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DNA POLYMERASE IN NUCLEI ISOLATED FROM HERPES SIMPLEX VIRUS TYPE-2-INFECTED CELLS

CHARACTERIZATION OF THE REACTION PRODUCT AND INHIBITION BY SUBSTRATE ANALOGS

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Nuclei isolated from herpes simplex virus (HSV) type 2-infected KB cells were examined for their capacity to serve as an in situ source of herpes DNA polymerase. In contrast to purified enzymes with added template, approx. 80% of the DNA synthesized in isolated nuclei was viral. The average size of DNA fragments labeled in vitro was $3.2 \cdot 10^6$ Da. Based on an increase in DNA density when nuclei were incubated in the presence of BrdUTP rather than dTTP, 16% of the nucleotides were added during the in vitro reaction. Sucrose gradient analysis of DNA polymerase activity in extracts of isolated nuclei demonstrated the nearly exclusive presence of herpes DNA polymerase. K_m concentrations for the four dNTPs were from 0.14 to 0.55 μM. DNA synthesis was inhibited competitively by the 5′-triphosphates of ara-A and ara-C ($K_i = 0.03$ and 0.22 μM, respectively) but not by the 5′-triphosphate of dideoxythymidine. aATP also served as a substrate ($K_m = 0.014$ μM) for the reaction. We conclude that nuclei from HSV-infected cells have significant advantages for the detailed study of inhibitors of herpesvirus replication.

Introduction

Studies on DNA synthesis in subcellular systems have contributed significantly to understanding the details of DNA replication. These systems have been used to examine the replication of both prokaryotic and eukaryotic DNA of cellular and viral origin. Although the replication of DNA from small and intermediate size viruses has been studied extensively in this manner (e.g., see Kornberg [1]), replication of DNA from larger viruses, such as the herpesviruses, has not been examined as thoroughly. The use of isolated nuclei should be very valuable in this regard because the nuclear matrix is intimately involved in DNA replication [2] and in herpes virogenesis [3]. In addition, much of the replication complex – including the natural template and DNA polymerases [4] – is functional [5–13].

In view of these observations, nuclei isolated from HSV-infected cells should have utility in studying the mode of action of selected inhibitors, especially antiviral drugs. Beside providing the replication complex not present in isolated en-

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Abbreviations: aATP, aCTP, BrdUTP, ddTTP, the 5′-triphosphates of 9-β-D-arabinofuranosyladenine, 1-β-D-arabinofuranosylcytosine, 5-bromo-2′-deoxyuridine and 2′,3′-dideoxythymidine; HSV, herpes simplex virus; $(I/S)_{50}$, ratio of concentrations of inhibitor to substrate which gives 50% inhibition; NTP, dNTP, a naturally-occurring nucleoside or deoxynucleoside triphosphate.

zymes, nuclei are permeable to substrates and inhibitors thereby permitting quantitative studies not possible in whole cells. This approach has been used with nuclei from HSV-1-infected cells [6,8,9,14,15] but to our knowledge nuclei from HSV-2-infected cells have not been utilized for the study of inhibitors of herpesvirus replication.

Therefore, we have characterized DNA synthesis in nuclei isolated from HSV-2-infected KB cells with regard to requirements for DNA synthesis, nature of the product and sensitivity to known inhibitors. We have found that isolated nuclei provide a readily obtainable in situ source of HSV-2 DNA polymerase. We also report that DNA synthesis occurs by addition of nucleotides to endogenous herpes DNA and that it is exquisitely sensitive to known inhibitors of herpes DNA polymerase. The nature of inhibition is qualitatively and quantitatively similar to that observed with highly purified enzymes.

Materials and Methods

Chemicals. [*methyl*-^{3}H]thymidine (20 Ci/mmol), [*methyl*-^{14}C]thymidine (56 mCi/mmol) and [α-^{32}P]dCTP (150 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL. [*methyl*-^{3}H]dTTP (60–80 Ci/mmol), [8-^{3}H]dATP, [8-^{3}H]dGTP, [5-^{3}H]dCTP and [2-^{3}H]aATP (all at 5–25 Ci/mmol) and unlabeled aATP were purchased from ICN, Irvine, CA. Unlabeled naturally-occurring dNTPs, rNTPs and aCTP were obtained as lithium, sodium or potassium salts from Calbiochem-Behring, La Jolla, CA, except for CTP and ddTTP which were obtained from P-L Biochemicals, Milwaukee, WI. All dNTPs were checked for purity using thin-layer chromatography [16]. Other materials were obtained from the following suppliers: spermine, spermidine and aprotinin – Sigma, St. Louis, MO; Hepes and pronase, B grade, nuclease free – Calbiochem-Behring; CsCl (highly purified) – Column One, Ann Arbor, MI; Tris (Reagent grade) – Brae Laboratories, New York, NY; calf thymus DNA – Worthington Biochemical, Freehold, NJ; and proteinase K (EC 3.4.21.14) from Beckman Instruments, Palo Alto, CA.

Cell culture techniques. Donor calf serum and fetal calf serum were obtained from KC Biological, Lenexa, KS. The source of KB cells; the routine growth and passage of cells; and techniques used for the enumeration of cells, the determination of cell viability, and the detection of mycoplasma contamination have been described previously [17,18]. Human embryonic lung cells, derived by Dr. C. Shipman Jr., were maintained and passaged in a manner similar to that used for KB cells except the medium was supplemented with 10% fetal bovine serum rather than calf serum.

Virus. HSV-2, strain X-79, was a gift from Dr. E.R. Kern, University of Utah. High-titer virus stock was prepared by infecting confluent KB cells at a multiplicity of 0.05 p.f.u./cell and harvesting virus 3 days later. Virus was titered on human embryonic lung cell monolayers grown by planting one-half to one million cells contained in 5 ml Eagle's minimum essential medium with Hanks' salts supplemented with 10% fetal bovine serum in each well of six-well Costar cluster dishes. The dishes were incubated in a humidified atmosphere consisting of 3% CO_2/97% air. After 24 h the cells were confluent and were inoculated with 0.2 ml of the virus suspension to be assayed. Macroscopic plaques were enumerated after 3 days.

Isolation of nuclei. Nuclei were prepared using a procedure patterned after that of Francke [13]. Confluent KB cell monolayers grown either in plastic roller bottles (Corning Glassware, Corning, NY) or glass 32-ounce prescription bottles were infected with HSV-2 at 5 or 10 p.f.u./cell. After 1 h adsorption at 37°C, the virus inocula were decanted and virus growth medium consisting of Eagle's minimum essential medium with Hanks' salts supplemented with 10% calf serum and 0.127 g/l L-arginine was added to each culture. 6–9 h later when virtually all cells exhibited cytopathic effect they were scraped from the flask surface into the medium and pelleted by centrifugation at 200 × *g* for 30 min. This and all subsequent steps in the isolation and handling of nuclei were performed at 0–4°C. Cells then were washed by suspension in buffer A (40 mM Hepes (pH 7.9)/250 mM sucrose/2 mM mercaptoethanol/50 mM KCl/0.3 mM spermine/0.5 mM spermidine/ 1 : 1000 aprotinin/1 mM $MgCl_2$). The cells were again centrifuged at 200 × *g* for 15 min and resuspended in buffer B (20 mM Hepes (pH 7.9)/2 mM mercaptoethanol/0.3 mM spermine/ 0.5 mM

spermidine/1 : 1000 aprotinin/1 mM $MgCl_2$). After 15 min in an ice bath, the swollen cells were lysed in 5 ml aliquots using five strokes of a stainless steel homogenizer (Kontes, 0.005 inch clearance). Nuclei were pelleted at $400 \times g$ for 15 min and resuspended in buffer B containing 0.3% Triton N-101. After standing for 15 min in an ice bath, with occasional agitation, the nuclei were pelleted and washed once in buffer A. Nuclei were monitored for purity using a transmitted light interference microscope (Zeiss). No intact cells were evident and most nuclei appeared to be free of cytoplasmic contamination. Isolated nuclei were suspended in a solution consisting of seven parts glycerol and three parts buffer A and stored in small aliquots at −76°C until needed. The preparations retained activity for several months under these conditions.

DNA synthesis in isolated nuclei. Frozen nuclei were thawed on ice, washed with buffer A (three cycles of suspension and centrifugation at $400 \times g$ for 10 min each) and counted with the aid of a hemocytometer. For incubation with other reaction mixture components, nuclei were finally suspended in buffer C (40 mM Hepes (pH 7.9)/2 mM mercaptoethanol/250 mM sucrose/80 mM potassium acetate/0.3 mM spermine/0.5 mM spermidine/1 : 1000 aprotinin/1 mM $MgCl_2$) so that 1 ml contained $5 \cdot 10^6$ nuclei. DNA synthesis was measured in a complete reaction mixture containing final concentrations of the following components in a total volume of 200 µl: 40 mM Hepes (pH 7.9); 2 mM mercaptoethanol; 250 mM sucrose; 80 mM potassium acetate; 0.3 mM spermine; 0.5 mM spermidine; 1 : 1000 aprotinin; 1 mM $MgCl_2$; 100 µM each NTP; 100 µM each of three unlabed dNTPs; 1 µM labeled dNTP, usually [^{3}H]dTTP (2.4 Ci/mmol); 0.1 mg/ml bovine serum albumin and $0.5 \cdot 10^6$ nuclei. Duplicate reaction mixtures were incubated at 37°C for selected periods of time in a water bath with constant agitation. Reactions were stopped by placing the reaction tubes in an ice bath and adding 0.5 ml 10 mM sodium pyrophosphate to each. 2 ml 7% $HClO_4$ in 10 mM sodium pyrophosphate were added to precipitate DNA. After at least 30 min in an ice bath, acid-insoluble material was collected on glass fiber filters (Reeve Angle) by vacuum filtration. The filters were washed five times with 0.05 M HCl in 10 mM sodium pyrophosphate, twice with 95% ethanol and dried under a heat lamp. 0.25% diphenyloxazole in toluene was added and the radioactivity present determined in a Beckman LS8100 liquid scintillation spectrometer.

Substrate degradation in isolated nuclei. The degradation of [^{3}H]dATP and [^{3}H]dTTP by nuclei was measured in reaction mixtures identical to those described above except the labeled substrate concentrations were near the K_m concentrations presented in Table II. Following incubations at 37°C for 5–30 min, assay tubes were transferred to an ice bath and 2 vol. cold 95% ethanol were added to each. After standing for a mimimum of 1 h at −20°C, precipitated materials were removed by centrifugation and a 10 µl portion of each supernatant fluid containing at least 10000 dpm was spotted along with unlabeled standards onto a polyethyleneimine-cellulose thin-layer sheet. The sheet was developed stepwise in increasing concentrations of LiCl in 1 M acetic acid [16], sectioned, eluted and counted as described previously [19]. Degradation in whole cell extracts was measured in the same manner near the K_m concentration of 1.5 µM using extraction and DNA polymerase assay conditions described previously [20].

DNA synthesis in whole cells. The amount and size of viral and cellular DNA synthesized in intact cells immediately prior to isolation of nuclei was examined by infecting KB cells with HSV-2 as described for the isolation of nuclei. 8 h after infection, medium was decanted and fresh medium containing [^{3}H]dThd (2 or 5 µCi/ml, 20 Ci/mmol) added. After a 30-min incubation at 37°C, cells were harvested and nuclei isolated as described above. Viral and cellular DNA were extracted and analyzed on CsCl and sucrose gradients as described in subsequent sections.

Preparation of [^{14}C]thymidine-labeled marker DNA. Monolayer cultures of uninfected KB cells were planted and grown to mid-log phase as described above. Fresh medium containing [^{14}C]thymidine (0.25 µCi/ml, 56 mCi/mmol) was added and incubation continued for 24 h. Cell sheets were washed with Hepes-buffered saline [21] and disrupted by the addition of 5% sarkosyl in TES (30 mM Tris-HCl (pH 8.0)/5 mM EDTA/50 mM NaCl). Proteinase K was added to a final concentration of 0.25 mg/ml and the mix-

ture incubated at 45°C for 2 h. DNA was extracted by means of a phenol/chloroform/isoamyl alcohol method. Isolated DNA was centrifuged in CsCl gradients, peak fractions were pooled, dialyzed twice against 2 vol. TES buffer and frozen at −20°C. A portion of the DNA preparation was sheared by sonication for 15 s at 20 kHz in a Bronwill sonicator (Bronwill Scientific, Rochester, NY) set to deliver 70 W of acoustical power through a needle probe. Size analysis of the resulting DNA fragments in a Beckman model E analytical ultracentrifuge by band sedimentation velocity [22] of a 10 μl aliquot (2.33 A_{260} units) through 1 M NaCl in TES buffer established an apparent sedimentation coefficient ($s_{20,w}$) of 9.7. Based on the method of Studier [23] this corresponds to an average molecular weight of $0.79 \cdot 10^6$.

Separation of DNA species. To separate viral from cellular DNA or BrdUMP-substituted DNA from unsubstituted DNA, $1 \cdot 10^6$ nuclei were incubated in twice the volume of the complete reaction mixture. Additional modifications are described in the legends to Figs. 2 and 3. Following incubation at 37°C, nuclei were chilled in ice and sarkosyl and proteinase K were added to final concentrations of 50 and 0.3 mg/ml. After incubation for 2 h at 45°C, DNA was sheared using a 25 gauge needle or a Pasteur pipette. CsCl, and where appropriate, [^{14}C]thymidine-labeled cellular DNA were added and the mixture centrifuged (72 000 × *g* at 15°C for 60 h) as described previously [17]. Relative amounts of DNA species were determined on the basis of incorporated label by integration after peak areas were fully resolved using a Dupont 310 Curve Resolver set to fit Lorentzian distribution curves.

Sedimentation of DNA synthesized in isolated nuclei. Isolated nuclei were incubated in the complete reaction mixture modified to increase the specific activity of [^{3}H]dTTP to 24 Ci/mmol. Reactions were terminated by adding 23 μl lysing buffer (100 mM Tris/250 mM disodium · EDTA/20% sarkosyl, pH 7.5) and 7 μl of 1% proteinase K. After incubating for 2 h at 45°C, the volume was brought to 250 μl with lysing buffer containing sufficient sodium dodecyl sulfate to give a final 2.5% concentration. (For alkaline gradients, lysing buffer contained NaOH to give a final concentration of 1 mM.) 10 μl of KB cell marker DNA labeled with [^{14}C]thymidine was added and the solutions (260 μl) were gently layered onto 5 ml 5–20% sucrose gradients containing 50 mM Hepes (pH 7.5)/1 mM NaCl/10 mM EDTA/0.15% sarkosyl (neutral gradients) or 0.3 M NaOH/0.7 M NaCl/10 mM EDTA/0.15% sarkosyl (alkaline gradients). Centrifugation was for 3 h (neutral gradients) or 4 h (alkaline gradients) at 189 000 × *g* at 20°C. Fractions were collected from the bottom of the gradients and acid-insoluble radioactivity was determined as described above.

Identification of DNA polymerases (EC 2.7.7.7) in isolated nuclei. Isolated nuclei ($3.6 \cdot 10^8$) were washed three times in buffer A as described above and then extracted using a modification of the method of Powell and Purifoy [24]. Washed nuclei were suspended in 1 ml of 20 mM Tris-HCl (pH 7.5) containing 0.5 mM dithiothreitol and left on ice for 30 min. All subsequent procedures were performed at 2°C unless otherwise noted. Nuclei were disrupted by sonication using a needle nose probe (18 × 10 s pulses, 70% maximum power). 1 ml of high salt extraction buffer (20 mM Tris-HCl (pH 7.5)/0.5 mM dithiothreitol/3.4 M KCl/10 mM EDTA/1 mg/ml bovine serum albumin) was added to the nuclear lysate and the preparation was vortexed. The extract was left on ice for 1 h with vortexing every 10 min, clarified by centrifugation at 21 000 × *g* for 30 min, and dialyzed twice against 500 ml of 50 mM Tris-HCl (pH 7.5) containing 0.5 mM dithiothreitol, 0.2% (v/v) NP-40 and 20% (v/v) glycerol. After a precipitate was removed by centrifugation, the nuclear extract (200 μl) was centrifuged through a 5–20% (w/w) sucrose density gradient containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 0.5 M NaCl at 149 000 × *g* for 20 h at 2°C. Fractions were collected from the bottom of the tube.

DNA polymerase activity in each gradient fraction was analyzed using three different assays. The polymerase-specific assays were modifications of those developed by Weissbach and associates [25–26]. [^{3}H]dATP (1500–4500 cpm/pmol) was used as labeled substrate; all other conditions have been published [20].

Results

Requirements for DNA synthesis

Incubation of isolated nuclei with the complete reaction mixture resulted in the incorporation of [^{3}H]dTTP into acid-insoluble material. No incorporation of label was observed when [^{3}H]thymidine was substituted for [^{3}H]dTTP. The amount of label incorporated varied significantly with changes in the concentration of Mg^{2+}. In the absence of added Mg^{2+} (plus 1 mM EDTA), there was no incorporation of label above background levels indicating that Mg^{2+} was essential for DNA synthesis. Concentrations of Mg^{2+} up to 10 mM increased the rate of incorporation during short incubations (5 min). Concentrations greater than 2.5 mM, however, resulted in either very little additional incorporation (5 mM) or an actual decline in acid-insoluble label (7.5 and 10 mM) when incubations were extended to 15 or 30 min. Therefore, all subsequent assays employed 1 mM Mg^{2+} which permitted linear incorporation for at least 5 min plus hyperbolic incorporation up to 1 h. A decreasing rate of incorporation in HSV-infected nuclei has been consistently reported by others [9,10,27] and has been shown to be associated with replicative DNA synthesis [13]. The reasons for limited synthesis are unknown but the need to avoid high Mg^{2+} concentrations suggests that the DNA product may be degraded by the HSV-induced DNAase(s) which are activated by Mg^{2+} [28]. Alternatively, essential factors may have been removed during the preparation of the subcellular fraction.

To determine the requirements for other components of the reaction mixture, DNA synthesis in the absence of these components was compared to synthesis in the complete system. Omission of potassium acetate, spermine plus spermidine, bovine serum albumin, or mercaptoethanol resulted in reduction of DNA synthesis (Table I). When ATP or all four NTPs were eliminated from the reaction, the rate of DNA synthesis was unchanged during the first 20–30 min of incubation. However, after longer incubation times there was a reduction in incorporated radioactivity. DNA synthesis in the complete system was highly dependent upon the presence of all four dNTPs. Omission of only one dNTP resulted in a marked reduction in DNA synthesis (Table I); omission of two dNTPs resulted in greater inhibition; omission of three dNTPs reduced label incorporation to background levels (data not shown). This high degree of dependence on a full complement of dNTPs probably reflected the known processivity of HSV DNA polymerase [29].

TABLE I

REQUIREMENTS FOR DNA SYNTHESIS IN ISOLATED NUCLEI

Nuclei were incubated for selected periods of time with reaction components omitted or added as shown. 5-min incubation periods were used unless otherwise indicated. Incorporation is reported as percent of label incorporated in complete reactions. This amounted to approx. 6000, 18000 and 20000 dpm/reaction at 5, 30 and 60 min, respectively. Standard procedures for in vitro DNA synthesis were used except for the minor differences listed below. All assays were performed in duplicate or triplicate.

Reaction mixture	Amount of incorporation (%)
Complete	100
– potassium acetate [a]	61
– spermine and spermidine [a]	71
– bovine serum albumin [a]	67
– mercaptoethanol [a]	51
– ATP [b]	79
– four rNTP [b]	60
– dATP or dTTP [b]	16
– dGTP or dCTP [b]	7
+ *N*-ethylmaleimide (5 mM) [a,c]	2

[a] 2 or 5 mM $MgCl_2$ plus 1 mM EDTA.
[b] 30 or 60-min incubation.
[c] Mercaptoethanol omitted.

Nature of DNA synthesized in vitro

In order to determine whether viral or cellular DNA was synthesized in isolated nuclei, the density of DNA was determined using CsCl gradients. Fig. 1A illustrates that most of the DNA synthesized had a density consistent with that expected for HSV-2 DNA. A minor peak corresponding in density to cellular DNA was also present. The pattern of DNA synthesized in isolated nuclei was similar to that for DNA synthesized in whole cells (Fig. 1B) which had been infected, incubated, labeled and extracted under conditions identical to those used with isolated nuclei. Separation and

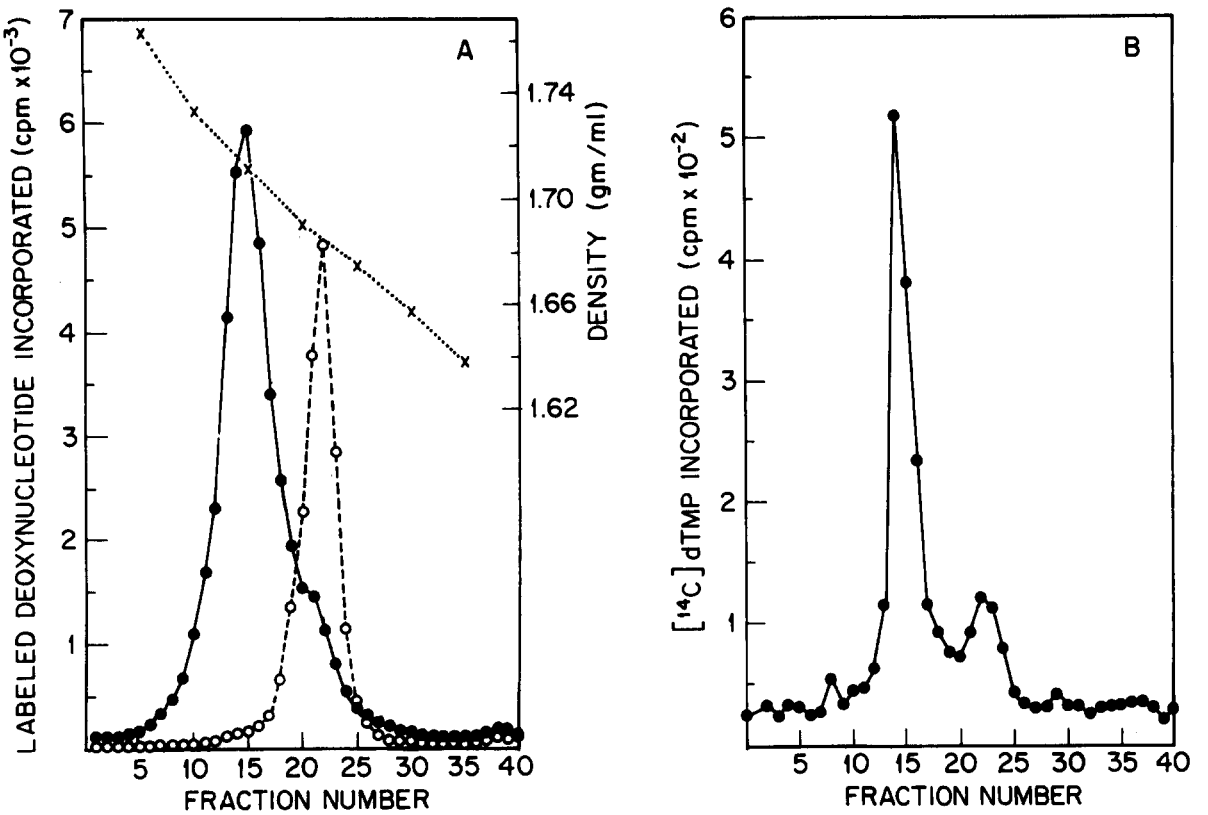

Fig. 1. Identification of viral and cellular DNA by isopycnic centrifugation in CsCl gradients. Panel A: DNA synthesized in isolated nuclei. Nuclei were incubated for 30 min at 37°C in a complete reaction mixture containing 1.25 μM [^{3}H]dCTP (20 Ci/mmol) and 100 μM dTTP to label DNA (●———●). DNA labeled with [^{14}C]dTMP from uninfected KB cells was used as a density marker (O— — —O). Panel B: DNA synthesized in intact cells. KB cells were infected with HSV-2 and incubated for 8 h in the absence of label and then for 30 min in the presence of [^{14}C]thymidine. DNA from nuclei and whole cells was extracted and sheared as described in Materials and Methods.

quantitation of the relative amounts of viral and cellular DNA by means of a curve resolver revealed that slightly more viral DNA was synthesized in isolated nuclei than in intact cells. Based on six replicate density gradient analyses, 77 ± 3% (mean ± S.D.) of DNA synthesized in isolated nuclei was viral compared to 69 ± 2% found in four analyses of intact cells. Therefore, with regard to the template utilized, DNA synthesis in isolated nuclei reflected synthesis in intact cells from which they were isolated. The peak of viral DNA synthesized in isolated nuclei (Fig. 1A) was broader than the peak from DNA synthesized in intact cells (Fig. 1B). The broadening may be due to the shorter size of DNA fragments labeled in isolated nuclei compared to DNA labeled in intact cells (see below).

To explore whether or not DNA synthesis in isolated nuclei was semiconservative, BrdUTP (100 μM) was substituted for dTTP and [^{3}H]dCTP was utilized as the source of label. For comparison, control nuclei were incubated with [α-^{32}P]dCTP and dTTP but no BrdUTP. The densities of the resulting labeled DNA were determined by equilibrium centrifugation in CsCl gradients. Fig. 2A shows that after a 5-min incubation, BrdUMP-substituted DNA shifted to a greater density than unsubstituted DNA. A more pronounced shift was observed after a 30-min incubation (Fig. 2B). The average density of the substituted DNA was 0.0049

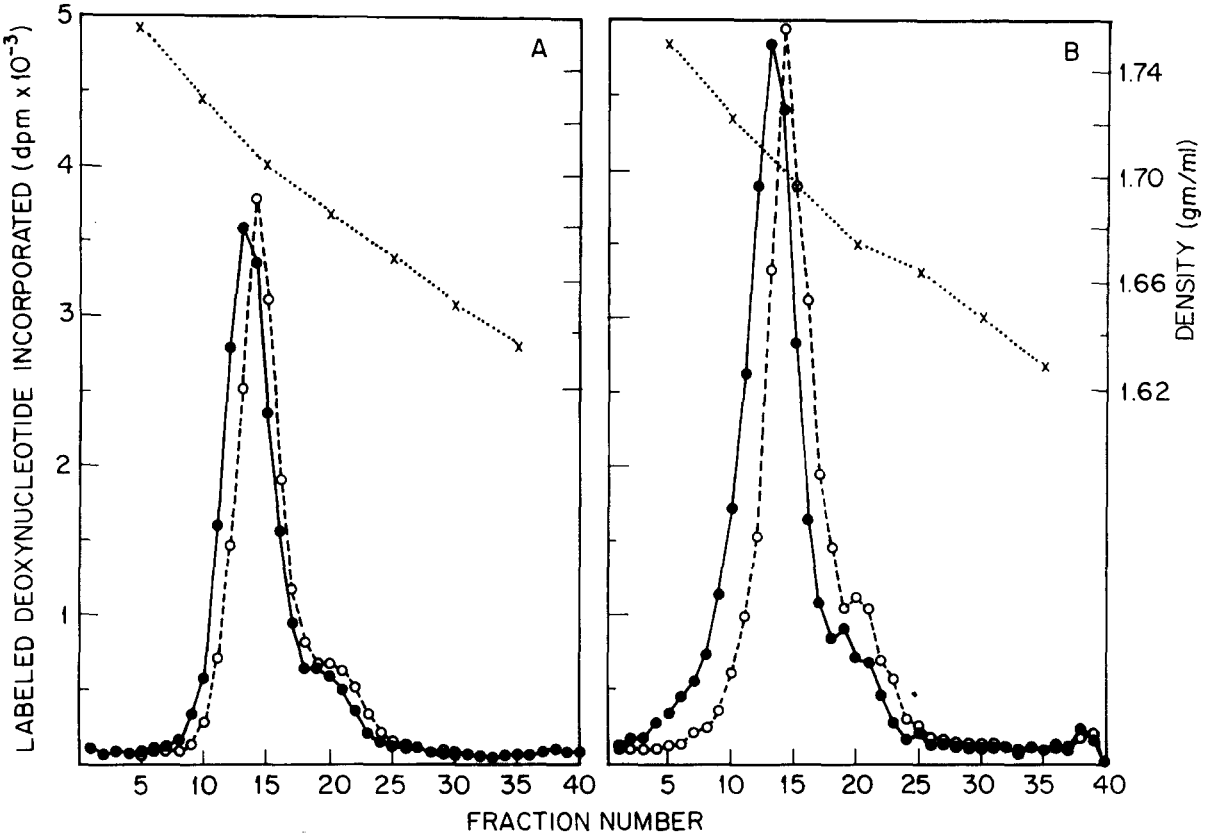

Fig. 2. Density of unsubstituted and BrdUMP-substituted DNA synthesized in isolated nuclei. Nuclei were incubated in complete reaction mixtures containing either 1.25 μM [α-^{32}P]dCTP (1 Ci/mmol) and 100 μM dTTP or 1.25 μM [^{3}H]dCTP (25 Ci/mmol) and 100 μM BrdUTP but no dTTP. Incubation was for 5 min (Panel A) or 30 min (Panel B). Equivalent amounts of each label were loaded into CsCl solutions and centrifugation was performed as described in Materials and Methods except DNA was not sheared. The same amount of density shift was observed in duplicate experiments in which DNA was sheared as described for the experiments depicted in Fig. 1. Unsubstituted DNA (O------O); BrdUMP-substituted DNA (●———●).

g/ml greater than unsubstituted DNA at 5 min and 0.0082 g/ml more dense after 30 min. Using the equation developed by Luk and Bick [30], we calculated that these density shifts correspond to the substitutions of 10 and 16% of dTMP by BrdUMP. This extent of substitution would be consistent with continuation of semiconservative replication which had been initiated in intact cells if only 10–20% of the DNA were replicated. However, because the DNA consisted of small fragments (see below), the same density shift could be caused by extensive repair of the DNA fragments. Thus, this amount of density shift does not constitute proof of semiconservative replication.

Size of DNA synthesized in isolated nuclei

After incubation of nuclei for 5 min in the complete reaction mixture, two size classes of DNA were evident in neutral sucrose gradients (Fig. 3). The larger DNA was heterogeneous with a peak at 15 S. According to the method of Burgi and Hershey [31], this corresponds to a molecular weight of $3.2 \cdot 10^6$. This class accounted for 56% of acid-insoluble radioactivity. The remaining DNA sedimented at less than 8 S. The two size classes of DNA were also evident when nuclei were labeled for 30 min (Fig. 3). All of the increase in radioactivity above the levels observed at 5 min was caused by an increase in the amount of the larger

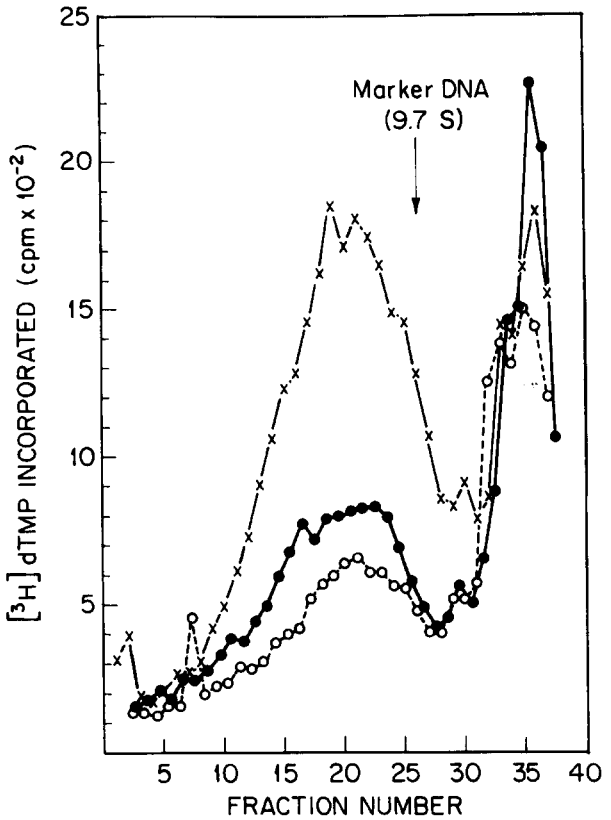

Fig. 3. Sucrose gradient analysis of DNA synthesized in isolated nuclei. Nuclei were incubated in a complete reaction mixture modified to increase the specific activity of dTTP to 24 Ci/mmol. After incubating for 5 min (●———●) or 30 min (×——— ×), reactions were terminated and analyzed on neutral sucrose gradients as described in Materials and Methods. In one set of reactions, a 100-fold excess of unlabeled dTTP was added after 5 min and the incubation continued for an additional 25 min (O------O). All reactions and gradients were run and analyzed in duplicate. Direction of sedimentation is from right to left.

class of DNA. Because no label was pelleted under these conditions, we conclude that no genome-size DNA nor concatemeric forms were present.

In order to determine if the marked increase in the amount of the larger class of DNA was a result of the ligation of smaller pieces of DNA, a pulse-chase experiment was performed. Nuclei were incubated in the complete reaction mixture for 5 min and then unlabeled dTTP was added to reduce the specific activity of [^{3}H]dTTP 100-fold. Following an additional 25-min incubation, the size distribution was essentially the same as that observed after 5 min except the total amount of incorporated label decreased by approx. 15%. The decrease occurred with all sizes of DNA except for a slight increase in intermediate size fragments (approx. 5 S, fractions 31–32 in Fig. 3). Virtually the same results were obtained when DNA was sheared prior to sedimentation through neutral sucrose gradients. Apparently, the size of DNA in nuclei already was small enough not to be affected by our shearing conditions. The observed labeling of the large fragments therefore, most likely represented nucleotide additions to pre-existing DNA and not ligation of the small fragments.

DNA labeled in isolated nuclei was also examined in alkaline sucrose gradients. Compared to DNA analyzed on neutral gradients, most denatured DNA was slightly smaller than marker (9.7 S) DNA indicating that nascent DNA was alkalilabile. Sizes of DNA ranged from 2 to 25 S. The size profile of DNA labeled for 2 min and chased for 28 min (addition of 100-fold excess of unlabeled dTTP) was virtually identical to the size profile of DNA labeled for 2 min (data not presented).

The relatively small size of DNA fragments labeled in isolated nuclei is perplexing since in intact cells replicating HSV DNA is found as high molecular weight concatemers [32]. In order to determine the effect of the in vitro incubation on DNA labeled in intact cells, infected cells were labeled with [^{3}H]thymidine for 1 h prior to isolation of nuclei. The nuclei were incubated in a reaction mixture in which none of the dNTPs were labeled. The size of labeled DNA extracted from these nuclei was determined by velocity sedimentation in both neutral and alkaline sucrose gradients. In both cases, most DNA labeled in intact cells sedimented to the lower one-half of the gradients and was, therefore, larger than DNA labeled in isolated nuclei but some sedimented at the same rate (data not presented). One possible explanation for this difference is that DNA labeled in isolated nuclei is more susceptible to endonuclease attack or shearing during extraction than is DNA labeled in intact cells.

Involvement of DNA polymerases in DNA synthesis

In order to determine which DNA polymerases were active in the synthesis of DNA described

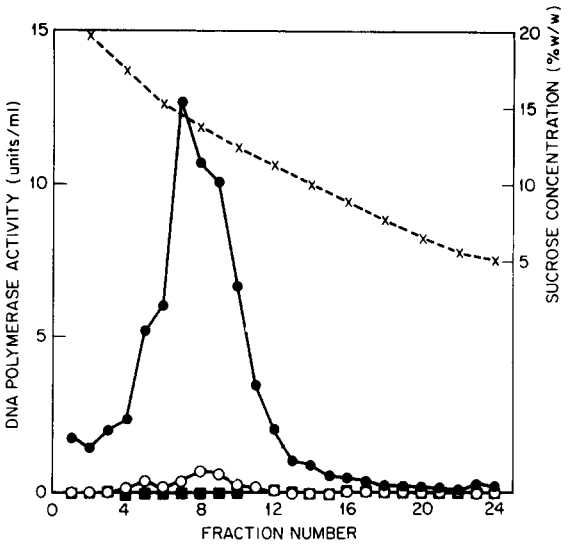

Fig. 4. Analysis of DNA polymerase activity in extracts of isolated nuclei. An extract of nuclei isolated from HSV-2-infected cells was sedimented through a 5–20% sucrose gradient for 20 h at 149000 × *g*. Each fraction was analyzed for DNA polymerase activity as described in Materials and Methods. Based upon our experience with HSV-1 DNA polymerase, the small peak of activity centered in fraction No. 8 may be HSV-2 DNA polymerase that cross-reacted in the α-polymerase assay (○——○). Units of polymerase activity are expressed as pmol [^{3}H]dAMP incorporated/min/ml under the specified assay conditions. ●——●, HSV DNA polymerase assay; ■——■, β-polymerase assay.

above, nuclear extracts were prepared and analyzed for DNA polymerase activities after velocity sedimentation through sucrose gradients. Fig. 4 illustrates that at least 95% of DNA polymerase activity in extracts from isolated nuclei was due to HSV-2 DNA polymerase. Only minor amounts of mammalian DNA polymerases α and β were detected. These results were confirmed by subsequent studies with known inhibitors of DNA polymerases. Tables I and III illustrate that DNA synthesis was strongly inhibited by *N*-ethylmaleimide, aATP and aCTP but not by ddTTP. These results are consistent with the known sensitivities of HSV and α DNA polymerase to *N*-ethylmaleimide, aATP and aCTP and β and γ polymerase to ddTTP [33]. We conclude, therefore, that HSV-2 DNA polymerase was responsible for all or nearly all the incorporation of [^{3}H]dNTPs into DNA.

Since HSV DNA polymerase is the active enzyme in isolated nuclei, Michaelis-Menton parameters for the dNTPs required for DNA synthesis were derived. The apparent K_m concentrations for all four dNTPs were significantly less than 1 μM (Table II) and are very similar to those reported for highly purified HSV-2 DNA polymerase [29]. They also are considerably lower than the 3–14 μM values derived using other readily obtained cellular preparations of crude herpes DNA polymerases (Ref. 34 and Reinke et al. unpublished data). A possible reason for this difference was a disparity in the rate at which dNTP substrates were degraded in isolated nuclei compared to extracts of HSV-2 infected cells used as a source of herpes DNA polymerase. For example, we found that 6% of [^{3}H]dATP and 10% of [^{3}H]dTTP were degraded during 10-min incubations with isolated nuclei compared to 80% degradation of [^{3}H]dATP in cell extracts. Thus, loss of substrate probably had negligible effect on K_m values for HSV DNA polymerase in nuclei, but the large amount of degradation in cell extracts may explain the higher K_m values obtained with this system. Isolation of nuclei from cytosol apparently removes most phophatase activity and results in K_m values similar to highly purified enzymes.

TABLE II

SUBSTRATE PARAMETERS FOR DNA SYNTHESIS IN ISOLATED NUCLEI

Nuclei were incubated in the complete reaction mixture for 4 min during which time incorporation was linear. The reaction mixture was modified so that the dNTP of interest was the limiting and radioactive substrate; concentrations ranged from 0.01 to 2.5 μM. Saturating concentrations (100 μM) of the other dNTPs were employed. The reciprocals of pmols of substrates incorporated were linearly regressed against the reciprocals of substrate concentrations; K_m and V_{max} values were calculated according to Lineweaver and Burk [47]. All regression lines fit data points with r^2 values > 0.96.

Substrate	K_m [a] (μM)	V_{max} [a] (pmol/min/10^6 nuclei)
dTTP	0.18	1.4
dATP	0.14	2.8
dCTP	0.55	1.6
dGTP	0.38	9.4

[a] Values presented are means calculated from results of three experiments with dTTP, two each with dATP and dCTP and one with dGTP. All assays were performed in duplicate.

Inhibition of DNA synthesis by fraudulent nucleotides

Since the affinity of HSV DNA polymerase for dNTP substrates in isolated nuclei appears to be remarkably similar to highly-purified enzymes, nuclei were used to examine the effect of fraudulent nucleotides on herpes DNA polymerase in situ. Inhibition was examined using aATP, the active nucleotide of an antiviral drug; aCTP, the active nucleotide of an anticancer drug; and ddTTP, an inhibitor of mammalian DNA polymerases β and γ. Fig. 5 illustrates that aATP and aCTP inhibited DNA synthesis in a manner competitive with the corresponding substrate. Little inhibition was noted with ddTTP (Table III). Inhibition by aATP appeared to be several-fold more potent than inhibition by aCTP based upon K_i concentrations. When, however, substrate affinity was considered by use of K_i/K_m or inhibitor/substrate ratios, aATP appeared to be only slightly more potent than aCTP as an inhibitor of herpes DNA synthesis (Table III). Use of the tritium-labeled compound demonstrated that aATP also served as a substrate for DNA synthesis. The K_m concentration (0.014 μM) was comparable to the K_i concentration but was considerably lower than the K_m for dATP (0.14 μM, Table II). The V_{max}, however, was only 2.2% of that noted for dATP indicating that despite high affinity for herpes polymerase, aATP has only limited substrate activity. These results are consistent with the limited incorporation of this antiviral drug into herpesvirus DNA [18] and its excision by herpes DNA polymerase [35].

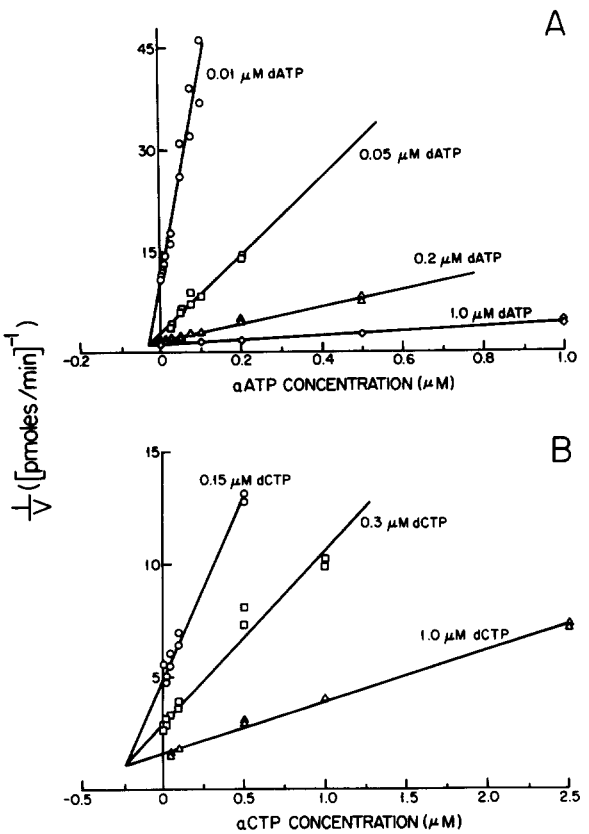

Fig. 5. Inhibition of nuclear DNA synthesis by fraudulent nucleotides. Nuclei were incubated for 4 min in the presence of aATP (Panel A) or aCTP (Panel B) using a protocol similar to that described in the footnote to Table II. Concentrations of the inhibitors and labeled substrates were varied as illustrated. The data are plotted according to the method of Dixon [49]. All regression lines fit the data points with r^2 values > 0.83.

TABLE III

INHIBITORY ACTIVITY OF FRAUDULENT NUCLEOTIDES

Inhibition parameter	aATP	aCTP	ddTTP
K_i (μM) [a]	0.03	0.22	[b]
K_i/K_m [c]	0.19	0.40	-
$(I/S)_{50}$ [d]	0.40	0.64	510
	(0.36–0.45)	(0.56–0.75)	(317–1053)

[a] K_i concentrations were determined as described in the legend to Fig. 5. Values are the average of two (aCTP, ddTTP) or three (aATP) separate experiments.

[b] Inhibition was too slight to provide accurate data.

[c] K_i/K_m ratios were derived using K_m concentrations reported in Table II.

[d] $(I/S)_{50}$ values were from dose-response curves derived by linearly regressing probit of response against log inhibitor/substrate ratio. Data from the replicate experiments used to calculate K_i concentrations were combined into a single curve for each nucleotide. Regression lines fit data points with r^2 values of 0.89, 0.90 and 0.55 for aATP, aCTP and ddTTP, respectively. $(I/S)_{50}$ values and corresponding 95% confidence intervals were calculated as described previously [48].

Discussion

We have characterized the synthesis of DNA in nuclei isolated from HSV-2 infected cells and found that it reflects DNA synthesis in intact cells. In

contrast to enzyme preparations, DNA synthesis in isolated nuclei utilized endogenous template and viral DNA was labeled to the same extent relative to cellular DNA as in intact cells. Furthermore, density labeling experiments showed that a large amount of substitution (16%) occurred in the isolated nuclei but not as much as would occur if a complete strand were synthesized (50%). Based upon the density shift and other experiments detailed above, an estimation of the number of dNTPs polymerized was possible. Since DNA preparations which were analyzed on CsCl gradients (Fig. 2, 30 min incubation) and on neutral sucrose gradients (Fig. 3, 30 min incubation) were labeled and sheared in the same manner, the fragments should have the same length. The sedimentation rate in neutral sucrose gradients indicated that the major size class of DNA had an average molecular weight of $3.2 \cdot 10^6$. Taking into consideration the G + C content of HSV-2 DNA (70%), we calculate that this molecular weight corresponds to approx. 9700 nucleotides. Because DNA synthesis was highly dependent on all four dNTPs (Table I), chain growth probably was processive. If dNTP addition was processive, then in order to 16% of dTMP to be substituted, 16% of the entire DNA fragment, or approx. 1500 nucleotides, would have been synthesized during the incubation. Therefore each DNA chain synthesized during the 30 min in vitro incubation could be approx. 1500 nucleotides, or 750 bp, long. This estimate is a maximum, however, because it assumes only one growing point per fragment.

Although the addition of nucleotides is 20–100-fold greater than that achieved with isolated enzyme preparations, it is still $< 1\%$ of the full genome length. This limited synthesis of DNA probably is due to the absence of factor(s) supplied by the cytoplasm in intact cells [36]. In addition, the smaller size of fragments labeled in the nuclei compared to DNA labeled in intact cells may be due to the absence of factors that would normally protect nascent DNA from degradation during the maturation process. The observation that DNA fragments labeled in intact cells immediately prior to isolation of nuclei are larger than the fragments labeled in vitro is evidence for this hypothesis.

Since most DNA polymerase activity extracted from isolated nuclei was the viral polymerase and because nuclear DNA synthesis was inhibited by known inhibitors of HSV DNA polymerase, we conclude that HSV-2 DNA polymerase was the major DNA polymerase in isolated nuclei. These observations confirm and extend the report of Bolden and coworkers [9] that phosphonoacetate and antiserum to HSV-1 DNA polymerase inhibited DNA synthesis in nuclei from HSV-1-infected cells. HSV-1 DNA polymerase has also been shown to be the major polymerase activity in isolated chromatin [37,38], in a soluble cell extract [39] and in a soluble replication complex [40]. In contrast, DNA polymerase α has been identified through inhibition data as the major polymerase active in nuclei from uninfected cells [41,42] and in the nuclear matrix from SV-40-infected cells [43].

DNA synthesis in HSV-2-infected nuclei responded to aATP, aCTP and ddTTP in the same manner as purified herpes DNA polymerase (Fig. 5). In fact, apparent K_m and K_i concentrations determined using isolated nuclei were similar to those reported only for highly purified enzymes. Substrate K_m concentrations (Table II) were virtually identical to those reported by Ostrander and Cheng [29] for highly purified HSV-2 DNA polymerase. The K_i value we found for aCTP was nearly the same as the value (0.12 μM) reported by Ostrander and Cheng. [29]. Our value for aATP (0.03 μM), however, was lower than the K_i value (0.42 μM) reported for the purified enzyme by these authors and the 0.5 μM value reported by Coen et al. [44]. The significantly lower K_i value for aATP that we consistently observed and our determination of a K_m value for aATP may reflect a difference in the properties of the HSV polymerase when associated with the in situ replication complex. This possibility illustrates the desirability of comparing kinetic data obtained with highly purified enzymes with data obtained from carefully characterized in situ systems such as isolated nuclei. The in situ systems may more accurately reflect the properties of the enzymes in intact cells.

Other work with uninfected cells supports our observations of K_m and K_i values near to or lower than those for purified enzymes. Krokan et al. [45] found low K_m values for dNTPs and also observed that aphidicolin inhibited DNA synthesis in isolated nuclei and DNA polymerase α by a similar

mechanism. Using permeabilized mouse cells, Müller and coworkers [46] found K_m (dATP) and K_i (aATP) values comparable to those seen with purified DNA polymerase.

Based upon the foregoing data, we believe that nuclei isolated from herpesvirus-infected mammalian cells provide a convenient in situ herpes DNA polymerase. The preparation is more easily obtained than are purified enzymes and has the advantage of providing the natural replication complex. Therefore, nuclei isolated from herpesvirus-infected cells are valuable for studying the action of inhibitors of DNA synthesis and of potential antiviral drugs.

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