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Cell-Free Synthesis of Herpes Simplex Virus DNA: Conditions for Optimal Synthesis†

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ABSTRACT: The reaction conditions for DNA synthesis in two cell-free systems from HSV-1 infected BHK cells are examined: (1) an unfractionated hypotonic lysate; and (2) purified nuclei. The lysate contains altered deoxynucleoside triphosphate pools at 15 h after infection when compared with uninfected lysates (2-fold increase for dCTP and dGTP, 25-fold increase for TTP, no change for dATP). The TTP pool is at its maximum at 12 h after infection at 31 °C. It is utilized in vitro for the synthesis of viral and cellular DNA in the lysate and is reduced to 1.5 μM in purified nuclei. The required concentrations of deoxynucleoside triphosphates for maximal synthesis in purifed nuclei are 10 μM for dATP and TTP, and 50 μM for cDTP and dGTP. The initial rate of synthesis in purified nuclei is independent from added ATP or the other three ribonucleoside triphosphates. Concentrations up to 0.5 mM ribonucleoside triphosphate stabilize the in vitro product against nucleolytic degradation and higher concentrations inhibit the initial rate. These effects are not specific for ATP or ribonucleoside triphosphates and can also be achieved with TTP, creatine phosphate, and inorganic pyrophosphate. The salt optimum for both systems is lower than for the corresponding uninfected systems. Cl^- is inhibitory in the nuclear system above 40 mM and in the lysate at any concentration. When used as acetate salt, K^+ was less inhibitory than Na^+ or NH_4^+. The Mg^{2+} optimum for cellular DNA synthesized in infected systems corresponds to that of uninfected systems and is lower than that for viral DNA (3 mM in the lysate, 5 mM in nuclei). Purified nuclei showed optimal synthesis between pH 7 and pH 8, where lysates are minimally active. The stimulation of synthesis in the lysate at pH 6 and pH 9 is most likely secondary to the action of nucleases. Both systems continue synthesis of viral and cellular DNA in the same proportion as labeled with [^{3}H]thymidine in vivo at different times after infection. At 12 h after infection at 31 °C the initial rate of viral DNA synthesis in the lysate is 9.6 pmol of deoxynucleotide per min per 10^7 nuclei at 31 °C. Purified nuclei are almost equally active (84% of the rate in the lysate), indicating a lack of requirement for cytoplasmic factors. Addition of cytosol from uninfected cells to purified nuclei is slightly inhibitory, while cytosol from infected cells stimulates initially and results in degradation of in vivo prelabeled and in vitro labeled viral DNA at later times.

The biochemical dissection of the complex process of DNA replication in prokaryotic organisms has been greatly facilitated by the use of mutants affecting the various enzymatic functions involved. A similar approach to DNA replication in eukaryotic cells is not possible at this time. But for a number of DNA viruses, which replicate in the nucleus of mammalian cells, conditional lethal mutants with defects in viral DNA synthesis are available. For the replication of viral DNAs, therefore, it appears experimentally feasible to approach the individual functions that comprise the replication complex biochemically by developing complementation assays based on cell-free DNA synthesis derived from mutant-infected cells. This approach has successfully been applied to the prokaryotic DNA virus, T4 (Alberts et al., 1975; Imae et al., 1976).

The study presented here was undertaken in order to establish optimal conditions for cell-free DNA synthesis systems derived from herpes simplex virus (HSV-1)[1]-infected BHK cells. This virus turns off host cell macromolecular synthesis early during lytic infection and presumably codes for most, if not all, functions required to replicate its DNA. Its large genome (100×10^6 daltons of double-stranded DNA) and the observation that more than half of the genetic complementation groups defined so far (Subak-Sharpe et al., 1974; Benyesh-Melnick et al., 1974) have a DNA-negative phenotype further support this notion. Among the new enzymatic activities related to DNA metabolism induced after infection are a DNA polymerase, an exonuclease (Keir and Gold, 1963),

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[1] Abbreviations used: HSV-1, herpes simplex virus, type I; pfu, plaque-forming units; Hepes, *N*-2-hydroxyethylpiperazine-*N*′,2′-ethanesulfonic acid; dThd, deoxythymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

and a deoxypyrimidine kinase (Dubbs and Kit, 1964). In addition, a characteristic set of new DNA binding nonstructural polypeptides has been demonstrated in infected cells (Bayliss et al., 1975), some of which are likely to be involved in DNA replication. In order to retain all factors potentially involved in HSV DNA replication, an unfractionated lysate consisting of hypotonically disrupted cells, capable of incorporating labeled deoxynucleoside triphosphates into DNA, is used, similar to the system previously described for polyoma-infected cells (Hunter and Francke, 1974). In addition, a system consisting of purified nuclei (Francke and Hunter, 1975) is investigated.

A number of studies on DNA synthesis in isolated nuclei from HSV-infected cells have been published during recent years (Kolber, 1975; Bolden et al., 1975; Becker and Asher, 1975; Biswal and Murray, 1974). In general, such systems are capable of synthesizing viral and cellular DNA and exhibit sensitivity to inhibitors and antiserum-specific for the viral DNA polymerase. Major problems are the small size of the in vitro product and the short duration of in vitro synthesis (Shlomai and Becker, 1975) which in some cases is followed by extensive degradation of the in vitro product (Biswal and Murray, 1974). Another drawback for the interpretation of results obtained from in vitro studies is the lack of knowledge about the replication process in vivo. Recent studies have demonstrated the existence of structures sedimenting faster than mature viral DNA as candidates for replicating intermediates (Ben-Porat et al., 1976; Shlomai and Becker, 1975), but little is known about the molecular mechanisms involved in replication.

As a basis for an in vitro approach to HSV DNA replication, the conditions for cell-free synthesis were studied. Optimal conditions for several parameters were found to differ from those of uninfected cells and the results presented are discussed in terms of the known intracellular changes occurring after infection. In an accompanying report, the structure of the in vitro product and the pattern of degradation of viral DNA favored under certain incubation conditions are examined.

Experimental Procedure

(1) *Cell Culture, Virus, and Growth Conditions.* BHK 21 (C13) cells were used for all experiments. They were grown as monolayers on 9-cm plastic dishes (Nunc) or glass roller bottles (Bellco) in Dulbecco-modified Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.) and 10% tryptose phosphate at 37 °C. Confluent monolayers yielded 2×10^7 cells per 9-cm dish.

HSV-1 (Glasgow strain) ts^+syn^+ was obtained from Dr. J. H. Subak-Sharpe. The virus was passaged once at low multiplicity (1 pfu/300 cells) and stored at −70 °C in 10% serum. Virus stocks prepared after a similar second passage were used for all experiments.

For infection, confluent monolayers of BHK cells were washed once with isotonic Tris buffer. Virus, diluted to the desired titer in isotonic Tris buffer containing 10% calf serum, was adsorbed to the cells for 1 h at 37 °C. The inoculum was then removed and fresh medium added. When extensive cytoplasmic effect was apparent (4 to 6 days at 37 °C), the cells were removed from the surface and after pelleting resuspended in 1 mL of supernatant medium per 2×10^8 cells. Cell-associated virus was liberated by sonication and clarified by low-speed centrifugation. The pellet of cell debris was resuspended in the same volume of supernatant medium; the suspension was resonicated and clarified. The two supernatant fractions were combined and stored in aliquots at −70 °C. Virus yields varied between 20 and 200 pfu per infected cell.

Virus stocks were assayed for plaque-forming ability by infecting monolayers of cells in 5-cm plastic dishes with appropriate virus dilutions as described above. After adsorption, 5 mL of growth medium containing 0.45% agarose was added to each dish and allowed to solidify. After 2 to 3 days at 37 °C, the agar was removed, the cell layer was fixed with 25% ethanol and stained with 1% crystal violet, and the plaques were counted.

High multiplicity infections for DNA synthesis experiments were done at 10 pfu per cell. After adsorption and removal of the inoculum, the cell layer was washed with isotonic Tris buffer and after addition of fresh medium the cells were incubated at 31 °C, generally for 12 h.

(2) *Cell-Free DNA Synthesis.* Preparation of the lysate system and purified nuclei was essentially as previously described for polyoma-infected 3T3 cells (Hunter and Francke, 1974; Francke and Hunter, 1975). The modified procedure necessary for optimal synthesis from HSV-infected cells was as follows. Hypotonic buffer contained 20 mM Hepes adjusted to pH 7.9 with KOH (0.5 M K^+ per 1 M Hepes), 1 mM $MgCl_2$, 1 mM dithiothreitol. Sucrose buffer contained 200 mM Hepes, pH 7.9, and 2.5 M sucrose. Isotonic buffer was a 9:1 mixture of hypotonic and sucrose buffers. Infected cells were washed with cold (4 °C) isotonic Tris buffer, swollen for 5 min with 5 mL of hypotonic buffer per 9-cm dish, drained, and scraped, yielding 150 to 250 μL of hypotonic lysate per 2×10^7 cells. Isotonic lysate consisted of 9 parts hypotonic lysate and 1 part sucrose buffer. Nuclei were prepared by diluting the hypotonic lysate sixfold with hypotonic buffer and treating with 0.04% NP-40 for 20 min. After pelleting the nuclei through 20% Ficoll in hypotonic buffer, they were washed two times with 20 volumes of isotonic buffer containing 50 mM KCl and finally resuspended in 1.1 volumes of isotonic buffer (1 volume equals the volume of the hypotonic lysate). Recovery of nuclei was 85% as judged by the recovery of viral DNA, prelabeled in vivo for 2 h at 31 °C with [^{3}H]dThd. Cytosol was obtained by homogenizing the hypotonic lysate in a tight fitting Dounce homogenizer, adding 0.1 volume of sucrose buffer and potassium acetate (pH 7.5) to 60 mM. After a low-speed spin, the supernatant was centrifuged for 2 h at 30 000*g* and 2 °C.

For in vitro DNA synthesis in the lysate and nuclei, the following additions were present (final concentrations, including the components of the isotonic buffer): 250 μM ATP; 100 μM each of CTP, GTP, UTP, dATP, dCTP, dGTP; 10 μM TTP, 1 mM dithiothreitol; 1 mM EGTA; 40 mM Hepes, pH 7.9; 5 mM $MgCl_2$; 80 mM potassium acetate (total K^+ = 100 mM); α-^{32}P- or ^{3}H-labeled TTP, as detailed with each individual experiment. The reaction was terminated by adding 900 μL of 20 mM Hepes, pH 7.9, with 5 mM EDTA, 100 μL of 20% $NaDodSO_4$, and 50 μL of Pronase (10 mg/mL, pretreated for 30 min at 60 °C in 0.2 M potassium acetate) to 100 μL of reaction. After at least 4 h at 31 °C, 100-μL aliquots were spotted onto Whatman 3 MM cellulose filter discs and batch washed (ca. 20 min at 4 °C for each wash) as follows: three times in 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate, two times in 5% trichloroacetic acid, and two times in 95% ethanol. After drying, they were counted in 3 mL of toluene containing 2,5-diphenyloxazole (5 g/L) using a Beckman LS200 scintillation counter. Counting efficiencies were 20% for ^{3}H and 90% for ^{32}P.

Both preparation conditions for lysate or nuclei and incubation conditions were modified occasionally. Deviations from the basic procedure described here will be cited with each experiment.

(3) *Centrifugation Techniques.* Viral DNA was separated

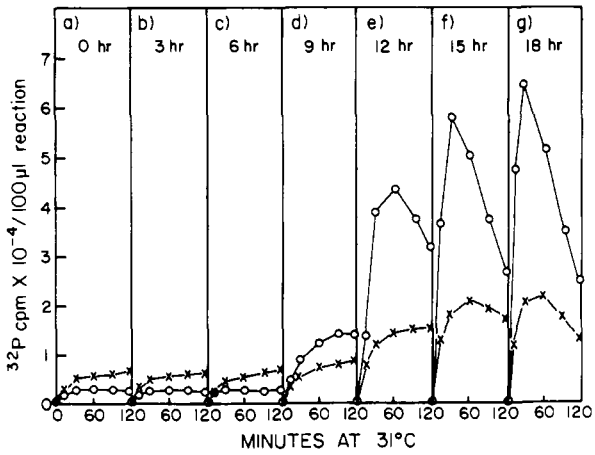

FIGURE 1: Incorporation of [α-^{32}P]TTP into lysates and purified nuclei from BHK cells, at various times after infection with 10 pfu of HSV-1 per cell at 31 °C. Preparation of lysates, purification of nuclei, and incubation conditions were as described in Experimental Procedure. For each set, eight 9-cm plates were labeled with 5 µCi of [^{3}H]dThd/mL for 1 h prior to the time after infection indicated. Lysate (0.6 mL) and 0.6 mL of nuclei, prepared at the times indicated, were incubated at 31 °C in the presence of [α-^{32}P]TTP (10 µM, 20 µCi/mL). Samples (100 µL) were removed during the reaction and the total incorporated ^{32}P radioactivity was determined. (X—X) Lysate, (O—O) nuclei, before (a) and at 3 (b), 6 (c), 9 (d), 12 (e), 15 (f), and 18 (g) h after infection.

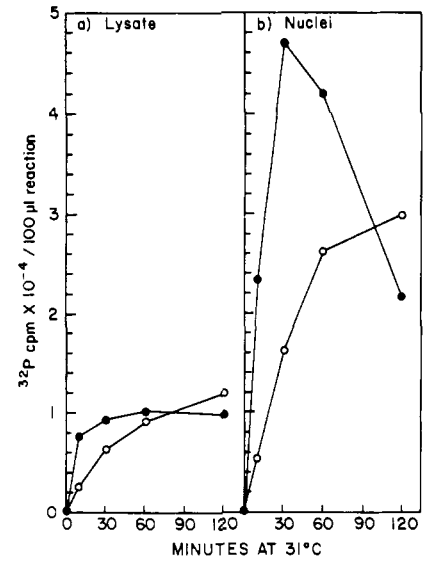

FIGURE 2: Viral and cellular DNA labeled in the lysate and purified nuclei from cells 15 h after infection with HSV-1. Aliquots of the samples taken at 10, 30, 60, and 120 min during the reaction shown in Figure 1f were centrifuged to equilibrium in CsCl density gradients as described in Experimental Procedure. The areas under the dense peak (viral DNA) and the light peak (cellular DNA) from the gradient profiles were integrated separately. (●—●) Viral DNA, (O—O) cellular DNA, labeled in the lysate (a) or purified nuclei (b).

from host cell DNA on the basis of density: 1 mL of sample (after NaDodSO$_4$ and Pronase treatment, see above) was added to 4.5 mL of a solution containing 1.233 g of CsCl per mL in H_2O and the density adjusted to 1.700 g/cm^3. Centrifugation was for 60 h at 34 000 rpm and 20 °C, using the 50 Ti angle rotor in a Beckman L2-50 ultracentrifuge. Twenty-five fractions were collected from the bottom of the tube directly onto cellulose filter discs and processed for determination of acid-precipitable radioactivity as described above. Recovery of input radioactivity was >90%. The values for viral and cell DNA throughout the results section represent the integrated radioactivity under the dense peak and the light peak, respectively. If further characterization of the viral DNA was performed, collection was into tubes and the viral DNA, located by assaying aliquots for radioactivity, was dialyzed against two changes of 10 mM Tris-Cl buffer, pH 8.0, 100 mM NaCl, 1 mM EDTA. The size of the DNA was determined by neutral sucrose gradient sedimentation: 200 µL of sample, containing the appropriate markers, were layered on top of a 3.6-mL gradient of 5 to 20% sucrose in 10 mM Tris, pH 8, 1 mM EDTA, and 1 M NaCl. Centrifugation was for 2.5 h at 15 °C and 45 000 rpm in a Beckman SW-56 rotor. Twenty-five to twenty-eight fractions were collected from the bottom of the tube and assayed for radioactivity. The sedimentation markers used were: HSV-1 DNA (55S prepared as described by Skare et al., 1975), polyoma DNA forms I and II (20S and 16S, respectively, kindly provided by W. Cogen), and polyoma DNA restriction endonuclease *Hpa*II, fragment 3 (7.6 S, kindly provided by Dr. M. Vogt).

(4) *Chemicals and Radioisotopes.* [*methyl*-^{3}H]TTP (13.4 Ci/mmol) and the four α-^{32}P-labeled deoxynucleoside triphosphates (130 to 135 Ci/mmol) were from New England Nuclear Corp. Ficoll 400 was from Pharmacia, dithiothreitol was from Calbiochem, and unlabeled ribo- and deoxyribonucleoside triphosphates were from Sigma.

Results and Discussion

Cell-Free DNA Synthesis at Different Times after Infection. The incorporation of [α-^{32}P]TTP into acid-precipitable

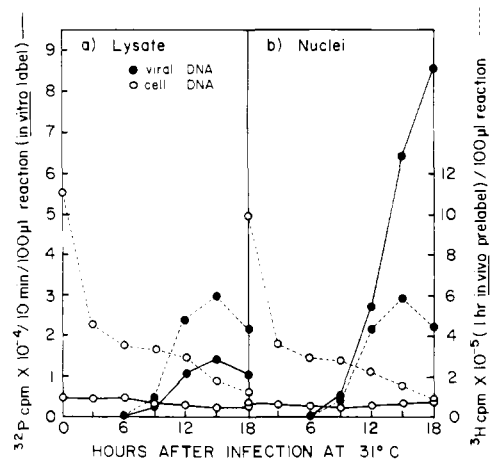

FIGURE 3: Comparison of viral and cellular DNA, labeled in vivo with [^{3}H]dThd and in vitro with [α-^{32}P]TTP, at various times after infection. Aliquots of the 10-min points of the reaction shown in Figure 1 were analyzed as described in the legend to Figure 2. (- -) In vivo prelabel ^{3}H, and (—) in vitro label ^{32}P, in viral (●) and cellular (O) DNA, after 10 min of incubation in the lysate (a) or purified nuclei (b).

products by lysates or nuclei from BHK cells at various times after infection with HSV is shown in Figure 1. Up to 6 h after infection the product consisted exclusively of cellular DNA, whereas, at later times, both viral and cellular DNA were labeled (shown for the 15-h postinfection sample in Figure 2). The identification of the two DNA types throughout this study is based on their different densities. It should be mentioned, though, that, at late times during infection, viral sequences are present in the cellular peak (Bigeleisen and Rush, 1976). Figure 2 demonstrates that at 15 h the short-term in vitro product is predominantly viral in nature; upon longer incubation, cell DNA continues to be synthesized while incorporation into viral DNA levels off and later the product becomes degraded to acid-soluble material. In order to compare the in vitro synthesis with the in vivo situation (Figure 3), the early in vitro products (10-min incubation) were therefore chosen.

TABLE I: Deoxyribonucleotide Pools in Uninfected and HSV-Infected Systems (μM).[a]

	dATP	dCTP	dGTP	TTP
(a) Lysate from uninfected BHK cells	4 (3-5)	7 (5-9)	2 (1-3)	4 (3-5)
(b) Lysate from BHK cells infected with HSV for 15 h at 31 °C	4 (3-5)	12 (9-15)	5 (3-7)	105 (90-120)

	Time after infection				
	0 h	6 h	9 h	12 h	15 h
(c) TTP pool in BHK cell lysate, infected with HSV at 31 °C	5 (3-7)	10 (5-15)	20 (14-26)	80 (65-95)	60 (40-80)

	For viral DNA synthesis	For cellular DNA synthesis
(d) TTP pool in BHK cell lysate after infection with HSV for 12 h	75 (60-90)	80 (55-105)
(e) TTP pool in purified nuclei from BHK cells, 15 h after infection with HSV	1.5 (0-3)	

[a-c] The pool sizes of the four deoxynucleoside triphosphates were determined graphically from the data shown in Figure 4 by plotting 1/incorporated cpm against the concentration of unlabeled triphosphate. The pool size was read at the intercept with the abscissa (numbers in parentheses indicate the lowest and highest values graphically possible for each of the points). TTP pools in c were measured in a different set of infected cells than used for b. [d] The TTP pools for viral and cellular DNA synthesis respectively were determined for the 12-h infection of the experiment in c. After separation by CsCl density gradient centrifugation, 1/cpm in viral or cellular DNA was plotted separately against the concentration of unlabeled TTP. [e] Nuclei from the same set of infected cells used for b were purified as described in Experimental Procedure, and the TTP pool was determined as above, except that the result from the incubation in the absence of unlabeled TTP was not used.

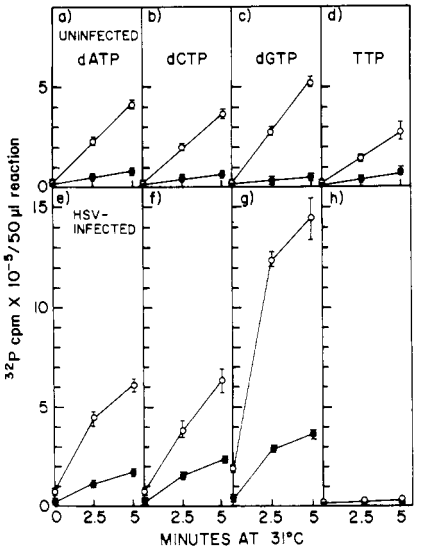

FIGURE 4: Determination of the deoxyribonucleoside triphosphate pools in lysates from uninfected BHK cells and after infection with HSV for 15 h at 31 °C, by isotope dilution. Six milliliters of lysate each, from uninfected and infected cells, were divided into 4 × 1.5 mL, containing the additions for in vitro synthesis as described in Experimental Procedure, omitting the triphosphate for which the pool size was to be determined; the three others were present at 100 μM. Each sample was divided into 4 × 350 μL and the missing triphosphate added to 0, 10, 50, and 250 μM, respectively. [α-^{32}P]dATP (129 Ci/mmol), dCTP (136 Ci/mmol), dGTP (136 Ci/mmol), and TTP (130 Ci/mmol) were added to the corresponding samples at 40 μCi/mL. Samples (50 μL) were removed in duplicate at 0, 2.5, and 5 min of incubation at 31 °C. Only the data for 0 and 10 μM unlabeled triphosphates are indicated in the figure. The pool sizes were determined as described in the footnote to Table I, using the data from all four concentrations of unlabeled triphosphate. [α-^{32}P]dATP (a, e); dCTP (b, f); dGTP (c, g); TTP (d, h); incorporated by uninfected (a-d) and infected (e-h) lysates with no (○) and 10 μM (●) unlabeled triphosphate present.

The cells used for the preparation of the in vitro systems shown in Figure 1 had been pulse labeled in vivo with [^{3}H]dThd for 1 h prior to the respective times. As demonstrated in Figure 3, the ^{3}H label is almost quantitatively recovered after purification of the nuclei. In vivo DNA synthesis is reduced by 3 h after infection and viral DNA becomes apparent by 9 h and reaches its maximum around 15 h. The 10-min in vitro reactions (^{32}P label in Figure 3) closely follow this pattern, indicating that the in vitro synthesis continues, at least initially, the in vivo process. Two deviations from this correlation are apparent in Figure 3: in vitro synthesis of cell DNA at the time of infection (compare Figure 1a) is not as high as expected from the in vivo prelabel; this is probably a consequence of the incubation conditions, which are not optimal for synthesis in uninfected systems. The other observation concerns the continued increase of viral DNA synthesis at 18 h (Figure 3b) in the nuclear system, while it declines in the lysate system (Figure 3a), suggesting that the lysate more faithfully reflects the in vivo process than do isolated nuclei.

For uninfected or polyoma virus-infected cells, isolated nuclei are less efficient in incorporating TTP into DNA than the lysate system and the requirement for cytoplasmic factors has been documented (Francke and Hunter, 1975). For HSV-infected cells, the opposite effect is observed in Figure 2. Both systems contained roughly equal amounts of prelabeled DNA. The different incorporation rates are largely due to the different TTP pools present in the two systems (see next paragraph), but the possible creation of additional primers and templates for DNA synthesis by the action of nucleases has to be borne in mind. The excess TTP incorporation by nuclei and nucleolytic activity in both types of systems critically depend on the time after infection (Figure 1) and the multiplicity of infection (data not shown) and are therefore most likely consequences of virus-induced functions. Because of the balance of synthesis and degradation and the variability of deoxynucleoside triphosphate pools in systems derived from HSV-infected cells, all results are presented as acid-precipitable radioactivity rather than amounts of nucleotides polymerized. An estimate of in vitro synthesis rates will be presented in the last paragraph.

Deoxyribonucleoside Triphosphates. HSV codes for a deoxypyrimidine kinase which is in part responsible for the major changes in the pool sizes of deoxynucleoside triphosphates occurring in infected cells (Jamieson and Bjursell, 1976b). Direct measurements of extractable pools (Jamieson and Bjursell, 1976a) have demonstrated increases for TTP (up to 20-fold), dCTP and dGTP (2- to 3-fold), and a decrease for dATP (3- to 4-fold). The experiments shown in Figure 4 and Table I were performed in order to determine whether these changes are reflected in the effective pool sizes for cell-free DNA synthesis in lysates from infected cells using the en-

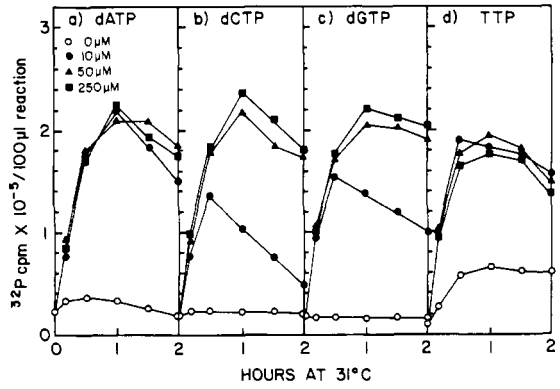

FIGURE 5: Dependence of DNA synthesis on added deoxynucleoside triphosphates in purified nuclei from BHK cells 15 h after infection with HSV at 31 °C. Eight milliliters of nuclei was divided into 4 × 2 mL containing the additions for DNA synthesis as described in Experimental Procedure, except the deoxynucleoside triphosphate to be tested was omitted from the respective samples: the labeled triphosphate was present at 50 μM and the two others at 150 μM. Labeling was with [α-^{32}P]TTP (130 Ci/mmol, 70 μCi/mL) in the sample where dGTP dependence was tested, and with [α-^{32}P]dGTP (136 Ci/mmol, 35 μCi/mL) in the three others. Each sample was divided into 4 × 500 μL and the omitted triphosphate added to 0, 10, 50, and 250 μM, respectively. Incubation was at 31 °C and 100-μL samples were removed at the times indicated. α-^{32}P incorporation in the absence (○) or presence of 10 μM (●), 50 μM (▲), or 250 μM (■) dATP (a), dCTP (b), dGTP (c), or TTP (d).

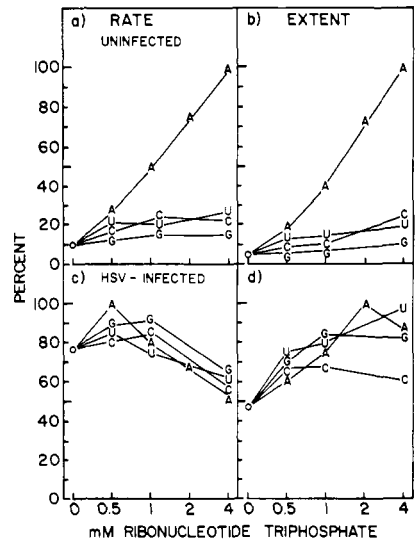

FIGURE 6: Influence of ribonucleoside triphosphates on DNA synthesis in purified nuclei. Four milliliters of nuclei each, from uninfected cells and after infection with HSV for 12 h at 31 °C, was purified and additions were made as described in Experimental Procedure, except that all ribonucleoside triphosphates were omitted. [^{3}H]TTP (125 μCi/mL) was present at 10 μM. To 250-μL aliquots, each of the four ribonucleoside triphosphates was added separately at the concentrations indicated. Each aliquot was made equimolar in $MgCl_2$ with respect to the respective ribonucleoside triphosphate, in addition to the 5 mM $MgCl_2$ present in all samples. Incubation was at 31 °C and 50-μL samples were removed at 0, 10, 30, 60, and 90 min. The results are presented as percent of maximum incorporation within each set. Rate of DNA synthesis (10 min incubation, a,c) and extent (60 min incubation, b, d) in nuclei from uninfected (a, b) and infected (c, d) cells in the absence (○) and presence of ATP (A), CTP (C), GTP (G), or UTP (U) at the concentrations indicated.

dogenous reaction of the system. The isotope dilution method employed has been described previously (Hunter and Francke, 1974). Figure 4 demonstrates some of the problems occurring specifically in infected lysates: With equal inputs of labeled triphosphate in the uninfected system, the incorporation in the absence of unlabeled triphosphate reflects the pool sizes quite adequately. In the infected system, the relative amount of high G-C viral DNA will contribute to the change in incorporated radioactivity, since incorporation into total DNA is measured. Isotope dilution with the same triphosphate circumvents this problem. Synthesis in the uninfected system was linear for the first 5 min, which was not the case for the infected system, indicating that even at early times of the in vitro reaction, the incorporated radioactivity might be a result of both synthesis and degradation. The pool sizes measured by this method for all four triphosphates are higher than the concentrations required for maximal rate of synthesis (compare Figure 5), ensuring that isotope dilution is not affecting the rate of synthesis, but its effect on degradation is not known. To minimize this potential complication, only the 2.5-min points from the infected system were used for the determination of the pool size. For reasons not understood, infected lysates have relatively high zero time background levels. These were therefore determined for each concentration separately and subtracted. Finally, to ensure that incorporation into DNA was measured, two samples for each triphosphate at two different concentrations were analyzed in CsCl density gradients; with a recovery of >90% in all cases, the incorporated radioactivity was either of viral DNA density (ca. 80%) or that of cellular DNA (ca. 20%). The pool sizes listed in Table I, expressed in μM concentrations in the lysate, illustrate the following points: The increase for dCTP, dGTP, and TTP are of similar magnitude as those of extractable pools (the experimental values for dATP were not significantly different, but scattered enough not to exclude a small decrease after infection, Table Ia,b). The drastic increase in the effective TTP pool after infection and its low value in purified nuclei (Table Ie) provide an explanation for the more efficient incorporation of labeled TTP by purified nuclei over the lysate systems (Figures 1-3). Its kinetic appearance after infection follows closely the course of viral DNA synthesis (Table Ic). Its decrease at late times after infection suggests, though, that the excess TTP incorporation by nuclei (18 h point in Figure 1b) over the lysate is likely to be due to other factors in addition to the removal of the TTP pool. The TTP pool is effective for both viral and cellular DNA synthesis (Table Id) and therefore does not seem to be compartmentalized. These conclusions are only valid for the in vitro situation, and they do not exclude the possibility of compartmentalized pools in vivo, utilization of different pools for viral and cellular DNA synthesis or locally much higher pools in intact cells than measured in the cell-free system, where they appear to be freely diffusible and to equilibrate quantitatively with exogenously added triphosphates.

To approach the question of whether the altered deoxynucleoside triphosphate concentrations are needed for optimal viral DNA synthesis, the requirement for their presence was investigated. This was done in the purified nuclear system, which is essentially free of triphosphate pools, and the results are illustrated in Figure 5. The full time courses of in vitro reactions in the absence and presence of three different concentrations for each of the four deoxynucleoside triphosphates are shown, since rate or extent of synthesis or stability of the product showed different requirements. In this experiment 85% to 95% of the product synthesized was viral DNA. Total dependence on added triphosphate was only obtained for dCTP and dGTP, while up to 10% synthesis was observed without dATP and up to 30% without added TTP. The latter may be due to the small but measurable TTP pool in purified nuclei (Table Ie). For dATP and TTP, 10 μM appears to be sufficient, while for dCTP and dGTP optimal conditions, most markedly for the extent of synthesis and the stability of the product, require higher concentrations. In view of the high G-C

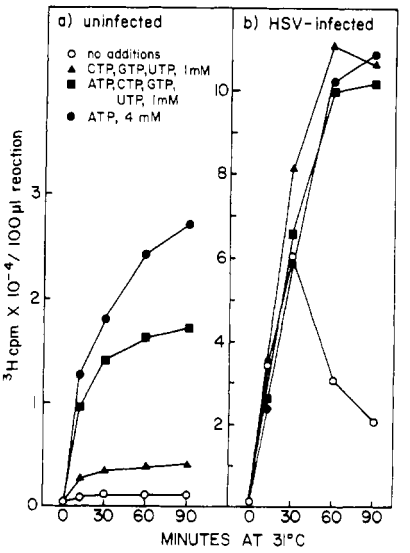

FIGURE 7: Influence of ATP and the other three ribonucleoside triphosphates on the [^{3}H]TTP incorporation kinetics in purified nuclei from uninfected and HSV-infected cells. Experimental details were as described in the legend to Figure 6, except that 100 µL aliquots were removed at the times indicated. ^{3}H cpm incorporated in the absence of ribonucleoside triphosphates (○), in the presence of 1 mM each of CTP, GTP, UTP (▲), 1 mM each of all four ribonucleoside triphosphates (■), and 4 mM ATP only (●) by nuclei from uninfected (a) and infected cells (b).

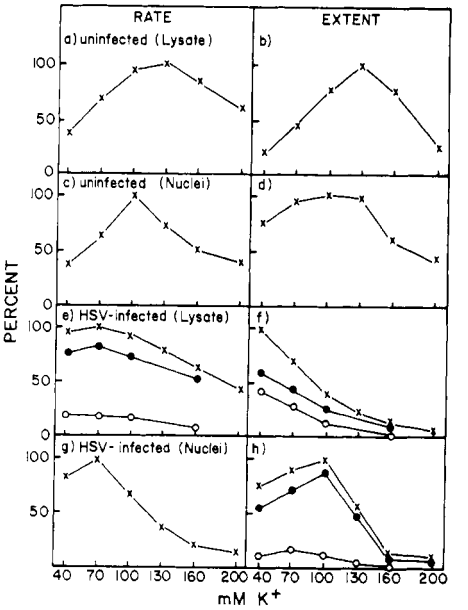

FIGURE 8: Influence of KCl on DNA synthesis in lysates and purified nuclei from uninfected and HSV-infected BHK cells. Preparation and incubation conditions were as described in Experimental Procedure, except that the KCl concentration was varied (the values include 20 mM K$^+$ from the buffer used and were brought to the indicated concentrations by the addition of KCl). [^{3}H]TTP (125 µCi/mL) was present at 10 µM. For each KCl concentration, 300-µL reactions were incubated at 31 °C and 50-µL samples removed at 0, 10, 30, 60, 90, and 120 min. Rate (10 min points, a, c, e, g) and extent (90 min points, b, d, f, h) of total DNA synthesized (X) in lysates (a, b, e, f) and nuclei (c, d, g, h) from uninfected (a–d) and HSV-infected (e–h) BHK cells, expressed as percent of maximum for each set. Separation of viral (●) and cellular (○) DNA was as described in Figure 2. These data are not included for the rate in infected nuclei (g) since 90% of these products were of viral density.

content of viral DNA and the pool size changes, discussed above, these observations appear reasonable, except that they do not provide an explanation for the high TTP pool. TTP (10 µM) is sufficient, demonstrating that the tenfold higher pool measured in the lysate is not required for optimal in vitro synthesis. At present, it cannot be decided whether the TTP is needed for a step in viral DNA replication in vivo, which the cell-free system is incapable of performing, or whether it serves some other purpose, not related to DNA synthesis, in infected cells.

Ribonucleoside Triphosphates. Most in vitro systems for DNA replication require ATP for optimal synthesis. As shown in Figure 6, this is also the case for nuclei from uninfected BHK cells and ATP cannot be substituted for by any of the other three ribonucleoside triphosphates (the continued stimulatory effect of ATP above 2 mM is not observed if an ATP regenerating system is included in the reaction, data not shown). For synthesis in HSV-infected nuclei, the effects of ribonucleoside triphosphates are different. Neither rate (75%) nor extent (45%) is strikingly dependent on their presence. At concentrations above 1 mM, all four triphosphates are somewhat inhibitory to the rate of DNA synthesis, but show a stimulatory effect on the extent. As evident in the time courses of in vitro labeling of DNA presented in Figure 7, the stimulatory effect is mainly due to stabilization of the in vitro product, which in the absence of the triphosphates undergoes extensive degradation. This protection can be provided equally well, at equimolar concentrations, by ATP alone, the three other ribonucleoside triphosphates, or by all four together (Figure 7a).

Apart from the four ribonucleoside triphosphates, the following compounds were found to protect the in vitro labeled viral DNA product equally well: TTP (at 4 mM), creatine phosphate (at 5 mM), and, to a lesser extent, inorganic sodium pyrophosphate (at 4 mM). The finding that in all three cases the stabilization of the product was accompanied by reduced synthesis rates suggested that the primary mode of action of these compounds was the inhibition of nucleolytic activities involved in the degradation of the product, and possibly also in triggering abnormally high synthesis rates in purified nuclei at late times of infection (see above).

The lack of ATP requirement for HSV DNA synthesis has been reported by Bolden et al. (1975), and the studies reported here support their observation. Attempts to stimulate DNA synthesis by adding more ATP during the in vitro reaction or by including creatine phosphate and creatine phosphokinase as an ATP regenerating system have had no effect in infected nuclei, while stimulating markedly in uninfected systems (data not shown), arguing against increased dephosphorylation of ATP in HSV-infected systems as a reason for the lack of stimulation by added ATP. For bacteriophage T4 (Alberts et al., 1975) and *E. coli* (Scott et al., 1977) in vitro DNA replication, the ATP requirement has been assigned to DNA-dependent ATPases. In both cases, their presence in the in vitro reaction increases the length of the product made. In view of the short size of the HSV DNA synthesized in isolated nuclei (see below), the lack of an ATP requirement does not exclude a potential role of ATP in the synthesis of long DNA strands. If this is the case, in vivo isolated nuclei must be lacking additional factors apart from ATP, since the size of the long-term in vitro product (analyzed by sucrose gradient sedimentation, data not shown) was not increased in the presence of high concentrations of ATP or any of the other phosphate compounds mentioned above, including TTP. Whether the larger size product synthesized under optimal conditions in the lysate system requires ATP cannot be tested because of the existing pools in that system.

Monovalent Cations and Anions. The DNA polymerase induced by HSV after infection (Weissbach et al., 1973) is resistant to salt concentrations up to 0.2 M. This property can

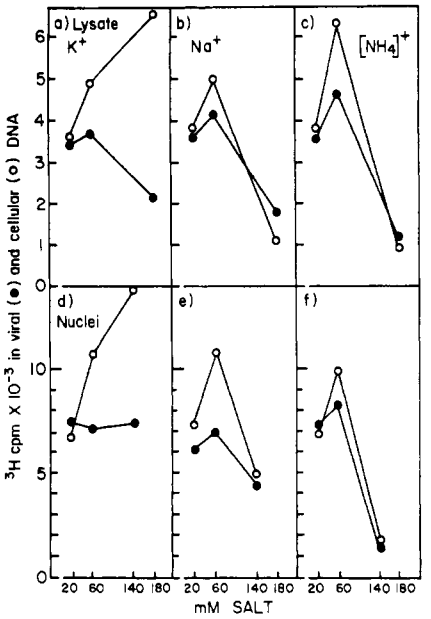

FIGURE 9: Influence of K^+, Na^+, and NH_4^+ as acetate salts, on synthesis in lysates and nuclei from HSV-infected BHK cells. Experimental conditions were as described in the legend to Figure 8, except that the Hepes buffer used for preparation and incubation of the systems had been adjusted to pH 7.9 with the hydroxide of the respective cations. These concentrations are included in the salt concentrations indicated. The acetate salts were brought to pH 7.5 with acetic acid prior to use. Reactions (100 μL), incubated at 31 °C for 90 min, were analyzed by CsCl density gradients. Viral (●) and cellular (○) DNA labeled in lysates (a-c) or nuclei (d-f) from HSV-infected cells in the presence of K^+ (a, d), Na^+ (b, d), or NH_4^+ (c, f) as acetate salts at the concentrations indicated.

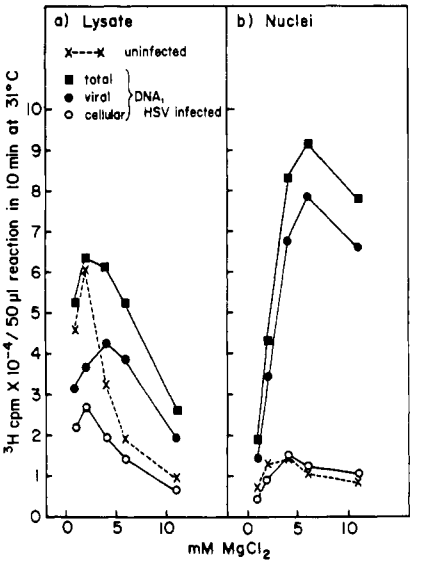

FIGURE 10: Effect of different concentrations of $MgCl_2$ on cell-free DNA synthesis. Experimental conditions were as described in the legend to Figure 9, using 80 mM K^+, 60 mM of which were added as acetate. The $MgCl_2$ concentrations indicated include 1 mM present in the hypotonic buffer. Only the data for the 10-min incubations are presented. Total DNA synthesized in uninfected (X) and infected (■) lysates (a) or nuclei (b) and relative amounts of viral (○) and cellular (●) DNA in the infected systems.

be used to assay selectively for the viral enzyme in extracts containing cellular polymerases which are inhibited by high salt concentrations. The influence of salt on the cell-free HSV DNA synthesis systems was therefore investigated. Using KCl (Figure 8), the salt optimum for HSV-infected systems is generally lower than in the corresponding uninfected systems, suggesting that the viral DNA synthesis observed in these systems does not result from the action of the viral polymerase exclusively, but most likely involves additional factors, sensitive to high salt. In the nuclear system, the optimum for viral DNA was reproducibly higher than for cellular DNA (Figure 8f), but was still inhibited to >90% at 160 mM KCl. In the lysate system (Figure 8f), synthesis was sensitive to very low concentrations of added KCl, indicating that optimal concentrations are already present. The marked inhibition by KCl in the lysate affects predominantly the long-term synthesis, having no effect on the initial rate. When the in vitro labeled DNA was analyzed by CsCl density gradient centrifugation (data not shown), viral DNA was stable up to 90 min, demonstrating that rather than the stimulation of nucleolytic activity, direct inhibition of synthesis is observed at high KCl concentrations. When using K^+, Na^+, and NH_4^+, both as chloride and acetate salt (data not shown), it became obvious that the inhibition is primarily due to the chloride ion, independent of the cation used, effective both in the lysate and—with a shift to higher concentrations—also in the nuclear system. Acetate salts were less inhibitory. Most strikingly, K^+ as acetate salt, stimulated both systems between 100 and 140 mM and showed very little inhibition, even at 180 mM. As demonstrated in Figure 9, this is mostly due to stimulation of cellular DNA in both systems (Figure 9a,d) but also to high salt resistant synthesis of viral DNA in the nuclear system. Using the chloride salts of Na^+ and NH_4^+, similar effects as with KCl (Figure 8) have been observed and are therefore not shown.

The basis for the inhibition by Cl^- and the stimulation by K^+ are unclear. But it is quite possible that at high potassium acetate concentrations a type of DNA synthesis is observed, which is predominantly a result of the viral DNA polymerase utilizing both viral and cellular DNA as templates. Analysis of the products by gradient sedimentation showed no difference in size between the various ionic concentrations. The only consistent observation was a larger product size under strongly inhibitory conditions, which may indicate a concomitant inhibition of nucleolytic activities.

Divalent Cations. The complication of DNA synthetic events by nucleolytic activities became especially obvious when the effect of divalent cations was investigated. Mg^{2+}, Mn^{2+}, and Ca^{2+}, or different combinations thereof, can act in a stimulatory or inhibitory way by favoring different types of nucleolytic processes, and are therefore presented in detail in the accompanying report. The stimulation by $MgCl_2$ alone is presented in Figure 10. In both systems the optimum for cellular DNA synthesized in HSV-infected samples is lower than for viral DNA and corresponds to that in uninfected samples. Viral DNA synthesis requires more Mg^{2+} in purified nuclei than in the lysate, demonstrating the removal of Mg^{2+}, or a compound for which Mg^{2+} can substitute, from the nuclei during purification. In purified nuclei, neither Ca^{2+} (optimum at 0.5 mM, 15% of maximal Mg^{2+} stimulation) nor Mn^{2+} (optimum at 1 mM, 40% of maximal Mg^{2+} stimulation) were found to be able to substitute for Mg^{2+}.

Influence of pH. In order to examine the effect of pH on cell-free HSV DNA synthesis, lysates and nuclei were prepared and incubated at a variety of pH values between 5.5 and 9.0, and the results compared with uninfected systems, as presented in Figure 14. In the case of infected cell lysates (Figure 11a), a rather complex situation revealed itself. The overall incorporation showed a minimum between pH 7 and 8, where the uninfected system was maximally active. At low pH, the synthesis of viral DNA was greatly favored, whereas high pH specifically stimulated cellular DNA synthesis. The size of the pH 6 viral DNA product (Figure 12) was almost exclusively

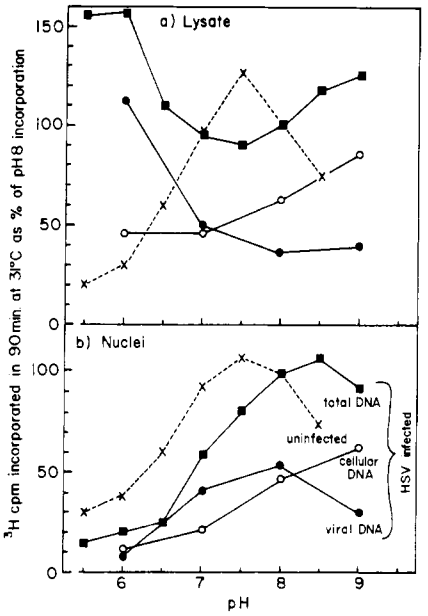

FIGURE 11: Cell-free DNA synthesis at varying pH. One-half milliliter of each, for every pH value, of lysate or nuclei was prepared as described in Experimental Procedure, except that the Hepes in all buffers had been adjusted to the indicated pH with KOH and then brought to 1 mol of K^+ per mol of Hepes with potassium acetate, previously adjusted to pH 7.0. Final K^+ concentration was 70 mM for lysates and 100 mM for nuclei. The effective pH in the lysates, as determined by spotting on indicator paper, ranged from <6.0 to >8.5. [^{3}H]TTP (100 μCi/mL) was present at 10 μM. Incubation was at 31 °C; 100-μL samples were removed at 0, 30, 60, 90, and 120 min. Results are presented at percent incorporation during 90-min incorporation (normalized to the pH 8.0 sample as 100%). Synthesis in nuclei (a) and lysates (b) from uninfected cells (X) and infected cells as total DNA (■) and relative amounts of viral (●) and cellular (○) DNA.

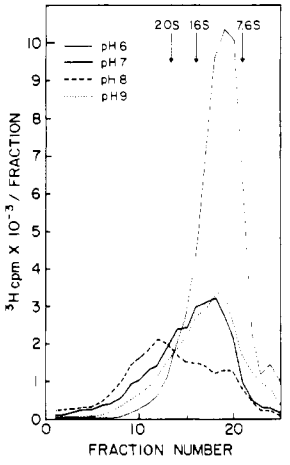

FIGURE 12: Size of viral DNA synthesized in lysates from HSV-infected cells at varying pH. Experimental details were as described in the legend to Figure 11. After preparative CsCl gradient centrifugation, the 90-min viral products were sedimented through sucrose gradients with ^{32}P-labeled marker DNA as described in Experimental Procedure. The four ^{3}H profiles were aligned, using the positions of the markers. Profiles for the pH 6 (—), pH 7 (—), pH 8 (- - -), and pH 9 (· · ·) products.

11 S under neutral conditions. This was the same size as the predominant long-term product in isolated nuclei (see Francke, 1977) and might therefore be a consequence of an endonuclease activated at low pH. The virus induced exonuclease (Morrison and Keir, 1968) has an optimum around pH 9. This enzyme might therefore be involved in the stimulation of cellular DNA synthesis at high pH, by exposing new templates in normally inactive chromosomal DNA. These speculative

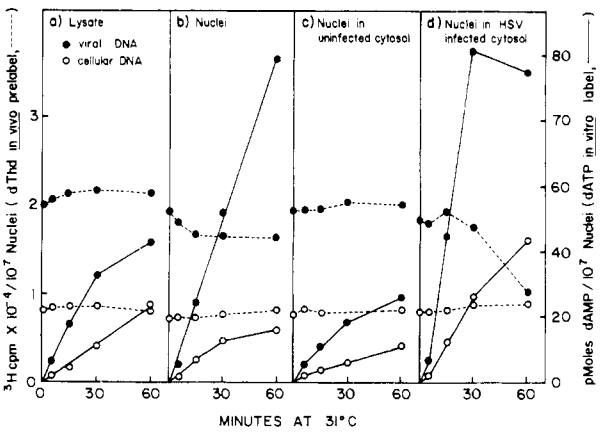

FIGURE 13: Comparison of synthesis in lysates to synthesis in purified nuclei in the absence or presence of cytosol from infected and uninfected BHK cells. Four milliliters of lysate was prepared from 25 9-cm dishes of infected cells which had been prelabeled with [^{3}H]dThd (10 μCi/mL in 5 mL of medium/dish) from 11 to 12 h after infection. Nuclei were prepared from 3 mL as described in Experimental Procedure, except that the isotonic buffer used for washing contained 60 mM KCl. Two milliliters of cytosol from infected cells and 1 mL of cytosol from uninfected cells were prepared from 20 and 10 9-cm dishes of unlabeled cells, respectively. Incubation conditions were as described in Experimental Procedure, except that TTP was present at 100 μM, dATP at 50 μM, and [α-^{32}P]dATP (127 Ci/mmol) at 75 μCi/mL. Samples (100 μL) were removed at the times indicated and analyzed by CsCl density gradient centrifugation. An incubation of cytosol from infected cells done under identical conditions incorporated <5% of the radioactivity incorporated in the presence of nuclei (not included in the figure). (^{3}H, - -) In vivo prelabeled and (^{32}P, —) in vitro labeled viral (●) and cellular (○) DNA in the lysate (a), purified nuclei in isotonic buffer (b), and cytosol from uninfected (c) and HSV-infected (d) cells.

explanations for the complex effect of pH on the lysate are supported by the size of the native viral DNA product (Figure 12). The optimum for size is close to pH 8, and deviations to either side result in shorter products. Since it is unlikely that extensive nuclease action is involved in in vivo viral DNA replication, it is assumed that synthesis in the lysate most resembles the in vivo situation when carried out between pH 7 and 8. This is also the optimum for viral DNA in the nuclear system, which contains greatly reduced nucleolytic activity. The size of the native viral DNA product in isolated nuclei did not change with pH, and the gradient profiles are therefore not shown.

Influence of Temperature. All experiments reported so far were performed at 31 °C. When examining the effect of temperature on HSV-1-infected BHK cell derived in vitro systems, the extent of synthesis was reduced at temperatures above 40 °C and the relative initial rates above 42 °C. The high temperature optimum (46 °C) reported for HSV-2 DNA synthesis in nuclei from infected HEL cells by Kolber (1975) is therefore likely to be a property of that particular virus-cell system used.

Quantitation of in Vitro DNA Synthesis. In order to compare the synthetic capacities of the lysate and the nuclear system quantitatively, the experiment shown in Figure 13 was performed. The infected cells had been prelabeled with [^{3}H]dThd 2 h prior to the preparation of the cell-free systems. The recovery of the prelabel in the purified nuclei was 95% compared with the lysate, ensuring that the same number of templates are available for in vitro synthesis in both systems. For in vitro labeling, [α-^{32}P]dATP was used at 50 μM, reducing the potential change in specific activity caused by endogenous pools to <10%. Under these conditions, the lysate incorporated dAMP into viral DNA at an initial rate of 1.6

pmol per min per 10^7 nuclei at 31 °C. When corrected for the base composition of HSV DNA, the rate (9.6 pmol of dXMP polymerized per min per 10^7 nuclei) amounts to about $1/8$th of that reported by Bolden et al. (1975) for total DNA synthesis in nuclei from HSV-infected HeLa cells at 37 °C, but is considerably lower than that reported recently by Schlomai et al. (1977) for nuclei from infected BSC-1 cells. Since the experiment shown in Figure 13 was performed at 31 °C and at 12 h after infection, it can be estimated approximately that the rate of viral DNA synthesis at its maximum (15 to 18 h after infection, compare Figure 1) can reach 20 to 30 pmol of dXTP per min per 10^7 nuclei, which—allowing for the difference in temperatures of the in vitro reaction—approaches the value reported by Bolden et al. (1975). Cheng et al. (1975) have estimated the synthesis rate in vivo for HSV-infected HeLa cells based on the measurement of the specific activity of the TTP pool after labeling with [^{3}H]dThd, and correlated it to the DNA content. Assuming that, at the peak of viral DNA replication there are about equal amounts of viral and cellular DNA present per infected cell, the maximal in vivo rate estimated by Cheng et al. would correspond to about 300 pmol of dXMP per min per 10^7 cells at 37 °C. This value may represent an overestimate, though, since it has not been shown yet that the intracellular TTP pool is utilized for DNA synthesis in vivo. If the pool is by-passed by external labeling with dThd, the specific activity of TMP incorporated into DNA could be higher than that of the TTP pool.

The initial rate of synthesis in the lysate (Figure 13) was essentially maintained in purified nuclei (84%, Figure 13b). While in the lysate the rate started to decrease after 15 min, isolated nuclei continued almost linearly for 1 h. Cytosol from uninfected cells (Figure 13c) inhibited extended synthesis below the level of the lysate, while cytosol from infected cells (Figure 13d) resulted in an increase of the rate after the first 5 min and in degradation at later times. The fate of the in vivo prelabel during the in vitro reaction demonstrates extensive degradation of viral DNA in the presence of infected cytosol, most likely due to the virus induced exonuclease, which is absent from uninfected cytosol. Limited degradation of prelabeled, but not of in vitro labeled, viral DNA was also observed in the nuclei alone. In both cases, it is likely that the excess in vitro synthesis over the lysate is secondary to nucleolytic activities, as discussed in detail in the following report (Francke, 1977). The stability of the prelabel in the lysate, which should contain the nuclease, suggests the presence of an inhibitor lacking from purified nuclei. The small increase in ^{3}H label during in vitro incubation in the lysate is normally seen after dThd prelabel and is probably due to the incorporation of [^{3}H]TTP from the pool inefficiently chased by the 100 μM unlabeled TTP added for the reaction. The main point illustrated by this experiment is that nuclei from HSV-infected cells do not appear to require cytoplasmic factors for optimal synthesis rates. This is in contrast to synthesis of cellular and viral DNA in polyoma (Francke and Hunter, 1975) and SV40-infected cells (DePamphilis et al., 1975). Viral DNA synthesis in nuclei from papovavirus-infected cells is reduced up to 15-fold in the absence of cytosol. The apparent independence of HSV from cytoplasmic factors might indicate that all functions needed for viral DNA synthesis are stably bound in the nucleus. On the other hand, it is not known at present whether all steps leading to mature viral DNA occur in vitro in the lysate, and therefore a potential requirement of certain steps for cytoplasmic factors—or for ATP and high TTP concentrations—can at present not be excluded.

During the course of these experiments, it became obvious that a major problem for cell-free HSV DNA synthesis systems is that of a potent nuclease, absent from uninfected systems. The nuclease can be so predominant as to result in rapid degradation (Figure 2), or can in a subtle way, act to stimulate DNA synthesis (Figures 11 and 13d). For "optimization" experiments of the nature reported here, the possible contribution of nuclease to the "optimum" for synthesis has to be considered. The conditions for preparation and incubation of lysate or nuclei described in Experimental Procedure are based not only on maximum incorporation rates, but also a maximum size of the product (as discussed in the following report (Francke, 1977)), since a maximum of synthesis that resulted in a decreased product size was considered to be an unlikely optimum for replicative DNA synthesis. The conditions described have resulted in no detectable degradation of either pre-existing or in vitro labeled viral DNA in the lysate and in a tolerably low level of degradation in isolated nuclei. It should be mentioned, though, that the conditions were successfully applied only to cells infected at a multiplicity of 10 pfu/cell for 12 h at 31 °C (or 6 h at 39 °C). Higher multiplicities or longer times of infection resulted in cell-free systems in which—despite carefully controlled conditions—degradation prevailed.

Acknowledgments

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Cell-Free Synthesis of Herpes Simplex Virus DNA: Structure of the in Vitro Product and Nucleolytic Degradation†

Bertold Francke

ABSTRACT: The size of viral DNA products synthesized in cell-free DNA synthesis systems from HSV-1 infected BHK cells under different incubation conditions is investigated. In the unfractionated concentrated lysate, preexisting viral DNA maintains its size (20–50 S) during the in vitro reaction and viral DNA synthesized in vitro cosediments with native prelabeled DNA. After denaturation with alkali, the viral DNA sediments between 10 and 40 S with a bias of the in vitro synthesized DNA for shorter size classes. In the absence of additional divalent cations other than Mg^{2+}, the lysate system shows practically no endogenous nucleolytic activity, while in diluted lysates endonucleolytic activity is observed. Endonuclease can be stimulated in concentrated lysates by the addition of Ca^{2+}, while the addition of Mn^{2+} stimulates predominantly an exonucleolytic type of degradation. Isolated nuclei can be rendered free of exonucleases by washing with 60 mM KCl, but they do retain endonuclease activity resulting in a native DNA product of 11 S in size for preexisting and in vitro synthesized DNA. Low ionic strength washes do not remove the exonuclease(s) and result in nuclear systems that rapidly degrade viral DNA. Degradation requires Mg^{2+}, but is not dependent on DNA synthesis. All nucleolytic events in vitro are specific for viral DNA, not affecting cellular DNA. Purified viral and cellular DNA are equally sensitive to degradation by the nuclease present in cytoplasm from infected cells. Cellular DNA contained in isolated chromatin is resistant. After reconstitution with chromatin proteins, both types of DNA become resistant to the nuclease. During preparation of chromatin from infected cells, viral DNA is preferentially removed. The differential degradation of viral and cellular DNA during in vitro synthesis therefore is most likely due to differential protection.

In the preceding communication (Francke, 1977), two systems for cell-free DNA synthesis derived from herpes simplex virus infected BHK cells have been described: an unfractionated lysate and purified nuclei. During the experimental evaluation of the various parameters affecting viral and cellular DNA synthesis in both systems, it became clear that nucleolytic activities can play an important role during the in vitro reaction. The action of nucleases during the in vitro synthesis may have different effects, depending on the type of nucleolytic activity and the extent of degradation: extensive exonuclease action would result in degradation of the in vitro product to acid-soluble material, while an endonuclease alone (by nicking), or in combination with limited exonuclease action (resulting in gaps), might act to stimulate DNA synthesis by creating new primers and exposing new templates. Indications for both have been obtained (Francke, 1977): at late times after infection extensive degradation of preexisting and in vitro labeled DNA was observed, and the addition of cytosol from infected cells to isolated nuclei triggered an excess of DNA synthesis over that observed in the unfractionated lysate. The type and extent of nucleolytic activity predominant under certain incubation conditions is therefore expected to influence greatly the structure of the in vitro product. In this communication, an analysis is presented of the viral DNA product synthesized under conditions that minimize degradation and under a variety of conditions that favor certain types of nucleolytic activities. Whenever observed, the in vitro degradation affected exclusively viral DNA, both preexisting and synthesized in vitro. This apparent specificity is of interest since it might reflect a difference in the intracellular environment of the two types of DNA in infected cells.

Experimental Procedure

Biological procedures, preparation, and incubation conditions for cell-free DNA synthesis systems, CsCl density gradient centrifugation, velocity sedimentation in neutral sucrose gradients, and the sources of chemicals and radioisotopes were as described in the preceding report (Francke, 1977). Velocity sedimentation in alkaline sucrose gradients was as follows: 3.6 mL of linear 5–20% sucrose in 10 mM EDTA,[1] 0.25 M NaOH and 0.75 M NaCl were overlayered with 200 μL of sample, dissolved in 0.25 M NaOH, and centrifuged in a Beckman

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[1] Abbreviations used: HSV-1, herpes simplex virus, type I; pfu, plaque-forming units; Hepes, *N*-2-hydroxyethylpiperazine-*N*′,2′-ethanesulfonic acid; dThd, deoxythymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.