

Intracellular Chelation of Iron by Bipyridyl Inhibits DNA Virus Replication

RIBONUCLEOTIDE REDUCTASE MATURATION AS A PROBE OF INTRACELLULAR IRON POOLS*

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The efficient replication of large DNA viruses requires dNTPs supplied by a viral ribonucleotide reductase. Viral ribonucleotide reductase is an early gene product of both vaccinia and herpes simplex virus. For productive infection, the apoprotein must scavenge iron from the endogenous, labile iron pool(s). The membrane-permeant, intracellular Fe²⁺ chelator, 2,2'-bipyridine (bipyridyl, BIP), is known to sequester iron from this pool. We show here that BIP strongly inhibits the replication of both vaccinia and herpes simplex virus, type 1. In a standard plaque assay, 50 μM BIP caused a 50% reduction in plaque-forming units with either virus. Strong inhibition was observed only when BIP was added within 3 h post-infection. This time dependence was observed also in regards to inhibition of viral late protein and DNA synthesis by BIP. BIP did not inhibit the activity of vaccinia ribonucleotide reductase (RR), its synthesis, nor its stability indicating that BIP blocked the activation of the apoprotein. In parallel with its inhibition of vaccinia RR activation, BIP treatment increased the RNA binding activity of the endogenous iron-response protein, IRP1, by 1.9-fold. The data indicate that the diiron prosthetic group in vaccinia RR is assembled from iron taken from the BIP-accessible, labile iron pool that is sampled also by ferritin and the iron-regulated protein found in the cytosol of mammalian cells.

Organisms have an ambiguous relationship with iron (1–3). Iron is essential to replication and growth, yet at the same time iron is cytotoxic. Iron is nutritionally essential due to its role as prosthetic group in a variety of enzymes and electron transfer proteins required for energy metabolism, for a variety of metabolic interconversions, and for the biosynthesis of the deoxyribonucleotides required for DNA synthesis and repair (4). Cytotoxicity is due to the efficiency by which iron, as Fe²⁺, can support the production of oxygen radicals, particularly the hydroxyl radical, HO· (3). Consequently, cells and organisms tightly regulate the uptake, efflux, and compartmentalization of iron so as to modulate the amount of iron accumulated and to ensure that by the appropriate sequestration, the cytotoxic potential of the iron that is absorbed is appropriately suppressed (4–6).

Pathogens and their hosts share this ambiguous relationship with iron (7–13). However, pathogens live within the context of the iron-restricted milieu that the host maintains as a key to the suppression of the cytotoxic potential of iron. Bacterial pathogens adapt to this iron-limited environment in several ways. One is the production of chelating agents that possess exceptionally large affinities for iron as either Fe²⁺ or Fe³⁺ (9, 11, 14). Another strategy is to divert transferrin iron by producing transferrin receptors that compete with the host's (11, 12). The dependence on the host's supply of iron that pathogens exhibit makes them susceptible to the potential bacteriostatic effects of host iron limitation or chelation. A large body of evidence indicates that manipulation of host iron status does lead to a modulation of the proliferation and virulence of many bacteria and protozoa (10, 13, 15, 16).

In contrast to such organisms, viruses have not evolved mechanisms for actively scavenging host iron. In part, this is no doubt due to the fact that viruses produce limited metabolic machinery except for that required for the replication of their genome. On the other hand, DNA viruses are directly dependent on iron for their proliferation as a result of the essential role that iron plays in the catalytic center of ribonucleotide reductase (RR)¹ (17–19). RR is encoded in most if not all large DNA viral genomes (*e.g.* pox and herpes viruses) and is produced early in infection to support the production of the dNTPs required for viral DNA synthesis (20–23). This pattern is found in the host as well, because mammalian RR is cell cycle-regulated with strong induction of its synthesis in S phase concurrent with genome replication (24).

Eukaryotic and viral ribonucleotide reductase is a heterodimeric protein (17–19, 25). The vaccinia virus subunits are referred to as R1 and R2. R1, the large subunit (87 kDa), binds NTPs and is regulatory in nature (26). R2, the small subunit (37 kDa), contains the active site of the enzyme that includes a diiron core and a catalytic tyrosyl radical (27). The genes encoding these two subunits are found on the *HindIII* F and I genomic fragments, respectively (26, 27). Both are early genes in that they are temporally expressed prior to DNA replication in the vaccinia infectious cycle (28). That a DNA virus requires the pool of dNTPs provided by RR in support of its replicative cycle is indicated by the effect of hydroxyurea (HU). HU inactivates ribonucleotide reductases by quenching the catalytic tyrosyl radical. HU blocks vaccinia replication in cultured cells (29).

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¹ The abbreviations used are: RR, ribonucleotide reductase; HU, hydroxyurea; BIP, 2,2'-bipyridyl; VSV, vesicular stomatitis virus; m.o.i., multiplicity of infection; hpi, hours post-infection; BPS, bathophenanthroline disulfonic acid; Ara-C, cytosine arabinoside; HSV-1, herpes simplex virus type 1; LIP, labile iron pool; CA, calcein; SIH, salicylaldehyde isonicotinoyl hydrazone; IRE, iron-response element; DFO, desferrioxamine; PAGE, polyacrylamide gel electrophoresis.

The vaccinia infection, and the essential role of RR in it, provides a useful biologic system in which to explore the mechanism by which iron-dependent enzymes obtain the metal that is critical to their activity. In effect, infection introduces in a regulated fashion a gene that encodes an iron-dependent enzyme whose biologic function (support of DNA synthesis and viral replication) and enzyme activity (NDP/NTP reduction) can be readily assayed. Analysis of the iron acquisition pathway employed by the viral RR will permit us to identify the iron pool(s) and proteins involved. This provides us with a useful model system that will permit the analysis of how iron is trafficked to iron apoproteins, and perhaps, how modulation of this trafficking could specifically impact on the replication of cell pathogens.

In this study, we have used the membrane-permeant, Fe^{2+} chelator, 2,2'-bipyridyl (BIP), as a probe of whether and how cellular iron is required for a productive infection by vaccinia virus in cultured cells. BIP has been established to interact with the "labile" iron pool within eukaryotic cells (30–32). This pool accounts for ~20% of the newly arrived iron in a cell (30) and appears to be the pool that is sensed by regulatory factors such as iron-response element-binding protein (IREPB/IRP1, cytosolic aconitase) (5, 33, 34) and, in the yeast *Saccharomyces cerevisiae*, the iron-regulated transcription factor, Aft1p (35, 36). We show here that this pool appears also to support a productive viral infection by providing the iron required for the activation of the vaccinia RR.

EXPERIMENTAL PROCEDURES

Cells and Virus—Vaccinia virus, strain WR, herpes simplex virus type 1, strain KOS, and the African green monkey kidney cell line BSC40 were used for this study. Monolayer cultures were grown Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% calf serum (HyClone, Logan, UT). Viral stocks were prepared from 48-h infected monolayers; these were titered in a standard plaque assay (37). This same assay was used to determine viral yield in both vaccinia and herpesvirus infections. A plaque assay was used also to demonstrate that BIP did not inhibit a vesicular stomatitis virus (VSV) infection. VSV is an RNA virus.

Analysis of Protein Synthesis in Virus-infected Cultures—The labeling procedure of Niles *et al.* (38) was used to follow protein synthesis in vaccinia-infected cells. Confluent monolayers of BSC40 were inoculated with vaccinia virus at a multiplicity of infection (m.o.i.) of 20 for 30 min. The medium was aspirated and replaced with virus-free medium. At varying times after infection, the medium was removed; the cells were washed once with prewarmed phosphate-buffered saline, and the cells were pulse-labeled for 15 min with 0.8 ml of prewarmed phosphate-buffered saline containing 100 $\mu\text{Ci/ml}$ [^{35}S]methionine (>1175 Ci/mmol, PerkinElmer Life Sciences). The label was removed, and the cells were moved into 1.0 ml of SDS-electrophoresis sample buffer (39). The labeled proteins were fractionated on a 10% SDS-PAGE gel and were subsequently visualized in the gel by autoradiography.

Analysis of DNA Synthesis—The protocol described by Condit and Motyczka was used (37). Duplicate 60-mm dishes of BSC40 monolayers were infected at an m.o.i. of 10. At varying times post-infection, the cells were pulse-labeled for 15 min with 15 μCi of [^3H]thymidine (>80 $\mu\text{Ci/mmol}$, PerkinElmer Life Sciences). Cells were scraped from the dishes into 1 ml of water and mixed with 10% trichloroacetic acid. The precipitates were collected and washed on glass fiber filters and counted in a Beckman scintillation spectrophotometer.

Analysis of RNA Binding Activity of Iron-response Element-binding Protein, IRP1—Cell extracts were prepared from control cells, and cells were treated with BIP (100 μM) for 3 h and used in an RNA gel shift assay for IRP1 (40) using as probe a 92-base ribooligonucleotide containing the IRE from the human ferritin L chain mRNA (41, 42). The extracts were prepared by detergent lysis (0.5% Nonidet P-40) in 10 mM Hepes (pH 7.5) containing 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and RNasin (40 units, Promega, Madison, WI). The riboprobe was transcribed from plasmid pTZ18RM1 (see Ref. 42, kindly supplied by Dr. William Walden) with T7 RNA polymerase, labeled with [α - ^{32}P]UTP following the standard procedure (Technical Manual TM016, Promega Corp., Madison, WI), and purified on a 10% polyacrylamide gel containing 8 M urea. The binding reactions (40,

42) were resolved on a 6% polyacrylamide gel; the gel was dried and analyzed using a Bio-Rad PhosphorImager and Molecular Analyst software.

Analysis of Vaccinia Ribonucleotide Reductase Synthesis and Stability—The labeling and immunoprecipitation protocols were as described by Howell *et al.* (28). Duplicate 60-mm dishes of BSC40 monolayers were infected at an m.o.i. of 10. At varying times post-infection, the cells were pulse-labeled for 1 h with 80 μCi [^{35}S]methionine, harvested, and washed. A cell extract was prepared in a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, 0.008% bromphenol blue, and 7.5% glycerol. Extract was incubated with polyclonal antibody to either the R1 or R2 subunit of vaccinia virus (kindly supplied by Dr. Christopher Mathews), and the immunocomplexes were isolated by adsorption to protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). After washing, the pellet was resuspended in 50 μl of Laemmli buffer and boiled, and 25 μl was applied to a 10% SDS-PAGE gel. The labeled R1 and R2 subunits were visualized by autoradiography.

Analysis of Vaccinia Ribonucleotide Reductase Activity—The assay described by Slabaugh *et al.* (43) was followed. Monolayers of BSC40 cells (100-mm dishes) were infected at an m.o.i. of 10. At various times post-infection, the medium was removed, and the dishes were placed on ice. All further manipulations were performed at 4 °C. Following washing in a 25 mM Hepes buffer containing 10 mM dithiothreitol, cell extracts were prepared in a hypotonic Hepes lysis buffer with the assistance of a Dounce homogenizer. Aliquots (20 μl) of these extracts were used in a ribonucleotide reductase assay mixture containing 25 μM (~200 cpm/pmol) [^3H]cytidine 5'-diphosphate (>20 Ci/mmol, Amersham Pharmacia Biotech). After 30 min the reaction mixtures were quenched by the addition of 10 M perchloric acid. The mixture was clarified by centrifugation, and 40 μl of the supernatant was transferred to a fresh tube that was tightly capped. Following boiling to convert all nucleotides to monophosphates and clarification of this mixture by centrifugation, a marker solution containing 20 mM each of CMP, dCMP, and dUMP was added, and the total nucleotides were fractionated by chromatography on plastic-backed cellulose thin layer plates. dUMP is a secondary product as a result of the dCMP deaminase activity present in BSC40 cell extracts. The markers permitted visualization of the reaction products under UV light; the corresponding regions of the plates were removed and counted in a Beckman scintillation spectrophotometer.

RESULTS

Bipyridyl Inhibits Vaccinia Virus Production—Confluent monolayers of BSC40 cells were inoculated with vaccinia virus at an m.o.i. of 10, either in the absence of 2,2'-bipyridyl (control) or in the presence of BIP from 10 to 100 μM . Initial tests demonstrated that at 100 μM BIP did not markedly inhibit the growth of these cells to confluency and did not cause significant changes in cell morphology or survival in confluent cultures (data not shown). Plates were titered for the number of viable virus produced after 48 h of infection (Fig. 1). The data show that BIP caused a 3–4-log decrease in viral yield with the sharpest decrease occurring between 40 and 80 μM .

Bipyridyl Inhibits Production of Intermediate and Late Vaccinia Proteins but Not of Early Gene Products—Early viral gene expression precedes DNA replication. Both are required for intermediate and late gene expression. A survey of protein production during a vaccinia infection serves as a temporal measure of the progression of the infectious cycle. To determine where in this cycle the BIP-dependent inhibition of virus formation occurred, cells were pulse-labeled with [^{35}S]methionine at different times, *i.e.* hours post-infection (hpi). Radiolabeled proteins were separated by SDS-PAGE and observed by autoradiography (Fig. 2). Analysis of protein synthesis in the control infected cells (no BIP) demonstrated the onset of viral early protein production (examples designated by *E* in Fig. 2) within 3 hpi followed by the shut-off of synthesis of these and host proteins at 6 hpi. The loss of host and early virus gene expression precedes onset of the viral late protein synthesis that is dependent on the initiation of viral DNA replication. The effect of BIP (100 μM in this experiment) was striking. BIP did not inhibit the production of viral early proteins. However, it delayed the shut-off of viral early and host protein synthesis and

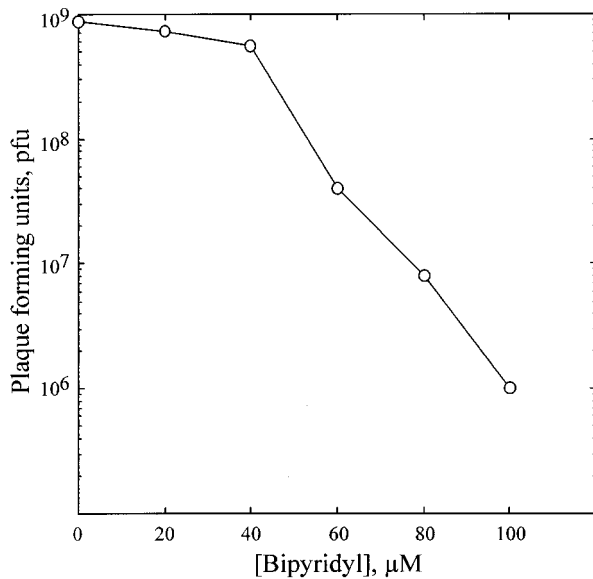


FIG. 1. **Bypyridyl inhibits vaccinia virus infection in a concentration-dependent manner.** Confluent monolayers of BSC40 cells were infected by vaccinia virus at an m.o.i. of 10 in the presence of the [BIP] as indicated. Virus-infected cells were collected at 48 hpi, and the viral yield was determined in a standard plaque assay. Each value in the figure is derived from a minimum of five dilutions. The results shown are representative of four separate experiments.

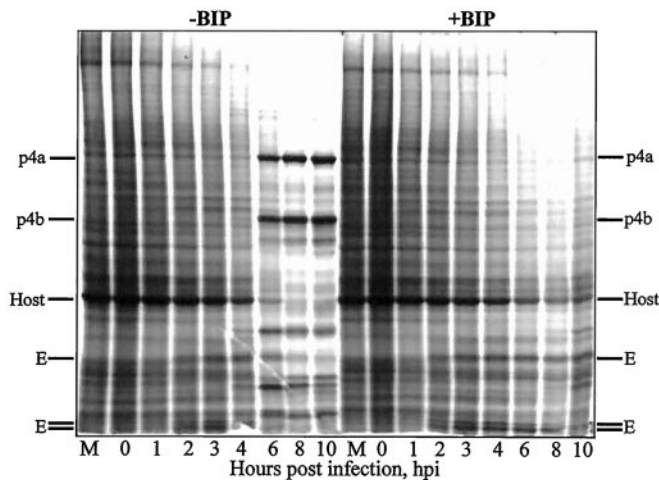


FIG. 2. **Bypyridyl blocks the synthesis of late but not early vaccinia virus proteins.** Confluent monolayers of BSC40 cells were infected at an m.o.i. of 20. The +BIP culture media contained 100 μM BIP added at the time of infection. The cultures were subsequently pulse-labeled with [^{35}S]methionine for 30 min at the times post-infection indicated in the figure. The mocked-infected cells (M) were labeled at 10 h. Cells were washed, collected, and cell extracts fractionated on SDS-PAGE; the fractionated proteins were visualized by autoradiography. E, examples of vaccinia early proteins; p4a and p4b, examples of vaccinia late proteins. A host protein, actin, is also indicated (Host). Note the shut-off of host protein synthesis concurrent with the onset of vaccinia late gene expression; both are inhibited by BIP. The figure was prepared from a scanned, digitized file of the autoradiograph.

prevented the accumulation of late viral proteins. This phenotype would be consistent with the inhibition by BIP of viral DNA replication.

Bipyridyl Inhibits Vaccinia DNA Replication—Viral DNA replication was assessed directly by measuring the rate of [^3H]thymidine incorporation into DNA at different times post-infection. A typical time course of labeling is shown in Fig. 3. Although the non-infected control cells (closed triangles) exhibited a constant level of [^3H]thymidine incorporation (average value over the 9-h experiment, 12,650 cpm), the vaccinia-in-

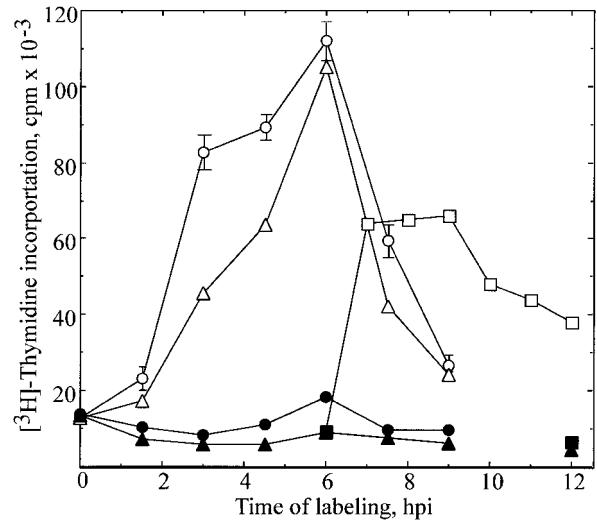


FIG. 3. **Bypyridyl blocks vaccinia virus-dependent DNA synthesis in a reversible manner.** Confluent monolayers of BSC40 cells were infected at an m.o.i. of 10. Cells were pulse-labeled with [^3H]thymidine for 15 min at the times indicated in the figure. The cells were washed and collected, and labeled polynucleotides were precipitated with trichloroacetic acid. The precipitates were collected on glass fiber filters that were counted in a Beckman scintillation spectrophotometer. The samples were as follows: vaccinia-infected cells, no additions (open circles); vaccinia-infected cells, 40 μM BIP (open triangles); vaccinia-infected cells, 100 μM BIP (closed circles); mock-infected cells (closed triangles). The recovery of DNA synthesis in BIP-treated cells also was examined. BIP-treated (100 μM), vaccinia-infected cells were pulse-labeled at 6 and 12 hpi (closed squares). At 6 hpi, duplicate cultures were washed free of BIP and then pulse-labeled at the times indicated in the figure (open squares). The recovery of DNA synthesis in these cells was then determined as above. The bars on the values for the control infected cells represent the S.E. ($n = 3$). The values for the BIP-treated, infected cells are averages of duplicate samples from one experiment that is representative of three separate experiments. The ranges in these values was 5–12%.

ected cells showed a 10-fold increase in incorporation at 3–6 hpi that then declined to control levels at 9 hpi (open circles). BIP inhibited this virus-dependent burst of DNA synthesis in a concentration-dependent manner that closely paralleled the concentration-dependent effect on viral yield seen in Fig. 1. Thus, 40 μM BIP inhibited [^3H]thymidine incorporation by <10% (open triangles), whereas 60 μM BIP inhibited >90% (closed circles) with little further inhibition at 100 μM BIP (labeling not different from uninfected control cells, closed triangles). This result also was fully consistent with the inhibition by BIP of late protein synthesis (Fig. 2) since expression of late genes, but not early ones, requires concurrent DNA synthesis. In summary, both labeling experiments indicate that BIP inhibits viral DNA synthesis and that this results in a block to further progression of the infectious cycle including expression of viral intermediate and late genes.

Bipyridyl Inhibition of Vaccinia DNA Replication and Virus Production Is Reversible—This block was reversible as indicated by the additional results shown in Fig. 3. At 6 hpi, the BIP (100 μM) was removed from a set of cultures, and DNA synthesis was measured in these now BIP-free cells. Within 1 h, DNA synthesis began and followed a time course similar to the virus-infected cell controls (cf. open squares to open circles). This recovery of virus-dependent DNA synthesis led to a productive infection since the viral yield from these cultures was equivalent to the yield from cultures that had never been treated with BIP (2.8 versus 2.5×10^9 plaque-forming units, respectively). Viral infected cells from which the BIP had not been removed did not exhibit this DNA synthesis pattern at any time over the 12-h experiment (closed squares) and exhib-

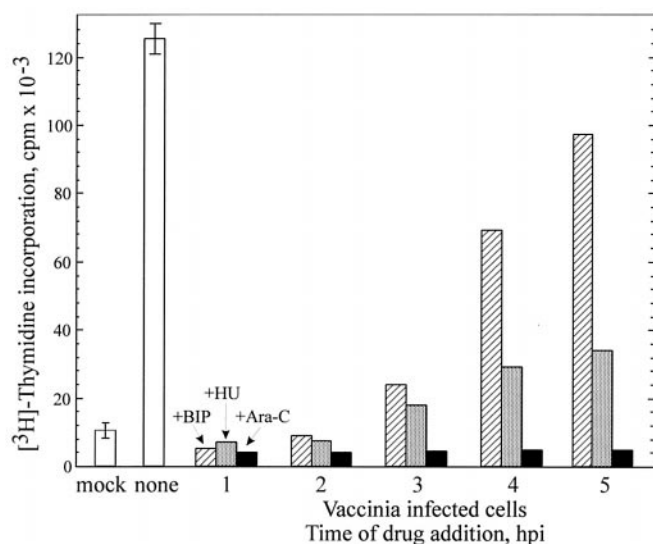


FIG. 4. Bipyridyl, hydroxyurea, and Ara-C inhibit viral DNA synthesis at different times post-infection. Confluent monolayers of BSC40 cells were infected with vaccinia virus at an m.o.i. of 10. The DNA synthesis in all cultures was then determined by pulse labeling with [^3H]thymidine as above. Inhibitors were added at the times post-infection indicated in the figure. The additions were as follows: BIP, 100 μM (stippled bars); HU, 10 mM (shaded bars); Ara-C, 100 $\mu\text{g}/\text{ml}$ (solid bars). The values are averages of duplicate cultures for each sample and are representative of two separate experiments. Typical ranges, which varied from 4 to 11% in this experiment, are illustrated by the bars on the mock-infected and infected cell controls.

ited the 3-log decrease in viral yield shown in Fig. 1.

Bipyridyl Inhibition of Vaccinia DNA Replication and Virus Production Occurs Early in the Infectious Cycle—BIP appears to block virus production by inhibiting viral DNA replication. Thus, BIP appears to work early in the infectious cycle. If the BIP effect was restricted to an early step in infection, then at some time point post-infection, BIP would no longer be able to inhibit. This inference was tested by measuring both incorporation of [^3H]thymidine at 6 hpi and viral yield at 48 hpi in cells that were treated with BIP (100 μM) at various times post-infection. In Fig. 4 the DNA synthesis in these cells was compared with that in uninfected cells (*mock*) and in control infected cells (*none*, no BIP added). The data demonstrate that to inhibit strongly DNA synthesis at 6 hpi, BIP must be added by 3 hpi (stippled bars). A similar result was observed in regards to the inhibition of viral yield (Fig. 5, closed circles). The results were completely consistent with the inference above that BIP acted early in infection and that the mechanism of BIP inhibition of virus production was linked to the BIP inhibition of DNA synthesis.

To probe further the mechanism of the BIP inhibition, the temporal effect of other inhibitors on viral DNA synthesis and yield was determined. In particular, HU inhibits active RR by reducing the ferric iron core. This results in the loss of the tyrosyl radical essential to the catalytic activity of this enzyme (27). RR is an early protein produced by vaccinia and other large DNA viruses and is required for a productive infection (20, 26, 27). Alternatively, we employed cytosine arabinoside (Ara-C), which directly inhibits DNA polymerization.

The temporal sensitivity of viral-dependent DNA synthesis to these two inhibitors is shown in Fig. 4. The corresponding inhibition of viral yield is shown in Fig. 5. Unlike BIP, HU inhibited DNA synthesis by 70% even if added at 5 hpi (Fig. 4, shaded bars), whereas Ara-C retained full inhibition when added at this time (solid bars). A similar pattern was observed in regard to viral yield (Fig. 5). HU (open triangles) and Ara-C (open squares) were equally effective in inhibiting viral yield

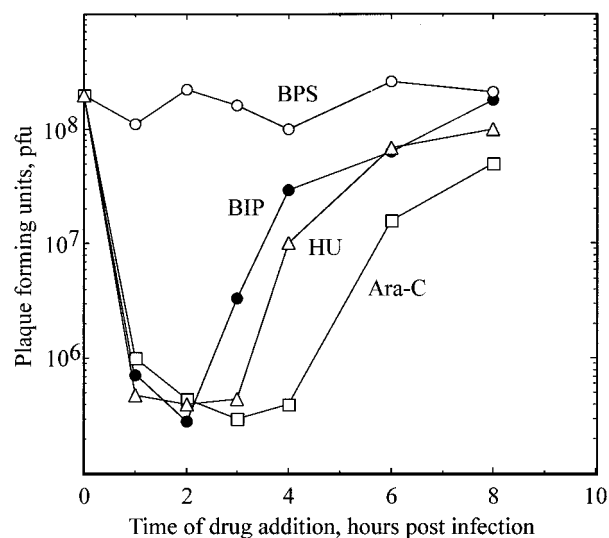


FIG. 5. Bipyridyl, hydroxyurea, and Ara-C inhibit viral replication at different times post-infection; a membrane-impermeant Fe^{2+} chelator, BPS, does not inhibit. BIP (100 μM , closed circles), hydroxyurea (10 mM, open triangles), and Ara-C (100 $\mu\text{g}/\text{ml}$, open squares) were added to vaccinia-infected monolayers of BSC40 cells at the times post-infection indicated in the figure. The viral yield in these infected cells was then determined at 48 hpi by a standard plaque assay. The inhibition of vaccinia virus infection by the membrane-impermeant Fe^{2+} chelator, BPS (100 μM), was also tested. This reagent was without inhibitory effect (open circles). The data in the figure are representative of three separate experiments.

when added up to 2 hpi. However, differences were observed when these inhibitors were added at later times. In contrast to BIP, HU was fully effective when added up to 3 hpi, while in contrast to both of the other compounds Ara-C was fully effective even when added 4 hpi.

The premise underlying our original prediction that BIP would inhibit vaccinia infection was that this lipophilic, membrane-permeant Fe^{2+} chelator would readily diffuse into cells and sequester iron within the cells that otherwise would be available in support of viral replication. The data show clearly that BIP does inhibit viral replication. A membrane-impermeant iron chelator was used also to show that the inhibitory effect of BIP was due to the chelation of intracellular iron only. Thus, the anionic Fe^{2+} chelator, bathophenanthroline disulfonic acid (BPS) was tested as an inhibitor of viral yield. As the data show, BPS was completely without effect on the production of viable virus (Fig. 5, open circles).

Bipyridyl Treatment Increases the RNA Binding Activity of IRP1—The result with BPS was fully consistent with the inference that the inhibition of viral replication due to BIP resulted from an effect that BIP had on the partitioning of intracellular iron and not on extracellular processes that result in iron accumulation. This inference was tested by comparing the RNA binding activity of the BCS40 IRP1 in cell extracts derived from control and BIP-treated cells. Thus, cells were incubated with 100 μM BIP for 3 h; cell extracts were isolated and used to program a standard RNA binding assay (40) using as probe a ribooligonucleotide derived from the human ferritin L chain mRNA (41, 42). The gel shift analysis of these binding reactions is shown in Fig. 6 (*inset*). Quantitative PhosphorImager analysis of the gel demonstrated that BIP treatment resulted in an average 1.9-fold increase (± 0.1) in this binding activity based on a linear least squares treatment of the PhosphorImager data (Fig. 6, *graph*). This result quantitatively reproduced the 1.8-fold increase in IRP1 binding activity in K562 cells due to the membrane-permeant Fe^{2+} chelator, salicylaldehyde isonicotinoyl hydrazone (SIH) (32). This effect

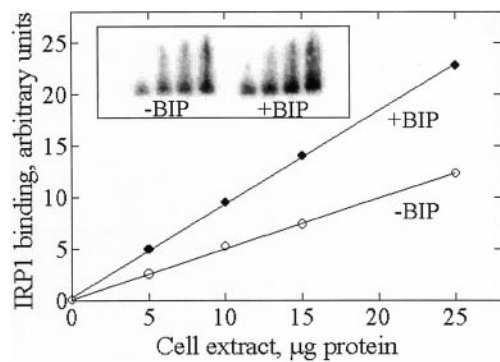


FIG. 6. Bipyridyl treatment increases the RNA binding activity of IRP1. BSC40 cells were treated with BIP (100 μM) for 3 h. Cell extracts were prepared from the BIP-treated and control cells and used to program RNA binding assays. The binding assays were prepared in 50 mM Hepes (pH 7.6), 5% glycerol, 0.2 M KCl, 5 μg of tRNA, 1 mM dithiothreitol, and 30 units RNasin and included probe (25,000 cpm) and the quantity of cell extract indicated. The mixtures were subsequently resolved on an agarose gel that was dried and developed by phosphorimaging using a Bio-Rad instrument and Molecular Analyst software. The IRP1-binding values in the graph represent the adjusted counts registered by the PhosphorImager. The digitized phosphorimage is shown in the *inset*. Based on the fitted slopes of the binding curves, the fold increase in IRP1 binding activity in the BIP-treated cells was 1.9 ± 0.2 . These slopes were determined by linear least squares analysis using Prism 3.0 software from GraphPad (Cambridge, MA).

has been correlated directly to the chelation by SIH of intracellular iron in these cells (32).

Bipyridyl Treatment Does Not Inhibit Ribonucleotide Reductase Subunit Synthesis but Blocks Enzyme Activation—The results above were consistent with the hypothesis that BIP inhibited vaccinia virus production by blocking the build-up of the dNTPs necessary for viral replication. The working model proposes that this block was due to an inhibition of the iron-activation of apoRR and, furthermore, that this inhibition was a consequence of the BIP chelation of the intracellular iron otherwise accessible for this purpose.

This assumption that the target of the BIP treatment was the viral ribonucleotide reductase was tested directly. First, pulse-labeling and immunoprecipitation experiments were carried out to determine the pattern of R1 and R2 synthesis and turnover during infection and BIP treatment. These data are shown in Fig. 7. They are fully consistent with the labeling results shown in Fig. 2 in that the synthesis of these two early gene products was not inhibited by BIP treatment (Fig. 7A). In fact, in cells treated with BIP, the synthesis of these proteins was extended up 10 hpi. In contrast, R1 and R2 synthesis in the untreated, virus-infected control cells had declined significantly at this time concurrent with initiation of intermediate and late gene expression (*cf.* Fig. 2). Furthermore, BIP had no effect on R1 and R2 protein stability. Thus, protein produced at 4 hpi exhibited no turnover in 3 h whether or not BIP was present (*cf. lanes 1 and 2 to lanes 3 and 4, Fig. 7B*). In general, the data show clearly that BIP-treated virus-infected cells exhibit normal synthesis and stability of RR subunits.

On the other hand, BIP treatment strongly reduced the ribonucleotide reductase activity in virus-infected cells (Fig. 8). In this experiment, cell extracts were prepared from a panel of cell samples that included mock-infected and BIP-free controls. In addition, the BIP was removed from some cultures at 6 h as above, with extracts prepared at 9 hpi. These extracts were then assayed for their total ribonucleotide reductase activity using [^3H]CDP as substrate (43). The data are presented in terms of the virus-derived RR activity, that is the values given have been corrected for the endogenous, virus-independent contribution to the total.

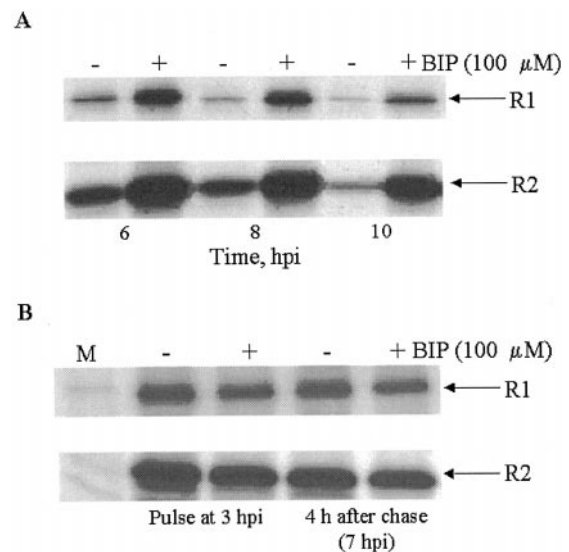


FIG. 7. Bipyridyl does not inhibit ribonucleotide reductase subunit synthesis nor alter subunit stability. A, vaccinia-infected cells were pulse-labeled with [^{35}S]methionine at the times post-infection indicated in the figure. The vaccinia R1 and R2 subunits synthesized at these times post-infection were detected separately by immunoprecipitation with rabbit polyclonal antisera to VVR1 and VVR2, respectively, followed by SDS-PAGE and autoradiography. B, BSC40 cells were infected in the absence or presence of 100 μM BIP and then pulse-labeled for 30 min with [^{35}S]methionine at 2.5–3 hpi. Following a chase with a 100-fold excess cold methionine (cells collected immediately at ~ 3 hpi) or following a chase period of 4 h (cells collected at 7 hpi), cell extracts were prepared, and the vaccinia R1 and R2 subunits were analyzed as above. The figure was prepared from scanned, digitized files of the autoradiographs.

The viral-dependent values for cells that had not been treated with BIP are similar to what has been reported previously using this same assay method (*solid bars at 6 and 9 hpi, -BIP*) (43). The decline in RR activity over this period parallels the decrease in the synthetic rate as shown in Fig. 7A (*-BIP samples*). In contrast, the RR activity in the BIP-treated cells was strongly reduced at 6 and, most dramatically, at 9 hpi (*stippled bars*). In the set of cultures from which BIP had been removed, the virus-dependent RR activity had recovered by 9 hpi to 65% of the peak activity normally observed in infected cells that had not been BIP-treated (*open bar, compare with solid bar, 6 hpi*). This was consistent with the observation that the DNA synthesis in such cultures also recovered in this time frame (Fig. 3). These observations are fully consistent with the hypothesis that vaccinia virus replication and infectivity can be inhibited by iron chelation and that the viral target of this chelation is ribonucleotide reductase. Importantly, in control assays, we showed that BIP added to either the cell extracts or to the assay mixtures themselves had no effect on the RR activity measured in these extracts (data not shown). This result indicates that the vaccinia ribonucleotide reductase is not inactivated by an iron chelator like BIP. This stands in sharp contrast to the sensitivity of the mammalian enzyme to such reagents (18, 44). Furthermore, the insensitivity of vaccinia RR to BIP *in vitro* is consistent with the fact that BIP addition to infected cells at 3 hpi or later had no effect on either viral-dependent DNA synthesis (Fig. 4) or infection (Fig. 5).

However, the data show that following removal of BIP, RR activity did increase. The immunoprecipitation results show that RR subunit synthesis is sustained in these BIP-treated cells and that RR subunits are stable (Fig. 7). The question arises whether the increase in RR activity seen between 6 and 9 hpi following removal of the BIP was due to metal addition to extant apoRR subunits, or to maturation of newly synthesized

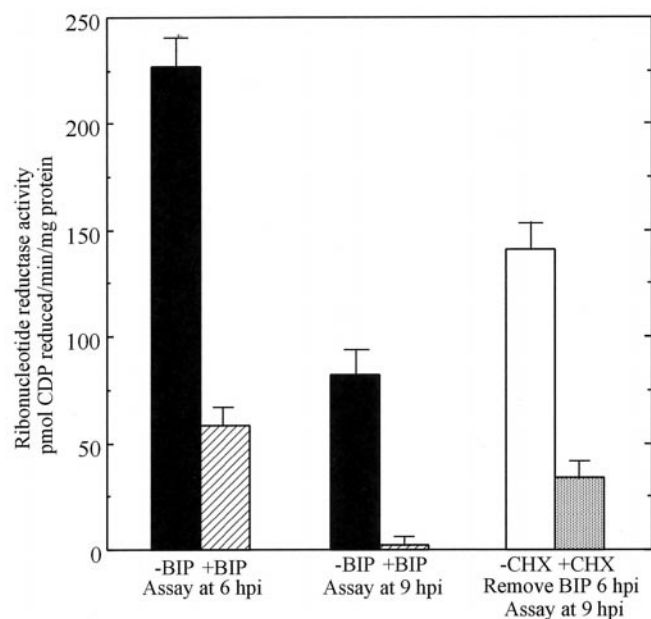


FIG. 8. Bipyridyl inhibits the activation of vaccinia ribonucleotide reductase; the recovery from this inhibition is inhibited by cycloheximide. Confluent monolayers of BSC40 cells were infected by vaccinia virus at an m.o.i. of 10 in the absence or presence of 100 μ M BIP. At 6 hpi, cells were collected, and cell extracts were assayed for ribonucleotide reductase activity by the production of [3 H]dCMP/dUMP from [3 H]CDP (1st two bars). Infected cells from a second set of cultures were collected at 9 hpi and analyzed for enzyme activity also (2nd set of bars). A third set of BIP-treated infected cultures was handled in the following manner. One-half of this set was treated with cycloheximide (CHX, 100 μ g/ml) for 15 min, and then both halves were washed free of BIP, including cycloheximide in the wash buffer and restored media of the cycloheximide-treated cultures. Cell extracts were subsequently prepared from these two sets of cultures at 9 hpi and assayed for reductase activity (last set of bars). All values shown have been corrected for the endogenous, virus-independent activity that was 14 ± 5 units (pmol of CDP reduced/min/mg protein). The S.E. in these corrected values are given in the figure. Note that BIP added *in vitro* to either the extracts or to the assay mixture had no effect on the reductase activity measured (data not shown).

ones, or both. Treatment of the cells with cycloheximide prior to BIP withdrawal was carried out to address this question. The result is shown in Fig. 8, shaded bar. Thus, cycloheximide completely inhibited the recovery of RR activity between 6 and 9 hpi. This result indicated that the recovery typically observed following removal of the BIP required protein synthesis. Whether this lack of recovery was due to inhibition of new RR synthesis, or to inhibition of the synthesis of some other factor required for iron activation of apoRR, or both, could not be deduced from this result.

Bipyridyl Inhibits HSV-1 but Not VSV Infection—Vaccinia is a large DNA virus that replicates in the cytoplasm. To determine if the BIP effect was specific to such viruses, as our hypothesis would require, and/or to viruses that replicate in the cytoplasm only, the effect of BIP on herpes simplex virus type 1 (HSV-1) and vesicular stomatitis virus (VSV) replication was determined. Unlike vaccinia, HSV-1 is a DNA virus that replicates in the nucleus; the data in Fig. 9A show that in comparison to vaccinia it is equally sensitive to BIP with a closely parallel concentration dependence (compare closed squares to closed circles). In contrast, VSV, an RNA virus, was completely insensitive to BIP. This was demonstrated by a simple plaque assay as illustrated in Fig. 9B and confirmed by more quantitative viral yield analysis (data not shown). These comparisons indicate that BIP most likely inhibits proliferation of DNA viruses only and indicate further that the cell locale of the viral replisome is not a factor in this inhibitory activity.

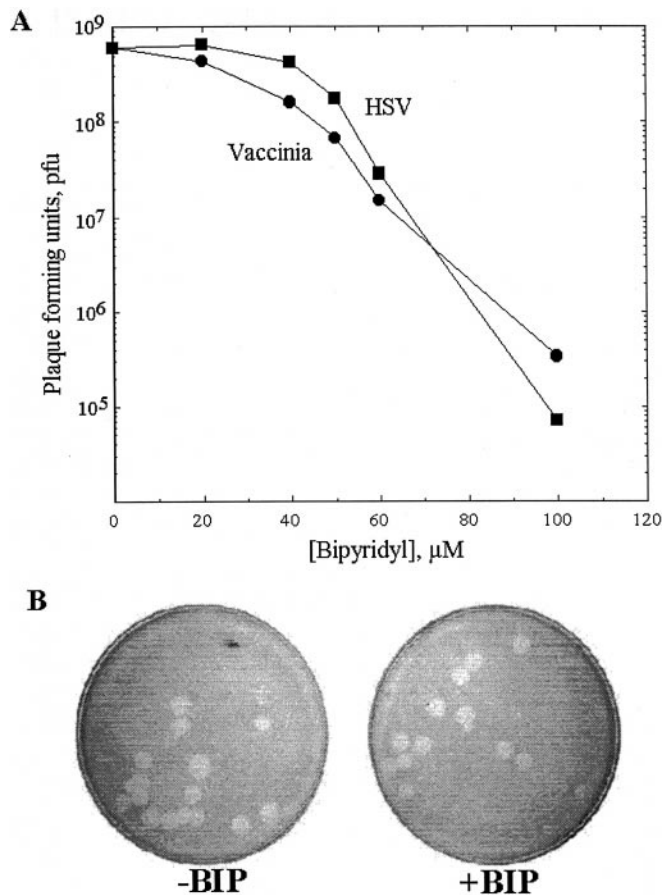


FIG. 9. Bipyridyl inhibits HSV-1 infection as effectively as vaccinia infection but not vesicular stomatitis virus infection. A, confluent monolayers of BSC40 cells were infected with either vaccinia virus or herpes simplex virus type 1 at an m.o.i. of 10 in the absence or presence of the [BIP] as indicated in the figure. Cells were removed at 48 hpi and assayed for virus production by a standard plaque assay. The values are representative of three separate experiments. B, confluent monolayers of BSC40 cells were infected with the equivalent of 20 plaque-forming units of VSV in the absence or presence of 100 μ M BIP. The image of these monolayers was obtained at 48 hpi. This image was generated by direct digitization of the plates using Bio-Rad Gel Doc hardware.

DISCUSSION

A variety of data suggests that eukaryotic cells contain a pool of iron that is accessible to membrane-permeant, iron-chelating agents like BIP. The most direct measurement of this "labile iron pool" (LIP) in mammalian cells is given by a membrane-permeant fluorescent iron chelator such as calcein (30–32, 45). The fluorescence of calcein (CA) is quenched upon iron binding; the amount of CA-bound iron is subsequently determined by the amount of CA fluorescence recovered upon addition of a competing, membrane-permeant iron chelator like BIP or SIH. Although there is some controversy about the specificity of CA for Fe^{2+} versus Fe^{3+} (45), the data clearly show that chelators like BIP and SIH are Fe^{2+} -specific indicating that in the cell CA is chelating Fe^{2+} . This chelatable Fe^{2+} constitutes ~20% of the total iron in both mammalian and yeast cells in culture (30, 35). Most reasonably, the major fraction of the remaining iron in these cells would be in ferritin (mammalian cells) (4, 32, 34) or in the yeast vacuole (46, 47). In most cell types, except for mature erythrocytes, only a minor fraction of total cell iron is bound as a prosthetic group in iron proteins. Among these would be ribonucleotide reductase, whether the endogenous enzyme or a pathogen-encoded one, e.g. the vaccinia virus RR.

One cytoplasmic protein that samples the LIP in mammalian cells is the iron-regulatory element-binding protein or cytosolic

aconitase (reviewed in Refs. 4 and 5). IRP1 is active as an RNA-binding protein when in an iron-depleted state; when iron-replete, IRP1 is equivalent to aconitase with its 4Fe-4S cluster and cannot bind to RNA. IRP1 binding to ferritin mRNA inhibits translation initiation of this transcript, whereas binding to the mRNA for the transferrin receptor stabilizes this message thereby enhancing receptor synthesis. Both transcripts contain an iron-response sequence element (IRE) that is specifically recognized by IRP1. Cabantchik and co-workers (32) demonstrated that the IRP1 isolated from SIH-treated K562 cells had a 2-fold increase in IRE binding activity when assayed *in vitro* in an electrophoretic mobility shift assay. In these cells, SIH treatment reduced the LIP by 75% providing evidence that the increase in IRP1 binding activity was due to the chelation of iron from this pool. We have demonstrated that BIP in BSC40 cells causes a quantitatively equivalent increase of IRP1 binding activity and that this increase parallels a decrease in the apparent iron activation of vaccinia RR. Although we did not measure a decrease in the LIP due to BIP directly, we do note that in the BCS40 cells the decrease in RR activation caused by BIP treatment was ~75% also.

Experiments in *S. cerevisiae* suggested that this organism has a pool of Fe²⁺ that is linked to the regulation of the transcription factor, Aft1p. Under conditions of low cell iron, Aft1p activates expression of the cohort of genes that encode the proteins responsible for the uptake and trafficking of iron in yeast (36). Iron, then, negatively regulates Aft1p transcriptional activity much as it does the RNA binding activity of IRP1, although the mechanism of this negative regulation of Aft1p is not known. Analogous to the effect of SIH and BIP on IRP1 RNA binding activity in mammalian cells, in yeast BIP strongly activates the transcriptional activity of Aft1p. This pool of BIP-accessible Fe²⁺ accounts for ~18% of the total cell iron based on the amount of BIP-extractable iron in the yeast cell (35). This percentage is comparable to the ~20% of total delivered iron in K562 cells that is associated with the LIP in those cells (30–32). In summary, eukaryotic cells appear to have a pool of Fe²⁺ that, at the least, is linked to the iron-dependent modulation of the activity of factors that regulate the production of iron-handling proteins. In as much as this pool can be buffered by production of ferritin H chain, for example, indicates that this pool supports both regulatory and storage functions (32). Linking these two functions is physiologically reasonable since the targets of the iron-dependent regulation are those genes that encode the proteins responsible for maintaining the size of this pool (5).

The data here add to this picture in that they suggest that this pool supplies iron for an iron-dependent enzyme, as well, since BIP blocks the activation of the vaccinia ribonucleotide reductase. The inhibition of this activation parallels the extent to which BIP and SIH reduce the LIP in mammalian cells in culture (30, 31, 34). Both reagents reduce the signal from this pool of Fe²⁺ by 75%; as noted, BIP reduced the RR activity by an identical percentage (227 versus 58 units, Fig. 8). The close quantitative comparison may indicate that the apoRR directly samples the BIP-accessible Fe²⁺ pool in BSC40 cells. Such a mechanism would be appropriate for a cytoplasmically produced and targeted protein like the vaccinia RR that is most likely associated with the cytoplasmic viral replisome.

A large body of evidence links the severity and/or progression of cellular pathogenesis to cellular iron status (2, 8, 10, 13, 15, 16, 48). However, little is known about possible underlying mechanisms of this linkage. One common theme is a decreased level of DNA synthesis whether of host or pathogen-specific DNA. For example, treatment of several human cancer cell lines with desferrioxamine (desferoxamine, DFO) strongly in-

hibited DNA synthesis in these cells. However, unlike BIP or SIH, DFO is not readily membrane-permeant; in addition, as its name implies, DFO binds Fe³⁺. Experimentally, DFO added to the growth medium of K562 cells does not chelate iron out of the intracellular calcein-iron complex (30, 32). Therefore, DFO exerts its effect by inducing a chronic inhibition of cell iron accumulation rather than an acute sequestration of iron from the LIP. Nonetheless, the probable iron limitation in the LIP, as would occur in cells that were chronically iron-deprived, did correlate with a decreased DNA synthesis and, most reasonably, the cell proliferation that attends metastatic disease (15, 16). However, in none of these experiments was the decrease in DNA synthesis and/or cell proliferation correlated with a decrease in RR activity as we have established here.

Clearly, reduction in the size of the LIP, whether caused by chronic limitation of cell iron accumulation or by direct, acute withdrawal of Fe²⁺ from the pool by a membrane-permeant ferrous ion-chelating agent, results in a decrease in RR activity. Nyholm *et al.* (44) and Cooper *et al.* (18) have demonstrated that addition of membrane-permeant or -impermeant iron chelators to cultured mouse cells resulted in a decrease in the intracellular concentration of RR-active sites. This decline was shown by the decreased EPR signal due to the tyrosyl-free radical component of the RR-active site. The formation and stability of this radical depend on the diiron prosthetic group in this active site. We were not able to detect this RR radical in vaccinia-infected BSC40 cells by EPR (data not shown). At least in the work by Nyholm *et al.* (44), however, this signal was detectable only in RR-overproducing cells. Nonetheless, the RR activity and protein analyses that we show here provide similar information, namely that BIP treatment results in the accumulation of inactive RR subunits.

Thus, the results shown here are the first to link iron chelation from the LIP with the loss of RR activity and the resulting block in the infectious cycle of a cell pathogen. Importantly, we have shown that unlike the mammalian RR, which appears to have a labile diiron active site (18, 44), the vaccinia enzyme is resistant to inactivation by an iron chelator. In consequence, BIP, which does not inactivate the vaccinia RR, inhibited only if added within the first 3 h post-infection. This was in contrast to HU, which inactivates RR and therefore was effective in blocking vaccinia DNA synthesis and infectivity even when added at 4–5 hpi. Thus, our *in vivo* data strongly indicate that the BIP-mediated withdrawal of iron from the LIP of the infected cells prevents activation of the vaccinia RR rather than leading to its inactivation. The lack of active vaccinia RR subsequently blocks the onset of viral-dependent DNA synthesis and the viral replicative and infectious cycle.

This cycle appears stalled at the interface between early and intermediate gene expression. The fact that in the presence of the BIP-mediated arrest R1 and R2 subunit synthesis persisted for up to 10 hpi (these are otherwise early gene products) is good evidence for this inference, and so is the fact that the onset of DNA synthesis immediately followed BIP withdrawal up to several hpi. This arrested infectious state remained poised for resumption of a productive infection up to ~16 hpi. The viral replicative capacity in the BIP-treated cells sharply declined at later times post-infection. In summary these data provide a fairly clear view of how manipulation of the labile iron pool in mammalian cells alters the progression of one type of cellular pathogenesis. They also raise the question of whether such manipulation with a simple organic compound like BIP might have some therapeutic use.

Our work does not directly provide evidence for the mechanism by which the diiron center of active RR is assembled, only that the required iron most likely is derived from the LIP.

There is limited, and controversial, evidence from *S. cerevisiae* that eukaryotic cells may produce a chaperone for the iron targeted for RR-active site assembly (17, 25). In the yeast genome *RNR1* and *RNR3* encode large subunit isoforms, whereas *RNR2* and *RNR4* encode isoforms of the small subunit that contains the diiron cluster and the catalytic tyrosyl free radical (17, 25, 49, 50). Rnr2p clearly is the small subunit isoform that is found in active RR in yeast. However, the function of Rnr4p is not fully known. Chabes *et al.* (17) have suggested that Rnr4p is an alternative subunit in active RR. In contrast, Nguyen *et al.* (25) propose that Rnr4p, which they demonstrated was not catalytically active, could serve as an iron chaperone in the efficient assembly of the diiron cluster in apoRnr2p. In yeast, analogous copper chaperones are essential for the trafficking of copper to their respective target proteins (51). If Rnr4p does assist in the targeting of iron to Rnr2p, a mammalian homologue could be involved in the targeting of iron from the LIP to the viral R2 protein. However, none of the mammalian genome data bases contain genes encoding yeast Rnr4p-like proteins. This leaves open the question of whether *S. cerevisiae* is unique in terms of the mechanism of iron targeting to apoRnr2p, and, if so, of how this targeting to the endogenous or vaccinia virus R2 is achieved in mammalian cells.

We performed one experiment that might have provided some information on this point. By releasing the infected cells from the BIP-dependent block in the presence of cycloheximide, we wished to determine if protein synthesis was required for the subsequent activation of viral RR. Cycloheximide treatment did inhibit activation. However, there are two reasonable explanations for this effect. First is simply that only newly synthesized R2 subunits can be activated. The second explanation is that the synthesis of an accessory protein is required for this activation. We cannot distinguish between these two mechanisms, nor can we exclude the possibility that both are involved. The Western blot analysis showed that R1 and R2 subunits were present and stable in the BIP-treated cells and that upon BIP withdrawal R1 and R2 synthesis continued in the absence of concurrent cycloheximide treatment. However, the work of Nyholm *et al.* (44) and Cooper *et al.* (18) noted above indicated that mammalian apoR2 could be reactivated *in vivo* suggesting that the vaccinia R2 subunits accumulating in the infected, BIP-treated cells could have been activated also. If this inference is correct, then our cycloheximide result suggests that concurrent synthesis of some protein(s) is required for this activation. Experiments in progress are designed to confirm that iron-depleted vaccinia RR can be reactivated *in vivo* and to subsequently identify the cell protein(s) that is required for this diiron cluster assembly process.

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