obtained by plasmaphoresis as previously described (17). Partially purified, concentrated Dane particle preparations were made from plasma by differential centrifugation and sedimentation in discontinuous sucrose density gradients as previously described (17).

Synthesis and isolation of radioactive Dane particle DNA. DNA polymerase reactions were carried out with concentrated preparations of Dane particles and ^3H- or ^{32}P-labeled deoxynucleoside triphosphates (dNTP's) for 4 h at 37°C as previously described (17). In some experiments the radioactive DNA was isolated by a modification of the method previously described (18). The Dane particle cores that contain the ^3H-labeled DNA in the reaction mixture were sedimented through a 30% sucrose solution. The pellet containing the cores was resuspended in 50 μl of TNE (0.01 M Tris-hydrochloride, pH 7.5, 0.15 M NaCl, and 0.001 M EDTA) with 1 mg of Pronase per ml and 2% sodium lauryl sarcosinate and incubated at 37°C for 1 h. The DNA was then sedimented in a preformed CsCl density gradient. The gradient fractions containing radioactive DNA were pooled and dialyzed against 0.01 M Tris-hydrochloride, pH 7.5, and 0.01 M EDTA before use. In other experiments the initial reaction mixture was made up to 0.01 M EDTA, 1% sodium dodecyl sulfate, and 1 mg of Pronase per ml and incubated at 37°C for 1 h. Radioactive DNA was then isolated by two phenol extractions and ethanol precipitation, and the DNA was dialyzed as above.

Specific radioactivity of Dane particle DNA. When Dane particle DNA concentrations were too low to be measured by A_{260}, PM-2 DNA was added to give a known final concentration, and the relative number of the two sizes of molecules (0.78 μm for Dane particle and 2.88 μm for PM-2) was determined by electron microscopy (18) (200 molecules counted). The specific activities of all DNA preparations from DNA polymerase reaction mixtures containing two ^3H-labeled dNTP's were between 2×10^6 and 10×10^6 cpm/μg.

S_1 nuclease. S_1 nuclease was purified from a crude extract of *Aspergillus oryzae* (purchased from Enzyme Development Corp.) by the method of Vogt (27). Peak fractions of enzyme activity from a Sephadex column were used.

Measuring DNA reassociation. In most experiments radioactive Dane particle DNA with unlabeled DNAs were denatured in 0.005 M Tris-hydrochloride, pH 7.5, and 0.005 M EDTA by heating to 100°C for 10 min and then rapidly cooled in an ice-water bath. NaCl and Tris-hydrochloride, pH 7.5, were then added to give final concentrations of 0.4 and 0.1 M, respectively. In other experiments the DNA was denatured and fragmented (24) by incubating for 10 min at 100°C in 0.3 M NaOH, cooling, and neutralizing with HCl. NaCl and Tris-hydrochloride, pH 7.5, were immediately added to give final concentrations of 0.4 and 0.1 M, respectively. To observe reassociation, the denatured DNA was incubated at 68°C, and four portions were removed at zero time and at different times of incubation. Each portion was added to 10 volumes of S_1 buffer (0.03 M sodium acetate, pH 4.6, 0.05 NaCl, 0.001 M $ZnCl_2$, and 5% glycerol) and stored at −60°C. All portions were later thawed and incubated at 37°C for 1 h with S_1 nuclease, and the acid-precipitable radioactivity was determined as previously described (18). An amount of S_1 nuclease was used that would convert more then 96% of heated chicken cell ^3H-labeled DNA at the same concentration used in the reassociation experiments to an acid-soluble form when incubated under the same experimental conditions.

RESULTS

Amount of DNA synthesis in Dane particle preparations. During a DNA polymerase reaction with Dane particle preparations, the rate of nucleotide incorporation into DNA is nearly linear for 3 or 4 h, after which it rapidly decreases (10). For the experiments described here, 4 h was chosen as a reaction time since at this time the reaction is more than 80% complete. In three experiments with different Dane particle preparations the amount of synthesis during 4-h reactions was calculated from the amount of radioactive nucleotide incorporated. The newly synthesized DNA was 29, 36, and 45% of the total circular DNA isolated from the Dane particle preparations after the reactions.

Reassociation of Dane particle DNA. Previous experiments have shown that none of the DNA synthesized in a Dane particle DNA polymerase reaction is rendered acid soluble by S_1 nuclease before heating. After denaturation by heating, the DNA product is completely susceptible to S_1 nuclease (18). This suggests that the new DNA is in a highly ordered or base-paired form. Incubation of heated Dane particle ^3H-labeled DNA at 68°C resulted in complete reassociation to an S_1 nuclease-resistant form (Fig.

1). The rate and extent of reassociation was the same for DNA denatured by heating in neutral or in alkaline solution as described above. Experiments with Dane particle DNA at different concentrations showed that the half-time for reassociation ($T_{1/2}$) was inversely proportional to the DNA concentration (Fig. 2), as previously shown for reassociation of other DNAs (2). The reassociation rate for Dane particle ^{3}H-labeled DNA was the same whether reassociation was carried out in the presence of carrier DNA from *Escherichia coli*, salmon sperm, or calf thymus.

To determine the amount of unique base sequence in the DNA synthesized in a Dane particle DNA polymerase reaction, the rate of reassociation of the radioactive DNA reaction product was compared with the rates for uniformly labeled viral DNAs of known size. Figure 3 shows the time course plotted by the method of Britten and Kohne (2) for the reassociation of Dane particle ^{3}H-labeled DNA made radioactive in an extensive DNA polymerase reaction (4 h) and for ϕX174 rf ^{3}H-DNA, PM-2 ^{32}P-DNA, and SV40 ^{3}H-DNA uniformly labeled during replication of the viruses in culture. The known size of PM-2 DNA (7), SV40 DNA (4), and ϕX174 rf DNA (21) and the experimentally determined C_0t required for half reassociation ($C_0t_{1/2}$) of the Dane particle ^{3}H-labeled DNA were used to calculate the amount of unique base sequence (i.e., the DNA size for viral DNAs without repeated base sequences) as shown by Britten and Kohne (2). The $C_0t_{1/2}$ values for four different Dane particle ^{3}H-labeled DNA preparations ranged from 1×10^{-4} to 3.2×10^{-4} mol $\times$ s/liter, corresponding to unique sequences of 625 and 1,250 nucleotide pairs, respectively. This represents between 25 and 50% of the 2,450 nucleotide pairs estimated to be present in a molecule the size of the double-stranded circular DNA previously shown to be the template for the Dane particle DNA polymerase reaction (18).

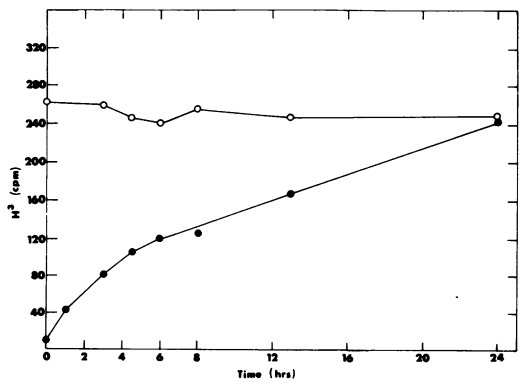

FIG. 1. *Reassociation of Dane particle ^{3}H-labeled DNA. ^{3}H-labeled DNA (15,000 cpm) and calf thymus DNA (850 μg/ml) in 1.2 ml were denatured by heating to 100°C and incubated at 68°C, 20-μl aliquots were removed, and the acid-precipitable radioactivity was determined without (○) or after (●) S_1 nuclease treatment as described in the text. Each point represents the average of two aliquots.*

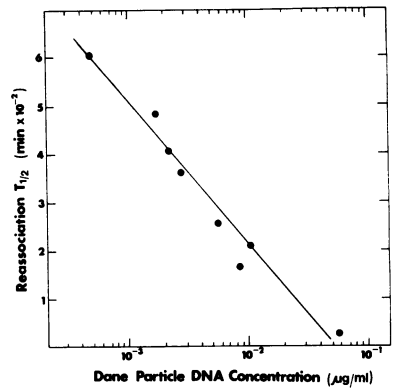

FIG. 2. *DNA concentration dependence of the $T_{1/2}$ for reassociation of Dane particle ^{3}H-labeled DNA. The $T_{1/2}$ for reassociation of Dane particle ^{3}H-labeled DNA at different known concentrations was determined in reassociation experiments as described in the legend to Fig. 1 and the text.*

Presence of Dane particle DNA base sequences in HBV-infected liver DNA. To test DNA extracted from liver tissue of HBV-infected patients and uninfected patients for the presence of Dane particle DNA base sequences, the effects of liver DNA in high concentrations (5 mg/ml) on the rate of reassociation of Dane particle ^{3}H-labeled DNA was determined. Figure 4 shows that liver DNA from an infected patient greatly accelerated the rate of reassociation of Dane particle DNA made radioactive in a DNA polymerase reaction. The DNA from uninfected liver did not change the rate compared with that in the presence of salmon sperm DNA. Figure 5 shows that the reassociation $T_{1/2}$ of Dane particle ^{3}H-labeled DNA in the presence of DNA from the liver tissue of each of six HBV-infected patients was shortened by a factor of 2 or more as compared with that in the presence of salmon sperm DNA, indicating that DNA from all of the infected livers contained Dane particle DNA base sequences. The lowest of these points represents the results with DNA extracted from hepatoma tissue with some surrounding liver obtained from a patient chronically infected with HBV. The others are with DNA from liver tissue of patients with chronic active hepatitis and chronic HBV infection. No change in reassociation $T_{1/2}$ was detected with DNA from the liver tissue of nine uninfected

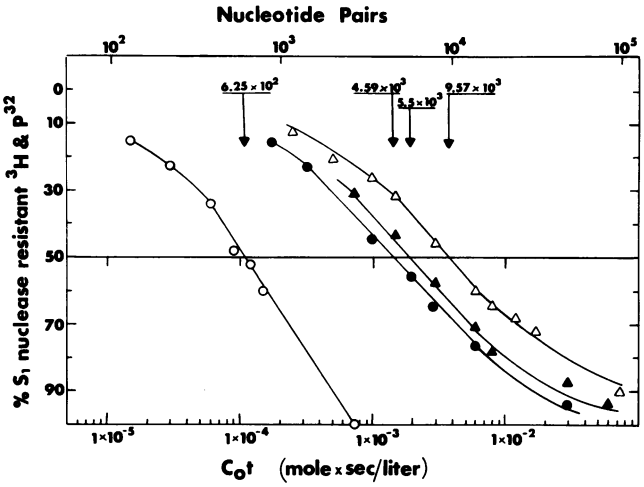

FIG. 3. *Reassociation of Dane particle ^3H-labeled DNA (○), SV40 ^3H-labeled DNA (●), φX174 rf ^3H-labeled DNA (▲), and PM-2 ^{32}P-labeled DNA (△) in the presence of 1 mg of calf thymus DNA per ml. DNA reassociation was carried out as described in the legend to Fig. 1 and the text. Each point represents the average of two determinations.*

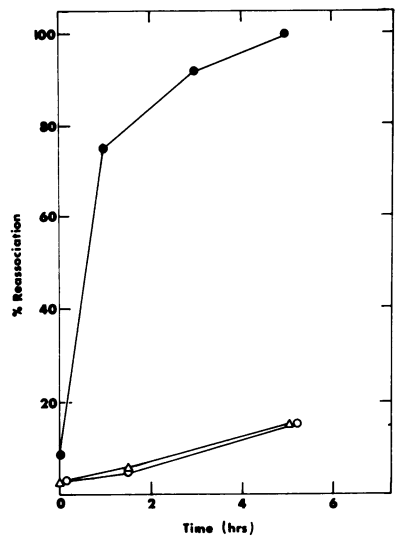

FIG. 4. *Reassociation of Dane particle ^3H-labeled DNA in the presence of 5 mg of DNA per ml from HBV-infected liver 357 (●), uninfected liver 10 (○), and salmon sperm (△). The DNAs were denatured at 100°C in 0.3 M NaOH, and reassociation was carried out as described in the text. Each point represents the average of two aliquots.*

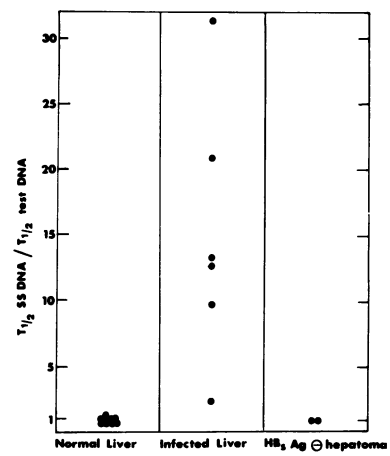

FIG. 5. *Effect of DNA from uninfected liver, HBV-infected liver and hepatoma tissue negative for HB_sAg on reassociation of Dane particle ^3H-labeled DNA. Each point represents the $T_{1/2}$ for reassociation of ^3H-labeled DNA in the presence of 5 mg of salmon sperm DNA per ml divided by the $T_{1/2}$ for reassociation in the presence of the test DNAs at 5 mg/ml. Reassociation experiments were carried out as described in the legend to Fig. 4 and the text.*

patients as compared with the $T_{1/2}$ in the presence of salmon sperm DNA. With the radioactive DNA probe used, the presence of as little as 0.1 copy of the DNA synthesized in the Dane particle DNA polymerase reaction per liver cell DNA equivalent was excluded in the uninfected tissue. Since the reassociation $T_{1/2}$ for a given DNA is directly proportional to the DNA concentration (2), the concentration in the liver DNA preparations of the DNA sequences synthesized in a Dane particle DNA polymerase reaction can be calculated (8) from the $T_{1/2}$ of Dane particle ^3H-labeled DNA in the presence of the liver DNA. Figure 2 shows the reassociation $T_{1/2}$ for ^3H-labeled DNA made radioactive in a Dane particle DNA polymerase reaction determined at different Dane particle concentrations. The data in Fig. 2 and the reassociation $T_{1/2}$ values for Dane particle ^3H-labeled

DNA in the presence of the DNAs from HBV-infected liver were used to calculate the concentration of Dane particle DNA base sequences in the liver DNA preparations. From these values, the average number of copies of the DNA synthesized in the Dane particle DNA polymerase reaction (about one-third of the circular 1.6 $\times$ 10^6-dalton Dane particle DNA molecule) per liver cell DNA equivalent was calculated (Table 1).

Size of liver DNA containing Dane particle DNA base sequences. To determine the size of the DNA molecules containing Dane particle DNA base sequences in HBV-infected liver, infected liver DNA was denatured with alkali and fractionated by sedimentation in an alkaline sucrose density gradient, and the effect of DNA from different regions of the gradient on reassociation of Dane particle ^{3}H-labeled DNA was determined. Figure 6 shows the sedimentation of 10 mg of DNA from infected liver with ϕX174 ^{3}H-labeled DNA as a sedimentation marker in an alkaline sucrose density gradient. Under these conditions, ϕX174 DNA has a sedimentation coefficient of 18*S* (15, 16) and ^{3}H-labeled DNA from Dane particles sediments at 11*S* (W. S. Robinson and L. I. Lutwick, in press). Although most of the liver DNA detected by A_{260} sedimented at 34*S* or less (gradient regions B, C, and D), a significant amount of DNA was recovered by alcohol precipitation from region A (greater than 34*S*). Linear DNA sedimenting at 34*S* under these conditions has a molecular weight of 13 $\times$ 10^6 (22). We have had difficulty isolating DNA with a higher average molecular size from postmortem liver, and this may be because of postmortem autolysis. Figure 7a shows that DNA from all four gradient regions (A, B, C, and D) significantly accelerated Dane particle ^{3}H-la-

TABLE 1. *Amount of the Dane particle ^{3}H-labeled DNA sequences in HBV-infected liver*

HBV-infected liver DNA prepn	Dane particle ^{3}H-labeled DNA sequence copies/cell[a]
353	12
354	55
355	67
356	58
357	70
358	57

[a] (Dane particle ^{3}H-labeled DNA sequences [micrograms per milliliter] $\div$ 4.16 $\times$ 10^5 [molecular weight of Dane particle ^{3}H-labeled DNA])/(total liver DNA [micrograms per milliliter] $\div$ 3.92 $\times$ 10^{12} ["molecular weight" of diploid mammalian cell DNA]) (25).

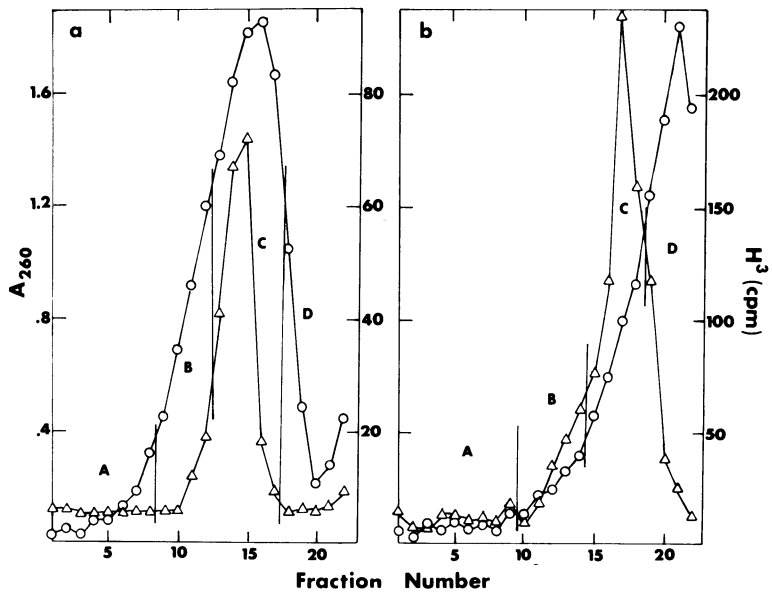

FIG. 6. *Fractionation of liver DNA by alkaline sucrose density gradient sedimentation. A total of 10 mg of DNA from HBV-infected liver 355 (a) and uninfected liver 10 plus 300 μg of Dane particle DNA (b) in 1.5 ml of 0.9 M NaCl, 0.3 M NaOH, and 0.01 M EDTA with ϕX174 ^{3}H-labeled DNA were layered over 5 to 20% sucrose density gradients containing 0.9 M NaCl, 0.1 M NaOH, and 0.005 M EDTA. Centrifugation was at 27,000 rpm and 25°C for 9.5 h in a Spinco SW27 rotor. Fractions collected from the bottom of each tube were assayed for acid-precipitable ^{3}H (△) and A_{260} (○). The fractions from each part of the gradients designated A, B, C, and D were pooled and neutralized with HCl, and the DNA was recovered by ethanol precipitation.*

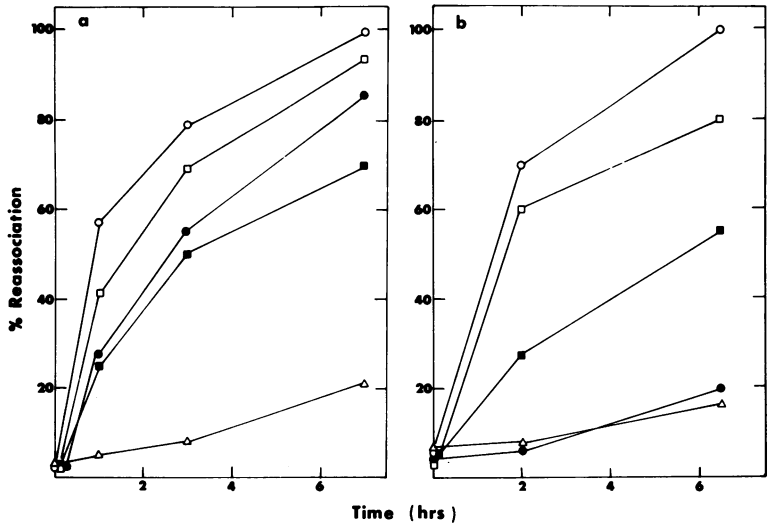

FIG. 7. *Reassociation of Dane particle ^{32}P-labeled DNA in the presence of DNA from the fractions shown in Fig. 6a (a) and 6b (b). DNA from the fractions in Fig. 6 (A, ●; B, ■; C, □; and D, ○) and salmon sperm DNA (△), each at 1 mg/ml, plus Dane particle ^{32}P-labeled DNA were denatured by heating at 100°C, and DNA reassociation was carried out as described in the legend to Fig. 1 and the text. Each point represents the average of duplicate aliquots.*

beled DNA reassociation, suggesting that Dane particle DNA sequences were present in liver DNA with molecular sizes greater than 13×10^6 as well as in smaller molecules.

As a control, 10 mg of DNA from an uninfected liver mixed with 300 μg of DNA extracted from Dane particles and ϕX174 ^{3}H-labeled DNA were sedimented in an alkaline sucrose gradient (Fig. 6b) to establish the distribution of monomeric Dane particle DNA in such a gradient. The reassociation experiment shown in Fig. 7b shows that no Dane particle DNA base sequences were detected in the DNA sedimenting faster than 34*S* (region A, Fig. 6b). The reassociation $T_{1/2}$ values in Fig. 7 suggest that the 300 μg of added Dane particle DNA (Fig. 6b) is comparable to the amount of Dane particle DNA found in the infected liver DNA used in the experiment shown in Fig. 6a. Thus, concentrations of monomeric Dane particle DNA detectable by ^{3}H-labeled DNA reassociation do not sediment faster than 34*S*, suggesting that the Dane particle DNA sequences sedimenting faster than this in DNA from infected liver are not in the form of monomeric Dane particle DNA, but are in more rapidly sedimenting alkali-stable DNA molecules.

Dane particle DNA base sequences in RNA of infected liver. Total tissue RNA extracted from HBV-infected liver and from uninfected liver were tested at 8 mg/ml for their effect on the rate of conversion of denatured Dane particle ^{3}H-labeled DNA to an S_1 nuclease-resistant form. Figure 8 shows that the rate in the presence of RNA from infected liver was greatly accelerated as compared with the rate in the presence of RNA from uninfected liver. The latter reaction proceeded at the rate of Dane particle ^{3}H-labeled DNA in the presence of calf thymus DNA. The possibility that the ^{3}H-la-

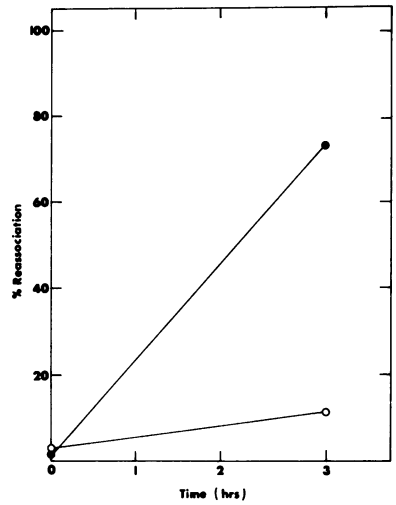

FIG. 8. *Hybridization of Dane particle ^{3}H-labeled DNA with RNA from HBV-infected liver (●) and from uninfected liver (○). Each RNA at 8 mg/ml with ^{3}H-labeled DNA was denatured by heating at 100°C, and the conversion of ^{3}H-labeled DNA to an S_1 nuclease-resistant form during incubation at 68°C was measured by the method described in the legend to Fig. 1 and the text for DNA reassociation. Each point represents the average of duplicate aliquots.*

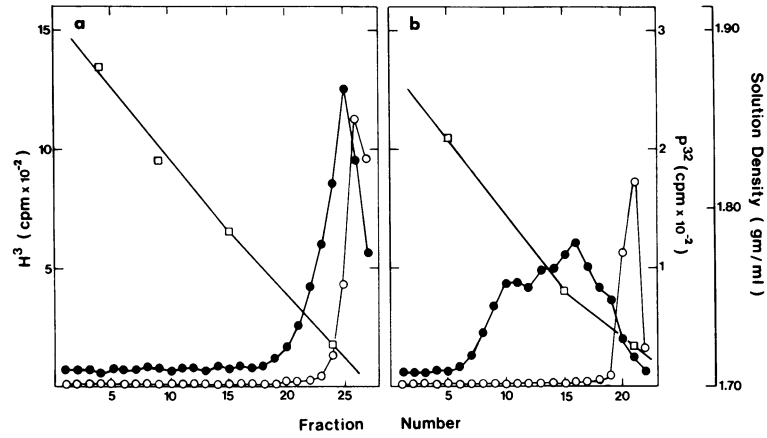

FIG. 9. *Equilibrium CsCl density gradient centrifugation of Dane particle ^{32}P-labeled DNA after hybridization with RNA from uninfected liver (a) and from HBV-infected liver (b). Portions from the 3-h hybridization reactions shown in Fig. 8 were added to undenatured ^{3}H-labeled DNA from the Dane particles and made up to 4.5 ml with 0.01 M Tris-hydrochloride, pH 7.5, and 0.01 M EDTA, and 6.75 g of solid CsCl was added to each. Both were centrifuged at 42,000 rpm and 25°C for 36 h in a Spinco 50 Ti rotor, and the acid-precipitable ^{32}P (●), ^{3}H (○), and solution density (□) were determined for fractions collected from the bottom of each tube.*

beled DNA was annealing with DNA contaminating the RNA preparation from infected liver rather than RNA was tested by analyzing the buoyant density of the annealed ^{3}H-labeled DNA. Figure 9 shows the distribution of the ^{3}H-labeled DNA from the hybridization reactions at 3 h shown in Fig. 8 after centrifugation to equilibrium in CsCl density gradients. The ^{3}H-labeled DNA from the reaction with RNA from uninfected liver (Fig. 9a) is in the position in the gradient expected for denatured DNA. The ^{3}H-labeled DNA from the hybridization reaction with RNA from HBV-infected liver (Fig. 9b) has a higher buoyant density as expected for DNA-RNA hybrids. These experiments indicate that Dane particle DNA base sequences can be detected in RNA from HBV-infected liver, but not in RNA from uninfected liver.

DISCUSSION

At a given DNA concentration the reassociation $T_{1/2}$ is proportional to the amount of unique base sequence, i.e., the size of a specific DNA that does not have regions of repeated base sequence (2). We used this reaction to measure the size of the new (i.e., radioactive) DNA made in a Dane particle DNA polymerase reaction. The $C_0t_{1/2}$ values for the newly synthesized DNA in different experiments were consistent with unique DNA of 625 to 1,250 nucleotide pairs (Fig. 3). This is one-fourth to one-half the size of the circular double-stranded DNA shown to be the template for the Dane particle DNA polymerase reaction (18). It is not clear how much of the variation observed in the apparent complexity of the newly synthesized DNA was actually due to differences in the amount of DNA template copied in reactions with different Dane particle preparations and how much was due to wider limits in the error involved in determining DNA concentrations by electron microscopy (see above), a method required by the small amounts of DNA available for most experiments. In one experiment with a high enough concentration of Dane particle DNA, the concentration determined by electron microscopy was the same as that determined by A_{260}, indicating that DNA concentrations can be accurately determined with electron microscopy. The reassociation kinetics (Fig. 3) are consistent with a uniformly labeled unique DNA sequence, suggesting that the DNA polymerase uniformly copies a region (or regions) of Dane particle DNA rather than copying some sequences more frequently than others.

The amount of DNA synthesis determined by measuring nucleotide incorporation was 29 to 45% of the total DNA extracted from Dane particles in different experiments. Thus, the total amount of new DNA synthesis is the same fraction of the total DNA extracted from Dane particles as the reassociation value for the complexity (i.e., size) of the new DNA is of the size of the circular Dane particle DNA molecule. This suggests that about 25 to 50% of the Dane particle's circular DNA molecule is probably copied one time during the reaction. The degree of complexity found for the newly synthesized

DNA indicates that the template for the reaction is a uniform population of molecules with respect to base sequence, as expected for a viral DNA, rather than a heterogeneous population, as might be expected if random host DNA moleculse were packaged in Dane particles. Our conclusion that one-fourth to one-half of the Dane particle DNA molecule is copied during the DNA polymerase reaction is in agreement with the results of Summers et al. (23), who found new DNA (i.e., radioactive nucleotides) in restriction endonuclease fragments corresponding to a similar fraction of the molecule.

The reassociation of Dane particle ^{3}H-labeled DNA was also used to test for the presence of the same base sequences in DNA and RNA extracted from HBV-infected and uninfected liver. The presence of these base sequences in DNA added to a ^{3}H-labeled DNA reassociation reaction will accelerate the rate of reassociation in proportion to the increase in the concentration of these sequences (2, 8). The actual concentration of the sequences can be calculated from the $T_{1/2}$ for reassociation after determining this value for known concentrations of the DNA sequences in a reconstruction experiment (Fig. 2). A knowledge of the concentration of these sequences in a preparation of liver DNA permits a calculation of the number of copies of the Dane particle DNA sequences per liver cell DNA equivalent as done in Table 1. The values (from 12 to 70 copies of Dane particle ^{3}H-labeled DNA sequences per cell DNA equivalent) are a minimum estimate of the copy number per infected cell since the calculation uses the concentration of DNA from all cells and every cell in a liver may not be infected. In our experience the number of infected cells detected by immunofluorescent staining for HB_sAg in different infected livers is widely variable (from 1% to nearly all cells). This is in agreement with published findings (6, 9). If the immunofluorescent-positive cells are the same as the cells with viral DNA, then the values here for copy numbers per infected cell would be low and the correct values would depend in each case on the actual fraction of cells infected. The presence of Dane particle DNA sequences in uninfected liver could be excluded at the level of 0.1 copy/cell. The presence of Dane particle DNA sequences in infected liver but not in uninfected liver is consistent with the DNA being viral DNA.

How much of the Dane particle DNA sequences detected in DNA extracted from infected liver is from within cells or how much is extracellular, such as in Dane particles known to be present in the plasma of infected patients, is not clear. The presence of these sequences in alkali-stable, rapidly sedimenting DNA molecules suggests that some of the sequences are probably covalently attached to host chromosomal DNA. This is consistent with Dane particle DNA being viral DNA integrated into host DNA molecules as is the case with other DNA viruses (20). Alternatively, the rapidly sedimenting sequences could represent Dane particle DNA forms such as double-stranded cyclic coils (26) or catenated oligomeric forms (3) that sediment more rapidly under alkaline conditions than the open circular form so far described in Dane particle DNA preparations (18; Robinson and Lutwick, in press).

The presence of Dane particle DNA base sequences in the RNA of infected liver but not uninfected liver is consistent with the transcription of viral DNA in infected liver cells.

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