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DNA Polymerase Requirements for Parvovirus H-1 DNA Replication In Vitro

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An in vitro system using nuclei from parvovirus H-1–infected cells was used to characterize the influence of inhibitors of mammalian DNA polymerases on viral DNA synthesis. The experiments tested the effects of aphidicolin, which is highly specific for DNA polymerase α, and 2',3'-dideoxythymidine-5'-triphosphate (ddTTP), which inhibits cellular DNA polymerases in the order γ > β > α. Both aphidicolin and ddTTP were inhibitory, indicating that both polymerase α and a ddTTP-sensitive enzyme are required for viral DNA synthesis. This was seen more clearly in kinetic measurements, which indicated an initial period of rapid DNA synthesis with the participation of polymerase α, followed by a period of less rapid, but more sustained, rate of DNA synthesis carried out by a ddTTP-sensitive enzyme, probably polymerase γ. One interpretation of the results is that polymerase α functions in a strand displacement stage of the viral DNA replication mechanism, whereas polymerase γ serves to convert the displaced single strands back to double-strand replicative form.

The genome of nondefective parvoviruses contains approximately 5,000 nucleotides in the form of a linear single-stranded DNA with short hairpins at both 5' and 3' termini (2, 3, 18, 27, 28). The intracellular replicative form is double stranded, with or without a terminal cross-link, and has a protein covalently associated with the 5' termini (25). Most of the genome coding capacity is required for the viral coat proteins, and as with other small DNA viruses, it is assumed that the viral replication mechanism is borrowed essentially completely from the host cell. We chose to study the replication of a member of this group, parvovirus H-1 (27), as a model for host DNA replication, and we report here studies on the polymerase requirements for viral DNA replication. The experiments were carried out in an in vitro system of nuclei isolated from virus-infected cells and used inhibitors to distinguish between activities of mammalian DNA polymerases.

MATERIALS AND METHODS

Cells and preparation of nuclei. NB cells (human embryonic kidney cells transformed by simian virus 40) (25, 27) were grown as monolayers in roller bottles (1,585 cm^2 each) in modified Eagle medium with 10% calf serum. Medium was changed at ~80% confluency (~8 × 10^7 cells per bottle), and 4 h later parvovirus-H-1 was added (20 infectious units per cell). At 18 h postinfection, the medium was removed, and the roller bottles were chilled and rinsed twice with 0.145 M NaCl, 1 mM potassium phosphate buffer (KPO_4), pH 7.6, 5 mM KCl, 10 mM Tris-hydrochloride, pH 7.6, 10 mM $MgCl_2$, and 0.5 mM $CaCl_2$. The cells were scraped out in 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Na^+), pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM KPO_4, pH 7.6, 0.2 M sucrose, and 0.1% bovine plasma albumin (3 ml/10^7 cells) with a rubber policeman. The cells were washed once with the same solution and once with 20 mM HEPES (Na^+), pH 7.6, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 M sucrose; they were then resuspended in twice their volume of the last solution without sucrose, and after 30 min on ice they were disrupted with a Dounce homogenizer. Nuclei were separated from cytosol by centrifugation (10 min, 900 × *g*), suspended in an equal volume of 30 mM HEPES (Na^+), pH 7.6, 10 mM KCl, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM dithiothreitol, and 50% glycerol, and stored in small portions at −70°C.

DNA synthesis in isolated nuclei. The standard incubation mixture for DNA synthesis contained 50 mM HEPES (Na^+), pH 7.6, 10 mM $MgCl_2$, 2 mM KPO_4, pH 7.6, 10 mM KCl, 20 mM phosphoenolpyruvate (Na_3), 50 μM dCTP, 50 μM dATP, 10 μM dGTP, 10 μM [^{3}H]dTTP (specific activity, 3.3 mCi/μmol), 0.2 mM each of GTP, CTP, and UTP, 5 mM ATP, 1 mM dithiothreitol, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 0.06% Nonidet P-40 (Shell), with 6 × 10^5 to 7.5 × 10^5 nuclei in a total volume of 30 μl. Dimethyl sulfoxide, used as solvent for the aphidicolin, had no effect on DNA synthesis in nuclei (nor with purified enzymes) at the maximum concentration achieved (1.7%). For analysis in gels, the radioactive label was [α-^{32}P]dCTP, and the concentration of dCTP was decreased to 10 μM and that of dTTP was increased to 50 μM. All incubations were at 37°C and were carried out for 60 min except where stated otherwise. Acid-insoluble counts were determined by mixing the sample with 2 ml of a solution

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containing 0.1 M potassium pyrophosphate, 5 mM EDTA, 50 μg of salmon sperm DNA per ml, and 0.5 M NaOH. The mixture was heated for 10 min at 60°C, and the DNA was precipitated in the cold with 2 ml of 3 M HCl and collected on Whatman GF/C glass-fiber filters. The filters were washed extensively with 10 mM HCl and acetone, dried, and counted in toluene scintillation fluid.

Purification of DNA polymerases. DNA polymerase α was partially purified from cytosol of cultured human lymphoblasts (8866) (33) by gradient elution (0.02 to 0.4 M KPO_4, pH 7.6) on a DEAE-cellulose column. The activity (197 nmol of total deoxynucleoside monophosphate per mg per 30 min) was identified by its peak elution at 0.14 M KPO_4, the predominant amount of the activity, its inability to utilize poly(rA)·oligo(dT), sensitivity to *N*-ethylmaleimide and aphidicolin, and insensitivity to 2',3'-dideoxythymidine-5'-triphosphate (ddTTP) (see below).

DNA polymerase β was purified from the fraction that did not adsorb to DEAE-cellulose (in 0.02 M KPO_4) (above) by adsorption (0.15 M KPO_4, pH 7.6)/step elution (0.3 M KPO_4) from a phosphocellulose column. The activity (13 nmol of total deoxynucleoside monophosphate per mg per 30 min) was identified as polymerase β by its chromatographic behavior (nonadsorption to DEAE-cellulose), ability to utilize poly(rA)·oligo(dT), resistance to *N*-ethylmaleimide and aphidicolin, and sensitivity to ddTTP.

DNA polymerase γ was purified from extracts of human placenta by salt gradients on columns of DEAE-cellulose (elution at 0.07 M KPO_4, pH 7.6) and native DNA cellulose (elution at 0.35 M KCl). The activity (82 nmol of total deoxynucleoside monophosphate per mg per 30 min) was identified as polymerase γ by chromatographic behavior, ability to use poly(rA)·oligo(dT), sensitivity to ddTTP and *N*-ethylmaleimide, and insensitivity to aphidicolin. The procedure is described in greater detail elsewhere (15a).

DNA polymerase assays. All DNA polymerase assays used for comparison with nuclear DNA synthesis were carried out with the same incubation mixture used for nuclei except for substitution of DNA polymerase and activated DNA (29) (0.4 mg/ml) in place of nuclei. All incubations were for 60 min at 37°C (reactions were linear for at least 60 min).

For purposes of enzyme purification and characterization (including the specific activities cited above) other than comparison with nuclei, enzyme assays were carried out under more specific conditions (15a). Briefly, the assays employed the following: for DNA polymerase α, activated DNA as primer-template in the absence of salt (other than 50 mM Tris buffer); for DNA polymerase β, activated DNA primer-template in the presence of 0.1 M KCl and *N*-ethylmaleimide; and for DNA polymerase γ, poly(rA)·oligo(dT) primer-template in the presence of Mn^{2+}.

Isolation of DNA. Three different methods of isolating the in vitro-labeled DNA were used. Method 1 followed the detergent lysis/NaCl precipitation procedure of Hirt (9). The Hirt supernatant was treated with pronase (grade CB, Calbiochem) (2 mg/ml) in sodium dodecyl sulfate (2%) for 90 min at 60°C and then with additional pronase (2 mg/ml) for 16 h at 37°C; this was followed by extraction with phenol (three times) and precipitation with ethanol in the presence of 1 M LiCl. In method 2 the reaction was stopped by EDTA (25 mM), and the nuclei were lysed with sodium dodecyl sulfate (2%) in a final volume of 100 μl. The remainder of the procedure (treatment with pronase, phenol and ethanol precipitation) was the same as for the preceding. The DNA was dissolved and sheared by repeated suction through a Pasteur pipette. Method 3 employed a modification of the Hirt procedure in which the sample was lysed in 0.5% sodium dodecyl sulfate (1 ml/5 × 10^6 cells) and kept at 0°C for 4 h (without NaCl); this was followed by sedimentation of high-molecular-weight DNA (together with precipitated detergent) at 103,000 × *g* for 10 min at 2°C. The supernatant was treated with RNase A (50 μg/ml) and then with pronase/sodium dodecyl sulfate, phenol, and ethanol as in the preceding.

Velocity sedimentation. Centrifugation was carried out in 5 to 20% sucrose gradients in a Beckman SW60 Ti rotor at 20°C. Neutral gradients contained 1 M NaCl, 10 mM HEPES, pH 7.6, 0.2% Sarkosyl (Geigy), and 3 mM EDTA; alkaline gradients contained 0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA.

Gel electrophoresis. Electrophoresis was carried out in 1% agarose horizontal slab gels in 40 mM Tris base, 20 mM acetic acid, 2 mM EDTA, and 0.5 μg of ethidium bromide per ml. Gels containing ^{32}P-labeled DNA were photographed on Kodak XR-5 film.

RESULTS

DNA synthesis in nuclei of infected and uninfected cells. (i) Kinetics of DNA synthesis. Synthesis of DNA was measured in nuclei incubated in a mixture containing buffer, salt, deoxynucleoside triphosphates, and ribonucleoside triphosphates. Nuclei of uninfected NB cells incorporated [^3H]dTMP into acid-insoluble material in an approximately linear fashion for about 20 min and at a diminishing rate for an additional 40 min (Fig. 1A). About two-thirds of the total synthesis was completed during the first 20 min. With a generation time of 22 h (37°C) and cell DNA content of about 6.3 pg, the rate of cellular DNA synthesis in vivo was approximately 14 pmol of total nucleotide per min per 10^6 cells. The initial rate of DNA synthesis in isolated nuclei of uninfected cells was about 0.3 pmol of total nucleotide per min per 10^6 cells, which corresponds to approximately 2% of the in vivo rate. In nuclei of H-1–infected cells, the initial rate was about five times as high and total incorporated nucleotide was four times as high as in uninfected nuclei. The amount of total DNA product synthesized in vitro corresponds to ~2,000 replicative form (RF) molecules per cell or ~1 to 2% of the total amount of RF present.

Omission of rATP (but not phosphoenolpyruvate) from the incubation mixture reduced synthesis by 40 to 45% (in the presence of phosphoenolpyruvate, deoxynucleoside triphosphate concentration is sustained). However, in either infected or uninfected systems, there was

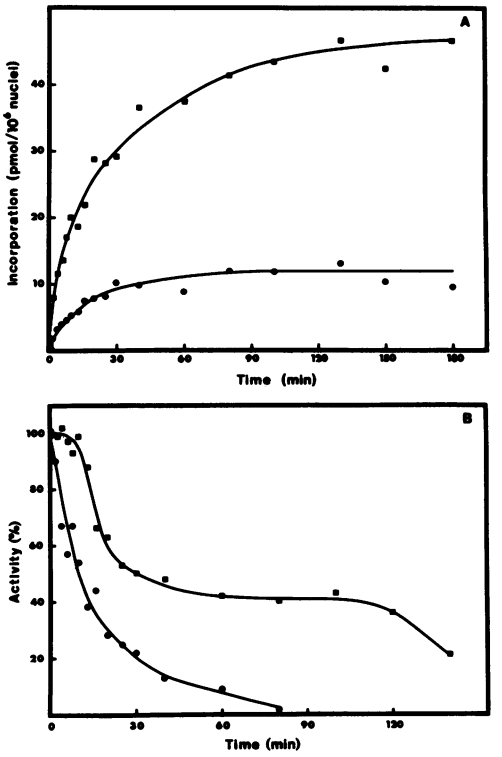

FIG. 1. DNA synthesis in nuclei of parvovirus-infected and uninfected NB cells. (A) Nuclei were incubated in the presence of components necessary for in vitro DNA synthesis (Materials and Methods) in a final volume of 0.6 ml. At each time point, 30 μl of reaction mix was withdrawn, and the incorporated radioactivity was determined. (B) Incubation was carried out as in A except that only unlabeled deoxynucleoside triphosphates were present until, at the times indicated on the abscissa, [^{3}H]dTTP was added and incubation was continued for an additional 30 min. Symbols: ■, infected nuclei; ●, uninfected nuclei.

minimal effect on synthesis (<10%) from omission of the other three ribonucleoside triphosphates or Nonidet P-40 or from inclusion of Ca^{2+} (0.5 mM), Ap_4A (0.1 mM), spermidine (0.5 mM) plus spermine (0.3 mM) (23), or cytosol (equivalent to about 7×10^5 cells).

In addition to quantitative differences, DNA synthesis in infected nuclei showed two kinetic phases, whereas with uninfected nuclei there was a single kinetic phase. During the initial 10 to 15 min, DNA synthesis occurred at a high rate, completing approximately 50% of the overall synthesis in this time. Thereafter, over a 10- to 15-min period, the rate rapidly fell to a fraction of the initial rate, at which it was maintained for another 80 to 100 min. The biphasic appearance of DNA synthesis in infected nuclei was more clearly demonstrated by pulse labeling at various times during the incubation (Fig. 1B). In nuclei of uninfected cells, the rate of DNA synthesis decreased rapidly during the first 15 to 20 min and at a diminishing rate from 20 to 60 min. DNA synthesis in nuclei of infected cells also declined rapidly after an initial high rate of nucleotide incorporation, similar to DNA synthesis in the uninfected system. However, in contrast to the uninfected system, instead of decreasing further, DNA synthesis in the infected nuclei entered a stable phase (between 20 and 120 min) during which the rate of DNA synthesis remained relatively constant. In nuclei of both uninfected and infected cells, the rates of DNA synthesis estimated by pulse labeling (Fig. 1B) correlated well with total DNA synthesis measured by cumulative incorporation of label from deoxynucleoside triphosphate (Fig. 1A).

(ii) [^{3}H]dTTP is incorporated into viral DNA. To test whether the incorporation of labeled deoxynucleotides into the DNA of infected nuclei occurred in chromosomal (host) or in viral DNA, in vitro-labeled material isolated from the nuclei of H-1-infected cells was analyzed by sedimentation through sucrose gradients. In a neutral gradient(s), the ^{3}H-labeled DNA product cosedimented with an internal marker of ^{14}C-labeled H-1 RF DNA isolated from H-1-infected cells labeled in vivo, the major peak of which corresponds to 16S double-stranded RF (26, 34) (Fig. 2A). In alkali the predominant 16S monomer length and smaller amount of 20S dimer (derived in part from "hinged" RF) molecules were both present in the in vitro product and again were not distinguishable from the in vivo material (Fig. 2B). In the experiment illustrated (Fig. 2), the DNA was prepared by a procedure

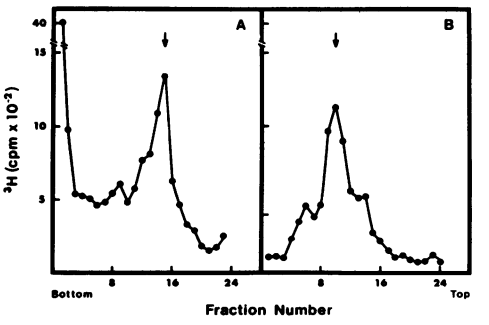

FIG. 2. Sucrose gradient centrifugation of parvovirus H-1 DNA synthesized in vitro. After incubation of nuclei from infected NB cells, the DNA was extracted (method 1; Materials and Methods) and analyzed by centrifugation on (A) a neutral sucrose gradient (45,000 rpm for 180 min) or (B) an alkaline sucrose gradient (50,000 rpm for 270 min). Sedimentation is from right to left; arrows indicate the position of internal marker DNA consisting of 16S ^{14}C-labeled double-strand monomer RF (neutral gradient) or 16S ^{14}C-labeled viral single strand (monomer) (alkaline gradient).

in which all of the DNA was analyzed after deproteinization (Materials and Methods); the results were not distinguishable from those obtained with a procedure that separates high-molecular-weight (cellular) DNA by detergent lysis and precipitation with salt (9).

A portion of the labeled material, prepared either in vivo or in vitro, sedimented much faster than RF and appeared at the bottom of the gradient. In the alkaline gradients, however, no DNA observed was larger than 20S, the size of a single-strand dimer of H-1 DNA (26, 34) (Fig. 2B). Similar evidence for material of unusual structure or high molecular weight was obtained by neutral gel electrophoresis (see below), and again, the same samples in alkaline gel electrophoresis contain only normal monomer- and dimer-length molecules. Those results, together with the *Hae*III restriction endonuclease pattern of the rapidly sedimenting portion (data not shown), indicate that the latter is composed of multiple copies of viral DNA. The sedimentation behavior was not changed by various alterations in the procedure, including the following, tested separately or in combination: more extensive treatment with protease, presence or absence of high salt, and presence or absence of the 60°C step (Materials and Methods), which could enhance renaturation of complementary strands. Intracellular "oligomeric" parvoviral DNA has been noted before (39). Neither the structure of the rapidly sedimenting material (whether random aggregates or ordered multimeric structures) nor the origin (whether formed during the replication process or as an artifact of the DNA isolation procedure) is established at this time.

It is concluded that the incorporation of labeled nucleotides into the DNA of isolated nuclei of H-1–infected cells occurs primarily into viral DNA and that the in vitro synthesis closely resembles in vivo events, in which there is little or no host cell DNA synthesis detectable 18 h after infection (unpublished data).

Influence of inhibitors of DNA polymerases on DNA synthesis in isolated nuclei: (i) Effect of aphidicolin. The influence of aphidicolin, which specifically inhibits polymerase α, on DNA synthesis in isolated nuclei was measured as a function of increasing drug concentration. Figure 3A shows that chromosomal DNA synthesis in uninfected nuclei was strongly inhibited by the drug (~25% residual activity at 1 μg/ml). DNA synthesis in infected nuclei was inhibited to a lesser extent (~40% activity remaining at 1 μg/ml). This difference between infected and uninfected nuclei was reproducible in repeated experiments. With the same drug concentrations and incubation conditions, purified DNA polymerase α was inhibited to a greater degree than DNA synthesis in uninfected nuclei, whereas the activities of DNA polymerases β and γ were not affected (Fig. 3B).

The results with aphidicolin indicate a requirement for polymerase α in chromosomal DNA synthesis, in agreement with previous studies with this inhibitor (14, 16, 17, 20, 41). Polymerase α also appears to have an important function in parvoviral DNA synthesis, as well; however, the lower sensitivity to aphidicolin of viral DNA synthesis compared with DNA synthesis in uninfected nuclei suggests the possibility of additional DNA polymerase requirements for viral DNA synthesis or the substitution of another DNA polymerase for α, or both.

(ii) Effect of ddTTP. Under the conditions of these experiments, ddTTP inhibited polymerase γ most strongly (30% residual activity at ddTTP/dTTP = 1), had a smaller effect on polymerase β (50% activity at ddTTP/dTTP = 1), and affected polymerase α only at high ratios (ddTTP/dTTP > 1) (Fig. 4B). (The inhibitory effects of ddTTP are more pronounced and appear to discriminate better between the enzymes, under *optimal* as-

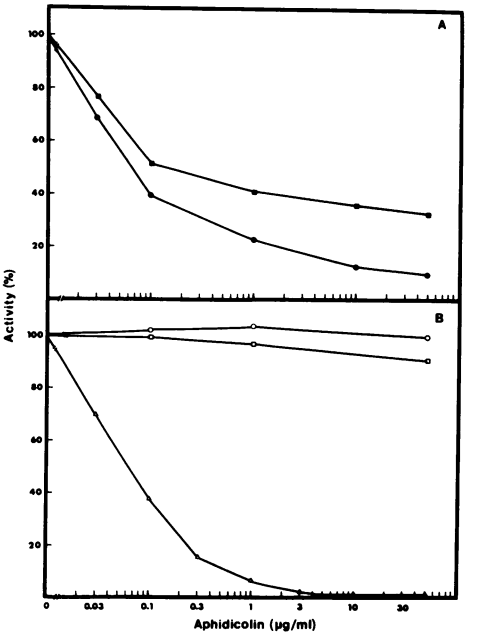

FIG. 3. Effect of aphidicolin on DNA synthesis in isolated nuclei and on DNA polymerases. (A) Effect of aphidicolin on parvovirus H-1–infected (■) and uninfected (●) nuclei (Materials and Methods). The amount of total deoxynucleoside monophosphate incorporated into DNA in the absence of aphidicolin was 6.2 pmol for infected nuclei and 2.4 pmol for uninfected nuclei. (B) Effect of aphidicolin on DNA polymerases α (△), β (○), and γ (□). Incubation conditions were the same as in A except for replacement of nuclei by activated DNA (330 μg/ml). Total deoxynucleoside monophosphate incorporated without inhibitor was 20.3 pmol for polymerase α, 9.8 pmol for polymerase β, and 9.2 pmol for polymerase γ.

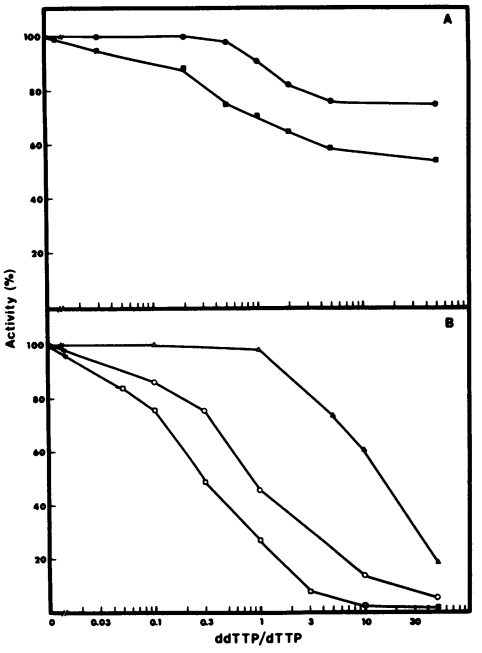

FIG. 4. Effect of ddTTP on DNA synthesis in isolated nuclei and on DNA polymerases. (A) Effect of ddTTP on parvovirus H-1-infected (■) and uninfected (●) nuclei. The amounts of total deoxynucleoside monophosphate incorporated into DNA in the absence of ddTTP were 5.5 pmol for infected nuclei and 2.2 pmol for uninfected nuclei. (B) Effect of ddTTP on DNA polymerases α (△), β (○), and γ (□). Incubation conditions were the same as for A except for replacement of nuclei by activated DNA (330 μg/ml). Total nucleotide incorporated without inhibitor was 22.1 pmol for polymerase α, 12.3 pmol for polymerase β, and 9.6 pmol for polymerase γ.

say conditions for each enzyme (1, 6, 10, 16, 22, 35) compared with the conditions selected for DNA synthesis in isolated nuclei, as used here.)

At ddTTP/dTTP = 1, a ratio at which polymerase γ-mediated DNA synthesis should be inhibited by at least 70 to 80%, uninfected nuclei were affected only slightly or not at all, whereas under the same conditions, synthesis of viral DNA was inhibited by about 30% (Fig. 4A). As in the experiments with aphidicolin, this difference between uninfected and infected systems was reproducible. The results with ddTTP indicate a role for DNA polymerase γ or β (or both) in parvoviral DNA replication in vitro. The results also suggest, but do not prove, that neither γ nor β polymerase is an obligatory requirement for normal cellular DNA synthesis in vitro (1, 6, 16, 35, 38, 41).

(iii) Drug sensitivity of the phases of viral DNA synthesis in vitro. The two phases of viral DNA synthesis in vitro were analyzed for differential sensitivity to the two inhibitors. The inhibitory effect of aphidicolin was seen only in the first phase of the reaction (Fig. 5), during which the rate of DNA synthesis was highest (Fig. 1B). During the second phase, which commenced after about 30 min, DNA synthesis was insensitive to this drug.

In contrast to the selective inhibition of the early phase by aphidicolin, ddTTP inhibited the entire period of DNA synthesis in infected nuclei to an equivalent degree. After a small increase of the inhibitory effect during the first 10 min, the influence of this drug became relatively constant, with suppression of DNA synthesis to about 40 to 45% of the activity without inhibitor. This is a considerably greater inhibitory effect than was observed with the standard 60-min incubation (Fig. 4). This can be accounted for by removal of the early phase of synthesis, which was relatively resistant to ddTTP (Fig. 5) and, because rate of synthesis was particularly high (Fig. 1), had a strong influence on total synthesis for the first 60 min. The change in drug sensitivity was not observed during the time course of DNA synthesis in uninfected nuclei; DNA synthesis was almost completely inhibited by aphidicolin and essentially unaffected by ddTTP throughout the reaction (data not shown).

Gel analysis of H-1 DNA synthesized in the in vitro reaction. Agarose gel electrophoretic analysis of the pulse-labeled product showed that most of the label was incorporated into monomer RF and material of higher molecular weight (Fig. 6B and C), with a distribution similar to

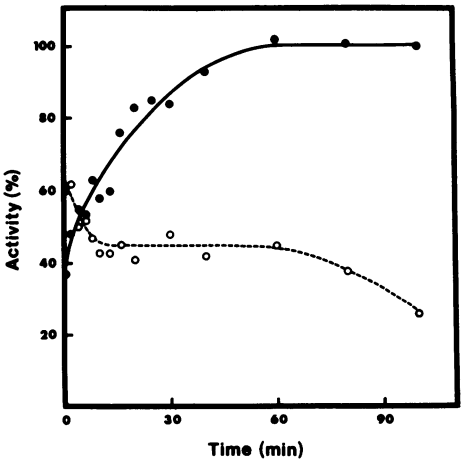

FIG. 5. Effect of aphidicolin and ddTTP on the rate of DNA synthesis in infected nuclei. Incubations were begun with only unlabeled deoxynucleoside triphosphates, without inhibitor or in presence of either aphidicolin (3 μg/ml) or ddTTP (ddTTP/dTTP = 1); at the times indicated on the abscissa [^{3}H]dTTP was added, and incubation was continued for an additional 30 min. Results are expressed as activity with inhibitor/activity without inhibitor × 100. Symbols: ●, aphidicolin; ○, ddTTP.

that of the total DNA in the sample (Fig. 6A). A large proportion of the DNA, both total (Fig. 6A) and in vitro-incorporated label (Fig. 6B and C), did not migrate into gels. This could not be avoided or reduced by modifications in the DNA isolation procedure, discussed above in relation to the rapidly sedimenting fraction (neutral sucrose gradients). It is assumed that the rapidly sedimenting fraction corresponds to the fraction that does not enter gels. Some of the labeled material migrating more slowly than RF may also consist of replicative intermediates with a partially displaced strand, but with the methods employed here it is not possible to distinguish these from a background of other slowly migrating species.

Radioactive label was also incorporated into material migrating between single-stranded viral and double-stranded RF DNAs. This is the region in the gel in which partially single-stranded and partially double-stranded monomer-length molecules would be found and, therefore, should contain intermediates in the process of conversion of displaced single strands to RF. The relative proportions of these replicative intermediates (between single-stranded viral and double-stranded RF) were higher in the later phase than in the earlier phase of the reaction (Fig. 6B, lanes a and d), suggesting that conversion of displaced strands to RF molecules may be a more prominent reaction in the later phase of viral DNA synthesis in vitro. This is seen more clearly in products reflecting comparable amounts of total incorporation (Fig. 6C). The differential effects of inhibitors of DNA polymerases, described above (Fig. 5), are well visualized by this approach. Aphidicolin inhibited DNA synthesis strongly early in the reaction but had little effect on DNA synthesis in the late phase (Fig. 6B, lanes b and e). In contrast, ddTTP had less effect than aphidicolin in the early phase, but caused very strong inhibition late in the reaction (Fig. 6B, lanes c and f).

An interesting feature of the in vitro-synthesized product is the occurrence of distinct bands between viral and RF DNA (Fig. 6B and C). Similar bands have been seen in the product of conversion of viral single strands to RF by DNA polymerase γ (15a), a reaction equivalent to the conversion of displaced strands back to RF. The bands are assumed to result from discrete pause sites in the chain elongation process, possibly due to secondary structure of the template DNA (11). Faint bands seen migrating more rapidly than single-stranded viral DNA may result from single-strand-specific nuclease acting on the partially replicated displaced strands or from release of partially completed new strands from replicative intermediates by branch migration of displaced strands.

DISCUSSION

We describe here an in vitro system with isolated nuclei from parvovirus H-1-infected NB cells and its use in studying the effects of DNA polymerase inhibitors on viral DNA synthesis. The nuclear system resembles that which has been used in our own and other laboratories to study DNA replication in nuclei from uninfected or virus-infected cells (1, 5, 6, 16, 30, 33–35, 38, 41). Previous work includes studies on adeno-associated virus (7) and bovine and rat

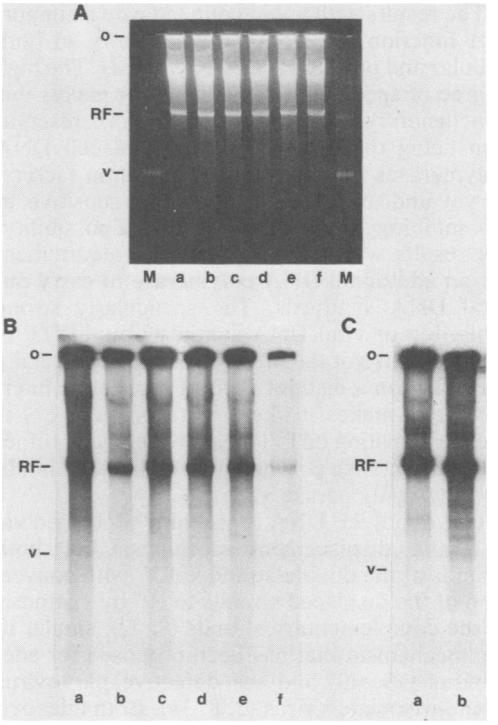

FIG. 6. Gel analysis of H-1 DNA synthesized in isolated nuclei. Incubations were begun with only unlabeled deoxynucleoside triphosphates until, at indicated times, the [α-^{32}P]dCTP was added and incubation was continued for either 10 or 30 min. Inhibitors, when present, were included from the beginning of the reaction. Viral DNA was extracted by method 3 and was analyzed by gel electrophoresis (Materials and Methods). (A) Photofluorograph of ethidium bromide-stained gel and (B) radioautograph of the same gel: ^{32}P labeling from 0 to 10 min (lanes a–c) or 60 to 70 min (lanes d–f). Lanes a and d were without inhibitor; b and e contained aphidicolin (3 μg/ml); c and f contained ddTTP (ddTTP/dTTP = 1). Lane "M" shows single-stranded viral ("v") and double-stranded RF ("RF") DNA markers; "o" indicates position of sample wells. (C) ^{32}P labeling from 0 to 10 min (lane a) or 45 to 75 min (lane b). Total incorporation (^{32}P) was (pmol): (B) a, 12.0; b, 3.2; c, 8.9; d, 5.3; e, 5.1; f, 1.2; (C) a, 13.3; b, 15.2.

(Kilham) parvoviruses (23). In contrast to single-stranded viral DNA synthesized in the latter system, the product in our system is predominantly double-stranded monomer RF (in both systems the in vitro process conforms closely to in vivo events at the time infected cells were collected for the isolation of nuclei). In addition, the DNA product is intact without the need for spermidine or spermine (23). As in most or all isolated nuclear systems, virus infected and uninfected, there is little or no evidence for reinitiation of synthesis, although in the present results the viral DNA synthesis is sustained longer than host chromosomal DNA synthesis.

The results with aphidicolin indicate an important function for DNA polymerase α, in both cellular and parvoviral DNA synthesis. The high degree of specificity of this inhibitor makes this conclusion rather secure, the principal reservation being the possibility of animal cell DNA polymerases or other DNA replication factors, as yet undiscovered, that are also sensitive to this inhibitor. If one rules out such a possibility, the results with ddTTP point to a requirement for an additional DNA polymerase to carry out viral DNA synthesis. The particularly strong inhibition of viral DNA synthesis by ddTTP in the late phase of the in vitro reaction, especially the effect on a distinct class of replicative intermediates, makes it likely that polymerase γ is the participating ddTTP-sensitive enzyme rather than polymerase β, which is more weakly inhibited than polymerase γ.

Parvoviral RF DNA is probably replicated via a strand displacement mechanism for both strands of the double-stranded RF, with conversion of the displaced strands to RF by synthesis of the complementary strands (8, 32), similar to the mechanism that has been proposed for adenovirus (19, 40) and the defective parvovirus adeno-associated virus (2, 8, 31). Both adenovirus (24) and the nondefective parvovirus (25) also have a protein, of as yet unknown function, covalently associated with both 5′ termini of the double-stranded DNA. Studies with inhibitors (1, 16, 17, 20, 35–37) and analysis of adenovirus replication complexes (1, 4, 15, 37, 42) have led to suggestions that polymerase γ is required for adenovirus DNA replication, probably in addition to polymerase α. Adeno-associated virus replication complexes also contained predominantly polymerase γ (7). In vitro experiments on adenovirus DNA synthesis indicate involvement of polymerase α, probably in the strand displacement mechanism (12).

In the system described here, there were early and late phases, distinguished by rate of synthesis, response to inhibitors, and forms of DNA synthesized. The results are consistent with polymerase α-dependent (aphidicolin-sensitive) strand displacement synthesis on H-1 RF occurring primarily during the initial ~20 min of incubation. The relatively constant ddTTP-sensitive synthesis that continued for ~120 min may correspond to the conversion of displaced single strands to RF by "gap-filling" or complementary strand synthesis by polymerase γ (or, less likely, polymerase β).

We have recently demonstrated the ability of purified polymerase γ to carry out the synthesis in vitro of RF from single-stranded viral DNA (15a), a reaction quite similar or identical to the synthesis of complementary strand on displaced single strands. The product of the reaction between polymerase γ and viral single strands included, in addition to full-length double-stranded RF, several discrete partially double-stranded products, similar to what was observed here as a feature of ddTTP-sensitive synthesis (Fig. 6). The obvious similarities between the conversion of viral single strands to parental RF and the conversion of displaced single strands to RF allows the expectation that both will have the same enzymatic mechanism, and the facile accomplishment of the former reaction in vitro by purified polymerase γ causes us to propose that this enzyme serves for both reactions, in vivo. If this is correct, it would explain the insensitivity to ddTTP of the in vitro parvoviral system of Pritchard et al. (23), since strand displacement was the principal reaction taking place under their conditions. The same function, synthesis of the complementary strand on displaced single strands by polymerase γ, may also account for the ddTTP sensitivity of adenovirus DNA synthesis. We believe that the assignments of enzyme function proposed here best account for a number of experimental observations, but we acknowledge that they are provisional and await, in particular, additional information about the capability for complementary strand synthesis that has been reported for polymerase β (13) and about the augmented functions described for polymerase α acting with accessory factors (12, 21).

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