dine-32. In contrast, only histidine-32 is ethoxyformylated on reaction of α-lactalbumin at high concentrations (37 mg/mL) with diethylpyrocarbonate. These differences in modification as a function of α-lactalbumin concentration probably reflect the fact that histidine-68, which is not essential for supporting the activity of α-lactalbumin and is, indeed, absent in human α-lactalbumin, cannot react because of the aggregation of α-lactalbumin at high protein concentrations (Kronman et al., 1967).

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Synthesis of Herpes Simplex Virus DNA in Isolated Chromatin†

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ABSTRACT: Herpes simplex virus DNA synthesis was studied in isolated chromatin (HSV chromatin) of African green monkey kidney (RC-37) cells after HSV type 1 infection. After optimizing the in vitro system, HSV chromatin was shown to synthesize both viral and cellular DNA at ratios identical with those seen in vivo. After 30 min of DNA synthesis in vitro, the DNA products were identical in size to the prelabeled parental DNA. More than 60% of the newly synthesized single-stranded DNA fragments sedimented with a sedimentation constant of greater than 10 S. HSV DNA polymerase was found to be responsible for the synthesis of 80% of all in vitro made viral and most likely also cellular DNA sequences.

In a previous report (Knopf & Weissbach, 1977), we have demonstrated that the properties and requirements of the chromatin system for DNA synthesis generally resemble those of isolated nuclei systems. Besides the fact that isolated chromatin synthesizes a larger amount of DNA, it furthermore seems to be attractive in that it functions as a natural template for exogenous DNA polymerase and in that no compartments hinder the free passage of macromolecules to the site of DNA synthesis. Therefore, it may provide a possible tool for examining the role of the known DNA polymerases and it also can lead to the discovery of proteins and factors required in DNA replication. In this report we tried to further characterize the system with the intention to show if some of the previously reported parameters for DNA synthesis can be extended to chromatin isolated from infected cells. As an experimental system, we chose the herpes virus infected cell, primarily because herpes simplex virus DNA replication occurs in the nucleus of the cells (Morgan et al., 1954) and also because HSV[1] induces its own DNA polymerase (Keir et al.,

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1966). There are several reports of HSV DNA synthesis in vitro using isolated nuclei systems (Bell, 1974; Becker & Asher, 1975; Biswal & Murray, 1974; Bolden et al., 1975; Kolber, 1975). We examined if chromatin isolated from HSV-infected cells (HSV chromatin) shares the main characteristics reported for nuclei systems. We asked if HSV chromatin is capable of synthesizing viral and cellular DNA sequences at similar ratios as observed in vivo and characterized the in vitro products. In addition we tried to analyze which of the DNA polymerases of the infected cell is responsible for synthesis of cellular and viral DNA sequences in HSV chromatin.

Materials and Methods

[^{3}H]Deoxynucleoside triphosphates and [^{3}H]- and [^{14}C]-thymidine were purchased from Amersham/Buchler Corp. Phenylmethanesulfonyl fluoride was obtained from Sigma Chemical Co. and Sarcosyl NL-97 from the Ciba-Geigy Corp. Diethylaminoethylcellulose (DE-52) was supplied by Whatman. Sephadex G25 (coarse) was purchased from Pharmacia. Triton X-100 was obtained from Serva. Phosphonoacetic acid was a gift of E. Heimer and A. Cook from the Hoffmann-La Roche Research Laboratories. Proteinase K was obtained from Boehringer GmbH. Pancreatic RNase (DNase free) was supplied by Worthington.

Growth of Cells and Virus Infection. African green monkey kidney cells (RC-37, Italdiagnostic Products) were grown in monolayer cultures as previously described (Schröder et al., 1976). RC-37 cells were infected at 20 plaque forming units per cell with HSV type 1 strain ANG, Heidelberg, passaged five times at a MOI of 0.01 obtained by Dr. C. H. Schröder. At suitable times after infection (zero time of infection was the moment of virus addition), medium (modified Eagle's medium supplemented with 2% fetal calf serum) was removed and cells were harvested by scraping into reticulocyte standard buffer (Yamashita & Green, 1974) with the aid of a rubber policeman, centrifuged for 10 min at 1500*g*, and immediately used for chromatin isolation. To obtain prelabeled, infected cells, monolayer cultures were grown in the presence of [^{14}C]thymidine (2.5 μCi/mL) from 7 to 8 h postinfection.

Chromatin Isolation. Nuclei of uninfected and HSV-infected RC-37 cells were prepared according to Yamashita & Green (1974). EDTA (2.5 mM) was included in the reticulocyte standard buffer. Fresh nuclei (2×10^7) were suspended in 2 mL of buffer I containing 1 mM each of Tris-HCl (pH 8), dithiothreitol, EDTA, and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$). After centrifugation at 15000*g* for 5 min at 0 °C, the pellet was resuspended by gentle Pasteur pipetting in 1 mL of buffer II (buffer I except with 0.1 mM EDTA). Aliquots of the swollen gel-like chromatin isolate were used immediately for the described studies. Mock and HSV chromatin preparations showed a DNA to protein ratio of 0.2 to 0.3.

Assay for DNA Synthesis in Chromatin. The reaction mixture contained, in a final volume of 100 μL, 50 mM Tris-HCl buffer (pH 8), 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 10 units/mL of pyruvate kinase, 0.5 mM each of CTP, GTP, UTP, and 2.5 mM ATP, 0.1 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity, 400 cpm/pmol), and the stated amount of chromatin. A chromatin content of 10 μg of DNA was assumed to be equivalent to 10^6 nuclei. The incubation was performed at 37 °C and aliquots of the reaction mixture were spotted onto Whatman GF/C glass filters and treated to determine acid-insoluble radioactivity as described by Bollum (1968).

Cytoplasmic Extracts. Supernatants obtained from nuclei preparations containing about 6×10^7 cell equiv per mL were cleared by a high-speed centrifugation (Spinco rotor, type 50 Ti, 42000 rev/min, 2 °C, 1 h).

Whole Cell and Chromatin Extracts. HSV-infected cells and HSV chromatin preparations containing 2×10^8 cell equiv were homogenized by vigorous douncing with a narrow fitting pestle in 0.25 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, 1 mM $PhCH_2SO_2F$, and 0.5% (v/v) Triton X-100. After high-speed centrifugation (Spinco rotor, type 50 Ti, 42000 rev/min, 0 °C, 1 h), supernatants were poured onto a Sephadex column (G-25 coarse, 30 mL) and eluted in 0.01 M potassium phosphate (pH. 7.5) containing 1 mM dithiothreitol. Fractions containing DNA polymerase activity were pooled and loaded onto a DEAE-cellulose column (6 mL) equilibrated with Sephadex elution buffer. After a wash with 3 column volumes of the same buffer, the column was eluted in 1 mL fractions with 7 column volumes of a linear gradient from 0.01 to 0.4 M potassium phosphate (pH 7.5) containing 1 mM dithiothreitol and 20% (v/v) glycerol.

Assay of DNA Polymerases α, β, and γ. Aliquots (10 μL) of the DEAE-cellulose fractions were assayed under optimal conditions using 250 μg/mL activated salmon sperm DNA as template for the α- and β-polymerases and 50 μg/mL of $(dT)_{12-18}$·poly(A) for the γ-polymerase assays as already described (Knopf et al., 1976).

Purification and Assay of HSV DNA Polymerase. HSV DNA polymerase was purified up to the phosphocellulose chromatography step as previously described (Weissbach et al., 1973). The enzyme exhibited a specific activity of about 4500 units per mg of protein. The reaction mixture contained, in a final volume of 100 μL, 50 mM Tris-HCl buffer (pH 8.5), 50 μg of bovine serum albumin, 0.5 mM dithiothreitol, 7.5 mM $MgCl_2$, 100 mM ammonium sulfate, 0.1 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity, 400 cpm/pmol), and 25 μg of activated salmon sperm DNA. Incubations were performed at 37 °C for 20 min. A unit is defined as that amount of enzyme catalyzing the polymerization of 1 nmol of nucleotide per h under standard assay conditions.

CsCl Equilibrium Density Gradient Centrifugation. Chromatin reaction mixtures were stopped by chilling in ice and by addition of 25 mM EDTA and were then incubated for 1 h at 55 °C in the presence of 1% Sarkosyl NL-97 and 0.5 mg/mL of proteinase K. After phenol and chloroform–isoamyl alcohol (24:1) extraction, DNA was precipitated with alcohol in the presence of 1 M LiCl and redissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA. The DNA solution (5.3 mL) was then adjusted to a density of 1.71 g/cm^3 with CsCl and centrifuged for 50 h to equilibrium at 42000 rev/min at 25 °C in a Spinco type 50 Ti rotor.

Alkaline Sucrose Sedimentation. Aliquots of chromatin reaction mixtures were treated for alkaline sucrose gradient centrifugation as previously described (Knopf & Weissbach, 1977).

Neutral Sucrose Sedimentation. Aliquots of chromatin reaction mixtures were treated as described for CsCl density sedimentation, except that after chloroform–isoamyl alcohol extraction the aqueous phase was dialyzed against 10 mM Tris-HCl buffer (pH 8) containing 10 mM EDTA and 0.1%

[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HSV, herpes simplex virus; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; ANG, Angelotti; MOI, multiplicity of infection; p.i., post infection.

Table I: Fate of Prelabeled DNA during HSV Chromatin Isolation[a]

isolation step	[^{14}C]thymidine-labeled DNA	
	acid precipitable (cpm)	% of total DNA
whole cell	2.24×10^6	100.0
nuclei supernatant	0.23×10^6	10.3
chromatin wash	4200	0.2
chromatin	2.00×10^6	89.3

[a] HSV type 1 infected RC-37 cells (2×10^7) were prelabeled with 2.5 μCi/mL of [^{14}C]thymidine (55 mCi/mmol) between 4 and 8 h post infection. Nuclei as well as chromatin were then prepared as described under Materials and Methods. Aliquots of the indicated fractions were acid precipitated onto glass filters and further processed as previously described (Bollum, 1968). Dried glass filters were incubated with 0.5 mL of a tissue solubilizer and counted in 10 mL of a toluene-based scintillation cocktail.

Sarcosyl NL-97. Samples (0.2 mL) were then loaded on top of 5–25% (w/v) sucrose gradients containing 1 M NaCl, 10 mM Tris-HCl (pH 8), 10 mM EDTA, and 0.1% Sarcosyl NL-97. Centrifugation was carried out in a Spinco SW 65 rotor at 47 800 rev/min for 25 h at 15 °C.

In general, gradients were dripped from the bottom and fractions collected directly onto glass filters and further processed to determine acid-precipitable radioactivity as previously described (Bollum, 1968). All data were computed for ^{3}H- and ^{14}C-channel overlap. DNA and protein concentrations were determined as previously described (Knopf & Weissbach, 1977).

Results

Preparation of HSV Chromatin. Chromatin was isolated from HSV type 1 infected RC-37 cells as previously described for HeLa chromatin (Knopf & Weissbach, 1977). When we examined the fate of DNA prelabeled with [^{14}C]thymidine between 4 and 8 h post infection during the original isolation procedure, we observed that about 10% of the label was lost by the last two chromatin washes. We shortened therefore the washing procedure as described under Materials and Methods. Using the modified isolation procedure, we could reduce the lose of prelabeled DNA to 0.2%, and the HSV chromatin preparation contained now about 90% of the total, 4–8 h p.i. [^{14}C]thymidine-prelabeled DNA of HSV-infected cells (Table I).

Characterization of in Vitro DNA Synthesis of HSV Chromatin. Chromatin was isolated from RC-37 cells at 8 h after mock and HSV type 1 infection. The requirements of both chromatin preparations for endogenous DNA synthesis in vitro were compared (Table II). In a similar fashion to that reported for isolated nuclei systems (Bolden et al., 1975; Kolber, 1975), HSV chromatin synthesized DNA at a 35-fold higher rate than the corresponding chromatin from uninfected cells (mock chromatin). In the absence of three deoxynucleotides, the DNA synthesis activity of HSV chromatin was reduced to 8%. Optimal DNA synthesis was observed in the presence of all four ribonucleoside triphosphates and a triphosphate generating system, whereas in their absence DNA synthesis was reduced 5-fold for HSV chromatin and 2.3-fold for mock chromatin, respectively. In the absence of ATP and the generating system, DNA synthesis of mock and HSV chromatin was 2.2- and 2.5-fold lower. In the presence of pancreatic RNase, the DNA synthesis activity of both HSV as well as mock-chromatin was inhibited about 2-fold. Addition of salt had a pronounced influence upon the DNA synthesis activity of HSV chromatin and was found to be less stimulatory for DNA synthesis of mock chromatin (Table II and Figure 1). With the reaction conditions described under Materials and Methods, a broad optimal pH spectrum of pH 8–10 was found for HSV chromatin. The optimal pH for mock chromatin was 7.5. The optimal reaction temperature was determined to be 41 °C for HSV chromatin and 38.7 °C for mock chromatin. At 25 °C the endogenous DNA synthesis activity of HSV chromatin was 10-fold lower.

Table II: Requirements of HSV Chromatin System for DNA Synthesis[a]

reaction mixture	activity (%)	
	mock chromatin	HSV chromatin
complete, + 2.5 mM ATP	100	100
complete, + 0.5 mM ATP	75	83
−ATP, phosphoenolpyruvate, pyruvate kinase	46	40
−ATP, CTP, GTP, UTP, phosphoenolpyruvate, pyruvate kinase	43	19
−CTP, GTP, UTP	67	63
−dATP, dCTP, dGTP	31	8
$-MgCl_2$	13	6
+RNase A, 250 units	40	58
+KCl, 100 mM	71	314
$+(NH_4)_2SO_4$, 20 mM	121	260
$+KPO_4$, 50 mM	93	386

[a] Chromatin reaction mixtures (100 μL) containing 33 μg/mL DNA equivalents of mock or HSV chromatin isolated 8 h post infection and omitting ammonium sulfate were incubated for 20 min at 37 °C as described under Materials and Methods. The complete system incorporated 1.82 pmol of deoxynucleotides with mock chromatin and 64.1 pmol with HSV chromatin, respectively.

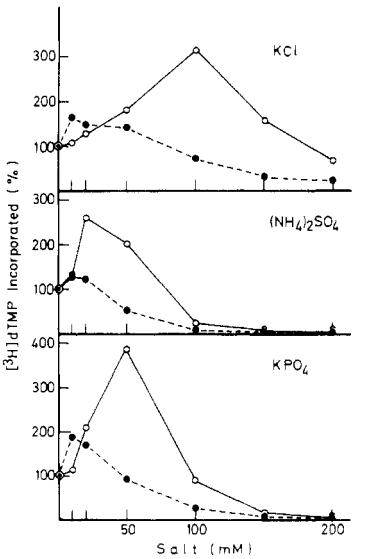

FIGURE 1: Influence of salt on DNA synthesis activity of isolated chromatin. Chromatin assays containing 33 μg/mL DNA equiv of mock (●) or HSV chromatin (○) were performed at different salt concentrations as described in Table II.

DNA Synthesis Activity of Chromatin Isolated at Various Times during HSV Replication. The DNA synthesis activity of chromatin isolated at 4, 8, and 12 h after HSV infection was compared. The time course of DNA synthesis in these chromatin preparations is presented in Figure 2. Whereas mock chromatin synthesized DNA at a rate of 10 pmol of deoxynucleotides per 10 min and 10^6 nuclei, HSV chromatin showed rates of 85, 130, and 220 pmol when prepared from infected cells after 4, 8, and 12 h, respectively. The observed rates of DNA synthesis for HSV chromatin were similar to those reported for isolated nuclei systems (Bolden et al., 1975).

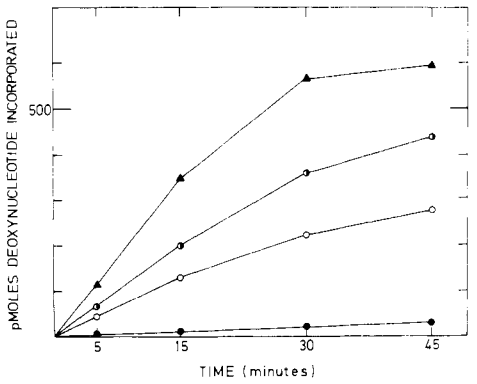

FIGURE 2: Kinetics of DNA synthesis in isolated chromatin. Chromatin was isolated from mock and HSV type 1 infected RC-37 cells and was assayed as described under Materials and Methods. Symbols: mock chromatin isolated 4 h p.i. (●) and HSV chromatin isolated 4 h p.i. (○), 8 h p.i. (◑), and 12 h p.i. (▲), respectively. Data are expressed as total deoxynucleotides incorporated into an acid-insoluble form and normalized per 1×10^6 nuclei equiv.

Table III: Effect of Addition of HSV Polymerase and Cytoplasm on DNA Synthesis Activity of Isolated Chromatin[a]

chromatin		% act. after addition of		
source	concn (nuclei equiv)	HSV polymerase	HSV cytoplasm	mock cytoplasm
HSV	5×10^5	147	146	100
	1×10^6	120	120	98
mock	5×10^5	920	930	105
	1×10^6	890	900	145

[a] Chromatin reaction mixtures containing various amounts of mock and HSV chromatin isolated 6 h post infection were incubated for 20 min at 37 °C with or without HSV DNA polymerase (1 unit), HSV or mock cytoplasm (6×10^5 cell equiv in 10 μL) prepared as described under Materials and Methods. One hundred percent values for 10^6 nuclei equiv are 3.8 pmol of incorporated deoxynucleotides for mock chromatin and 244.8 pmol for HSV chromatin, respectively.

Therefore, it could appear that the endogenous in vitro DNA synthesis activity reflected the actual cellular DNA synthesis occurring at the time of chromatin preparation. Since CsCl density gradient analysis of the DNA of 10- and 20-min, [^{3}H]thymidine-pulse-labeled HSV-infected RC-37 cells revealed that the rate of HSV DNA synthesis was at a maximum between 8 and 12 h after infection, this idea seemed to be further supported.

Effect of Addition of HSV Polymerase and Cytoplasm. We have previously shown (Knopf & Weissbach, 1977) that chromatin represented a template for exogenously added DNA polymerases. Similarly, DNA synthesis of mock as well as HSV chromatin was stimulated by addition of both HSV DNA polymerase (phosphocellulose enzyme) and HSV cytoplasm. Addition of HSV DNA polymerase to HSV chromatin resulted in slight stimulation, whereas when added to mock chromatin DNA synthesis was stimulated ninefold (Table III). A similar stimulation of DNA synthesis was observed when HSV cytoplasm was added to mock or HSV chromatin. Addition of mock cytoplasm to either mock or HSV chromatin gave no corresponding stimulatory effect on DNA synthesis activity.

Which Are the DNA Polymerases Responsible for the DNA Synthesizing Capability of HSV Chromatin? To obtain information about the DNA polymerase content of HSV chromatin and of whole infected RC-37 cells prepared 8 h post infection, high salt extracts were performed, and DNA polymerases were purified by DEAE-cellulose chromatography as described. Then specific DNA polymerase assays for α, β, and γ (Knopf et al., 1976) and for HSV DNA polymerase (see Materials and Methods) were applied to determine the total activity of each of the DNA polymerases present. As the main result we found that HSV chromatin contained HSV DNA polymerase as well as α-, β-, and γ-polymerase (Table IV). α- and HSV DNA polymerase were present to a relatively lower amount with respect to β-, and γ-polymerase found in the whole cell. The HSV DNA polymerase activity represented about 90% of the total DNA polymerase activity and was, therefore, the dominant DNA synthesizing activity in HSV chromatin isolated 8 h post infection. This result was supported by the finding that phosphonoacetic acid shown to inhibit HSV as well as α-polymerase (Mao et al., 1975; Bolden et al., 1975) inhibited exclusively the DNA synthesis of HSV chromatin (Figure 3). The DNA synthesizing activity of HSV chromatin was inhibited to 85% with 10 μg/mL of phosphonoacetic acid, whereas no inhibitory effect was noted on the DNA synthesis activity of mock chromatin. This result indicated again that HSV DNA polymerase was the major DNA synthesizing activity in HSV chromatin.

Table IV: Distribution of DNA Polymerases in HSV-Infected RC-37 Cells and in HSV Chromatin[a]

DNA polymerase	distribution in	
	whole cell	chromatin
α	3.9	1.3
β	1.1	1.2
γ	1.0	1.0
HSV	91.0	23.0

[a] The content of α-, β-, γ-, and HSV DNA polymerase of whole cell and chromatin extracts prepared 8 h post infection from HSV type 1 infected RC-37 cells (2×10^8 cells) was determined after DEAE-cellulose chromatography as described under Materials and Methods. The data are listed as relative distribution of the individual polymerases, whereby the γ-polymerase content (total units/h) was taken as 1. The total γ-polymerase content was 84 units/h in whole cells and 19 units/h in HSV chromatin, respectively.

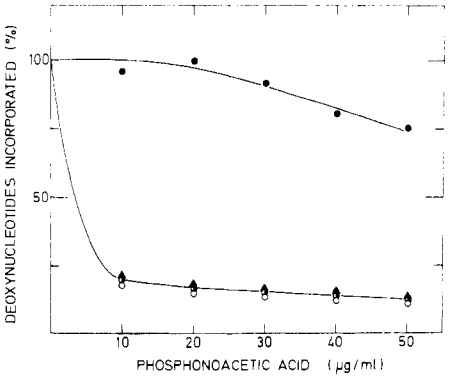

FIGURE 3: Effect of phosphonoacetic acid upon DNA synthesis of isolated chromatin. Chromatin reaction mixtures containing either mock chromatin isolated 4 h p.i. (●) or HSV chromatin isolated 4 h p.i. (○), 8 h p.i. (◑), and 12 h p.i. (▲) at a concentration of 500 μg/mL DNA equiv were incubated for 20 min in the presence of phosphonoacetic acid as described under Materials and Methods.

Characterization of the in Vitro DNA Product. To compare the ratio of viral and cellular DNA sequences synthesized by HSV chromatin with that found in vivo, we pulse-labeled infected cells between 7 and 8 h post infection with [^{14}C]-thymidine prior to chromatin preparation. Then DNA synthesis was performed in vitro in the presence of ^{3}H-labeled deoxynucleotides for 30 min at 25 °C. The in vitro product

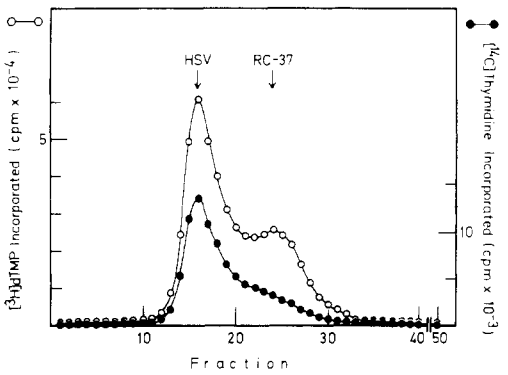

FIGURE 4: Characterization of DNA sequences synthesized by HSV chromatin. Eight hour HSV chromatin was isolated from RC-37 cells prelabeled from 7 to 8 h p.i. with 2.5 μCi/mL [^{14}C]thymidine (55 mCi/mmol). Chromatin reaction mixtures (0.5 mL) containing 600 μg/mL DNA equiv and [^{3}H]dTTP at a specific activity of 2000 cpm/pmol were incubated for 30 min at 25 °C, and the DNA was isolated as described. Aliquots containing 60 μg of DNA were subjected to neutral CsCl equilibrium density gradient centrifugation as described under Materials and Methods. Arrows indicate the position of [^{3}H]HSV DNA (1.726 g/cm^3) and [^{14}C]RC-37 DNA (1.700 g/cm^3) which were sedimented together in a parallel tube. Symbols: (●) [^{14}C]thymidine prelabel and (○) [^{3}H]dTMP-labeled in vitro product.

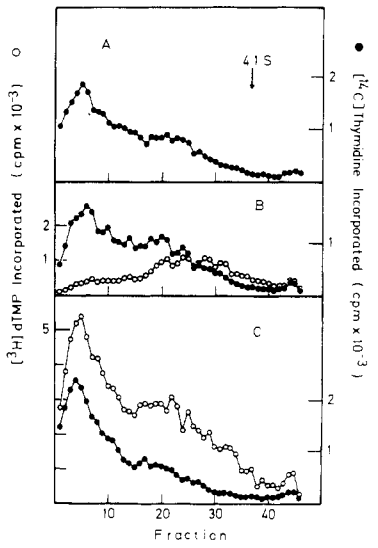

FIGURE 5: Size analysis of HSV chromatin DNA products by alkaline velocity sedimentation. HSV chromatin was isolated from prelabeled cells and assays were performed as described in the legend of Figure 4. Aliquots containing 17 μg of DNA equiv were withdrawn after 5 and 30 min of DNA synthesis in vitro and subjected to alkaline velocity sedimentation as described under Materials and Methods. Sedimentation profiles derived from (A) unincubated [^{14}C]thymidine prelabeled HSV chromatin (●), (B) after 5 min and (C) after 30 min of DNA synthesis in vitro (○). Sonicated calf thymus DNA (4.1 S) was used as an external marker.

was isolated and examined by CsCl equilibrium density gradient centrifugation as described. Quantitative evaluation of the sedimentation profiles revealed that 67.8% of the in vivo prelabeled DNA banded in the position of viral DNA (Figure 4). The in vitro DNA product, 65.5%, was also found in the density position of viral DNA. From this result we concluded that HSV chromatin synthesized both viral and cellular DNA sequences at ratios identical with that seen in vivo.

Size Analysis of the in Vitro DNA Product. HSV chromatin was isolated 8 h post infection from cells prelabeled as described in the section above, and the DNA synthesis was performed for 0, 5, and 30 min at 25 °C. The DNA products of aliquots of the chromatin reaction mixture were analyzed

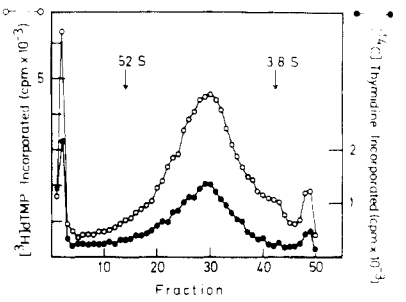

FIGURE 6: Size analysis of HSV chromatin DNA products by neutral velocity sedimentation. HSV chromatin was isolated and assays were performed as described in Figure 5. An aliquot containing 17 μg of DNA equiv was withdrawn after 30 min of DNA synthesis in vitro and subjected to neutral velocity sedimentation as described under Materials and Methods. [^{3}H]HSV DNA whose sedimentation constant was determined according to Studier (1965) and sonicated calf thymus DNA were used as external standards. Symbols: (●) [^{14}C]thymidine prelabel and (○) [^{3}H]dTMP-labeled in vitro product.

by velocity sedimentation under alkaline and neutral conditions. Figure 5 shows the results obtained by alkaline velocity sedimentation of the HSV chromatin products. After 5 min of in vitro synthesis (Figure 5B), 5B), the newly synthesized single-stranded DNA sedimented with an average sedimentation coefficient of 10 S. In contrast, the bulk of the prelabeled parental DNA sedimented with larger than 10 S. After 30 min, the in vitro DNA products sedimented identically as the prelabeled parental DNA (Figure 5C), and the majority of the DNA fragments (about 60%) exhibited a sedimentation constant of larger than 10 S. It should be emphasized that no dramatic degradation both of the parental DNA and of the newly made DNA was observed. The sedimentation pattern of the prelabeled DNA of the unincubated HSV chromatin (Figure 5A) was identical with that at 30 min of DNA synthesis (Figure 5C). The newly synthesized DNA after 30 min of DNA synthesis was further analyzed by neutral velocity sedimentation (Figure 6). Again the in vitro DNA products sedimented identically as the in vivo prelabeled DNA. The majority of the DNA sedimented with 27 S corresponding to about 15 × 10^6 daltons (Studier, 1965).

Discussion

With the impetus to obtain an in vitro system to study HSV DNA replication, we tried to adapt the previously introduced chromatin system (Knopf & Weissbach, 1977). After applying some modifications for isolation of HSV chromatin and for assaying DNA synthesis in vitro, we succeeded in elaborating a HSV chromatin system. Control experiments with prelabeled cells indicated that HSV chromatin preparations contained the same amount of DNA as isolated, intact nuclei (Table I). Optimal DNA synthesis was achieved in the presence of all four ribonucleoside triphosphates, a triphosphate generating system, and salt. The main differences between mock and HSV chromatin were found in the requirements for salt (Figure 1), pH, and temperature. The presented HSV chromatin system exhibited a quite striking similarity to isolated nuclei systems. The rates of DNA synthesis in vitro of HSV chromatin corresponded to those obtained from isolated nuclei of HSV type 1 infected HeLa cells (Bolden et al., 1975). The ratio of viral and cellular DNA synthesized by HSV chromatin was identical both with the ratio found in vivo (Figure 4) and with the ratio observed in isolated nuclei systems of HSV type 1 and type 2 infected cells (Bolden et al., 1975; Kolber, 1975). The in vitro DNA products of HSV chromatin were of considerably large size. More than 60%

of the single-stranded DNA sedimented with greater than 10 S after 30-min synthesis, and the in vitro made DNA showed an identical sedimentation pattern as the in vivo prelabeled parental DNA (Figure 5C). Furthermore, also under neutral conditions, the majority of the in vitro synthesized DNA sedimented together with the parental DNA and revealed a size corresponding to about one-sixth of the viral genome (Figure 6). A comparable size was obtained in isolated nuclei of HSV-2 infected HEL cells (Kolber, 1975).

HSV DNA polymerase was found to be most likely responsible for the synthesis of 80% of all in vitro made DNA sequences as shown by the following: 90% of the total DNA polymerase activity of HSV chromatin could be ascribed to HSV DNA polymerase (Table IV). DNA synthesis in HSV chromatin was inhibited to about 85% by phosphonoacetic acid, whereas DNA synthesis in mock chromatin remained unaffected (Figure 3). DNA synthesis of mock chromatin was stimulated about six- to sevenfold more than HSV chromatin in the presence of both HSV DNA polymerase and HSV cytoplasm (Table III), indicating that HSV DNA polymerase might be involved in the synthesis of cellular DNA sequences in vitro. While this work was in progress, further evidence on this subject was presented by immunological studies (Yamada et al., 1978).

In summary, it should be emphasized that, as shown earlier with HeLa chromatin, chromatin isolated from HSV-infected cells also proved useful as an in vitro system for studying DNA synthesis. Even though HSV chromatin DNA synthesis shares the main characteristics of isolated, intact nuclei, it still remains a superior system by its accessibility for macromolecules in studying regulatory and accessory proteins in viral DNA synthesis.

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