JOURNAL OF VIROLOGY, Nov. 1986, p. 506–514
0022-538X/86/110506-09$02.00/0

Vol. 60, No. 2

Hydroxyurea-Resistant Vaccinia Virus: Overproduction of Ribonucleotide Reductase

MARY B. SLABAUGH* AND CHRISTOPHER K. MATHEWS

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

Received 2 May 1986/Accepted 10 August 1986

Repeated passages of vaccinia virus in increasing concentrations of hydroxyurea followed by plaque purification resulted in the isolation of variants capable of growth in 5 mM hydroxyurea, a drug concentration which inhibited the reproduction of wild-type vaccinia virus 1,000-fold. Analyses of viral protein synthesis by using [^{35}S]methionine pulse-labeling at intervals throughout the infection cycle revealed that all isolates overproduced a 34,000-molecular-weight (MW) early polypeptide. Measurement of ribonucleoside-diphosphate reductase (EC 1.17.4.1) activity after infection indicated that 4- to 10-fold more activity was induced by hydroxyurea-resistant viruses than by the wild-type virus. A two-step partial purification which yielded >90% of the induced ribonucleotide reductase activity in the fraction obtained by 35% saturation with ammonium sulfate resulted in a substantial enrichment for the 34,000-MW protein from extracts of wild-type and hydroxyurea-resistant-virus-infected, but not mock-infected, cells. In the presence of the drug, the isolates incorporated [^{3}H]thymidine into DNA earlier and at a rate substantially greater than that of the wild type, although the onset of DNA synthesis was delayed in both cases. In the absence of the drug, the attainment of a maximum viral DNA synthesis rate was accelerated after infection by drug-resistant isolates. The drug resistance trait was markedly unstable in all isolates. In the absence of selective pressure, plaque-purified isolates readily segregated progeny that displayed a wide range of resistance phenotypes. The results of this study indicate that vaccinia virus encodes a subunit of ribonucleotide reductase which is a 34,000-MW early protein whose overproduction confers hydroxyurea resistance on reproducing viruses.

Encoded within the large (180 kilobase pair) double-stranded DNA genome of the prototypic poxvirus vaccinia is genetic information sufficient to render the host cell nucleus dispensable for viral DNA replication (27). Even the requirement for a nuclear function involved in the maturation of progeny virus (15, 24) has been circumvented in mutants selected for resistance to α-amanitin, which carry out the entire infection cycle in enucleated cells (36, 37).

Nuclear independence, then, suggests that all enzymes and substrates required for replication are either provided by viral genes or are preexistent in the host cell cytoplasm. Indeed, among the early products of viral transcription are a DNA polymerase (17, 23, 34, 35) and two strongly expressed proteins with affinity for DNA (26, 33), which may be analogous to the bacteriophage T4 gene 32 helix-destabilizing protein. A nicking-closing enzyme, topoisomerase I, has been identified as a major constituent of virion cores (2) and is induced in enucleated cells after infection (25). Each of these proteins is reportedly associated with the large nucleoprotein aggregates termed virosomes, which are thought to be the sites of poxvirus replication (16, 25, 26).

Deoxyribonucleotide substrates for viral replication could be provided via salvage or de novo pathways. The importance of the de novo pathway in cellular replication is evidenced by the rapidity with which DNA synthesis in the nucleus is halted after the addition of hydroxyurea (HU), an inhibitor of ribonucleoside-diphosphate reductase (EC 1.17.4.1). Unlike polymerase α, ribonucleotide reductase has been localized to the cytoplasmic compartment by immunocytochemical techniques (3, 10). The cellular enzyme, then, might be expected to provide sufficient precursors for viral DNA synthesis. However, ribonucleotide reductase activity is detected with difficulty in nonreplicating cells, suggesting that viruses infecting such cells would be obliged to either induce cellular reductase activity or encode the enzyme.

We recently described a novel ribonucleotide reductase activity which is induced after vaccinia virus (VV) infection of primate cells (31, 32). The extent of enzyme induction is proportional to the multiplicity of infection (MOI), and peak enzyme activity correlates with maximum viral DNA synthesis rates after a synchronous infection. As compared with the host cell reductase, the induced activity is more responsive to positive activators and less dependent on Mg^{2+}, but similar in sensitivity to inhibition by dATP, dTTP, and HU.

To obtain more compelling evidence for the viral origin of the induced reductase, we sought to isolate VV strains which are resistant to HU. The choice of this drug as the selective agent was based on the knowledge that it inactivates the free radical which is present in the active site of ribonucleotide reductase (28). The ensuing depletion of deoxynucleoside 5′-triphosphate (dNTP) precursors blocks replicative DNA synthesis with minor impact on RNA and protein synthesis.

In the present report, we describe the isolation and characterization of VV variants which were selected by repeated passages in the presence of 5 mM HU, a concentration of drug sufficient to inhibit wild-type (wt) virus replication at least 1,000-fold. As compared with wt VV, these isolates exhibited overproduction of the virally induced ribonucleotide reductase and enhanced synthesis of a 34,000-molecular-weight (MW) protein.

MATERIALS AND METHODS

Virus and cells. VV, strain WR, and BSC40 monkey kidney cells were maintained essentially as previously de-

* Corresponding author.

scribed (15, 31), except that routine plaque assays were performed without agar overlays and plaques were visualized by staining monolayers with methylene blue (20 min, 0.5% methylene blue in 50% methanol) and destaining with two 10% methanol washes.

Reagents. [5-^{3}H]cytidine 5'-diphosphate, trisodium salt (20 Ci/mmol), L-[^{35}S]methionine (1,115 Ci/mmol), and [*methyl*-^{3}H]thymidine (80 Ci/mmol) were purchased from New England Nuclear Corp. and used without further purification. HU and 1-β-D-arabinofuranosylcytosine (araC) were obtained from Calbiochem-Behring, and stock solutions of 250 and 1 mM, respectively, were prepared in phosphate-buffered saline (PBS) or water, sterilized by filtration, and stored at −20°C. Stock solutions were thawed only once.

Assay of ribonucleotide reductase. Preparation of the extracts and assays of ribonucleotide reductase were performed as described previously (31).

Isolation of HU-resistant VV. Growth medium was replaced, 1 h before the infection of BSC40 cells, with medium containing HU. For the initial passage in HU, cells were infected with unmutagenized VV at 1 PFU per cell and harvested after 48 h in the presence of 1 mM HU by scraping the plastic plate with a rubber policeman. Cells and associated virus were collected by low-speed centrifugation, suspended in PBS (2.5 ml/100-mm-diameter dish), and stored at −80°C. For the determination of virus yield, crude stocks were subjected to three cycles of freeze-thaw and sonicated for 2 min in a water bath.

Initially, virus titers were not determined before the infection in the next passage. Rather, the crude stock was split 1:5 and applied to cell monolayers. However, once plaquelike areas were observed on plates in the presence of 5 mM HU, we wished to decrease the MOI to less than 1 PFU per cell to avoid the possible rescue of wt genomes by HU-resistant virus. This required determining titers before reinfection. The HU concentration was increased to 5 mM at passage 2 and 10 mM at passage 6. After two passages in this latter concentration of the drug, the stock was plated at a high dilution under a 0.8% agarose overlay, with or without 10 mM HU. After 48 h, monolayers were stained with neutral red as described previously (15), and well-isolated plaques were picked with sterile pipettes and flushed into 0.6 ml of PBS. After two freeze-thaw cycles, the entire volume was deposited onto monolayers of cells in dishes (diameter, 60 mm) for one-step amplification with or without HU. After 48 to 72 h, virus isolates were harvested and evaluated.

Two assays were used in evaluating HU resistance. (i) Reproductive efficiency was determined by the passage of virus in the presence of various concentrations of the drug and is defined as the number of infective progeny produced in the presence of HU divided by the number produced in its absence. In practice, the numerical value of reproductive efficiency was a function of the MOI and the length of the infection, as well as the HU concentration. (ii) Plaquing efficiency (PE) is defined as the ratio of the number of plaques produced in the presence of the drug divided by the number in its absence. The amount of reduction in plaque diameter as a result of drug treatment, on the other hand, is taken to be a measure of reproductive efficiency rather than PE.

After drug-resistant VV variants were isolated, we investigated whether the pretreatment of host cells with HU had any effect on the PE of HU-resistant virus. The drug was omitted from one or more of the following periods: pretreatment (2 h), absorption (1 h), or growth (48 h). The postabsorption growth period was the only determinant of PE in the presence of the drug. The pretreatment of cells with HU, therefore, was discontinued, although the drug was included during inoculation periods longer than 1 h.

Analysis of protein synthesis. Cells in dishes (diameter, 35 mm) were infected with virus at 20 PFU per cell. In some experiments, araC (25 μM) or HU (5 mM) was included to prevent the synthesis of late viral proteins. At various times after infection, medium was replaced with prewarmed medium containing 5% dialyzed fetal bovine serum, 1/10 the normal concentration of methionine, and [^{35}S]methionine (25 μCi/ml). After the incorporation period (20 to 60 min), radioactive medium was removed, and infected monolayers were rinsed once with PBS and immediately solubilized in 0.2 ml of sodium dodecyl sulfate (SDS) sample buffer (18). Total incorporation was determined by trichloroacetic acid precipitation of 10-μl portions. Before electrophoresis, samples were sonicated 20 s to reduce viscosity (Ultrasonics model W-10, setting 3) and heated at 100°C for 2 min. Volumes containing 1×10^4 to 5×10^4 acid-precipitable cpm were subjected to electrophoresis on 10 or 12% acrylamide-SDS slab gels (18), processed for fluorography (4), and exposed to X-Omat film (Eastman Kodak Co.) at −80°C for 2 to 4 days.

Partial purification of ribonucleotide reductase. BSC40 cells in dishes (diameter, 150 mm) were infected at 10 to 30 PFU per cell with virus for 8 h in the presence of araC (25 μM). Previous time course experiments had determined that this infection protocol maximized the induction of enzyme activity. Infected cells were scraped from the dishes, collected by low-speed centrifugation, washed once with cold PBS, and suspended at 1.5×10^8 cells per ml in buffer A (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0], 2 mM $MgCl_2$, 2 mM dithiothreitol). Cells (on ice) were disrupted by three 10-s bursts of sonic oscillation with intermittent cooling periods. All subsequent steps were at 4°C. Debris was pelleted by centrifugation for 10 min at 12,000 × *g* in a microcentrifuge (Brinkmann Instruments, Inc.). Solid streptomycin sulfate was added to the supernatant to a final concentration of 1% and stirred for 20 min before the removal of precipitated material by centrifugation. To the supernatant, solid ammonium sulfate was added to 35% saturation. After a 30-min equilibration period, the precipitate was collected by centrifugation, dissolved in a minimal volume of buffer A (approximately 1/10 of the original volume), and dialyzed against two changes of buffer B (20 mM Tris-chloride [pH 7.4], 2 mM $MgCl_2$, 2 mM dithiothreitol) for 2 h. The dialysate was clarified by brief microfuge centrifugation and stored at −80°C in small portions. Partially purified preparations of reductase were stable for at least 10 months at −80°C.

For some experiments, viral proteins were radiolabeled by including [^{35}S]methionine (5 μCi/ml) in the growth medium from 2 to 7 h postinfection.

Analysis of viral DNA synthesis. BSC40 cells were grown to confluence in dishes (diameter, 35 mm). To avoid stimulation of cellular DNA synthesis by the addition of fresh medium, conditioned medium was removed from the dishes before the initiation of experiments and was used to prepare viral inocula and growth and labeling media. The cells were infected with virus at 10 to 15 PFU per cell. At intervals, growth medium was replaced with prewarmed labeling medium containing [^{3}H]thymidine (0.5 ml per dish; 2.5 μCi/ml). After a 20-min labeling period, the dishes were placed on ice, the radioactive medium was quickly removed, and the cells were scraped into 2 ml of ice-cold 10% trichloroacetic acid. Precipitated material was collected onto glass fiber filters,

washed extensively with cold 5% trichloroacetic acid, and counted in a toluene-based scintillation fluid.

RESULTS

Enrichment for HU-resistant VV. Since the ribonucleotide reductase activity which is induced after the infection of cells by VV is inhibited in vitro by HU (31), we reasoned that the isolation of HU-resistant virus might provide mutations in the putative viral reductase gene(s). Before attempting mutant isolation, we determined the ability of VV to replicate in the presence of increasing concentrations of HU. At 0.2 mM HU, 50% inhibition of viral growth was achieved, and yields in 1 and 5 mM HU were approximately 1 and 0.1%, respectively, of control values.

To select VV variants resistant to HU inhibition, wt virus was passaged repeatedly in the presence of the drug. After an initial passage of the virus in 1 mM HU, the drug concentration was increased to 5 mM, and the MOI was kept at less than 1 PFU per cell to avoid the rescue of wt genomes by HU-resistant variants. Under these conditions, virus yields from early passages were very low (passage 3: output PFU per input PFU, <0.1), but increased rapidly with repeated selection (passage 6: output PFU per input PFU, 1,000), suggesting enrichment for the resistant virus. Plaque assays in the presence and absence of the drug indicated that the stock obtained from passage 6 contained approximately 40% resistant virus (PE, 0.4). Two additional passages in 10 mM HU preceded the initial plaque isolation.

Plaque isolation of HU-resistant VV. To obtain pure stocks of HU-resistant virus, serial dilutions from passage 8 were plated under an agarose overlay. Well-isolated plaques were picked, amplified, and screened as described in Materials and Methods. Plaque reduction assays revealed that the collection of 20 isolates contained a diversity of phenotypes ranging from wt sensitivity to resistance to 10 mM HU. As scored with respect to the ability to plaque in 5 mM HU, the incidence of resistance was much greater in isolates picked from plates which were under drug selection at the time of plaque isolation (70%) as compared with plaques isolated in the absence of drug (10%).

Quantitative plaque reduction and 24-h growth reduction assays performed with several HU-resistant isolates after one-step amplification in the absence of the drug (Fig. 1) confirmed that these stocks were at least 1,000-fold more resistant to HU inhibition than was wt VV; PEs, however, were only 0.2 to 0.3 in 5 to 10 mM HU.

Isolates HU1 and HU19, picked from plates containing and lacking HU, respectively, were selected for further study. An additional passage on BSC40 cells without the drug yielded preparations of high titer (2×10^9 PFU/100-mm-diameter plate), but with PEs of 0.1 in 5 mM HU. We hypothesized that the impact of HU on PE might be the result of adverse effects of the drug on host cells, which could reduce the probability of establishing a productive infection. However, the PE determined for a given stock was affected neither by whether cells were pretreated with HU nor by the presence or absence of the drug during the 1- to 2-h inoculation period (data not shown). The apparent decline in PE which was observed during passage of virus stocks in the absence of selective pressure suggested that the isolates were either contaminated with wt virus or were subject to a high rate of reversion.

A second round of plaque isolation, carried out in the absence of the drug, confirmed that both HU1 and HU19 stocks contained HU-sensitive virus. Of 10 isolates characterized, 3 were indistinguishable from the wt, 3 were able to plaque in 5 mM HU, and 4 displayed intermediate levels of resistance. The PE of the HU-resistant isolates was 0.1. The passage of two of these isolates, HU1-1 and HU19-2, three times in 5 mM HU, however, resulted in PEs of 0.5 but yields of 2×10^8 PFU/100-mm-diameter plate (1/10 of normal wt yields). To characterize these populations, a third plaque purification experiment was performed in which plaques were picked from plates with or without the drug, but were amplified in the presence of the drug. Using this protocol, we obtained seven HU-resistant and three HU-sensitive isolates from plates lacking HU. Since the parent HU1-1 and HU19-2 stocks had been derived from well-isolated plaques in the

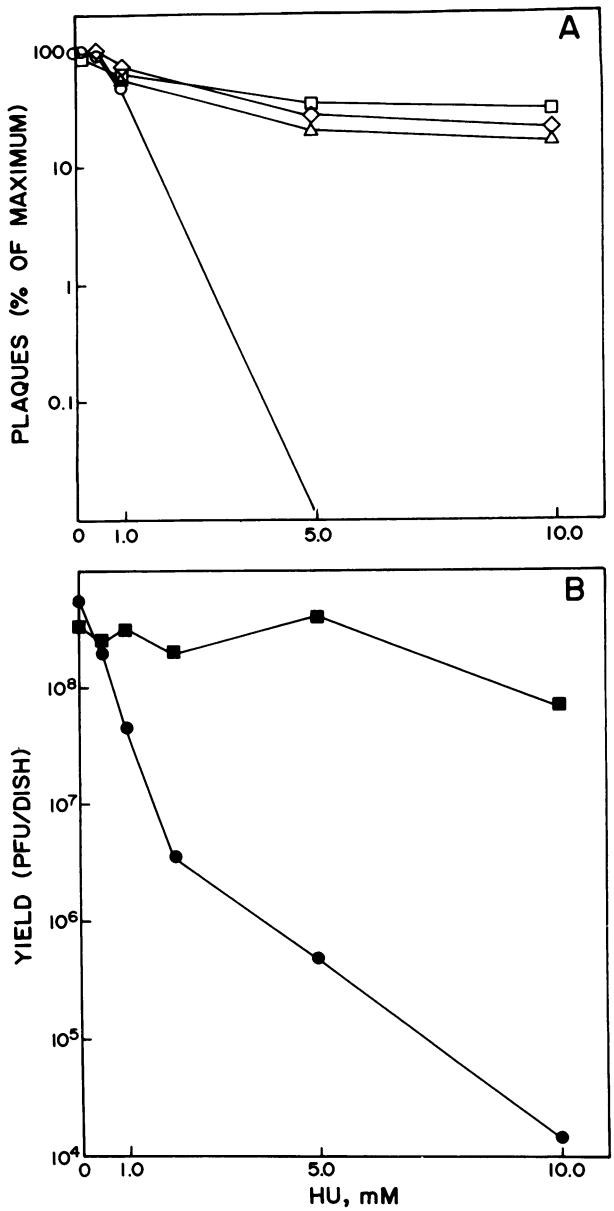

FIG. 1. Effect of HU on wt (○, ●) and HU^r (□, ■, ◇, △) VV. (A) Plaque reduction assay. wt VV and plaque isolates HU1, HU10, and HU19 were amplified 48 h without the drug, diluted, and plated on cell monolayers. Plaque numbers (irrespective of plaque size) were scored after 48 h. (B) Growth reduction assay. Cells were infected with wt or HU1 virus at 0.1 PFU per cell, harvested after 24 h, and titrated by plaque assay.

TABLE 1. Instability of HU resistance phenotype

Virus stock and passage[a]	PFU (PE)[b]	
	+HU	No addition
HU1-1-6		
1	5.6×10^7 (0.82)	7.0×10^7 (0.20)
2	2.2×10^7 (0.86)	4.0×10^7 (0.17)
3	1.8×10^7 (0.48)	5.6×10^7 (0.12)
HU19-2-4		
1	2.0×10^8 (0.50)	7.7×10^8 (0.26)
2	1.7×10^8 (0.10)	1.1×10^9 (0.13)
3	0.3×10^8 (0.44)	1.4×10^9 (0.07)

[a] PE before passages: HU1-1-6, 0.68; HU19-2-4, 0.55.

[b] Cell monolayers in dishes (60-mm diameter) were infected with virus at 0.1 PFU per cell in the presence or absence of HU and harvested at 48 h postinfection. In each series, cells in passage 1 were infected with initiating-virus stock, cells in passage 2 were infected with virus from passage 1, no addition, and cells in passage 3 were infected with virus from passage 2, no addition.

absence of the drug, these results strongly suggested that the HU-sensitive revertants arose in HU-resistant populations with high frequency. Not surprisingly, all of the plaques isolated from HU-containing plates were scored as resistant to 5 mM HU. The PEs of all HU-resistant isolates, 0.5 to 0.7, reflected those of the parent HU1-1 and HU19-2 stocks.

Instability of HU resistance phenotype. To directly investigate the apparent instability of the HU resistance phenotype, representative isolates from the third round of plaque purification were passages three times in succession without HU. At each step, the titer and PE of progeny virus were determined. The effect of renewed selection after passage without HU was also evaluated. We observed a progressive decline in PE in both HU1-1-6 and HU19-2-4 (Table 1). As before, reimposition of drug selection resulted in restoration of PEs to high values in a single passage, suggesting strong selection for resistant virus. The two isolates, however, exhibited divergent reproduction phenotypes. Whereas yields of infective progeny from HU19-2-4 virus increased as the degree of resistance declined, HU1-1-6 virus produced only 10% of normal wt titers, even when the PE had declined to 0.1, suggesting that this variant strain was debilitated in ways other than the unstable drug resistance trait. Further plaque purifications revealed that all HU1 derivatives shared the inability to achieve high-titer growth.

Comparison of wt and HU1-1-5 growth curves. One explanation for the low yields from HU1-1 series isolates would be a delayed time course of productive infection. However, although the appearance of HU1-1-5 progeny virus was retarded (e.g., HU1-1-5 required 4 h longer than the wt to multiply input titers 10-fold), the maximum yield was obtained by 48 h (Fig. 2). These data also show that HU decreased yields of wt virus by a factor of 1×10^3 to 2×10^3 at all time points, but reveal that VV was able to replicate very slowly in the presence of 5 mM HU.

Ribonucleotide reductase activity in wt and HU-resistant VV. One mechanism of resistance to drug inhibition is overproduction of the target enzyme. Another mechanism is mutational alteration of the enzyme, rendering it no longer susceptible to the inhibitor. To investigate whether HU-resistant VV exhibited either phenotype with respect to ribonucleotide reductase, we compared the activities of this enzyme in extracts of BSC40 cells after their infection by wt VV or HU-resistant isolates. Previous work has shown that the extent of induction of virus-specific ribonucleotide reductase is directly proportional to an MOI of between 1 and 10 PFU per cell (31). Therefore, cell monolayers were infected with 1 to 4 PFU per cell, and the data were subsequently normalized to reflect equivalent MOIs after determination of the exact titers of inoculations by plaque assay.

The results of a 10-h time course analysis in which wt VV and HU1-1-5 isolates were compared is shown in Fig. 3. Cells infected by the HU-resistant virus accumulated four- to fivefold more reductase activity than was induced by wt VV. Since HU1-1-5 virus was selected by virtue of its ability to reproduce in the presence of HU, we expected that we would observe the superinduction (14) of reductase by araC but not HU, because the extended synthesis of early VV gene products in the presence of DNA synthesis inhibitors is attributed to a lack of late gene expression. However, both HU (Fig. 3) and araC (data not shown) elicited an additional twofold increase in reductase activity after infection by HU-resistant virus. One explanation for these results is that drug-resistant variants overcame HU inhibition of DNA synthesis slowly (see Fig. 7), probably reflecting the time required to accumulate substantial intracellular levels of reductase.

The in vitro HU sensitivity of BSC40 host cell and virus-induced ribonucleotide reductase was measured in crude extracts. Although HU1-1 and HU19-2 viruses induced approximately 4- and 10-fold, respectively, more of the virus-specific enzyme activity than did wt VV, HU inhibited the activities from all sources in a similar manner

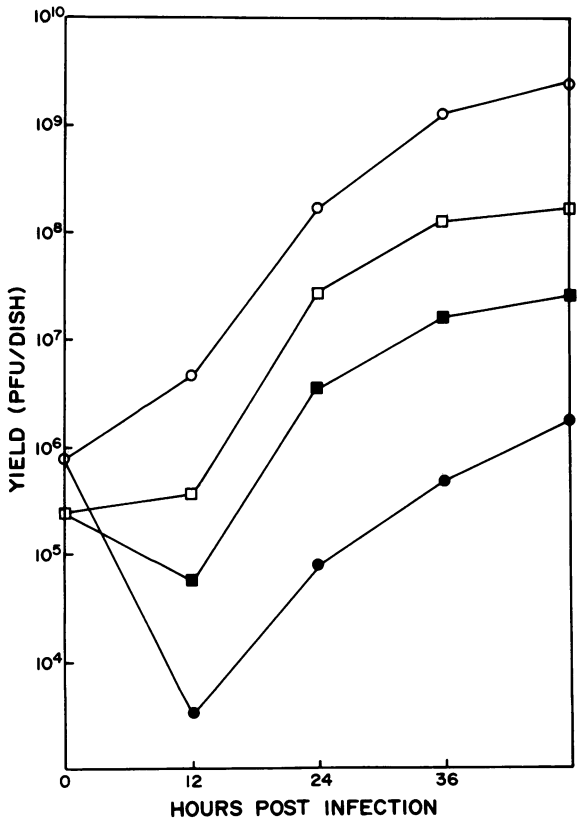

FIG. 2. Growth curves for wt (○, ●) and HU^r (□, ■) VV in the presence (●, ■) and absence (○, □) of 5 mM HU. Cell monolayers in dishes (diameter, 60 mm) were infected at <0.3 PFU per cell and harvested at the indicated times postinfection. The yield and PE with and without 5 mM HU were determined for each dish.

(Fig. 4). It is noteworthy that 50% inhibition required 2 to 3 mM HU, whereas 50% inhibition of wt-virus growth was achieved at 0.2 mM HU. Although one interpretation of this discrepancy is that there may be an additional site of action for the drug in vivo, a more likely explanation is spontaneous reactivation of the enzyme in vitro by regeneration of the tyrosyl radical in the presence of O_2, iron, and dithiothreitol (28). We concluded from these experiments that both series of HU-resistant isolates induced severalfold more ribonucleotide reductase activity per infecting unit than did wt VV. We obtained no evidence, however, for the altered sensitivity of the overproduced enzyme to HU.

Overproduction of 34,000-MW protein by HU-resistant viruses. To see whether the HU resistance phenotype correlated with detectable alterations in the synthesis of one or more viral proteins, [^{35}S]methionine-pulse-labeled extracts from mock-, wt-, or variant-infected cells were examined by the fluorography of SDS-polyacrylamide gels. Preliminary studies (data not shown) revealed that isolates of the HU1-1 series consistently exhibited increased labeling of three early minor proteins with MWs of 34,000, 28,000, and 26,000 and decreased labeling of two early major proteins with MWs of 32,000 and 25,000. HU19-2 series isolates, however, appeared identical to wt VV except for the enhanced labeling of a 34,000-MW protein. Late protein labeling patterns of wt, HU1-1, and HU19-2 viruses were indistinguishable.

Synthesis of the 34,000-MW protein (Fig. 5, arrows) was seen to peak at 3 h after infection by either wt or HU19-2 virus, as shown by the time course analysis. Relative to the wt, increased synthesis of this protein by HU19-2, however, was detectable as early as 1 h postinfection (Fig. 5, compare lanes 2 and 7). In the presence of 5 mM HU, late proteins were not expressed by either virus, but synthesis of the 34,000-MW protein (and other early proteins) continued.

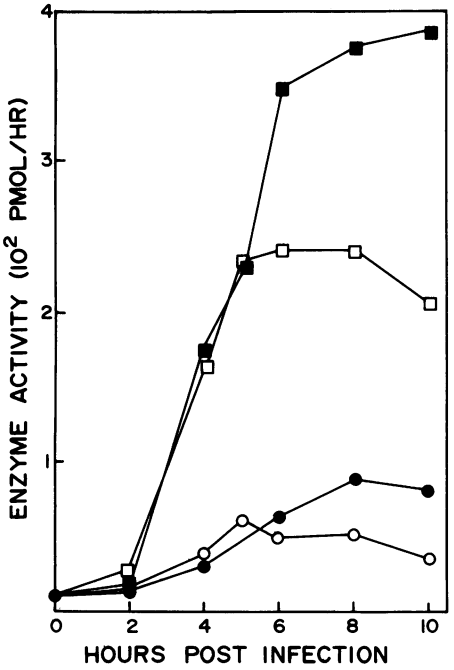

FIG. 3. Time course of induction of ribonucleotide reductase activity by wt (○, ●) and HU^r (□, ■) VV. Cell monolayers in dishes (diameter, 100 mm) were infected at ca. 1 PFU per cell in the presence (●, ■) or absence (○, □) of 5 mM HU. At the indicated times, crude extracts were prepared and assayed for reductase activity. Data points show the average of duplicate reductase assays.

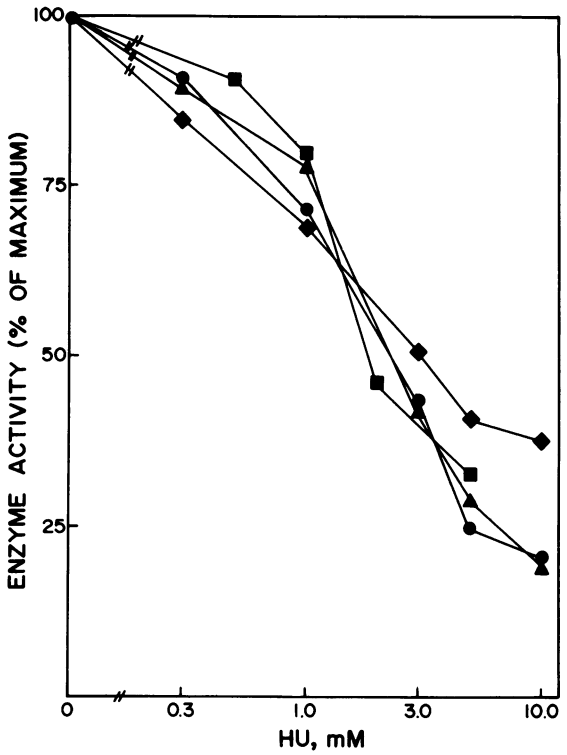

FIG. 4. In vitro HU sensitivity of host cell and virus-induced ribonucleotide reductase activity. Cells in dishes (diameter, 100 mm) were mock infected (◆) or infected with wt VV (●), HU1-1 (■), or HU19-2 (▲) at ca. 5 PFU per cell. At 4.5 h postinfection, extracts were prepared and reductase assays were performed in duplicate (average value plotted) in the presence of various concentrations of HU. The maximum enzyme activities induced by HU1-1 and HU19-2 viruses were 4-fold and 10-fold, respectively, that induced by wt VV.

Taken together, the increased induction of ribonucleotide reductase activity and the enhanced synthesis of a 34,000-MW protein by all HU-resistant isolates examined suggests that this peptide might be a viral counterpart to the smaller of the two subunits of cellular ribonucleotide reductase. It is tempting to speculate that the pleiotropic nature of early protein synthesis aberrations observed in HU1-1 series isolates is responsible for their reducted virulence.

Cofractionation of virus-induced ribonucleotide reductase activity and 34,000-MW protein. To determine whether the 34,000-MW protein is a viral subunit of ribonucleotide reductase, we fractionated extracts prepared from infected cells by procedures similar to those used by others to obtain partially purified preparations of this enzyme (12). In preliminary studies, we determined that most of the reductase activity in extracts from uninfected BSC40 cells precipitated at ammonium sulfate concentrations above 35% saturation, whereas more than 90% of the virus-specific reductase was recovered in the 35% ammonium sulfate pellet. The sensitivity of the enzyme activities to Mg^{2+} and the positive activator 5'-adenylylimidodiphosphate (an ATP analog) was tested after sequential ammonium sulfate cuts, and the differential response to the effectors observed (data not shown) confirmed our previous study (32) and indicated that

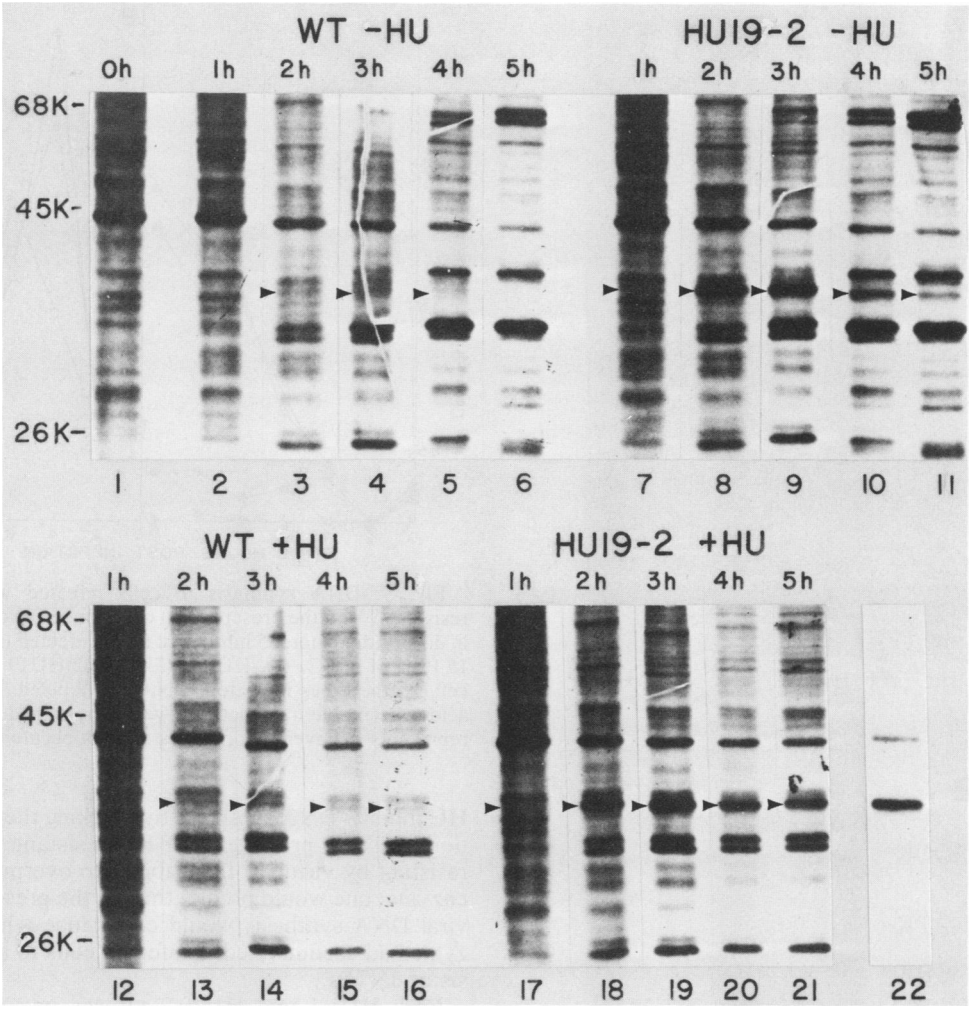

FIG. 5. Time course of protein synthesis in wt- and HU^r VV-infected cells with (top) and without (bottom) 5 mM HU. Cell monolayers in dishes (diameter, 35 mm) were infected at 20 PFU per cell. Polypeptides were pulse-labeled with [^{35}S]methionine for 1 h at the time points indicated, harvested directly into SDS sample buffer, and electrophoresed on 12% SDS-polyacrylamide gels. The 34,000-MW protein is identified by the ammonium sulfate pellet fraction prepared from HU^r VV-infected cells (lane 22).

the 10- to 20-fold increase in enzyme activity recovered in the 35% ammonium sulfate pellet after virus infection was attributable to the induced reductase. The results of a representative two-step fractionation are shown in Table 2. This protocol resulted in an eightfold increase in specific activity and separated the enzyme from a substrate-diverting 5′-nucleotidase activity which is present in crude extracts (data not shown). In this form, the enzyme was stable for several months when stored at −80°C. Enzyme activity declined approximately 15%, however, each time a preparation was rethawed.

The fractionation protocol was next applied to [^{3}S]methionine-labeled extracts, and the behavior of the overproduced 34,000-MW protein was monitored by electrophoretic analysis of the various fractions. Less than 10% of the ^{35}S-labeled protein in infected or uninfected cells was recovered in the ammonium sulfate pellet fraction (Table 2). However, this fraction was highly enriched for the

TABLE 2. Fractionation of ribonucleotide reductase activity and acid-precipitable radioactivity in extracts from virus-infected cells

Fraction	Enzyme activity[a]			Acid-precipitable radioactivity (cpm [10^3]) (%)[b]:		
	Activity (pmol min^{-1} [%])	Protein (mg) (%)	Sp act	BSC40	BSC40 + wt VV	BSC40 + HU^r VV
Clarified extract	1,800 (100)	146 (100)	12.3	3,220 (100)	2,026 (100)	3,153 (100)
Streptomycin sulfate pellet	32 (2)	71 (49)	0.5	700 (22)	524 (26)	778 (29)
Ammonium sulfate pellet	1,605 (90)	17 (12)	94.4	182 (6)	134 (7)	250 (8)
Ammonium sulfate supernatant	32 (2)	15 (10)	2.1	709 (22)	353 (17)	516 (16)

[a] Cells (1.5×10^9) were infected with 10 PFU of wt VV per cell for 7 h in the presence of 25 μM araC.

[b] Cells (6×10^7 per group) were mock infected or infected with 20 PFU of wt VV or HU19-2 virus per cell as described in footnote *a*, except that [^{35}S] methionine was present from 2 to 7 h postinfection.

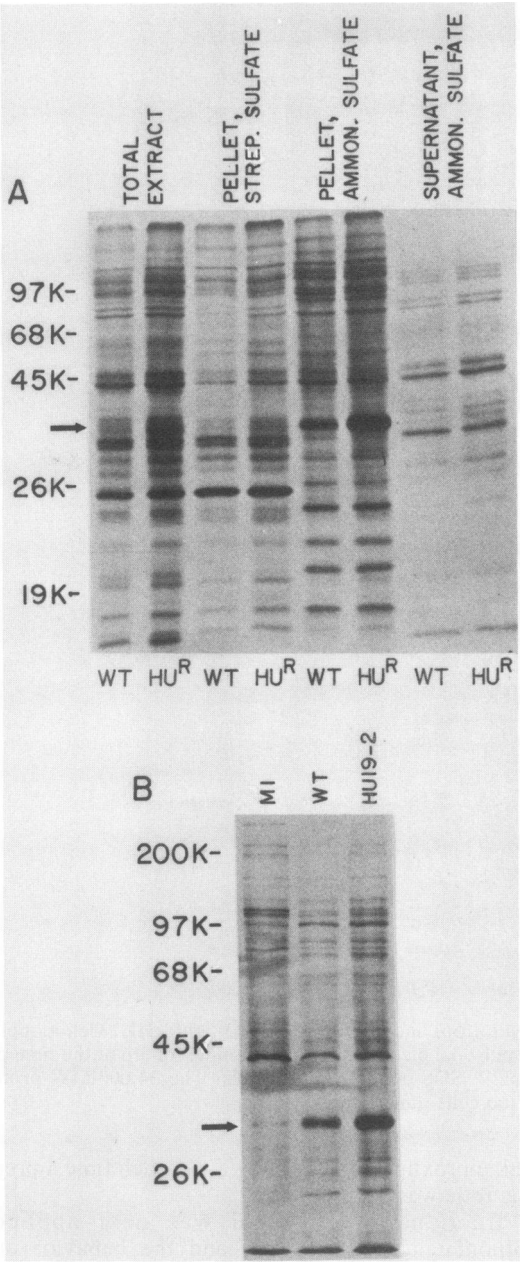

FIG. 6. Fractionation of radiolabeled cell lysates after infection with wt or HU^r VV (A) and comparison of ammonium sulfate pellet fractions from mock-, wt- or HU^r-VV-infected cells (B). Cell monolayers in dishes (diameter, 100 mm) were mock infected or infected with 20 PFU per cell in the presence of araC (25 μM) for 7 h. Proteins were labeled with [^{35}S]methionine (5 μCi/ml) from 2 to 7 h postinfection. Samples containing 1×10^4 to 3×10^4 acid-precipitable cpm were subjected to electrophoresis on 12% (panel A) or 10% (panel B) polyacrylamide gels. ^{14}C-labeled marker proteins (data not shown) were run in parallel lanes. The position of the overexpressed 34,000-MW protein is indicated by the arrows.

34,000-MW protein (Fig. 6), and its increased synthesis by the HU-resistant variant was apparent (Fig. 6 A and B, arrows). Uninfected host cells yielded very little radiolabeled 34,000-MW protein in the ammonium sulfate pellet fraction (Fig. 6B).

DNA synthesis in cells infected by wt and HU-resistant VV.

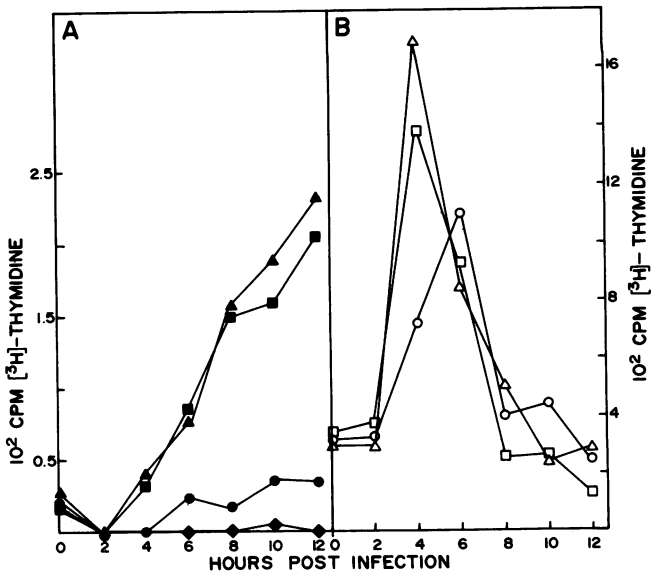

FIG. 7. DNA synthesis in cells infected with wt and HU-resistant VV in the presence (A) or absence (B) of 5 mM HU. Cells in dishes (diameter, 35 mm) were mock infected (◆) or infected with 15 PFU of wt (○, ●), HU1-1, (□, ■), or HU19-2 (△, ▲) virus per cell. At the times indicated, DNA was labeled and harvested, and acid-precipitable radioactivity was determined. Each data point represents the average of values from duplicate dishes.

HU inhibits DNA synthesis by blocking the de novo production of dNTP precursors. If HU-resistant viral isolates are resistant by virtue of their ability to overproduce the target enzyme, one would predict that, in the presence of the drug, viral DNA synthesis would commence when sufficient enzyme had accumulated in infected cells to allow the synthesis of dNTPs.

Both HU1-1 and HU19-2 viruses began to incorporate [^{3}H]thymidine into an acid-precipitable form at 4 h postinfection in the presence of 5 mM HU (Fig. 7A). DNA synthesis rates increased linearly for at least 12 h postinfection. Under these conditions, DNA synthesis in wt-infected cells was not detectable until 6 h postinfection and plateaued at a rate six- to sevenfold lower than that achieved by the HU-resistant variants. Uninfected cells were 99 to 100% inhibited at all time points.

We were surprised to see that, in the absence of HU, both drug-resistant isolates achieved maximal rates of DNA synthesis earlier in the infection cycle than did wt VV (Fig. 7B). This result, which was confirmed in a duplicate experiment, suggests that a rate-determining factor for viral DNA synthesis is not limiting after infection by HU-resistant viruses.

HU was a potent in vivo inhibitor of DNA synthesis in host cells. When HU was added to cell medium at the same time as [^{3}H]thymidine, incorporation of the isotope was decreased 90 to 95%. Since the labeling period was only 20 min, this verified that the supply of cellular dNTP precursors was depleted rapidly upon inhibition of ribonucleotide reductase.

DISCUSSION

HU inhibits DNA synthesis in both procaryotes and eucaryotes by inactivating the tyrosyl free radical in the smaller of the two subunits of ribonucleotide reductase, thus starving the cell of dNTP substrates. HU-resistant mamma-

lian cells have been isolated by passaging cells in increasingly higher concentrations of the drug. HU resistance has been attributed both to gene amplification, leading to overproduction by gene dosage effects, and to alterations in the enzyme subunit, rendering it less susceptible to inactivation (reviewed in reference 38), although definitive data are lacking. In one case, a high degree of resistance was transferred to sensitive cells by transfection of metaphase chromosomes from HU-resistant cells (20). In the present report, we describe variants of VV that were resistant to the DNA synthesis inhibitor HU. Relative to wt VV, the resistant isolates overproduced a 34,000-MW protein that was a product of early viral transcription and induced substantially more ribonucleotide reductase activity than wt VV at comparable MOIs. The 34,000-MW protein and the induced reductase cofractionated through two purification steps, suggesting that this protein may be a viral counterpart to the M2 subunit of the mammalian enzyme.

Ribonucleotide reductase induced by another group of large DNA viruses, namely the herpesviruses, has been described in some detail. Based on genetic, immunological, and enzymatic data, 140,000- and 38,000-MW proteins encoded in the genomes of herpes simplex virus types 1 and 2 have been identified as reductase subunits (1, 7, 22). Epstein-Barr virus encodes 93,000- and 34,000-MW proteins which are also believed to represent reductase subunits (13). The 34,000-MW vaccinia peptide is therefore similar in size to the smaller of the herpesvirus gene products. Sequence homology has been detected among reductase genes from sources as diverse as *Escherichia coli*, clam, mouse, and herpesvirus (6, 30), indicating conservation of this important enzyme.

A striking characteristic of the vaccinia HU-resistant isolates characterized in our study was the instability of the drug resistance phenotype. As assessed by plaquing efficiency in the presence and absence of HU, the degree of resistance declined by approximately 50% at each passage without selective pressure. Previous levels of resistance, however, were restored in one passage upon rechallenge with HU. Even freshly plaque-purified isolates segregated nonresistant revertants with a high frequency.

The instability of drug resistance phenotypes in cell lines is associated with the early stages of gene amplification and the presence of extrachromosomal DNA. Interestingly, such aberrations are promoted by the transient exposure of cells to HU, which is proposed to induce random rereplication of the genome (5, 21, 29). We are presently analyzing DNA isolated from HU-resistant VV isolates, since one prediction of a gene amplification model, that homologous recombination between genomes carrying multiple gene copies would rapidly generate offspring that are both more and less resistant than parental genotypes, is consistent with the instability and variability observed in our isolates. If gene amplification is shown to be the mechanism underlying the resistance in VV, the ability to study this phenomenon in a viral system may facilitate the elucidation of the processes involved. In this regard, it is perhaps noteworthy that a single passage of wt VV in 5 mM HU resulted in a detectable increase in the number of progeny able to form minute plaques in the presence of this concentration of the drug.

Since mammalian ribonucleotide reductase has been shown to be a cytoplasmic enzyme (10) and since dNTP pools are apparently distributed throughout the cell (19), it is reasonable to ask what the role of a virally encoded reductase might be. Recent studies on the cell cycle regulation of the mammalian enzyme indirectly shed some light on this question. Ribonucleotide reductase in nondividing cells has been found to be virtually undetectable, either by immunostaining (10) or enzyme activity (8). In cycling cells, holoenzyme activity is maximal during S phase and decreases immediately after mitosis, a phenomenon that is attributed to the varying availability of the small subunit (11), which has been shown to have a half-life of 3 h in two cell types (9). Therefore, synthesis of a new enzyme may confer a decided advantage on viruses infecting quiescent or non-S-phase cells. Our observation that both variant strains studied achieved maximal rates of DNA synthesis sooner than did wt virus (in the absence of HU) supports the idea that a virus-encoded function is rate limiting for viral replication. Apropos to this line of reasoning, preliminary experiments indicated that the dGTP pool in vaccinia-infected BSC40 cells was at least twice that measured in uninfected cells (J. Leeds, unpublished data). The dGTP pool is consistently the smallest DNA precursor pool measured in a wide variety of cell types and may be rate limiting for DNA synthesis.

We were unable to detect increased synthesis of a protein which might represent a viral counterpart to the larger, effector-binding subunit of ribonucleotide reductase. The possibility that vaccinia encodes a free-radical-containing subunit which interacts with the constitutively expressed and long-lived M1 subunit (9) must be considered. We did note, however, that a protein with an approximate MW of 83,000, which cofractionated with both reductase activity and the 34,000-MW protein, was photoaffinity-labeled by [^{35}S]dTTP in extracts from VV-infected, but not uninfected, BSC40 cells (unpublished data).

Our purpose in selecting vaccinia variants resistant to HU was to develop tools for mapping and cloning the putative viral reductase gene(s). The tentative identification of an early 34,000-MW protein as the small subunit of the enzyme should facilitate this task.

ACKNOWLEDGMENTS

We thank Yu Wang for skilled technical assistance and Sue Conte for typing the manuscript.

This work was supported by National Science Foundation grant PCM 83-01748.

LITERATURE CITED

1. **Bacchetti, S., M. J. Evelegh, B. Muirhead, C. S. Sartori, and D. Huszar.** 1984. Immunological characterization of herpes simplex virus type 1 and 2 polypeptides involved in viral ribonucleotide reductase activity. J. Virol. **49**:591–593.
2. **Bauer, W. R., E. C. Ressner, J. Kates, and V. J. Patzke.** 1977. A nick-closing enzyme encapsidated in vaccinia virus: partial purification and properties. Proc. Natl. Acad. Sci. USA **74**:1841–1845.
3. **Bensch, K. G., S. Tanaka, S.-Z. Hu, T. Wang, and D. Korn.** 1982. Intracellular localization of human DNA polymerase α with monoclonal antibodies. J. Biol. Chem. **257**:8391–8396.
4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
5. **Brown, P. C., T. D. Tlsty, and R. T. Schimke.** 1983. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. Mol. Cell. Biol. **3**:1097–1107.
6. **Caras, I. W., B. B. Levinson, M. Fabry, S. R. Williams, and D. W. Martin, Jr.** 1985. Cloned mouse ribonucleotide reductase subunit M1 cDNA reveals amino acid sequence homology with *E. coli* and herpesvirus ribonucleotide reductases. J. Biol. Chem. **260**:7015–7022.
7. **Dutia, B. M.** 1983. Ribonucleotide reductase induced by herpes

simplex virus has a virus-specified constituent. J. Gen. Virol. **64**:513–521.
8. **Elford, H. L., M. Freese, E. Passamani, and H. P. Morris.** 1970. Ribonucleotide reductase and cell proliferation. J. Biol. Chem. **245**:5228–5233.
9. **Engström, Y., S. Eriksson, I. Jildevik, S. Skog, L. Thelander, and B. Tribukait.** 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. J. Biol. Chem. **260**: 9114–9116.
10. **Engström, Y., B. Rozell, H.-A. Hansson, S. Stemme, and L. Thelander.** 1984. Localization of ribonucleotide reductase in mammalian cells. EMBO J. **3**:863–867.
11. **Eriksson, S., A. Graslund, S. Skog, L. Thelander, and B. Tribukait.** 1984. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. J. Biol. Chem. **259**:11695–11700.
12. **Eriksson, S., and D. W. Martin.** 1981. Ribonucleotide reductase in cultured mouse lymphoma cells. Cell cycle-dependent variation in the activity of subunit protein M2. J. Biol. Chem. **256**:9436–9440.
13. **Gibson, T., P. Stockwell, M. Ginsburg, and B. Barrell.** 1984. Homology between two EBV early genes and HSV ribonucleotide reductase and 38K genes. Nucleic Acids Res. **12**: 5087–5099.
14. **Hruby, D., and L. A. Ball.** 1981. Control of expression of the vaccinia virus thymidine kinase gene. J. Virol. **40**:456–464.
15. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. J. Virol. **29**:705–715.
16. **Joklik, W. K., and Y. Becker.** 1964. The replication and coating of vaccinia DNA. J. Mol. Biol. **10**:452–474.
17. **Jones, E. V., and B. Moss.** 1984. Mapping of the vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected RNA. J. Virol. **49**:72–77.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Leeds, J. M., M. B. Slabaugh, and C. K. Mathews.** 1985. DNA precursor pools and ribonucleotide reductase activity: distribution between the nucleus and cytoplasm of mammalian cells. Mol. Cell. Biol. **5**:3443–3450.
20. **Lewis, W. H., and P. R. Srinivasan.** 1983. Chromosome-mediated gene transfer of hydroxyurea resistance and amplification of ribonucleotide reductase activity. Mol. Cell. Biol. **3**:1053–1061.
21. **Mariani, B. D., and R. T. Schimke.** 1984. Gene amplification in a single cell cycle in Chinese hamster ovary cells. J. Biol. Chem. **259**:1901–1910.
22. **McLaughlan, J., and J. B. Clements.** 1983. DNA sequence homology between two co-linear loci on the HSV genome which have different transforming abilities. EMBO J. **2**:1953–1961.
23. **Moss, B., and N. Cooper.** 1982. Genetic evidence for vaccinia virus-encoded DNA polymerase: isolation of phosphonoacetic acid-resistant enzyme from the cytoplasm of cells infected with mutant virus. J. Virol. **43**:673–678.
24. **Pennington, T. H., and E. A. C. Follett.** 1974. Vaccinia virus replication in enucleate BSC-1 cells: particle production and synthesis of viral DNA and proteins. J. Virol. **13**:488–493.
25. **Poddar, S. K., and W. R. Bauer.** 1986. Type I topoisomerase activity after infection of enucleated synchronized mouse L cells by vaccinia virus. J. Virol. **57**:433–437.
26. **Polisky, B., and J. Kates.** 1975. Viral-specific polypeptides associated with newly replicated vaccinia DNA. Virology **66**:128–139.
27. **Prescott, D. M., J. Kates, and J. B. Kirkpatrick.** 1971. Replication of vaccinia virus DNA in enucleated L-cells. J. Mol. Biol. **59**:505–508.
28. **Reichard, P., and A. Ehrenberg.** 1983. Ribonucleotide reductase—a radical enzyme. Science **221**:514–519.
29. **Schimke, R. T., S. W. Sherwood, A. B. Hill, and R. N. Johnston.** 1986. Overreplication and recombination of DNA in higher eucaryotes: potential consequences and biological implications. Proc. Natl. Acad. Sci. USA **83**:2157–2161.
30. **Sjöberg, B.-M., H. Eklund, J. A. Fuchs, J. Carlson, N. M. Standart, J. V. Ruderman, S. J. Bray, and T. Hunt.** 1985. Identification of the stable free radical tyrosine residue in ribonucleotide reductase. FEBS Lett. **183**:99–102.
31. **Slabaugh, M. B., T. L. Johnson, and C. K. Mathews.** 1984. Vaccinia virus induces ribonucleotide reductase in primate cells. J. Virol. **52**:507–514.
32. **Slabaugh, M. B., and C. K. Mathews.** 1984. Vaccinia virus-induced ribonucleotide reductase can be distinguished from host cell activity. J. Virol. **52**:501–506.
33. **Soloski, M. J., M. Esteban, and J. A. Holowczak.** 1978. DNA-binding proteins in the cytoplasm of vaccinia virus-infected mouse L-cells. J. Virol. **25**:263–273.
34. **Sridhar, P., and R. C. Condit.** 1983. Selection for ts mutations in specific vaccinia virus genes: isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, ts DNA polymerase. Virology **128**:444–457.
35. **Traktman, P., P. Sridhar, R. C. Condit, and B. E. Roberts.** 1984. Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J. Virol. **49**:125–131.
36. **Villarreal, E. C., and D. E. Hruby.** 1986. Mapping the genomic location of the gene encoding α-amanitin resistance in vaccinia virus mutants. J. Virol. **57**:65–70.
37. **Villarreal, E. C., N. A. Roseman, and D. E. Hruby.** 1984. Isolation of vaccinia virus mutants capable of replicating independently of the host cell nucleus. J. Virol. **51**:359–366.
38. **Wright, J. A.** 1983. Altered forms of mammalian nucleoside diphosphate reductase from mutant cell lines. Pharmacol. Ther. **22**:81–102.