JOURNAL OF VIROLOGY, Nov. 1984, p. 507–514
0022-538X/84/110507-08$02.00/0

Vol. 52, No. 2

Vaccinia Virus Induces Ribonucleotide Reductase in Primate Cells

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Received 30 April 1984/Accepted 31 July 1984

Infection of monkey kidney (BSC-40) cells with vaccinia virus strain WR resulted in a marked increase in ribonucleoside diphosphate reductase (EC 1.17.4.1) activity as measured by CDP reduction in cell-free extracts. After a synchronous infection, increased activity was detected at 2 h, peaked at 4 to 5 h, and then declined between 6 and 8 h to the endogenous cellular level. The induction, detectable at 0.5 PFU/cell, correlated strongly with multiplicity of infection to 10 PFU/cell and continued to increase to 50 PFU/cell. It paralleled the previously described induction of viral DNA polymerase and thymidine kinase, suggesting that the reductase may also be a product of early transcription of the viral genome. The inhibition of DNA synthesis throughout infection resulted in prolonged accumulation of reductase activity and delayed and incomplete down-regulation at 8 h, suggesting that repression involves late functions. Rescue of fluorodeoxyuridine-inhibited DNA synthesis with exogenous thymidine restored the normal pattern. Preferential association of the induced reductase with the cytoplasmic sites of vaccinia virus DNA replication (virosomes) was not detected. The induced enzyme is similar in several respects to other eucaryotic ribonucleotide reductases, but is distinct from host cell reductase in response to certain modulators of reductase activity (M. B. Slabaugh and Christopher K. Mathews, J. Virol. 52:501–506, 1984). Full activity required an activator, exogenous reducing equivalents, and iron. Hydroxyurea, EDTA, dATP, and dTTP inhibited CDP reduction, setting this reductase apart from T4 reductase, which is not inhibited by dATP, and from herpesvirus reductase, which requires no activation and is insensitive to deoxyribonucleoside triphosphate inhibition.

The cytoplasmic site of reproduction employed by the poxviruses provides a unique opportunity to study the expression and replication of a large extranuclear genome. In light of the extensive coding capacity of the viral DNA, it is not surprising that poxviruses are relatively independent of host cell functions. The enzymes required for RNA synthesis and processing, including the virus-specific RNA polymerase, are packaged within the virion, allowing transcription of the early class of genes to begin immediately upon uncoating of the core particle (for review, see reference 27).

That viral DNA synthesis is host nucleus independent is implied by the observations that neither physical removal of the nucleus before infection (15) nor specific inhibition of host transcription by α-amanatin (41) prevents replication of vaccinia virus DNA, although these procedures do block virion morphogenesis late in the productive growth cycle. Two activities involved in DNA metabolism, thymidine kinase and DNA polymerase, are now known to be virally encoded (14, 28, 44, 48) and are among the few products of early transcription that have been characterized at the functional level. Early induction of polynucleotide ligase (39), polyadenylic acid polymerase (2), and a deoxyribonuclease (26) have also been reported. However, the question of host enzyme participation in viral replication remains open, especially since enzymes of DNA precursor synthesis and polymerization are frequently detected in the cytoplasmic component of fractionated cells (35).

The hypothesis that viral DNA synthesis requires de novo synthesis of deoxyribonucleotides is supported by the observations that host DNA breakdown products are not utilized for viral replication (31), and that the salvage enzyme thymidine kinase is a nonessential activity under normal growth conditions (6). Ribonucleotide reductase is the only enzyme that catalyzes reduction of ribonucleotides to deoxyribonucleotides. Since reductase activity in eucaryotes is closely coupled to nuclear DNA synthesis and is nearly undetectable in nondividing cells, it seems likely that poxviruses might synthesize their own reductase. Two other large DNA viruses, bacteriophage T4 and herpesvirus, have in fact been shown to encode this activity (7, 16, 49). In both cases, the viral enzymes depart from the complex scheme of allosteric activation and regulation that is characteristic of cellular reductase (1, 7, 19–21).

We have studied ribonucleotide reductase (ribonucleoside diphosphate reductase, EC 1.17.4.1) activity in cell-free extracts from uninfected and vaccinia virus-infected BSC-40 cells. We report that infection resulted in increased CDP-reducing capability, the magnitude of which was dependent on the multiplicity of infection. The time course of the transient increase, which is maximal 4 to 5 h postinfection, coincided with the period of viral DNA synthesis (17) and declined to endogenous levels 6 to 8 h postinfection. Inhibition of DNA synthesis enhanced and prolonged the induction, suggesting that regulation required late functions. Unlike the activity induced by herpesviruses, the vaccinia virus-induced enyzme was sensitive to negative regulation by dATP and dTTP. However, studies of positive regulation (43) revealed differences between endogenous and induced enzymes, which led us to tentatively conclude that the induced activity is novel and probably virally encoded.

MATERIALS AND METHODS

Radiochemicals. [5-^{3}H]cytidine 5′-diphosphate, trisodium salt (>20 Ci/mmol) and [*methyl*-^{3}H]thymidine (70 Ci/mmol) were purchased from New England Nuclear Corp. and used without further purification. [*methyl*-^{3}H]thymidine triphosphate (45 Ci/mmol) was supplied by ICN Pharmaceuticals. Before use, the isotopes were taken to dryness under a stream of inert gas and immediately suspended in the reaction mixture.

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Reagents and supplies. Adenylylimido-diphosphate (β,γ-imidoadenosine 5'-triphosphate; AMP-PNP) was purchased from Sigma Chemical Co., as were other ribonucleotides, deoxyribonucleotides, and inhibitors. Tissue culture media were purchased from Irvine Scientific, and plasticware was from Corning Glass Works or Costar.

Virus and cells. Vaccinia virus strain WR was generously provided by D. Hruby and was propagated and titered on BSC-40 cells, a clonal derivative of BSC-1 cells selected for the ability to grow at 40°C. Cells were grown in monolayer culture in minimal essential medium supplemented with 5% heat-inactivated fetal calf serum. Cells were periodically tested for the presence of mycoplasma by using a DNA-fluorescent dye binding assay (3). For preparation of virus stocks, confluent monolayers in 100-mm plastic tissue culture dishes were infected with crude virus (0.1 PFU/cell) that had been treated 1:1 (vol/vol) with 0.25% trypsin in phosphate-buffered saline for 15 min at 37°C. Virus was diluted with ice-cold minimal essential medium–5% serum to quench the trypsin and then diluted further with phosphate buffered saline–1 mM $MgCl_2$–0.1% bovine serum albumin. Samples of 1.5 ml were added to dishes from which the culture medium had been aspirated. After a 3.5-h absorption period, 10 ml of minimal essential medium–5% serum was added. Cells were scraped from the dishes 48 h after infection and collected by low-speed centrifugation. Pellets were suspended in minimal essential medium without serum at 1 ml/dish and stored at −70°C in small working samples. The titer of crude stocks was determined by plaque assay as described by Hruby et al. (15) and was usually 2×10^9 to 4×10^9 PFU/ml.

Preparation of extracts for ribonucleotide reductase assay. Confluent monolayers of BSC-40 cells in 100-mm dishes from which medium had been aspirated were infected with vaccinia virus (generally at 10 to 20 PFU/cell) in 1.5 ml of phosphate-buffered saline–1 mM $MgCl_2$–0.1% bovine serum albumin. Before infection, crude stocks were trypsinized as described above. After a 1-h absorption period at 37°C, the inoculum was removed, and minimal essential medium–5% serum was added. If the effect of various drugs was to be studied, these compounds were present in the inoculum as well as the growth medium. At the designated time after infection, dishes were placed on ice, and the medium was aspirated. All manipulations from this point on were at 4°C. Cells were scraped from the dishes into phosphate-buffered saline and collected by low-speed centrifugation. The supernatant was aspirated, and the pellet was suspended in 1 to 2 ml of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.8)–10 mM dithiothreitol (DTT). Cells were again collected by centrifugation, the supernatant was aspirated, and the swollen cell pellet was suspended in lysis buffer at 5×10^7 or 1×10^8 cells per ml. Cells were disrupted either by sonication (three 10-s bursts, setting 4, Kontes cell disrupter), or Dounce homogenization with a tight-fitting pestle. Complete cell lysis was confirmed by microscopic examination. Either procedure yielded equivalent amounts of ribonucleotide reductase activity. Cellular debris was removed by centrifugation for 4 min in a refrigerated microfuge, and the supernatant was used directly as the enzyme source. Mock-infected BSC-40 cells harvested in the same manner provided endogenous ribonucleotide reductase.

Preparation of virosome fraction. Virosomal aggregates can be isolated by sucrose gradient centrifugation (5, 29). Since high concentrations of sucrose interfered with the standard ribonucleotide reductase assay, however, we sought conditions under which nuclei and virosomes could be separated by differential centrifugation. In preliminary experiments, we labeled uninfected cell nuclei by including [^{3}H]thymidine (0.1 μCi/ml) in growth medium for 16 h before harvest and specifically labeled viral DNA by infecting BSC-40 cells with vaccinia virus (10 PFU/cell) in the presence of [^{3}H]thymidine (0.5 μCi/ml). Aphidicolin (1 μM) was present during the 4-h infection to inhibit incorporation of the label into nuclei. Cells were scraped from dishes, collected by centrifugation, suspended in hypotonic buffer (25 mM HEPES [pH 7.8], 10 mM KCl, 2 mM magnesium acetate, 1 mM $CaCl_2$, 10 mM DTT) at 5×10^7 cells per ml and disrupted with 10 strokes of a Dounce homogenizer (tight pestle).

Centrifugation of homogenates for short periods (4 min) at low gravity forces followed by quantitation of acid-precipitable radioactivity in pellet and supernatant fractions revealed that virosomal DNA sedimented almost as rapidly as did host cell nuclei. After a centrifugation at $70 \times g$, 87% of nuclear DNA and 43% of viral DNA was pelleted. At $200 \times g$, 90% of nuclear DNA and 66% of viral DNA was sedimented; $500 \times g$ brought down 95 and 86%, respectively. A centrifugation at $2{,}100 \times g$ was sufficient to clear 98% of both nuclear and viral acid-precipitable radioactivity from homogenates.

For determination of enzyme association with virosomal aggregates, nuclei were first removed from homogenates of infected cells by centrifugation at $70 \times g$. The supernatant was subjected to centrifugation at $2{,}100 \times g$ to sediment remaining virosomes. Pellets were suspended in the homogenization buffer before assay for DNA polymerase and ribonucleotide reductase activity. Uninfected cells were carried through the same protocol.

Ribonucleotide reductase assay. Unless otherwise noted, the 40-μl standard reaction mixture for CDP reduction contained 100 mM HEPES (pH 7.8), 10 mM DTT, 10 mM NaF, 20 μM $FeCl_3$, 4 mM AMP-PNP, 2 mM magnesium acetate, 25 μM [^{3}H]CDP (100 to 200 cpm/pmol), and enzyme, usually added as one-half the final volume. After incubation in a 37°C water bath for 30 or 60 min, reaction tubes were transferred to ice, and reactions were terminated by adding 4.4 μl 10 M perchloric acid. Precipitated macromolecules were removed by centrifugation, and 40 μl of the supernatant was transferred to fresh microfuge tubes and capped snugly. The acidified solutions were heated in a boiling water bath for 20 min to hydrolyze phosphorylated nucleosides to the monophosphates. Tubes were cooled, and 4 μl of marker solution (CMP, dCMP, and dUMP, 20 mM each) was added. KOH (5 M) was added to neutralize the mixtures. Care was taken to ensure that the pH was >6 in each tube by spotting 0.5-μl samples on pH paper. Basic pH was not detrimental to the chromatography. After 15 min on ice, the large potassium perchlorate precipitate was spun down, and 20-μl samples of the supernatant were spotted in 2- to 3-μl increments on cellulose plastic-backed thin-layer chromatograms. A 15-cm-long wick prepared from Whatman 3MM paper was stapled to the top edge of the thin-layer chromatographic plate. With the wick folded back and supported by bent glass tubing, chromatograms were developed for 12 to 15 h with a solvent composed of ethanol–saturated sodium tetraborate–5 M ammonium acetate (pH 9.8)–250 mM EDTA (220:80:20:1, by volume) (36). The solvent was prepared fresh from stock solutions immediately before each run. Marker spots were identified under UV light, and pieces (2.5 by 1.5 cm) containing dCMP-dUMP were cut from the sheets and placed in scintillation vials.

Nucleotides were eluted with 1 ml of 0.5 N HCl for 15 to 20 min and then counted in 5 ml of Triton X-100 scintillation fluid.

In the presence of ATP or AMP-PNP, dCMP was the major reaction product detected after acid hydrolysis at 100°C. Under conditions that favored phosphatase activity, dUMP was generated due to high levels of deoxycytidylate deaminase activity present in BSC-40 cell-free extracts (C. Spiro, unpublished results). Conversion of product to dTMP was not detected. For routine assays, the elongated dCMP-dUMP spot was sectioned and place in one scintillation vial, a practice that did not depress counting efficiency. The number of counts per minute detected as dCMP-dUMP in reactions terminated at 0 min was routinely less than 0.5% of the total radioactivity applied. Enzyme activity is expressed either as picomoles per 10^6 cell equivalents or as picomoles per milligram of protein.

DNA polymerase assay. DNA polymerase was assayed by measuring the amount of [^{3}H]dTTP incorporated into acid-insoluble material. The reaction mixture contained, in a final volume of 200 μl, 50 mM HEPES (pH 7.8), 0.5 mM DTT; 5 mM $MgCl_2$; 100 μM each dATP, dCTP, and dGTP; 50 μM [^{3}H]dTTP (50 cpm/pmol); 50 μg of calf thymus DNA activated by heating to 100°C for 10 min followed by quick cooling; and the cell-free extract as the enzyme source. Reaction mixtures were incubated at 37°C for 30 min and stopped by the addition of 1 ml of ice-cold 1 M perchloric acid. Acid-precipitable material was collected on Whatman GF/C filters and washed repeatedly with cold 5% trichloroacetic acid. Filters were digested with 0.5 ml of Protosol (New England Nuclear) at 60°C for 30 min as specified by the manufacturer and then counted in Econofluor.

Other assays. Thymidine kinase in vaccinia virus-infected cell extracts was measured essentially as described by Hruby and Ball (13), except that the enzyme source was prepared as described above for the assay of ribonucleotide reductase. Protein concentrations were determined by a modification of the Folin phenol method (32).

RESULTS

Infection of BSC-40 cells with vaccinia virus produced a marked induction of ribonucleotide reductase activity as measured by conversion of [^{3}H]CDP to [^{3}H]dCDP by cell-free extracts. The virus-induced increase was detectable 2 h after a synchronous infection, increased still further with time up to 5 h, and then declined precipitously between 6 and 8 h (Fig. 1). Extracts from cells harvested 8 to 24 h postinfection exhibited no more activity than did uninfected host cell extracts (see Fig. 3B). When extracts from infected cells were assayed for thymidine kinase activity (Fig. 1), we observed the well-characterized pattern of rapid induction between 2 and 4 h followed by maintenance of plateau levels of activity for several hours (13, 18, 24). Measured 4 to 5 h after infection, the induction of ribonucleotide reductase was detectable with a virus input of 0.5 PFU/cell (Fig. 2). The extent of the induction was strongly dependent upon the multiplicity of infection up to approximately 10 PFU/cell and continued to increase somewhat as the multiplicity of infection was raised from 10 to 50. In most of the experiments reported in this communication, cells were infected at 10 to 20 PFU/cell.

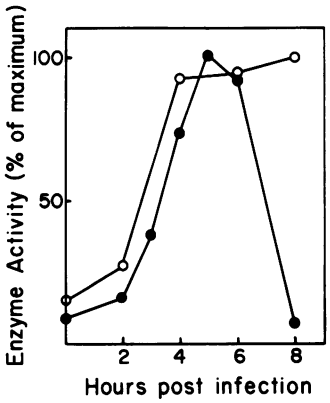

FIG. 1. Time course of the increase in ribonucleotide reductase activity (●) and thymidine kinase activity (○) in BSC-40 cells infected with vaccinia virus (10 PFU/cell). The reductase assays were performed in the presence of 10 mM AMP-PNP and 50 μM CDP. Magnesium acetate was omitted.

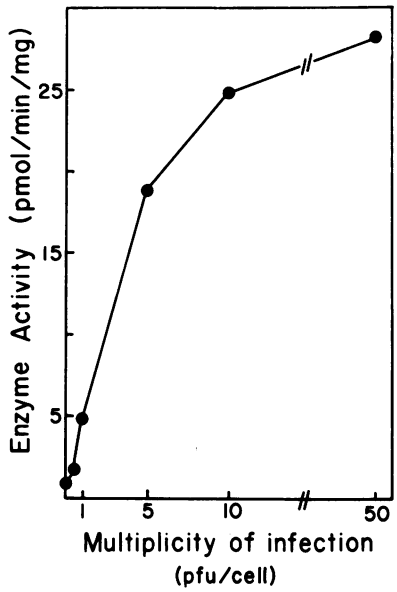

FIG. 2. Dependence of ribonucleotide reductase activity on the multiplicity of infection. Monolayers of BSC-40 cells were infected with virus at 0.5, 1, 5, 10, and 50 PFU/cell for 4.5 h. The activator (AMP-PNP) was present in the reductase reactions at 10 mM, and the substrate (CDP) was present at 50 μM. Magnesium acetate was omitted.

The induced activity in crude extracts was unstable in our hands. Preparations kept on ice in the presence of 10 mM DTT for 4 h or dialyzed against DTT-containing buffers for a similar time exhibited only 30 to 40% of the activity measured in fresh extracts. Furthermore, storage of either frozen infected cells or cell extracts at −20°C resulted in the loss of >95% of the induced activity within 1 week. Neither host cell reductase activity nor the virus-induced thymidine kinase activity displayed such lability. All experiments reported herein were performed with freshly prepared enzyme. Determination of stabilizing conditions for the induced reductase awaits further experimentation.

Regulation of reductase expression in vaccinia virus-infected cells. Vaccinia virus-induced thymidine kinase and DNA polymerase activities increase rapidly for 3 to 4 h after infection, then plateau abruptly, and are maintained at induced levels for 18 to 24 h (13, 24). Both genes are classified as "early" genes because they are transcribed by

the virion RNA polymerase immediately on infection. Early transcription is insensitive to inhibitors of protein or DNA synthesis. In a normal infection, transcription of early genes declines with time, and "late" genes are expressed after the onset of DNA replication. Repression of early gene expression is not well understood, but is thought to involve virally encoded functions since the presence of cycloheximide throughout an infection prevents shutoff of early transcription and expression of late genes.

In some virus-host systems, repression seems to be a late gene function, since inhibitors of viral DNA synthesis promote "superinduction" of thymidine kinase, DNA polymerase, and DNase (18, 25). However, in other virus-host combinations, neither DNA nor RNA synthesis inhibitors added as early as 30 min postinfection prevent repression of thymidine kinase (13, 50), suggesting that repression is expressed as an early gene function.

The overall pattern of ribonucleotide reductase induction differs from the other vaccinia virus-induced enzymes of DNA metabolism described to date in that the activity peaks at 4 to 5 h and then declines rapidly to endogenous levels. If this activity is a viral gene product, translational inhibition or inactivation of the enzyme (or both), as well as repression of transcription, may be involved in the regulation of its expression.

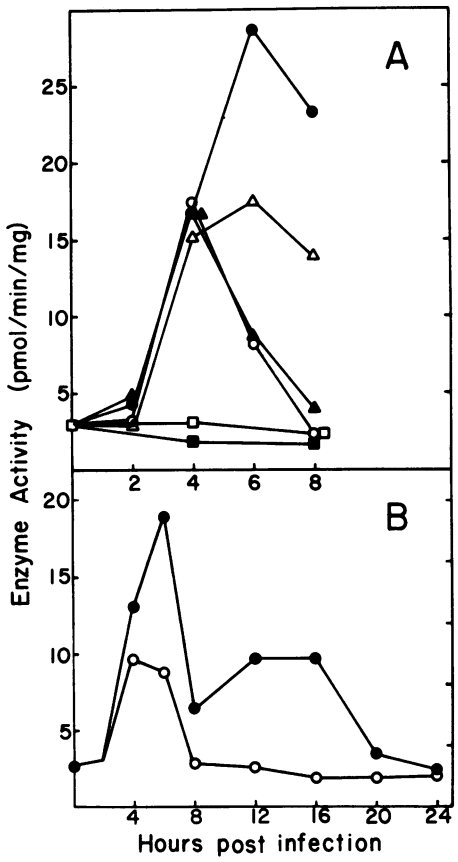

FIG. 3. Effect of inhibiting viral DNA synthesis on induction of ribonucleotide reductase. A and B present the results of two independent experiments. The reductase assay mixtures contained 5 mM AMP-PNP and 25 μM CDP. Magnesium acetate was omitted. After infection at 10 PFU/cell, monolayers of BSC-40 cells were harvested at the times indicated, and extracts were prepared and assayed immediately. araC (100 μM), FUdR (10 μM), and thymidine (100 μM) were added at the time of infection. Symbols: ○, vacinnia virus-infected cells; ●, vaccinia virus-infected cells plus araC; △, vaccinia virus-infected cells plus FUdR; ▲, vaccinia virus-infected cells plus FUdR and thymidine; □, uninfected cells; ■, uninfected cells plus FUdR.

We have investigated the effects of inhibiting viral DNA synthesis on the induction of ribonucleotide reductase. The presence of fluorodeoxyuridine (FUdR; 10 μM) or cytosine arabinoside (araC; 100 μM) in the growth medium disrupted the normal pattern of reductase regulation (Fig. 3A). Enzyme induction continued for 2 h beyond the normal time, and the decline in activity was delayed. Rescue of DNA synthesis from FUdR inhibition by thymidine (100 μM) completely restored the pattern observed in an uninhibited infection.

AraC was more potent than FUdR in superinducing reductase in this experiment, a result which may be due to partial rescue of FUdR-inhibited DNA synthesis by low levels of thymidine in the serum-supplemented growth medium. FUdR-inhibited vaccinia virus DNA synthesis has been shown to proceed at maximal rates in the presence of as little as 5 μM thymidine (40).

A 24-h time course carried out in the presence of araC revealed that enhancement of reductase specific activity was observed up to 20 h postinfection (Fig. 3B). Since inhibition of viral DNA synthesis prolongs "early" transcription, this could account for the extended period of reductase activity accumulation that we observed in the presence of both FUdR and araC. Repression or inactivation (or both) of this activity, however, is clearly a complex process, possibly involving transcriptional and translational controls. Understanding of these mechanisms in vaccinia virus may shed light on the analogous control of ribonucleotide reductase activity in eucaryotic cells.

Is vaccinia virus-induced reductase associated with virosomes? It has been reported that a large fraction (15 to 50%) of the DNA polymerase activity induced in vaccinia virus-infected cells can be sedimented with the cytoplasmic inclusions known as virosomes or viroplasm (18, 23). These nonmembrane-bound nucleoprotein aggregates are sites of poxvirus replication and virion assembly. Since ribonucleotide reductase provides precursors for DNA synthesis, it was of interest to determine whether the induced activity might also be associated with virosomes.

Virosomes can be isolated as rapidly sedimenting material on 38 to 50% sucrose gradients (5, 29). We found, however, that high sucrose concentrations were incompatible with the standard reductase assay; therefore, we prepared "nuclear" and "virosomal" fractions by differential centrifugation. In preliminary experiments, conditions for the sedimentation of virosomes were determined with extracts from cells infected for 4 h in the presence of [^{3}H]thymidine and 1 μm aphidicolin, a concentration of drug which inhibits the cellular, but not the viral, replicative polymerase. The results of these studies are described above. Extracts from infected and uninfected cells were first subjected to low speed centrifugation (70 × g, 4 min) to remove nuclei; the supernatants were recentrifuged (2,100 × g, 4 min) to pellet virosomal material. A large proportion (43%) of the virosomal DNA was found in preliminary experiments to sediment with nuclei.

We recovered approximately half of the total virus-specific DNA polymerase activity in the two pellet fractions (Table 1); 44% of the sedimentable activity was in the low-speed pellet. In contrast to these results, 95% of the ribonucleotide reductase activity in infected cells remained in supernatant fractions, suggesting that if reductase is complexed with the

TABLE 1. DNA polymerase and ribonucleotide reductase activities in cellular subfractions of uninfected and vaccinia virus-infected BSC-40 cells[a]

Activity	Fraction	Sp act (cpm)		
		BSC-40	Vaccinia virus plus BSC-40	Vaccinia virus specific
DNA polymerase[b]	70 × g pellet (nuclei)	777	3,187	2,410
	2,100 × g pellet (virosomes)	310	3,376	3,066
	2,100 × g supernatant	3,535	9,234	5,699
Ribonuclotide reductase[c]	70 × g pellet (nuclei)	25	158	133
	2,100 × g pellet (virosomes)	36	140	104
	2,100 × g supernatant	326	4,611	4,285

[a] The data from uninfected cell extract were subtracted from infected cell extract counts to derive vaccinia virus-specific values. The data tabulated are means of three independent DNA polymerase assays or four ribonucleotide reductase assays for each fraction. The background counts per minute (124 for the polymerase assay and 360 for the reductase assay) have been subtracted.
[b] Counts per minute for 2.5×10^6 cell equivalents.
[c] Counts per minute for 3.3×10^5 cell equivalents.

replication apparatus in vivo, the association does not survive our extraction and centrifugation conditions. Virus infection increased ribonucleotide reductase activity 12-fold over endogenous levels in this experiment. The increase in virus-specific polymerase activity measured in the same extracts was 2.4-fold. Nevertheless, viral DNA polymerase specific activity exceeded that of reductase by a factor of 4 to 6.

Effects of additions and subtractions to the standard reductase assay. Ribonucleotide reductases from a wide variety of organisms conform to the same basic design. The active enzyme is a complex of two nonidentical subunits: a large, nucleotide-binding, sulfhydryl-containing protein is associated with a smaller subunit carrying a stable, organic free radical, which, in the case of *Escherichia coli* and eucaryotes, is an iron (III)-tyrosyl center (for review, see reference 19). Hydrogens from the enzyme dithiol are transferred to the iron-tyrosyl radical and subsequently displace the C2'-OH of the ribonucleotide substrate in a reaction that is unique in biological systems and whose precise mechanism is still incompletely understood (38). In vivo, reduced thioredoxin, and in vitro, small-molecule dithiols such as DTT, serve as ultimate sources of reducing power.

TABLE 2. Effects of additions and deletions on vaccinia virus-induced CDP reduction[a]

Addition or deletion	Relative rate (%)
None	100
−NaF	89
$-FeCl_3$	72
−DTT	32
+ 1 mM EDTA	28
+ 5 mM EDTA	20
+ 0.1 mM hydroxyurea	96
+ 1 mM hydroxyurea	63
+ 10 mM hydroxyurea	22

[a] The complete reaction mixture contained 50 mM HEPES (pH 7.4), 10 mM DTT, 10 mM NaF, 20 μM $FeCl_3$, 10 mM AMP-PNP, 50 μM [5-^{3}H]CDP (100 cpm/pmol), and enzyme.

We have made a preliminary assessment of the vaccinia virus-induced enzyme with respect to these characteristics by noting the effects of various additions and deletions to the standard reaction mixture (Table 2). The omission of NaF, which was included as a generalized phosphatase inhibitor, had only slight effect. The omission of $FeCl_3$ resulted in a 30% loss of activity. This result indicates that the induced enzyme may bind iron comparatively loosely, since omission of iron in assays of mammalian reductase has no untoward effects (9). The inhibitory effects of EDTA constitute further evidence for metal involvement in the catalytic reaction. The omission of exogenous reducing equivalents drastically inhibited CDP reduction, and hydroxyurea, which destroys the tyrosyl free radical (8), inhibited the induced enzyme in a dose-dependent manner. Triton X-100 at 0.1 and 0.5% had little effect on activity (data not shown).

Effect of dTTP and dATP on vaccinia virus-induced ribonucleotide reductase. The discovery of the reductase induced by herpesviruses was preceded by the observation that herpes simplex virus type 1 replication in vivo is unimpeded by exogenous thymidine at concentrations sufficient to block host cell DNA synthesis (4). The inhibition of cellular replication by thymidine is due to the negative effect of the metabolite dTTP on CDP reduction. Several studies have now demonstrated that herpesvirus ribonucleotide reductase is an unusual enzyme that is highly resistant to dTTP and dATP inhibition (1, 15a, 20, 21).

An early study indicated that vaccinia virus replication might also be resistant to thymidine (12). To confirm this, we passaged vaccinia virus in the presence and absence of thymidine (2 mM, 0.1 PFU/cell). Thymidine reduced the yield of infectious virus by nearly 4 orders of magnitude (Fig. 4). The yield of virus in the presence of the nucleoside did not exceed the inoculum after 24 h. We conclude that vaccinia virus replication was indeed sensitive to thymidine, and that the results of the earlier study (equal infective titers from passage with or without thymidine) were the result of using a very high multiplicity of infection (which in itself reduces yield), since we also observed less than 1 order of magnitude inhibition by thymidine after infection at 10 PFU/cell.

We have directly tested the vaccinia virus-induced reduc-

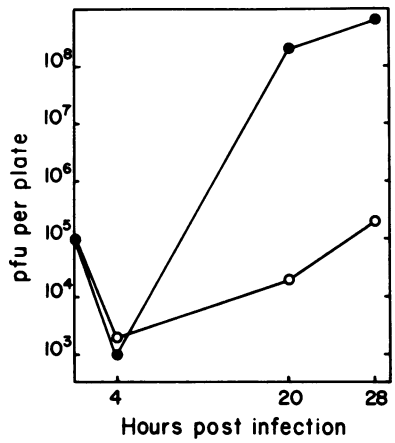

FIG. 4. Effect of thymidine (2 mM) on the one-step growth of vaccinia virus. BSC-40 monolayers in 35-mm dishes were pretreated with thymidine for 4 h before infection at 0.1 PFU/cell. Duplicate dishes were harvested at 4, 20, and 28 h postinfection. Virus growth in the presence (○) and absence (●) of thymidine was quantitated by a plaque titration assay.

tase for inhibition of CDP reduction by dTTP and dATP. Increasing concentrations of the deoxynucleoside triphosphates were added to assay mixtures containing enzyme from uninfected or infected BSC-40 cells. Although the CDP reduction in uninhibited reactions containing infected cell extract was 10 times greater than that in reactions containing the control cell extract, the induced activity was at least as sensitive as the BSC-40 host enzyme to both negative effectors (Fig. 5). The induced activity is somewhat more sensitive to dATP than to dTTP. The inhibition of the viral reductase by dTTP provides an explanation for the severe inhibition of production virus growth we observed in the presence of thymidine.

DISCUSSION

The finding that cytoplasmic ribonucleotide reduction is strongly enhanced by vaccinia virus infection adds yet another enzyme to the list of activities involved in DNA metabolism which are induced by the poxviruses. Induction of DNA polymerase (18), thymidine kinase (24), polynucleotide ligase (39), and deoxyribonuclease (26) has been previously reported. To date, only DNA polymerase and thymidine kinase have been unequivocally shown to be viral gene products through isolation of mutants and physical mapping of the genes (14, 28, 44, 48).

Ribonucleotide reduction occupies a position of central importance in replication, for this reaction is the only pathway providing de novo-synthesized deoxyribonucleotide precursors for DNA synthesis. The singular role of these small molecules, aside from regulation of their own synthesis (10) and possible effects on initiation of DNA synthesis (37), is incorporation into DNA. The tight coupling of cellular ribonucleotide reduction to DNA synthesis (22) suggests that ribonucleotide reductase may be involved directly in the regulation of DNA synthesis (45).

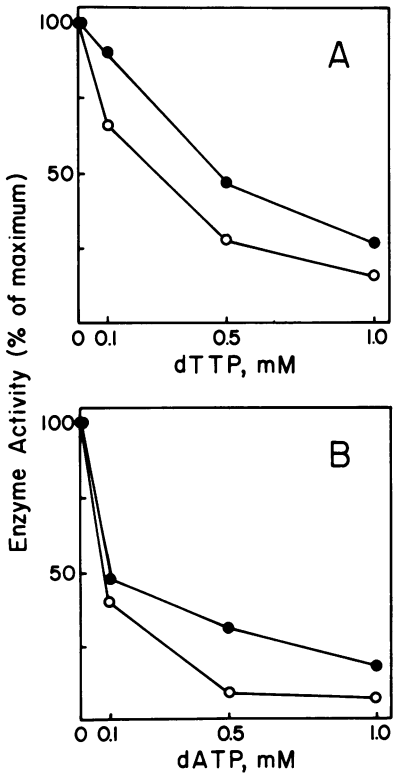

FIG. 5. Effects of dTTP (A) and dATP (B) on ribonucleotide reductase activity in extracts from infected (○) and uninfected (●) cells. The data are expressed as the percentage of uninhibited activity to facilitate comparison; 100% is 76 and 7 pmol/h per 10^6 cell equivalents for infected (10 PFU/cell) and uninfected cell extracts, respectively.

Upon infecting a cell, a virus could either take advantage of the resident cellular reductase, possibly augmenting the rate of reduction by derepression of enzyme synthesis or by metabolic activation, or encode a novel reductase of its own. The second alternative is the one utilized by two large DNA virus groups: T-even bacteriophages (49) and the herpesviruses (7). That vaccinia virus, with a genome even larger than those of herpesviruses, should also encode its own reductase seems likely, since replication occurs in the cytoplasm of the infected cell and is independent of the host nucleus (15). The time course of appearance of the induced reductase activity, the correlation with the multiplicity of infection, and the superinduction achieved on inhibition of DNA synthesis are all consistent with the idea that ribonucleotide reductase is an immediate-early viral gene product, analogous to thymidine kinase and DNA polymerase. Although the data reported herein do not prove such a hypothesis, we report in the accompanying paper (43) that the endogenous and induced enzymes respond differently to certain effectors of CDP reduction.

In view of their fundamental role in replication (and their possibly prebiotic evolution), it is perhaps not surprising that ribonucleotide reductases from a wide variety of organisms share common features. Although cobalamin- and manganese-dependent enzymes have been identified, the non-heme iron- and tyrosyl-free radical-containing, sulfhydryl proteins of *E. coli* and mammalian species are the most thoroughly understood. Based upon the following observations, we conclude that the vaccinia virus-induced reductase is of this class. (i) The requirement for exogenous DTT suggests that sulfhydryl groups are involved in the reductive reaction. (ii) The partial loss of activity observed on omission of $FeCl_3$ from the reaction mixture and susceptibility to hydroxyurea inhibition are diagnostic of a metal-stabilized free radical at the active site. (iii) The nonlinearity of CDP reduction at high enzyme dilutions (43) suggests a dissociating multisubunit system, characteristic of all known eucaryotic reductases.

In contrast to the apparently unregulated ribonucleotide reductase encoded by herpesviruses, the vaccinia virus-induced enzyme appears to retain allosteric regulation by deoxynucleoside triphosphates, thought to direct reduction toward each of the four alternate substrates (10). CDP reduction by virus-induced enzyme was at least as sensitive to the inhibitory effects of dATP and dTTP as was reduction by the endogenous enzyme. Further experimentation will be required to determine whether dTTP stimulates GDP reduction and whether dGTP stimulates ADP reduction. The selection of mutant S49 cells that contain reductases insensitive to dATP and dGTP (47) suggests that isolation of vaccinia virus mutants harboring altered reductase may be possible.

Although this communication is the first description, to our knowledge, of induction of ribonucleotide reductase after vaccinia virus infection, high levels of CDP reductase were detected in cell-free extracts of a monkey tumor induced by Yaba poxvirus (11). The significance of this result is difficult to evaluate, since virtually all tumors (as well as rapidly growing normal tissues) exhibit relatively high specific activities of ribonucleotide reductase. However, the detection of characteristic cytoplasmic inclusions

(virosomes) in thin sections of Yaba tumor cells (30) suggests the active participation of the virus in tumor growth. Another member of the poxvirus family which is tumorigenic in rabbits, Shope fibroma virus, has been shown to stimulate host cell replication (33, 42, 46). As with Yaba tumor virus, characteristic cytoplasmic inclusions are apparent in fibroma virus-infected cells. Whether elevated ribonucleotide reductase activity due to poxvirus infection might be a factor promoting or permitting host cell proliferation is a question of considerable interest, especially in light of the suggestion (for which no direct experimental evidence has yet been published) that the herpesvirus-induced ribonucleotide reductase may be a transforming or mutagenic agent or both (16).

ACKNOWLEDGMENTS

We thank D. Hruby and R. Condit for generous gifts of virus stocks and cells and for valuable discussions throughout this study.

This work was supported by Public Health Service grant AI15145-07 from the National Institutes of Health and by National Science Foundation grant PCM 83-01748. M.B.S. is a Leukemia Society of America Fellow.

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