Roles of Vaccinia Virus Ribonucleotide Reductase and Glutaredoxin in DNA Precursor Biosynthesis*

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To examine the possible role of the vaccinia virus glutaredoxin as a cofactor for viral ribonucleotide reductase, viral growth, DNA synthesis, and dNTP pools were measured in infections of B-SC-40 monkey kidney cells with wild type vaccinia virus and with mutants of vaccinia that lacked a functional reductase or glutaredoxin. In infections of untreated host cells, the lack of viral ribonucleotide reductase or glutaredoxin had only small effects upon virus growth. When host cells were pretreated with α-amanitin, which blocks host RNA polymerase II but not viral transcription, viral DNA synthesis was markedly reduced in infections with either of the mutants when compared with wild type infections. Relative to dNTP levels in wild type infections, pools of dCTP, but not of the other dNTPs, were significantly reduced in infections of amatin-treated cells with either mutant. The parallel depletion of dCTP in the two mutants suggests that the role of glutaredoxin may be to function as a cofactor for viral ribonucleotide reductase. The data suggest that both viral proteins become essential for DNA replication only when levels of the corresponding host cell proteins are depleted.

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‡The abbreviations used are: RNR, ribonucleotide reductase; kbp, kilobase pair; bp, base pair; dNTP, deoxyribonucleoside 5’-triphosphate.

Poxviruses, such as vaccinia virus, replicate their DNA in the cytoplasm of host cells. This means that the viral genome must encode most, if not all, of the proteins required for DNA synthesis, since the host enzymes for replication are localized in the nucleus. Indeed, vaccinia virus has been shown to encode many of the enzymes involved in the synthesis of DNA and its deoxyribonucleotide precursors (1).

Among such virally encoded enzymes is ribonucleotide reductase (RNR), which provides the precursors for DNA synthesis by the reduction of ribonucleotides to their corresponding deoxynucleotides. To carry out this reaction, ribonucleotide reductase presumably acts in conjunction with a hydrogen donor, which, in turn, reduces the enzyme. Among the cofactors known for various ribonucleotide reductases are the small thiol transferase proteins called the glutaredoxins (2).

It was recently shown that the vaccinia O2L open reading frame encodes a functional glutaredoxin, a 12-kDa protein that is expressed postreplicatively but which is packaged in the virions (3). Although the protein is synthesized after DNA replication has begun and may well have other roles in the virus growth cycle, one cannot rule out the possibility that it is acting as a hydrogen donor for ribonucleotide reductase.

To assess the role of the viral glutaredoxin in relation to the activity of the viral ribonucleotide reductase, viral growth, DNA synthesis, and dNTP pools were compared in infections of B-SC-40 monkey kidney cells with wild type vaccinia virus, and with two mutant viruses, M1λ, with an inactive ribonucleotide reductase R1 subunit, and vGRX− (gpt), a glutaredoxin mutant. M1λ, which has been shown to have no ribonucleotide reductase activity as measured by conversion of tritiated CDP to dCDP, has been reported to replicate at wild type levels, as measured by plaque-forming ability at 24 and 48 h postinfection (4). This would suggest that in the absence of functional viral RNR, viral replication could be sustained by a supply of dNTPs produced by the host cell’s ribonucleotide reductase. If this is true, then the loss of a functional glutaredoxin might have no discernible effect on viral replication, even if glutaredoxin was, in fact, a required cofactor for the viral ribonucleotide reductase. Thus, it is important to measure DNA synthesis and dNTP pools in the absence of an active host cell RNR. To achieve this, we have used α-amanitin treatment of host cells for 18 h prior to infecting them with virus. Amanitin has been shown to inhibit host RNA polymerase II but not viral transcription (5, 6). Therefore, we expect amanitin pretreatment to deplete the cell of relatively short-lived mRNAs, including those for RNR and glutaredoxin. In this report, we show that in the absence of host cell transcription, the synthesis of dNTPs, as well as of viral DNA, is greatly affected in mutant viruses for either ribonucleotide reductase or glutaredoxin.

EXPERIMENTAL PROCEDURES

Chemicals

Radiochemicals (tritiated dNTPs) were obtained from DuPont NEN. Klenow fragment of polymerase I and unlabeled dNTPs used in pool measurements were purchased from Life Technologies, Inc., while templates poly(d-dC) and poly(dA-dT) were obtained from Pharmacia Biotech Inc. The genius II nonradioactive nucleic acid labeling and detection kit was obtained from Boehringer Mannheim. All other chemicals were from Sigma.

Methods

Cells and Virus—B-SC-40 cells, a derivative of B-SC-1 cells selected for their ability to grow at 40 °C, were grown in Eagle’s minimal essential medium plus 5% calf serum. Vaccinia virus, WR strain (wild type), and mutant strains, M1λ and vGRX− (gpt), were maintained as described previously (7). M1λ was a generous gift from Dr. Dennis Hruby, Oregon State University.

vGRX Gene Disruption—The “gene knock-out” plasmid pGRX(gpt) was constructed from the previously described plasmid pVH(P/O) (3). pVH(P/O) is a Bluescript-SK plasmid vector containing the vaccinia virus grx gene within the 1.7-kbp HindIII P and O fragments of the vaccinia virus (strain WR) genome. First, the 3’-terminal 720-bp HindIII/PstI fragment was deleted from pVH(P/O). Then, the 3’-end AscI/Ball fragment within the 5’-proximal coding region of grx was replaced...
with the 2-kbp AclI/Smal chimeric DNA fragment of pGEM-gpt (9), which contains the Escherichia coli guanine phosphoribosyltransferase (gpt) gene under control of the vaccinia virus P7.5 promoter. Approximately 5 μg of precipitated calcium phosphate pGRX(gpt) was transfected into 5 × 10^6 CV-1 cells that had been infected 2 h earlier with 5 × 10^3 plaque-forming-units of vaccinia virus (strain WR). After 48 h at 37°C in a 5% CO₂ atmosphere, the cells were harvested and the virus progeny released by three rounds of freezing and thawing. Progeny virus was inoculated into fresh B-SC-1 cells that had been incubated for 24 h in selective medium consisting of modified Eagle’s medium containing 0.08 μM mycophenolic acid, 0.11 μM hypoxanthine, and 1.6 μM xanthine. Drug-resistant recombinant viruses were selected by 5 rounds of plaque purification as described previously (10).

DNA Blot Analysis—Vaccinia virus-infected B-SC-1 cells (2 × 10^5) were harvested after 48 h and suspended in 2 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 M β-mercaptoethanol, 2 mM EDTA, 0.5 mg/ml proteinase K, 1% sodium dodecyl sulfate, and 20% sucrose). After 3 h at 37°C, DNA was isolated by extraction with phenol and precipitated with ethanol. The DNA was digested with Xbal and resolved by electrophoresis on an 0.8% agarose gel, transferred to a GeneScreen Plus membrane (DuPont NEN), and hybridized with a ^32P-labeled grx DNA probe at 65°C for 16 h in 50 mM Tris-HCl, pH 7.5, 1 mM NaCl, 10 μg/ml salmon sperm DNA, 1% sodium dodecyl sulfate, and 10% dextran. The probe was prepared by polymerase chain reaction of grx coding sequences with the primers ATGCCCGGGAATTGTGACAAACGG and ATGACATGTCGGCGACATGATTA and [α-^32P]dCTP (Amer-sham Corp). The membrane was washed at 75°C for 1 h in 0.1 × SSC containing 1% sodium dodecyl sulfide and an autoradiograph made.

Immunoblot Analysis—Proteins from vaccinia virus-infected B-SC-1 cells (1 × 10^6) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Binding to a rabbit polyclonal antibody prepared against recombinant glutaredoxin was performed in phosphate-buffered saline containing 0.08% agrose gel, transferred to a GeneScreen Plus membrane (DuPont NEN), and hybridized with a ^32P-labeled DNA probe at 65°C for 16 h. The DNA probe was prepared by random hexamer priming a polymerase chain reaction-derived 800-bp vaccinia virus DNA fragment that should hybridize to a DNA probe. Bottom, Southern blot autoradiograph showing the XbaI fragments that hybridized to grx probe. Wild type vaccinia virus DNA, lanes 1–3, independent recombinant viruses after five successive plaque isolations in gpt selective medium. Arrows show the sizes of XbaI DNA fragments.

RESULTS

Construction and Characterization of grx Deletion Mutants—The E. coli gpt gene was used as an insertion marker (3) to inactivate the grx gene of vaccinia virus. Recombinant viruses were isolated by 5 rounds of plaque purification in the presence of gpt selective medium. The viral DNA was digested with XbaI, and Southern blot analysis was performed with a grx probe. As depicted in Fig. 1, there were three predicted types of recombinant DNA genomes. (i) and (ii) represent the two types of unstable single crossover recombinant genomes and (iii) the stable double crossover mutant. Of 18 virus isolates examined, 5 were the desired double recombinants. The Southern blot (Fig. 1) shows the DNA patterns for two double recombinants (2 and 4) and five that are mixtures of single or single and double crossover recombinants.

Immunoblot analysis was performed to confirm the absence of grx expression. As shown in Fig. 2, cells infected with wild type vaccinia virus or recombinant viruses 1, 3, 5, 6, and 7 produced an M, 12,000 protein that reacted with rabbit polyclonal antibody to glutaredoxin. By contrast, cells infected with recombinant viruses 2 and 4 did not produce an immunoreactive protein. Thus, recombinant viruses 2 and 4 had neither an intact grx gene nor detectable expressed glutaredoxin and were, therefore, used for further experiments. No difference was noted in the size or appearance of wild type and mutant plaques that formed on B-SC-1 cells, indicating that a functional grx was not necessary for replication in tissue culture.

Lack of Viral Ribonucleotide Reductase or Glutaredoxin Does Not Inhibit Virus Growth When Host Cell Transcription Is Permitted—To test whether the lack of the ribonucleotide reductase large subunit or glutaredoxin affected the growth of vaccinia virus in culture, the replication of the M1α and vGRX^- (gpt) mutants in B-SC-40 cells was compared with that of wild type virus. Subconfluent cultures of B-SC-40 cells were infected at a multiplicity of infection of 1 and the infected cells harvested at various times postinfection. The titers of virus obtained from each of the strains infecting B-SC-40 cells are shown in Fig. 3. The data showed that although there is an initial lag in viral replication in infection by each of the two mutants, by 12 h postinfection there is no discernible difference between mutant and wild type virus growth. One set of plates of cells infected with each strain was observed at 48 h postin-
The sizes in kDa of protein markers are shown on the left. The arrow points to glutaredoxin.

Infections of untreated B-SC-40 cells. Fig. 4 shows viral DNA synthesis in infections of untreated B-SC-40 cells. Fig. 5B shows dNTP pools in infections of cells pretreated with amanitin. It is evident that dNTP pools are dramatically reduced in all cases, including the mock-infected cells. A comparison of pool sizes of individual nucleotides in infections of wild type and mutant viruses showed no significant differences in dATP, dTTP, or dGTP levels among the three strains. In striking contrast, however, the levels of dCTP were drastically lower in the infections with the two mutant virus strains when compared with the corresponding wild type virus.

**DISCUSSION**

Under conditions where host cell RNA polymerase II is inactivated, it is possible to examine the effects of mutations in viral genes that are not apparent in infections of untreated cells. The experiments to measure growth of the mutant viruses (in terms of plaque-forming ability) could not be done using amanitin-treated host cells, as it has been shown that host cell functions are required for viral assembly, though not for viral DNA replication (12, 13). Viral growth, measured in infections of untreated cells, showed relatively small differences at early time points and no difference after 12 h postinfection between wild type and either of the mutant viruses (in terms of plaque-forming ability). It is evident that the mutant viruses were 84 and 98% of wild type for M1 and M1 versus vGRX virus is shown in Fig. 4A. DNA accumulation increased steadily with time up to 8 h in all three strains. The levels of DNA accumulation at this time in infections of untreated cells were 1 and 14%, respectively, of the corresponding wild type levels.

**dNTP Pool Sizes Are Altered in Infections of Amanitin-treated B-SC-40 Cells**—To investigate the possibility that the reduced DNA synthesis seen in the mutant viruses was a consequence of reduced availability of precursors for DNA synthesis, dNTP pools were measured in wild type, M1, and vGRX virus infections of cells that had or had not been treated with amanitin. Infected cells were harvested at 6 h postinfection at a time when DNA synthesis is still actively occurring in wild type virus infections. Fig. 5A shows that dNTP pools are essentially the same in wild type, M1, and vGRX virus infections of untreated B-SC-40 cells. Fig. 5B shows dNTP pools in infections of cells pretreated with amanitin. It is evident that dNTP pools are dramatically reduced in all cases, including the mock-infected cells. A comparison of pool sizes of individual nucleotides in infections of wild type and mutant viruses showed no significant differences in dATP, dTTP, or dGTP levels among the three strains. In striking contrast, however, the levels of dCTP were drastically lower in the infections with the two mutant virus strains when compared with the corresponding wild type virus.

**Viral DNA Synthesis Is Markedly Reduced in M1 and vGRX Viruses**—To compare viral DNA synthesis in the presence and absence of cellular ribonucleotide reductase and glutaredoxin, infections with wild type, M1, and vGRX viruses were performed on B-SC-40 cells that had been treated with a-amanitin for 18 h prior to infection. Control infections were carried out on untreated B-SC-40 cells.

Viral DNA synthesis in infections of untreated cells with wild type, M1, and vGRX virus is shown in Fig. 4A. DNA accumulation increased steadily with time up to 8 h in all three strains. The levels of DNA accumulation at this time in the mutant viruses were 84 and 98% of wild type for M1 and vGRX virus, respectively. Fig. 4B shows viral DNA synthesis in infections of a-amanitin-treated cells. DNA accumulation in infections with wild type virus were comparable in amanitin-treated and untreated cells at all time points measured. In contrast, infections of amanitin-treated cells with either M1 or vGRX virus showed a marked reduction in DNA synthesis. At 8 h, the DNA accumulations in M1 and vGRX virus infections were 1 and 14%, respectively, of the corresponding wild type levels.

**Fig. 2. Immunoblot analysis of cells infected with recombinant viruses.** B-SC-1 cells were infected with the same recombinant viruses described in Fig. 1, and the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane, which was then incubated with rabbit polyclonal antibody to recombinant glutaredoxin followed by 125I-protein A. Lanes containing wild type and recombinant viruses correspond to Fig. 1. The sizes in kDa of protein markers are shown on the left. The arrow points to glutaredoxin.

**Fig. 3. Time course of virus growth.** B-SC-40 cells were infected with wild type (w.t.), M1, or vGRX virus at 1 plaque forming unit (p.f.u.)/cell. At the indicated times postinfection infected cells were harvested, and the titer of progeny virus was determined by plaque titration. Titeres are based on triplicate plaque counts, which generally agreed within 10%.

**Fig. 4. Viral DNA accumulation in infections of B-SC-40 cells, untreated (A) or treated with 6 μg/ml a-amanitin (B).** Infections with wild type (W.T.) and mutant viruses were carried out as for growth measurements. Infected cells were harvested at the indicated times after infection, lysed, and assayed for viral DNA accumulation using a quantitative dot blot hybridization method.
wild type virus in infections of B-SC-40 cells, measured at 24 and 48 h postinfection (4). This is somewhat unexpected, given the importance of ribonucleotide reductase in supplying precursors for DNA replication. A simple explanation of this observation could be that in infections with virus lacking a functional reductase, the host cell’s reductase might supply the virus with sufficient dNTPs to sustain growth. The growth experiments were done with subconfluent monolayers of B-SC-40 cells, which, although not synchronized, would have at least a subset of the population in active division and thus have functional ribonucleotide reductase activity. In the case of the vGRX (gpt) mutant, it is not entirely surprising to see no difference in growth compared with the wild type virus, since (a) it may not have any direct role in the production of dNTPs and (b) even if it did function as a cofactor for ribonucleotide reductase, a similar argument could be advanced as for M1L, namely that a host cell protein stands in for the missing viral glutaredoxin.

If host proteins were, indeed, substituting for viral gene products, then this should be apparent if host transcription was inhibited for long enough to ensure that existing host reductase or glutaredoxin had been turned over prior to virus infection. The experiments to measure DNA synthesis and dNTP pools were designed with this in mind. A comparison of DNA synthesis by the three strains of virus in untreated and amanitin-treated cells showed that wild type DNA synthesis was unaffected by the shutdown of host cell transcription, as might be expected for a virus that has all of the machinery required for the synthesis of DNA, as well as for its precursors. M1L and vGRX (gpt) virus, however, showed severely reduced levels of DNA synthesis under conditions where host cell proteins were presumably unavailable, while synthesizing close to wild type levels of DNA in host cells that had not been amanitin-treated.

While this observation supports the idea that cellular proteins are recruited by viruses mutant for reductase or glutaredoxin, it does not shed any light on the question of whether the similar reductions in DNA synthesis in the two mutants are because they function together in the production of dNTPs. The classical way to answer this question would be to make a double mutant virus that lacked both reductase and glutaredoxin. The prediction would be that the double mutant would have a phenotype no different from those of either of the single mutants if they both act through the same pathway. However, it is very likely that even if glutaredoxin were a cofactor for reductase, it also has other cellular functions (14). Thus, it would be difficult to analyze such a mutant and separate the effects of the losses of the different functions of glutaredoxin.

A simpler way to assess whether glutaredoxin is involved in the production of dNTPs is to measure dNTP pools, as we did, in an infection by a vGRX (gpt) mutant and compare them with pools in infections with a reductase mutant and with wild type. Such measurements showed that while dNTP pools were considerably reduced in all amanitin-treated cells, including those that were mock-infected, the M1L and vGRX (gpt) mutant infections showed a strikingly similar depletion of dCTP pools as compared with wild type infections. It is interesting that although wild type infections in amanitin-treated cells showed a large reduction in the pool sizes of all four nucleotides, there were apparently sufficient levels of dNTPs nevertheless for viral DNA synthesis to occur at levels comparable with those in untreated cells. The drastic reduction in DNA synthesis in the two mutants, then, seems related to the almost total depletion of the dCTP pools. The reason for this apparent selective depletion of dCTP pools in both mutants is not clear, but its parallel occurrence in both mutant infections indicates that the loss of reductase and the loss of glutaredoxin causes a very similar effect, supporting the idea that they may be working together in the production of dNTPs. It has been noted that the viral ribonucleotide reductase has a 3-fold lower Km for CDP compared with the cellular enzyme (8). Thus, under conditions where substrate levels are low, such as might be prevalent in the amanitin-treated cells, there would be a depletion of dCTP in infections with viruses lacking a functional reductase or reductase cofactor, while wild type virus would still be capable of reducing CDP efficiently and producing sufficient dCTP to sustain normal levels of DNA replication and growth.

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Fig. 5. Pool sizes of dNTPs in infections of B-SC-40 cells, untreated (A) or treated with 6 μg/ml α-amanitin (B). Infections with wild type (w.t.) and mutant viruses were carried out as for growth measurements. Infected cells were harvested at 6 h postinfection, and extracts were prepared. Pool sizes of the four dNTPs were measured by an enzymatic method. Error bars represent standard deviations in triplicate assays. In B, dCTP and dGTP were virtually undetectable in infections by M1L and vGRX (gpt) mutant viruses. M1L, mock infected.
