JOURNAL OF VIROLOGY, May 1978, p. 281-290
0022-538X/78/0026-0281$02.00/0

Vol. 26, No. 2
Printed in U.S.A.

Synthesis of Viral and Host DNA in Isolated Chromatin from Herpes Simplex Virus-Infected HeLa Cells

MICHIYUKI YAMADA,† GILBERT BRUN, AND ARTHUR WEISSBACH*
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Received for publication 6 December 1977

DNA synthesis in chromatin isolated from herpes simplex virus type 1-infected HeLa cells (HSV chromatin) was examined in vitro. The HSV chromatin was found to carry out an initial limited synthesis of DNA in vitro, 50 to 64 pmol of dTMP incorporated in 10^6 nuclei per 10 min, which is comparable to that found in nuclei isolated from HSV-infected cells. DNA synthesis in vitro proceeded for only 30 min, and both HSV DNA and host DNA were synthesized in significant amounts. The HSV and host DNA syntheses in isolated chromatin were inhibited to the same extent by anti-HSV antiserum or by phosphonoacetic acid. The results indicate that the HSV-induced DNA polymerase is most likely involved in the synthesis of host and HSV DNA in isolated chromatin, even though this chromatin contains small amounts of the host γ-polymerase in addition to the HSV-induced DNA polymerase. The HSV chromatin contains no detectable levels of DNA polymerases α and β, even though infected cells have normal, or increased, levels of these enzymes.

Nuclei isolated from uninfected and virus-infected eucaryotic cells have been used to study in vitro DNA replication. It has been demonstrated that DNA synthesis by isolated nuclei requires deoxynucleotides, ATP, and cytoplasmic fraction (see review [10]). Although DNA synthesis by isolated nuclei produces some abnormal distribution of DNA sequences (32), the nuclei system has been used successfully to detect stimulatory factors in the cytoplasm (4, 11) and to show the involvement of RNA in DNA replication (22, 23, 27, 28, 31).

Recently, the extraction and reconstitution of nuclear DNA synthesis in isolated mammalian cell nuclei has been attempted (26), and partial separation of a putative adenovirus DNA replication complex from adenovirus-infected nuclei (33) has been suggested. The finding that simian virus 40 DNA synthesis can occur in a nucleoprotein complex (9, 25) and that HeLa DNA synthesis is observed in isolated chromatin (12) has shown that chromatin is active for DNA synthesis in vitro. Chromatin may offer advantages over the isolated nuclei in its accessibility to DNA polymerases or other enzymatic factors (12, 15).

Previously, a number of groups have reported that nuclei from herpes simplex virus (HSV)-infected cells could synthesize HSV DNA in vitro (2, 6, 7, 14). This paper will show that a chromatin preparation isolated from the nuclei of HSV-infected HeLa cells can also replicate both HSV and host DNA and that the HSV-induced DNA polymerase is responsible for both the HSV DNA and the host DNA syntheses in this system.

MATERIALS AND METHODS

Radiochemicals. [*Methyl*-^{3}H]dTTP was purchased from Schwarz/Mann, and [*methyl*-^{3}H]thymidine, [α-^{32}P]dTTP and [5-^{125}I]dCTP were obtained from New England Nuclear Corp.

Chemicals. Deoxynucleoside triphosphates and ribonucleoside triphosphates were purchased from Sigma Chemical Co. Bromodeoxyuridine triphosphate was obtained from P-L Biochemicals, and Sarkosyl NL-97 was from the Ciba-Geigy Corp. Phosphonoacetic acid was a gift of E. Heimer and A. Cook from the Hoffman-La Roche Research Laboratories. Pancreatic DNase I and RNase were from Worthington Biochemical Corp., and RNase T1 and Pronase were from Calbiochem. HSV antiserum was the same as described previously (7). Ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) was obtained from Sigma Chemical Co.

Cell culture and virus infection. HeLa F cells were used for the proliferation of HSV type 1 (HSV-1) (Miyama strain) (20). HeLa F cells were grown in monolayer culture in a glass roller bottle (10 by 50 cm) or in 150-cm^2 culture flasks with F-11 medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum, 4 mM glutamine, and 1% streptomycin-penicillin. When the cells approached confluency, they were washed with phosphate-buffered saline (Grand Island Biological Co.) and infected with HSV at 10

† Present address: National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan.

PFU/cell for 30 min. The infected cells were grown in F-11 medium supplemented with 2% heat-inactivated calf serum, 4 mM glutamine, and penicillin-streptomycin. At 7 h postinfection, the infected cells were washed with phosphate-buffered saline and collected with scraping.

Preparation of nuclei. The nuclei from the HSV-infected cells were isolated by the method of Krokan et al. (17) with a modification. The collected cells were washed with 2 mM EGTA-3 mM $MgCl_2$-10 mM Tris-hydrochloride (pH 7.5) (buffer A). The cells (4×10^8, 1.2 g) were suspended in 3 ml of buffer A containing 0.5 mM dithiothreitol (DTT) and broken in a Dounce homogenizer with 10 strokes. After this, 1.5 ml of 0.3 M Tris-hydrochloride (pH 8.0)-2 mM EDTA-3 mM $MgCl_2$-0.15 M glucose-0.15% Triton X-100 (buffer B) containing 0.5 mM DTT was added to the suspension, which was again homogenized with five strokes. The homogenate was centrifuged at $1,000 \times g$ for 8 min, and the pelleted nuclei were washed with 2 ml of buffer C (a mixture of 2 volumes of buffer A and 1 volume of buffer B containing 0.5 mM DTT) by Dounce homogenization and centrifuged at $1,000 \times g$ for 8 min. The nuclei were suspended in 2 ml of buffer C (without Triton X-100), divided into small portions, and stored in liquid nitrogen.

Preparation of chromatin. The frozen nuclei were thawed, and a suspension of 420 μl (4.2×10^7 nuclei) was centrifuged at $1,000 \times g$ for 5 min. The pelleted nuclei were suspended in 315 μl of 1 mM EDTA (pH 7.0)-1 mM DTT, allowed to stand for 30 min at 0°C, and then centrifuged at $1,000 \times g$ for 5 min. The pellet was resuspended in 315 μl of the above solution, allowed to stand for 30 min at 0°C, and centrifuged at $1,000 \times g$ for 5 min. This pellet was taken up in 315 μl of 1 mM Tris-hydrochloride (pH 8.0)-0.1 mM EDTA-1 mM DTT-40% glycerol. There were no visible nuclear structures in this preparation under a light microscope. Usually, chromatin was prepared from fresh nonfrozen nuclei, and the chromatin fraction could be stored for several weeks in the liquid nitrogen refrigerator without showing any significant change in TMP incorporation.

Reaction mixture for chromatin DNA synthesis. The reaction mixture (100 μl) for DNA synthesis contained 5 μmol of Tris-hydrochloride (pH 8.1), 0.4 μmol of $MgCl_2$, 0.25 μmol of ATP, 5 nmol each of CTP, UTP, and GTP, 0.25 μmol of DTT, 1 nmol of [^{3}H]-dTTP (2 μCi/nmol), 10 nmol each of dCTP, dGTP, and dATP, and the chromatin preparation. The mixture was incubated at 37°C, and the determination of incorporation of dTMP into acid-insoluble materials was the same as described previously (32).

Alkaline sucrose gradient centrifugation. A reaction mixture (0.1 ml) obtained from a chromatin DNA synthesis reaction was lysed in the presence of 0.3 N NaOH-0.03 M EDTA-0.3 M NaCl-0.2% Sarkosyl NL-97 for 1 h at 4°C. The lysate was placed onto the top of the 5 to 20% (wt/vol) linear sucrose gradient (4.9 ml) containing 0.3 N NaOH, 0.7 M NaCl, and 2 mM EDTA. The centrifugation was carried out at 48,000 rpm for 4 h at 10°C in an SW50.1 rotor. Fractions of 0.18 ml were collected from the bottom of the tube and assayed for acid-insoluble radioactivity.

Neutral sucrose gradient centrifugation. The chromatin reaction mixture (0.1 ml) was treated with Pronase (1 mg/ml) in the presence of 0.1% sodium dodecyl sulfate (SDS)-25 mM EDTA overnight at 37°C. The solution was extracted with chloroform-phenol (1:1, by volume), and the DNA solution was placed onto the top of a 5 to 20% (wt/vol) linear sucrose gradient (4.9 ml) containing 1 M NaCl, 10 mM Tris-hydrochloride (pH 8.0), 2 mM EDTA, and 0.01% Sarkosyl NL-97. The centrifugation was carried out at 48,000 rpm in an SW50.1 rotor for 3 h at 10°C, and fractions of 0.17 ml were collected from the bottom of the tube. Acid-insoluble radioactivity was determined in each fraction.

CsCl equilibrium density gradient centrifugation. [^{3}H]DNA was prepared from chromatin reaction mixtures as described above. The DNA solution was brought up to 2 ml with 10 mM Tris-hydrochloride, (pH 8.0)-1 mM EDTA, and the solution was adjusted to a density of 1.700 g/cm^3 with 6 ml of 65% (wt/wt) CsCl solution in 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA. The DNA solution was centrifuged at 40,000 rpm at 20°C for 72 h in a 50Ti rotor. Micrococcal DNA was included as a density marker. Fractions of 0.160 ml were collected from the bottoms of the tubes, and the density of the fractions was determined from the refractive index. The fractions were assayed for acid-insoluble radioactivity.

DNA-DNA reassociation kinetics. The rate of reassociation of [^{3}H]dTMP-labeled HSV DNA was followed by using S_1 nuclease digestion. The DNA was sheared and denatured with or without unlabeled HSV DNA by placing it at 100°C for 15 min in 0.33 N NaOH. After neutralization with HCl, the solution of sheared denatured DNA was brought to 0.5 M NaCl-10 mM Tris-hydrochloride (pH 7.8)-1 mM EDTA. The DNA reassociation was performed at 72°C, and at various times portions were removed and duplex DNA formation was measured after S_1 nuclease digestion (32).

Extraction of DNA polymerases from chromatin. Chromatin, equivalent to 5×10^7 nuclei, was placed in 2.2 ml of 0.35 M KPO_4 (pH 7.5)-0.5 mM DTT and sonically treated twice for 15 s. The suspension was centrifuged at $10,000 \times g$ for 20 min. All of the DNA polymerase activity (>98%) was found in the supernatant fluid and was passed through a DEAE-cellulose column (2 ml), previously equilibrated with 0.35 M KPO_4 (pH 7.5)-0.5 mM DTT; the column was then washed with the same buffer. The pass through fraction, in which all of the DNA polymerase activity is found, was dialyzed against 0.02 M KPO_4 (pH 7.5)-0.5 mM DTT. After dialysis, the insoluble materials were removed by centrifugation at $10,000 \times g$ for 10 min, and the solution was chromatographed on a DEAE-cellulose column (21).

Assay for DNA polymerases. The DNA polymerase α, β, and γ assay conditions were the same as described previously (13, 21), except that 10 μM [^{3}H]dTTP (2 μCi/nmol) was used instead of 100 μM [^{3}H]dTTP and the reaction volume was 50 μl. For the HSV DNA polymerase assay, 0.25 M KCl was included in the α-polymerase assay mixture.

Preparation of HSV antiserum. Rabbit kidney cells were infected with 10 PFU of HSV-1 per cell. After 8 h, the cells were collected and used to prepare

antiserum in rabbits as described by Citarella et al. (8).

Preparation of DNA. HSV DNA was prepared from HSV virions isolated by the method of Spear and Roizman (24) with the following modification. HeLa F cells were infected with HSV-1 at 1 PFU/cell. At 37 h after infection, the cells were lysed in reticulocyte standard buffer (RSB = 10 mM Tris [pH 7.4]-10 mM KCl-1.5 mM $MgCl_2$) containing 0.2% Nonidet P-40 in a Dounce homogenizer. The suspension was centrifuged at 1,000 × *g* for 10 min, and the pellet was washed in the above solution by another Dounce homogenization. The supernatant and wash fractions were combined and loaded on the top of a discontinuous sucrose gradient (7 ml each of 25% [wt/wt] sucrose, 30% sucrose, 40% sucrose, and 52% sucrose in RSB). The tube was centrifuged at 25,000 rpm in an SW27 rotor for 1.5 h at 4°C, and the virus band was collected. The collected virus particles were treated with DNase I (4.4 μg/ml) and pancreatic RNase (29 μg/ml) at 22°C for 30 min and then diluted three times with RSB. The particles were centrifuged at 25,000 rpm in an SW27 rotor for 1 h at 4°C and suspended in 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA. The virus suspension was brought to 0.5% SDS and 1 mg/ml with Pronase and incubated at 37°C overnight. The solution was extracted twice with chloroform-phenol (1:1, by volume), and the DNA was precipitated with 2 volumes of alcohol in the presence of 0.1 M NaCl. The DNA was dissolved in 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA and treated successively with pancreatic RNase (52 μg/ml) and RNase T1 (5 U/ml) for 1.5 h at 37°C and then with Pronase (100 μg/ml) in the presence of 0.1% SDS for 30 min at 37°C. The DNA solution was extracted with chloroform-phenol and precipitated with ethanol as described above. The precipitated DNA was dissolved in 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA and subjected to CsCl density gradient equilibrium centrifugation. The DNA at the density of 1.725 g/ml in the CsCl gradient was collected and dialyzed against 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA.

To isolate DNA from nuclei or chromatin, the nuclear fraction was brought to 0.1% SDS-25 mM EDTA-10 mM Tris (pH 7.5)-1 mg of Pronase per ml, incubated overnight at 37°C, and then extracted with chloroform-phenol as described above.

RESULTS

Properties of HSV-infected chromatin. (i) DNA synthesis in isolated chromatin. DNA synthesis in chromatin from HSV-infected cells as a function of time is shown in Fig. 1. DNA synthesis in chromatin was linear for 10 min and continued for 30 min until it leveled off. By contrast, DNA synthesis in isolated nuclei from HSV-infected cells is linear for at least 60 min (7). The amount of dTMP incorporated in HSV chromatin was 50 to 64 pmol/10^6 nuclear equivalents of chromatin for 10 min under our conditions, and this initial rate of dTMP incorporation is about 80% of that found in isolated nuclei. Since DNA synthesis in chromatin is of shorter duration than that in nuclei, it may indicate that some components involved in DNA synthesis are missing or unstable. However, the addition of bovine serum albumin or a wash fraction from the chromatin preparation to the isolated chromatin could not extend the time of DNA synthesis in chromatin (Fig. 1). The rate of DNA synthesis by chromatin in vitro was linearly proportional to chromatin concentrations up to 1.5 × 10^7 nuclear equivalents per ml.

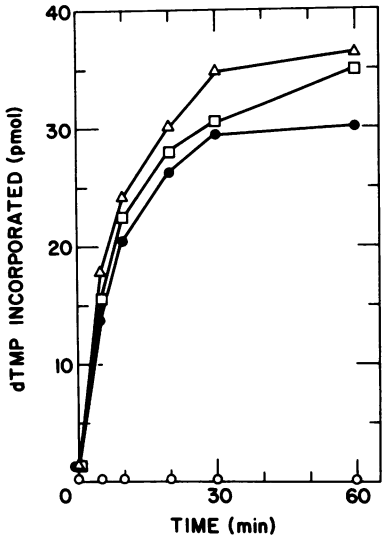

FIG. 1. *Time course of dTMP incorporation in chromatin isolated from HSV-infected HeLa cells. The reaction conditions for DNA synthesis were the same as described in the text, except that the reaction mixture was incubated for the stated times. Symbols: ●, chromatin equivalent to 3.3 × 10^5 nuclei; □, chromatin equivalent to 3.3 × 10^5 nuclei plus 50 μg of bovine serum albumin; △, chromatin equivalent to 3.3 × 10^5 nuclei plus a nuclear wash equivalent to 6 × 10^5 nuclei; ○, nuclear wash equivalent to 6 × 10^5 nuclei.*

(ii) Requirements for DNA synthesis. Table 1 shows the requirements for DNA synthesis by HSV chromatin. The chromatin system requires only $MgCl_2$ and all four deoxynucleoside triphosphates for DNA synthesis. Omission of one of four deoxynucleotides reduced the synthesis of DNA 80%. The presence of ATP, KCl, or DTT stimulated the DNA synthesis slightly. These requirements for chromatin DNA synthesis are similar to those in isolated nuclei (7).

(iii) Effects of pH and salts. DNA synthesis in isolated chromatin was maximum at pH 8.0 with Tris-hydrochloride buffer and was 25% of the maximum at pH 7.0. Synthesis in the pH range from 8.2 to 9.0 was 75 to 90% of that seen at pH 8.0. $MgCl_2$ at 4 mM showed a sharp optimum for DNA synthesis in this system. The

TABLE 1. *Requirements for DNA synthesis in chromatin isolated from HSV-infected HeLa cells*[a]

Reaction mixture	dTMP incorporated	
	pmol	%
Complete	10.6	100
- $MgCl_2$	0.2	2
- ATP	8.1	76
-GTP, CTP, UTP	12.1	114
- ATP, GTP, CTP, UTP	12.2	115
- DTT	10.1	95
- KCl	7.8	74
- dATP	2.0	19
- dATP, dCTP	2.3	22
- dATP, dCTP, dGTP	1.5	14

[a] The reaction mixture for DNA synthesis was the same as described in the text, except that the component was omitted where indicated. The chromatin equivalent to 2×10^5 nuclei was used. Assays were performed for 20 min as described in the text.

influence of various salts on DNA synthesis in HSV chromatin is shown in Fig. 2. NaCl and KCl at 20 mM concentrations stimulated the DNA synthesis slightly. All of the salts tested were inhibitory at 100 mM or higher concentrations, which are known to be optimal conditions for the HSV-induced DNA polymerase in vitro (30).

Involvement of HSV-induced DNA polymerase in HSV-infected chromatin DNA synthesis. (i) Effect of HSV antiserum on DNA synthesis in isolated chromatin. As shown in Fig. 3, the presence of HSV antiserum (prepared against HSV-infected rabbit cells) inhibited more than 90% of the DNA synthesis in isolated chromatin from HSV-infected cells, but did not inhibit the DNA synthesis in chromatin isolated from noninfected cells. Preimmune serum did not affect the DNA synthesis in chromatin isolated from either HSV-infected cells or noninfected cells. This result indicates that HSV-specified proteins are involved in DNA synthesis in HSV-infected chromatin in vitro. A likely candidate would appear to be the HSV-induced DNA polymerase (29), and this was tested with phosphonoacetic acid. Mao et al. (19) have demonstrated that the herpes-induced DNA polymerase can be inhibited by phosphonoacetic acid. Figure 4 shows the effect of phosphonoacetic acid upon isolated chromatin from HSV-infected cells and noninfected cells. Phosphonoacetic acid has a powerful inhibitory effect on DNA synthesis in HSV-infected chromatin, but almost no effect on chromatin derived from uninfected cells.

The effect of HSV antiserum on HSV DNA and host DNA syntheses in HSV-infected chromatin was further quantitated. It is known that both HSV DNA and host DNA can be synthe-

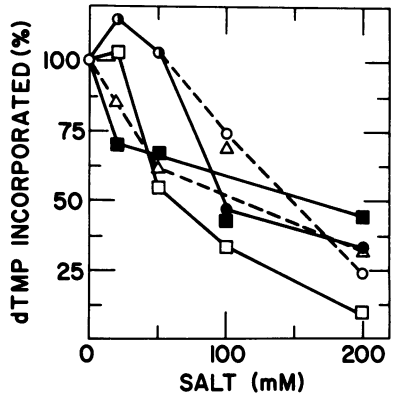

FIG. 2. *Effect of salt upon the dTMP incorporation in HSV-infected chromatin. Assays were performed with 2.6×10^5 nuclear equivalents of chromatin for 20 min in the presence of different concentrations of the following compounds: ●, KCl; ○, NaCl; △, NH_4Cl; □, KPO_4; ■, K_2SO_4. The 100% value was 13.4 pmol of dTMP incorporated, using the standard reaction conditions.*

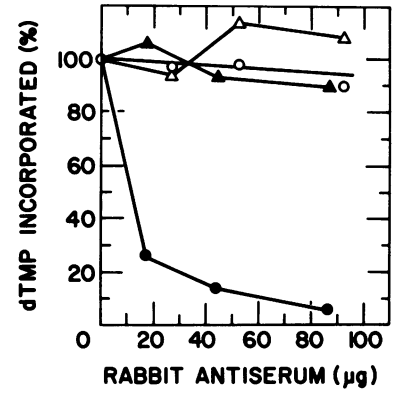

FIG. 3. *Effect of HSV antiserum on DNA synthesis in HSV-infected chromatin and noninfected chromatin. The preincubation mixture (18 μl) for the antiserum chromatin reaction consisted of 40 μg of bovine serum albumin, 2.6×10^5 nuclear equivalents of HSV chromatin or 1×10^6 nuclear equivalents of noninfected chromatin, and the various amounts of HSV antiserum or normal rabbit serum as indicated. Bovine serum albumin was added when necessary to maintain a constant protein concentration. The mixture was incubated for 15 min at 20°C, and then the reaction mixture for DNA synthesis was added. The incubation time for DNA synthesis in HSV chromatin was 10 min, and that for DNA synthesis in noninfected chromatin was 15 min. The 100% values were 18.4 pmol of dTMP incorporated for DNA synthesis in HSV-infected chromatin and 1.94 pmol of dTMP incorporated in noninfected chromatin. The specific activity of the [^{3}H]TTP was 10 μCi/nmol for noninfected chromatin and 2 μCi/nmol for HSV chromatin. Symbols: ●, HSV chromatin plus HSV antiserum; ○, HSV chromatin plus normal rabbit serum; ▲, noninfected chromatin plus HSV antiserum; △, noninfected chromatin plus normal rabbit serum.*

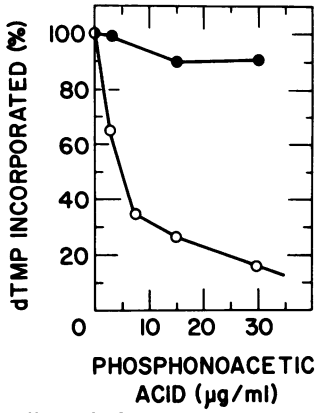

FIG. 4. *Effect of phosphonoacetic acid on DNA synthesis in HSV-infected and noninfected chromatins. The reaction mixture was the same as described in the text, except that various amounts of phosphonoacetic acid disodium were added as indicated. [^{3}H]TTP (10 μCi/nmol) was used as the label for DNA synthesis in noninfected chromatin, and [^{3}H]-TTP (2 μCi/nmol) was used for that in HSV-infected chromatin. Both chromatins used were equivalent to 2.6 × 10^5 nuclei. The incubation time was 10 min. The 100% values were 13.5 pmol of dTMP incorporated with HSV chromatin and 0.38 pmol of dTMP incorporated with noninfected chromatin. Symbols: ○, HSV chromatin; ●, noninfected chromatin.*

sized by isolated nuclei from HSV-infected cells (2, 3, 7). HSV-infected chromatin also synthesizes both host and viral DNA (Fig. 5A) in appreciable amounts. From the CsCl gradient studies shown in Fig. 5, the amount of newly synthesized HSV DNA and host DNA was calculated to be 64 and 36%, respectively, of total DNA made in HSV-infected chromatin. Control experiments carried out in vivo showed that HeLa cells, in a 10-min pulse with [^{3}H]thymidine at 7 h after infection with HSV-1 at 10 PFU/cell, synthesized both viral DNA and host DNA at a ratio of 92 parts HSV DNA to 8 parts host DNA.

We have shown above that DNA synthesis in HSV-infected chromatin is almost completely inhibited by the presence of HSV antiserum. It was therefore of interest to know whether the HSV antiserum could inhibit equally the HSV and host DNA syntheses in HSV-infected chromatin. The amounts of HSV and host DNAs synthesized in HSV-infected chromatin in the presence of various amounts of HSV antiserum were analyzed by banding the DNAs in CsCl density gradients (Fig. 5). These results are quantitated in Table 2. The incorporations of dTMP into HSV DNA and host DNA were inhibited at the same rate in the presence of increasing amounts of HSV antiserum (Fig. 5B and C). It is clear from these results that the

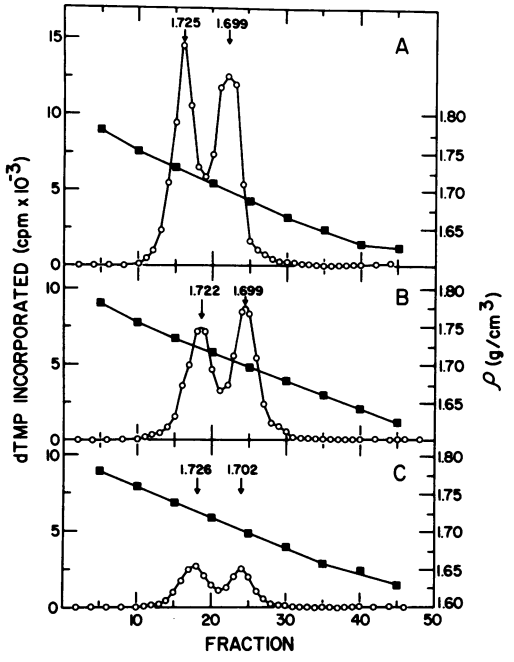

FIG. 5. *CsCl isopycnic centrifugation of DNA made in HSV-infected chromatin in the presence of HSV antiserum. HSV-infected chromatin equivalent to 2.6 × 10^5 nuclei was incubated with or without HSV antiserum under the same conditions as described in the legend to Fig. 3, and the mixture was incubated for DNA synthesis for 10 min under the same conditions as described in the legend to Fig. 3, using [^{3}H]dTTP with a specific activity of 10 μCi/nmol. DNA was isolated after the addition of HSV DNA (10 μg) and HeLa (10 μg) as carrier, as described in the text. The DNA was subjected to CsCl equilibrium density gradient centrifugation at 40,000 rpm for 72 h. (A) Without HSV antiserum; (B) 8.5 μg of HSV antiserum; (C) 42.8 μg of HSV antiserum.*

TABLE 2. *Effect of HSV antiserum on syntheses of HSV DNA and host DNA in HSV chromatin*

HSV antiserum (μg)	dTMP incorporated						(HSV DNA/total DNA) × 100
	HSV DNA		Host DNA		Total DNA		
	cpm × 10^{-3}	%	cpm × 10^{-3}	%	cpm × 10^{-3}	%	
0	86.2	100	91.5	100	177.7	100	48.5
8.55	53.3	61.8	62.7	68.5	116.0	65.2	45.9
42.80	21.3	24.7	17.8	19.4	39.1	22.0	54.4

syntheses of both the host and the HSV DNAs are inhibited equally by the HSV antiserum. From these data and the above studies with phosphonoacetic acid, we assume that the synthesis of both host and viral DNA in isolated HSV chromatin is mediated by the HSV-induced DNA polymerase.

(ii) DNA polymerases present in chromatin. To confirm that the HSV-induced DNA polymerase is involved in both HSV DNA and host DNA syntheses in isolated chromatin, the DNA polymerases present in HSV-infected chromatin or uninfected chromatin were analyzed by DEAE-cellulose column chromatography (Fig. 6). HSV-infected chromatin prepared by our technique contained mostly HSV-induced DNA polymerase (98% of total) and DNA polymerase γ (2% of total) (Fig. 6B), whereas chromatin from noninfected cells contained DNA polymerases α and λ (Fig. 6A) in expected amounts and a small amount of putative DNA polymerase β (nonadsorbed activity). The DNA polymerase activity observed with the α-polymerase assay conditions in the DEAE-cellulose chromatogram of HSV-infected chromatin extract (Fig. 6B) is ascribed to the residual activity of HSV DNA polymerase under these conditions (30) because this activity is not inhibited by antiserum prepared against the α-polymerase. It was shown above that DNA synthesis in HSV-infected chromatin was sensitive to phosphonoacetic acid and HSV antiserum, and it is also known that phosphonoacetic acid inhibits the HSV DNA polymerase but not the γ-polymerase (7). Therefore, we conclude that the HSV DNA polymerase is the only polymerase involved in both HSV and host DNA syntheses in isolated chromatin.

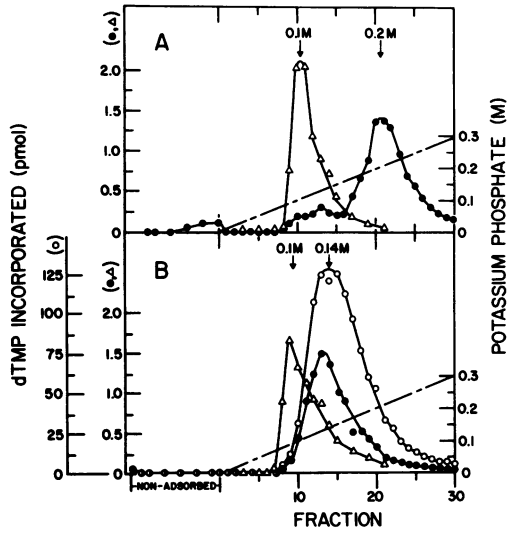

FIG. 6. *DEAE-cellulose chromatograms of extracts from HSV-infected and noninfected chromatins. The chromatin extract was prepared from 5 × 10^7 nuclear equivalents of chromatin, as described in the text, and chromatographed on DEAE-cellulose (1.25 ml) with a linear gradient elution of 10 ml between 0.02 M KPO_4 (pH 7.5)–0.5 mM DTT and 0.30 M KPO_4 (pH 7.5)–0.5 mM DTT. Fractions of 0.33 ml were collected. The amounts of dTMP incorporated with 4 μl of each fraction under α, γ, and HSV DNA polymerase assay conditions are shown. Symbols: △, γ-polymerase; ●, α-polymerase; ○, HSV polymerase. (A) Chromatogram of noninfected chromatin extract; (B) chromatogram of HSV-infected chromatin extract.*

(iii) DNA polymerases in herpesvirus-infected cells. The unexpected finding that chromatin isolated from HSV-infected cells did not contain DNA polymerase α and β activities led us to study the total DNA polymerase content of cells infected with herpesvirus. The results of this study are shown in Table 3, in which the total units of each host DNA polymerase and the HSV-induced DNA polymerase are shown at various times after infection with HSV-1. It is quite clear that HSV-1 infection for up to 24 h does not lead to the disappearance or diminution of any of the host DNA polymerases, but rather to a slight increase in the cells' content of these enzymes. This is of particular interest in view of the known inhibition of cellular protein synthesis after HSV-1 infection (5) and the moderate lability of the DNA polymerases, particularly

TABLE 3. *DNA polymerase activities in HeLa cells infected with HSV*[a]

Time postinfection (h)	DNA polymerase α (total units per 10^8 cells)	DNA polymerase β (total units per 10^8 cells)	DNA polymerase γ (total units per 10^8 cells)	HSV DNA polymerase (total units per 10^8 cells)
0	1,695	52	80	0
3	1,463	55	98	1,084
6	1,430	43	95	3,264
9	2,001	82	56	4,161
24	2,592	64	145	10,060

[a] The DNA polymerase activities were determined in both crude extracts directly and insoluble extracts after sucrose gradient velocity sedimentation, as previously described (5a), using the assay procedures listed in the text (12, 21). The soluble cellular extracts obtained after centrifugation of crude extracts for 45 min at 176,000 × *g* were loaded as 250-μl aliquots (0.33 mg of proteins) on 5-ml 5 to 20% (wt/wt) sucrose gradients made in 50 mM HEPES (pH 7.5)–0.35 M KCl–2 mM DTT–1 mM EDTA buffer. The gradients were run for 18 h at 44,000 rpm in an SW50.1 Beckman rotor, and 25 equal fractions were collected from the bottom of each gradient. Ten-microliter aliquots of each fraction (200 μl) were assayed for the four DNA polymerase activities, using specific assays for each. A unit is defined as that amount of enzyme catalyzing the polymerization of 1 nmol of nucleotide per h under standard assay conditions.

DNA polymerase β, in HeLa cells (21). The failure of HSV chromatin to contain two of the three host DNA polymerases may indicate profound changes occurring in the host nucleus after HSV infection and does not reflect the cellular content of these enzymes.

Properties of DNA made in HSV-infected chromatin. (i) Alkaline and neutral sucrose gradient centrifugations. HSV-infected cells were labeled with [^{3}H]thymidine from 2 to 7 h postinfection, and ^{3}H-labeled chromatin was prepared from the cells. DNA synthesis in vitro was performed under our standard conditions with ^{3}H-labeled chromatin and [α-^{32}P]dTTP. The DNAs were analyzed by alkaline or neutral sucrose gradient centrifugation. Figure 7 shows the size of ^{3}H- or ^{32}P-labeled DNA in alkaline sucrose velocity gradients. [α-^{32}P]dTMP-labeled DNA, synthesized for 1 min in vitro, had a single broad peak with the value of 8.1S, slightly smaller than the [^{3}H]thymidine-prelabeled chromatin DNA present in the incubation, which had a peak with a median value of 11S (Fig. 7A). When the chromatin was incubated with [α-^{32}P]dTTP for 1 min and then further incubated for 2 min with a 50-fold excess of unlabeled dTTP, there was no detectable elongation in size of ^{32}P-labeled DNA (Fig. 7B). As shown in Fig. 8, neutral sucrose gradient centrifugation of [α-^{32}P]dTMP-labeled DNA after a 3-min incubation in vitro showed that a major part of the DNA migrated with an S value of 13, at the same position as the ^{3}H-prelabeled DNA. The patterns and distributions of ^{32}P- or ^{3}H-labeled DNA after a 10-min incubation were the same as those observed in the DNAs after a 3-min

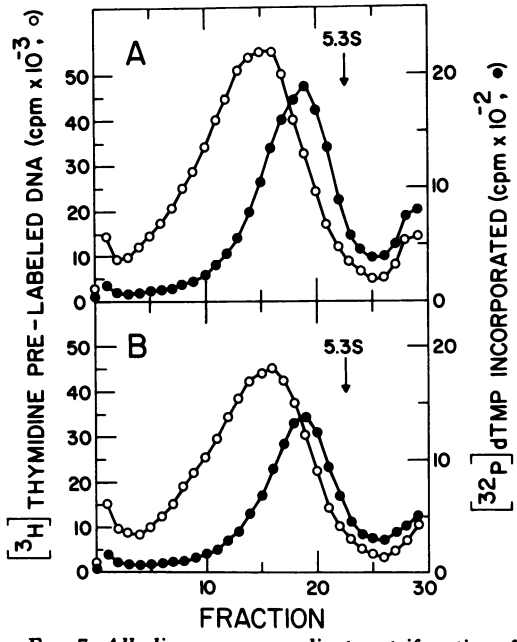

FIG. 7. *Alkaline sucrose gradient centrifugation of DNA made in HSV-infected chromatin. HSV-infected cells were labeled with [^{3}H]thymidine at 28 μCi/ml from 2 to 7 h postinfection, and the [^{3}H]thymidine-labeled HSV chromatin was prepared as described in the text. The chromatin equivalent to 5 × 10^5 nuclei was incubated with [α-^{32}P]dTTP (10 μCi/nmol) under the same conditions as described in the text. After the incubation, the chromatin was lysed and placed on linear sucrose gradients as described in the text. After centrifugation at 48,000 rpm for 4 h in an SW50.1 rotor at 10°C, 10-μl portions of each fraction were removed, and acid-insoluble radioactivity was measured as described in the text. (A) DNA synthesis in vitro in chromatin was performed for 1 min; (B) DNA synthesis in vitro in chromatin was performed for 1 min, followed by an incubation for 2 min with a 50-fold excess of unlabeled dTTP. Symbols: ○, [^{3}H]-thymidine-prelabeled DNA; ●, [α-^{32}P]dTMP incorporated in vitro. The marker DNA of 5.3S was a sonically treated calf thymus DNA.*

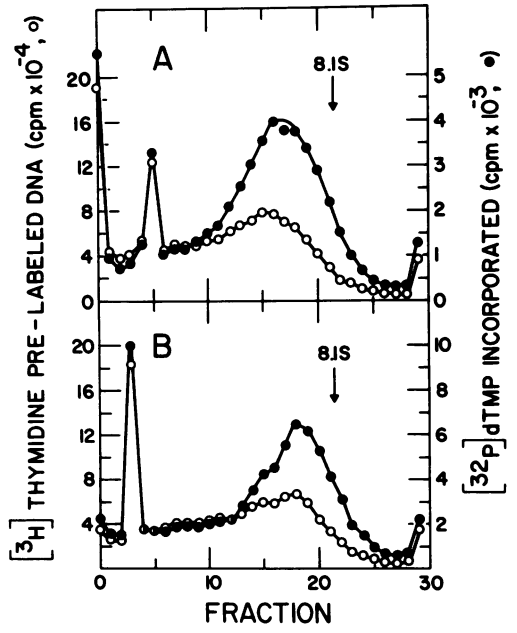

FIG. 8. *Neutral sucrose gradient centrifugation of DNA made in HSV-infected chromatin. [^{3}H]thymidine-labeled chromatin was prepared as described in the legend to Fig. 7. The chromatin equivalent to 5 × 10^5 nuclei was incubated with [α-^{32}P]dTTP (10 μCi/nmol) as described in the text. After the incubation, the DNA was prepared as described in the text, placed onto the top of a neutral sucrose gradient, and centrifuged at 48,000 rpm for 3 h at 10°C in an SW50.1 rotor. After the fractionation, 25-μl portions were removed from the fractions, and the acid-insoluble radioactivity was measured as described in the text. (A) 3-min incubation with chromatin; (B) 10-min incubation with chromatin. Symbols: ○, [^{3}H]thymidine-labeled DNA; ●, [α-^{32}P]dTMP incorporated in chromatin.*

incubation, and in both cases some material sedimenting at 24S was observed.

(ii) CsCl equilibrium density gradient centrifugation of DNA made in vitro. To obtain more information about the in vitro DNA synthesis, [^{3}H]thymidine-labeled chromatin was incubated with unlabeled BrdUTP (where "Brd" indicates "bromodeoxy"), dATP, and dGTP and [^{125}I]dCTP for 10 min, and the DNA was isolated and subjected to CsCl equilibrium density gradient centrifugation. As shown in Fig. 9, the newly synthesized BrdUMP-containing DNAs of either HSV or the host were heavier than the respective [^{3}H]thymidine-prelabeled DNAs. The difference in the densities between ^{3}H-labeled HSV DNA and BrdUMP-labeled HSV DNA was 0.008 g/cm^3. Based on the thymidine content of 16% in HSV DNA, the BrdUMP substitution in the newly synthesized DNA was calculated to be 14.5% of the total thymidine according to the equation of Luk and Bick (18). The difference in the densities of the ^{3}H-labeled host DNA and BrdUMP-labeled host DNA was 0.006 g/cm^3. This density difference was calculated to represent a 5.8% substitution of dTMP with BrdUMP in the new DNA. If the incubation in vitro with BrdUTP was carried out for 3 min instead of 10 min, the substitutions of dTMP with BrdUMP in the HSV DNA and the host DNA were 7.2, and 3.8% of the total dTMP, respectively. This large amount of synthesis is more compatible with a replicative mode of synthesis than with a repair mode.

(iii) Reassociation kinetics of HSV DNA made in HSV-infected chromatin. The [^{3}H]dTMP-labeled HSV DNA made by HSV-infected chromatin in vitro was separated from host DNA by CsCl equilibrium density gradient centrifugation, and the reassociation kinetics of this HSV DNA made in vitro were followed in the presence or absence of unlabeled HSV virion DNA (Fig. 10). The kinetics of reassociation of HSV DNA made in vitro showed a linear curve, with the possible indication of a break (Fig. 10A). This could indicate that 7 to 9% of a total HSV DNA made in vitro reassociated 1.5-fold faster than the rest of HSV DNA, and this is under further study. More importantly for our studies, these experiments permit us to calculate the absolute amount of HSV DNA present. From the known rate of reassociation of HSV DNA (16) and the data shown in Fig. 10, which compares the reassociation rate of chromatin DNA in the absence or presence of a known amount of exogenous HSV DNA, the amount of newly synthesized ^{3}H-labeled HSV DNA and the endogenous HSV DNA present in the chromatin can be determined. Thus, we estimate that, under our conditions, about 30% of the endogenous HSV DNA in the chromatin was further replicated in 20 min in vitro. Molecular hybridization analysis of the ^{3}H-labeled host DNA made by the isolated chromatin showed it to have the same distribution of sequences as normal, in vivo DNA.

DISCUSSION

Chromatin from herpesvirus-infected HeLa cells is capable of synthesizing both HSV and host DNA in vitro. The bulk of the newly syn-

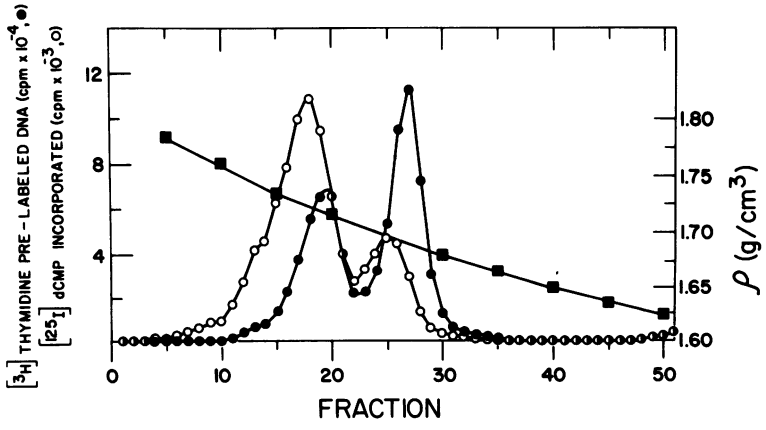

FIG. 9. *Isopycnic centrifugation of DNA made in chromatin in the presence of BrdUTP. [^{3}H]thymidine-labeled HSV-infected chromatin was prepared as described in the legend to Fig. 7 and then incubated for 10 min with 2.5 nmol of BrdUTP and 5 nmol of dCTP containing 20 pmol of [^{125}I]dCTP (20 μCi/20 pmol). The DNA was prepared from the chromatin as described in the text and subjected to CsCl equilibrium density gradient centrifugation at 39,000 rpm for 72 h at 19°C in a 50Ti rotor. Portions (25 μl) of each fraction were removed, and the radioactivity of acid-insoluble materials was measured. Symbols: ●, [^{3}H]thymidine-labeled DNA; ○, [^{125}I]dCMP incorporated in chromatin.*

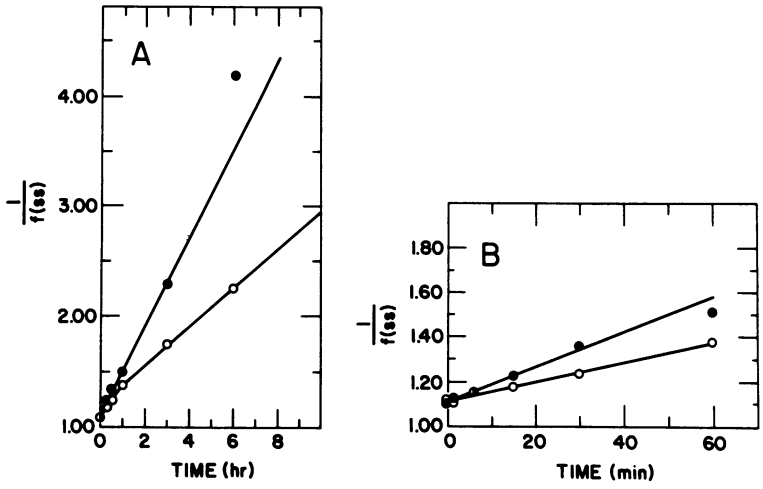

FIG. 10. *Reassociation kinetics of HSV DNA made in HSV-infected chromatin. The reaction mixture for DNA synthesis (0.3 ml) was incubated with 6.7 × 10⁷ nuclear equivalents of chromatin for 20 min with [³H]-dTTP. The ³H-labeled DNA was isolated as described in the text, and the ³H-labeled HSV DNA was separated from host DNA by two successive CsCl equilibrium density gradient centrifugations. The ³H-labeled HSV DNA, made in isolated chromatin, was reassociated at 72°C for the stated times with or without unlabeled HSV DNA, as described in the text. The amount of reassociated DNA was measured as described in the text. Symbols: ○, without the unlabeled HSV DNA; ●, with 622 ng of unlabeled HSV DNA per ml. $1/f_{(ss)}$ is a reciprocal of the fraction of single-stranded DNA present at a stated time. (B) is an amplification of the early reassociation kinetics shown in (A).*

thesized DNA in vitro is of a relatively small size, with a single-stranded chain length of about 900 to 1,000 nucleotides (8S). This is about one-half the size of the DNA strands present in the parental chromatin as isolated under our conditions. Our results are similar to those seen with isolated HeLa nuclei, which synthesize both short fragments of DNA (400 to 1,500 nucleotides) and larger fragments (5,000 to 9,000 nucleotides) in about equal amounts (23). The failure to detect longer DNA strands may then be caused by an interruption in the normal DNA replication process. On the other hand, our inability to detect the DNA synthesis of larger strands may reflect the presence of nucleases that can act rapidly in vitro since Francke (Biochemistry, in press) has found that lysates prepared from HSV-1-infected BHK cells can cause extensive nucleolytic cleavage of endogenous DNA under certain conditions. It is unlikely that any of the DNA synthesis observed in vitro with chromatin represents a significant repair process at nicks or small gaps. The substitution experiments with BrdUTP indicated that the ³H-labeled HSV DNA fragments produced in vitro consist of a preexistent fragment of HSV DNA of about 650 nucleotides, to which another 350 or so nucleotides have been added in vitro.

A significant proportion (36%) of the DNA made in vitro by chromatin from herpesvirus-infected cells consists of host sequences. It has been reported previously that isolated nuclei from either HSV- or vaccinia virus-infected cells will also synthesize some host DNA in vitro (7). The results obtained in our present studies clearly indicate that all of the HSV chromatin DNA synthesis is catalyzed by the HSV-induced DNA polymerase, since such synthesis is inhibited by HSV-specific antiserum or the drug phosphonoacetic acid. This was not unexpected since the HSV-induced DNA polymerase is known to be required for HSV DNA synthesis in vivo (1).

Furthermore, the analysis of the DNA polymerases in the HSV chromatin shows that the HSV-induced DNA polymerase was the predominant activity, although a small amount of the host γ-polymerase was also present. In this respect, the HSV chromatin clearly differs from chromatin prepared from uninfected cells which contains DNA polymerases α and γ and, to a much lesser extent, DNA polymerase β. Nor is the lack of the α- and β-polymerases in the HSV chromatin a reflection of the cellular content of these enzymes since we have shown here that the host DNA polymerases α, β, and γ are present at normal (or higher) levels in HSV-infected cells.

What remains to be explained is why the HSV chromatin synthesizes large amounts of host DNA in vitro (~36% of the total) and why all of the host DNA synthesis is catalyzed by the

HSV-induced DNA polymerase. This must be related to the difference in the DNA polymerase activities found in chromatin obtained from normal vis-à-vis HSV-infected cells and may indicate profound changes in vivo in chromatin structure and content that occur after HSV infection. An analysis of these chromatin changes may help us to understand the mechanisms by which HSV can shut off host DNA synthesis.

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