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Herpes Simplex Virus Type 1-Induced Ribonucleotide Reductase Activity Is Dispensable for Virus Growth and DNA Synthesis: Isolation and Characterization of an ICP6 *lacZ* Insertion Mutant

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Herpes simplex virus (HSV) encodes a ribonucleotide reductase consisting of two subunits (140 and 38 kilodaltons) whose genes map to coordinates 0.56 to 0.60 on the viral genome. Host cell lines containing the *Hpa*I F fragment which includes the reductase subunit genes of HSV type 1 strain KOS (coordinates 0.535 to 0.620) were generated. Transfection of these cells with a plasmid containing the immediate-early ICP0 gene resulted in the expression of ICP6; interestingly, ICP4 plasmids failed to induce expression, indicating an unusual pattern of ICP6 regulation. One such cell line (D14) was used to isolate a mutant with the structural gene of *lacZ* inserted into the ICP6 gene such that the *lacZ* gene is read in frame with the N-terminal region of ICP6. This mutant generated a protein containing 434 amino acids (38%) of the N terminus of ICP6 fused to β-galactosidase under control of the endogenous ICP6 promoter. Screening for virus recombinants was greatly facilitated by staining virus plaques with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal). Enzyme assays of infected BHK cells indicated that the mutant is incapable of inducing viral ribonucleotide reductase activity. Surprisingly, although plaque size was greatly reduced, mutant virus yield was reduced only four- to fivefold compared with that of the wild type grown in exponentially growing Vero cells. Mutant virus plaque size, yields, and ability to synthesize viral DNA were more severely compromised in serum-starved cells as compared with the wild type grown under the same condition. Although our evidence suggests that the HSV type 1 ribonucleotide reductase is not required for virus growth and DNA replication in dividing cells, it may be required for growth in nondividing cells.

Ribonucleotide reductase is a key enzyme in the DNA biosynthesis of eucaryotic and procaryotic organisms. By reducing ribonucleotides to their corresponding deoxyribonucleotides, the enzyme provides a major pathway in the formation of DNA precursors. *Escherichia coli* and mammalian ribonucleotide reductases are similar in that they exist as a complex of two nonidentical subunits and their activities are under stringent allosteric control (50). Herpes simplex virus type 1 (HSV-1), like other herpesviruses, encodes its own ribonucleotide reductase (10), which is composed of two nonidentical subunits (3, 18, 25, 40). Biochemical properties of the HSV enzyme differ from those of the mammalian counterpart in that it lacks an absolute requirement for Mg^{2+} and is insensitive to inhibition by dTTP and dATP (25, 29, 30, 39). The large subunit, designated ICP6 (23, 24), has a molecular weight of 140,000 and is encoded by a 5.0-kilobase (kb) mRNA which maps to the U_L region of the viral genome (coordinates 0.562 to 0.597) (1, 34). The small subunit has a molecular weight of 38,000 and is encoded by a 1.2-kb transcript whose 3′ end is shared by the 5.0-kb transcript of the large subunit (1, 34) (see Fig. 1).

Evidence that the HSV-1-encoded ribonucleotide reductase may be required for viral growth and DNA synthesis comes from the analysis of a temperature-sensitive (*ts*) mutant of strain 17, *ts*1207, which fails to induce reductase activity at the restrictive temperature and whose mutation has been mapped to the coding region of the large subunit of the enzyme (10, 40). At the nonpermissive temperature (NPT), however, DNA synthesis is not completely abolished (40). Preston and co-workers (40) suggest that this mutant is leaky and synthesizes sufficient amounts of functional enzyme for small amounts of DNA to be made. Alternatively, they suggest that the cellular reductase provides sufficient deoxyribonucleotide pools to support some viral DNA synthesis. Therefore, the question of whether or not the viral reductase is essential for HSV DNA synthesis remains unanswered.

To provide direct evidence for the requirement of the HSV ribonucleotide reductase in viral DNA synthesis and to circumvent the problems posed in the study of temperature-sensitive mutants, we set out to make host range mutants. This strategy was originally developed by Benjamin (4) using biochemically transformed cells to isolate and propagate polyomavirus transformation-defective mutants. In the present study, permissive cell lines containing the wild-type ribonucleotide reductase genes were constructed for the isolation and propagation of potentially lethal reductase mutants. We isolated a *lacZ* insertion mutation in the HSV-1 ICP6 gene utilizing a novel method first employed in an animal virus system by Chakrabarti et al. (5) and Panicali et al. (37) for the construction of vaccinia virus mutants. The structural gene for *E. coli* β-galactosidase (β-gal) was fused in frame with the amino-terminal region of the HSV-1 ICP6 gene such that a fusion protein was synthesized under the control of the endogenous ICP6 promoter. Isolation of a viral recombinant containing a *lacZ* insertion into this gene was greatly facilitated by screening for blue plaques after staining with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal). Studies with a *lacZ* insertion mutant indicated that viral ribonucleotide reductase activity is not essential for viral growth and DNA synthesis in cultured cells.

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MATERIALS AND METHODS

Cells and viruses. Procedures for the growth and maintenance of African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, Md.) have been previously described (54). BHK-21 clone 13 cells (American Type Culture Collection) were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum. D14 cells (see below) were propagated as described above, but with the addition of 250 μg of the antibiotic G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml. Resting, serum-starved cells were obtained primarily as described by Preston and co-workers (40). Briefly, cells were grown to confluency in medium containing 5% fetal calf serum, at which time the cells were washed with Tris-buffered saline and grown for 5 days in medium containing 0.5% fetal calf serum. HSV-1 strain KOS was used as the wild-type virus.

Plasmids and bacteria. Plasmids pSG124 and pSG1 containing the *Eco*RI A (coordinates 0.535 to 0.620) and *Eco*RI C (coordinates 0.744 to 1.000) fragments of HSV-1 strain KOS, respectively, were generously provided by R. Sandri-Goldin, University of California at Irvine, and M. Levine, University of Michigan, Ann Arbor (20). To construct plasmid pKHF, we subcloned the 13-kb *Hpa*I F fragment of HSV-1 strain KOS (coordinates 0.535 to 0.620) from pSG124 into the vector pUC19 by digestion of pSG124 with *Hpa*I and blunt-end ligation with T4 DNA ligase into the *Sma*I site of pUC19. To construct plasmid pKpX2, we subcloned the 2.3-kb *Xho*I fragment of pKHF (coordinates 0.564 to 0.580) into plasmid vector pKP54 (14) by ligating the fragment directly into the unique *Sal*I site of the vector. pKX2-βG3 was constructed by partial digestion of pKpX2 with *Bam*HI and ligation of a *Bam*HI fragment containing the *lacZ* gene from plasmid pDP503 (37) (generously provided by D. Panicali) into the *Bam*HI site indicated in Fig. 1 (see Results). The *Hin*dIII K fragment of KOS (coordinates 0.530 to 0.592) was subcloned from pKHF and ligated into the *Hin*dIII site of plasmid pKC7 to create plasmid pKC7K. pSV2neo contains the bacterial gene conferring neomycin resistance under the control of the simian virus 40 early promoter (46). Plasmid pK1-2 (kindly provided by N. DeLuca) contains the immediate-early gene ICP4 inserted into plasmid vector pUC8. Plasmid pW3-HSΔ8 (kindly provided by W. Sacks) contains the immediate-early ICP0 gene inserted into pUC13. All recombinant plasmids were propagated in *E. coli* UT481 or DH5 cells by standard procedures (32).

Transformation of Vero cells and isolation of cell line D14. Vero cells were transformed with plasmid pKHF and pSV2neo essentially as described by DeLuca et al. (8). pSV2neo (1 μg) was coprecipitated with pKHF (10 μg) and 29 μg of salmon sperm DNA in a final volume of 2 ml by the method of Graham and van der Eb (21). After a 20-min precipitation in $CaCl_2$, freshly trypsinized Vero cells (4×10^6) were added to the precipitate and incubated with continuous shaking at 37°C for 30 min. The cell-DNA mixture was suspended in 20 ml of culture medium, plated onto two 100-mm petri dishes, and placed at 37°C. After 4 h, resettled cells were shocked with 15% glycerol (in HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered saline) and incubated for several days until reaching confluency. Cells were then trypsinized, diluted 10-fold in medium containing 1 mg of G418 (a neomycin analog) per ml, and plated onto 10 100-mm petri dishes. The G418 concentration was decreased to 0.5 mg/ml after 5 days. Individual G418-resistant colonies were picked and screened as described in the Results.

Marker rescue analysis. Marker rescue experiments were performed by the procedure of Parris et al. (38) with the modifications described by Chiou et al. (7).

Marker transfer and isolation of HSV-1 ICP6 insertion mutant. Marker transfer experiments were done essentially as described above for marker rescue. Briefly, 200 to 1,000 infectious units (approximately 1 μg) of wild-type viral DNA were cotransfected with a 10-fold molar excess of the recombinant plasmid containing the mutation. For the isolation of mutant *hr*R3, the 5.3-kb insert of pKX2-βG3 was excised by cutting with *Xba*I and *Hin*dIII and used with infectious KOS DNA to cotransfect D14 cells. When widespread cytopathic effects were observed, progeny were harvested and titers were determined on permissive cells. Recombinant viruses were detected by the method of Chakrabarti et al. (5) and Panicali et al. (37). On day 3 or 4 after infection, plaques were simultaneously stained by adding 1 to 2 ml of a solution containing 300 μg of the chromogenic substrate X-gal per ml and neutral red (0.00125%) in Tris-buffered saline to a methylcellulose overlay. Plaques were visualized within 12 to 24 h at 34°C. Blue plaques were purified three times in D14 cells before stocks were made.

Viral and cellular DNA isolation. *hr*R3 and KOS DNAs were prepared from partially purified virions as described by Parris et al. (38). Cellular DNA was isolated as described by Weller et al. (55).

Analysis of cellular and viral DNA. Total cellular or viral DNA was digested with restriction endonucleases, separated by agarose gel electrophoresis in a Tris-borate-EDTA buffer, and transferred by the method of Southern (45) to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.) as suggested by the supplier. Recombinant DNAs used as probes for hybridization were labeled by the method of Feinberg and Vogelstein (15).

Indirect IF. Immunofluorescence (IF) was performed as follows. D14 cells were plated onto cover slips after transfections. After transfected cells were incubated at 37°C for 40 h, cover slips were washed three times with cold phosphate-buffered saline, fixed in 100% methanol for 5 min at −20°C, and washed again with phosphate-buffered saline. Cover slips were preincubated in 3% normal goat serum in phosphate-buffered saline for 15 min. Monoclonal antibodies 48S (specific to ICP6) and 58S (specific to ICP4) were kindly provided by M. Zweig (43). Monoclonal antibodies 48S and 58S were used in IF experiments at 1:100 dilutions in 1% normal goat serum. All incubations were done at room temperature. After preincubation, transfected cells were incubated in the presence of primary antibodies for 1 h. Cells were washed five times in 1% normal goat serum, incubated in horse anti-mouse immunoglobulin G conjugated to biotin (Vector Laboratories) for 30 min, washed again, incubated in avidin conjugated to rhodamine (Vector), and washed again. Cover slips were mounted in glycerol onto slides and visualized with a Zeiss standard microscope equipped for fluorescence microscopy with a Neofluar 40× objective.

Analysis of viral proteins. Vero or D14 cells were infected with KOS or mutant virus at a multiplicity of infection of 10 PFU per cell. Cells were labeled at 5 h with [^{35}S]methionine as previously described (S. K. Weller, E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer, Virology, in press) and were harvested at 16 h postinfection. Cells were washed three times with phosphate-buffered saline and scraped into 0.05 M Tris hydrochloride (pH 7.2)–0.15 M NaCl–0.1% sodium dodecyl sulfate–1% sodium deoxycholate–1% Triton X-100–0.1 mM protease inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma Chemical

Co., St. Louis, Mo.)–0.5 mM phenylmethylsulfonyl fluoride (Sigma) (RIPA buffer). The extracts were then sonicated for 45 s, and cell debris was removed by centrifugation for 5 min in an Eppendorf microcentrifuge. Samples either were solubilized directly in sample buffer containing 0.37 M Tris hydrochloride (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 10% sodium dodecyl sulfate, and 0.001% bromophenol blue (28) and electrophoresed or were used for immunoprecipitations. Immunoprecipitations and gel electrophoresis were done essentially as described by Huszar et al. (26) except that protein A-Sepharose CL-4B beads (Pharmacia, Inc., Piscataway, N.J.) were preadsorbed with unlabeled, KOS-infected extracts to reduce nonspecific background. For each immunoprecipitation reaction, 80 μl of extract was incubated with 80 μl of protein A-Sepharose beads and 5 to 10 μl of either 48S antibody or a 1:100 dilution of a β-gal monoclonal antibody (Promega Biotec, Madison, Wis.). Gel electrophoresis was done on 9% polyacrylamide (acrylamide-bisacrylamide, 30:0.8) containing 0.1% sodium sodium dodecyl sulfate. Gels were fixed in methanol-water-acetic acid (5:5:1, vol/vol/vol), treated with Autofluor (National Diagnostics), dried, and exposed at −70°C to Kodak XAR-5 film.

Ribonucleotide reductase assays. Ribonucleotide reductase assays were performed on extracts of *hr*R3- and KOS-infected BHK cells essentially as described by Huszar and Bacchetti (25). Before infection, cells were grown for 3 days in medium containing a reduced concentration of serum (0.5%) to decrease mock activity (T. Spector, personal communication). dATP (0.1 mM) was included in reaction mixtures to minimize background from the cellular reductase activity. Protein concentrations were determined with the Bio-Rad protein assay kit. Crude extracts of *hr*R3-, KOS-, and mock-infected BHK cells containing between 1 and 2 mg of protein were used in each assay. Viral enzyme activity was measured by monitoring the conversion of [^{3}H]CDP to [^{3}H]dCDP by ascending chromatography on plastic-backed cellulose as described by Slabaugh and co-workers (44).

Synthesis of viral DNA in infected cells. Analysis of viral DNA synthesis was performed as described by Aron et al. (2) except that proteinase K was used instead of pronase. Levels of [^{3}H]thymidine incorporation into viral DNA were measured on exponentially growing and serum-starved Vero and D14 cells.

RESULTS

Permissive hosts for ribonucleotide reductase mutants. Permissive host cell lines containing the genes for the HSV-1 ribonucleotide reductase were isolated for the propagation of mutants carrying potentially lethal defects (i.e., insertions or deletions). Since it is likely that many mutants of this type would be incapable of synthesizing any active protein under the nonpermissive condition (growth on untransformed cells), an accurate assessment of the role of the viral enzyme in DNA synthesis during lytic infection would be possible. The reductase subunit genes are situated within the 13-kb *Hpa*I F fragment of HSV-1 strain KOS (map coordinates 0.535 to 0.620). Figure 1 shows an expanded version of this region and the relative positions of the coterminal transcripts. To construct the cell lines, we cotransfected Vero cells as described in Materials and Methods with plasmid pKHF and a plasmid containing the gene conferring neomycin resistance (pSV2neo [38]). Transfected cells were grown in medium containing the antibiotic G418, an analog of neomycin. Drug-resistant colonies were picked and screened by Southern blot analysis for the presence of DNA from the *Hpa*I region as described in Materials and Methods. Total cellular DNA was isolated from several cloned cell lines, digested with either *Hin*dIII or *Bam*HI, subjected to electrophoresis, transferred to filters, and probed with labeled *Hin*dIII fragment K (coordinates 0.530 to 0.592). Two of the

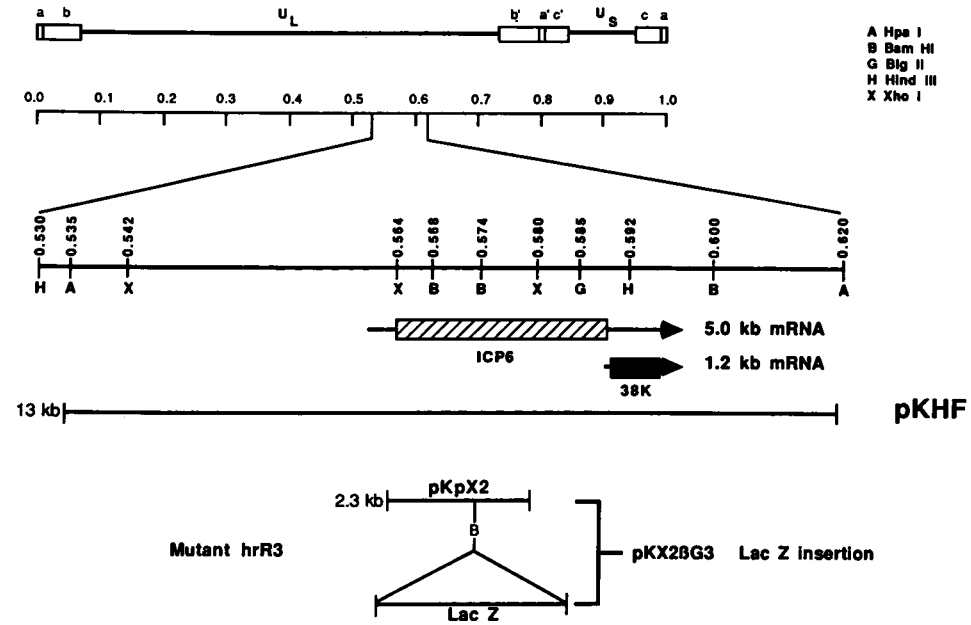

FIG. 1. Plasmids used in this study. This figure shows an expanded version of the region of the HSV-1 strain KOS genome containing the ribonucleotide reductase subunit genes. Shown are relative positions of transcripts and open reading frames for both subunits as mapped by McLauchlan and Clements (34). Plasmid pKHF contains the *Hpa*I F fragment of HSV-1 strain KOS. Subclone pKpX2, containing the 2.3-kb *Xho*I fragment of pKHF, was used to construct pKX2-βG3, which has the structural gene of *lacZ* inserted into the indicated *Bam*HI site.

positive cell lines, designated D14 and D16, were shown to contain 1 to 2 and 5 to 10 copies of the entire *Hpa*I region per haploid genome, respectively (data not shown).

ICP6 expression in D cell lines. D cell lines were screened for the expression of the large subunit of ribonucleotide reductase by indirect IF with a monoclonal antibody specific for ICP6 (48S; kindly provided by M. Zweig). No positive staining was detected in these experiments (data not shown). Since the cell lines were apparently not constitutively expressing ICP6 based on IF, it was suspected that the immediate-early regulatory function(s) ICP4 or ICP0 might be necessary for induction (13, 19, 36, 41). Plasmid pSG1, which contains both immediate-early genes (see Materials and Methods), was used to transfect D14 cells. IF with 48S antibody of cells fixed 36 h posttransfection revealed that 1 to 5% of the cells were induced to express ICP6 (Fig. 2A). Figure 2A also shows that ICP6 is localized to the cytoplasm.

To determine more specifically the nature of the ICP6 induction, we used individual clones containing the ICP4 (pK1-2) or ICP0 (pW3-HSΔ8) gene, or both together, to transfect D14 cells. ICP6 was induced in the cytoplasm of cells transfected with clones containing the ICP0 gene alone or in combination with ICP4 (staining pattern identical to Fig. 2A). However, when cells were transfected with the ICP4 clone alone, no ICP6 synthesis was observed (Fig. 2C). To confirm that ICP4 was actually expressed in cells transfected with pK1-2, we stained a separate cover slip with a monoclonal antibody specific to ICP4 (58S). Transfected cells were induced to express ICP4 in the nucleus (Fig. 2B). Thus, ICP6 expression is induced by ICP0 and not ICP4, which suggests an unusual pattern of regulation (see Discussion).

Isolation of ICP6 *lacZ* insertion mutant. An insertion mutant, *hr*R3, was constructed by the scheme outlined in Fig. 1. The coding region of the *lacZ* gene of *E. coli* was inserted into a *Bam*HI site (coordinate 0.574) in plasmid pKpX2 to construct plasmid pKX2-βG3 such that the structural gene of *lacZ* was read in frame with the N-terminal portion of ICP6. Since β-gal activity can be easily detected by blue color formation in the presence of the indicator dye X-gal, the *lacZ* gene was used to facilitate screening for mutants synthesizing the fusion protein. According to the ICP6 sequence information (kindly provided by J. McLauchlan and B. Clements), introduction of the *lacZ* gene into the *Bam*HI site at coordinate 0.574 within the ICP6 gene would result in the synthesis of a fusion protein containing 38% of the N terminus of ICP6 and the entire β-gal protein under the control of the endogenous ICP6 promoter. Since a stop codon exists at the end of the *lacZ* gene, the remaining portion of ICP6 would not be expected to be synthesized. The 5.3-kb *Xho*I fragment of pKX2-βG3 (Fig. 1) was introduced into infectious wild-type DNA by marker transfer into D14 cells, and recombinant viruses were identified by staining the plaques with X-gal. Blue plaques, appearing at a frequency of about 4%, were purified and propagated in D14 cells. One mutant virus, *hr*R3, was used in further studies.

Restriction analysis of *hr*R3. To confirm that *hr*R3 contains the *lacZ* gene at the appropriate position, DNAs from KOS and *hr*R3 were purified, digested with *Xho*I, and subjected to electrophoresis and Southern blot hybridization in duplicate as described in Materials and Methods (Fig. 3). Plasmids pKpX2 and pKX2-βG3 (containing the wild-type and mutant versions of this region, respectively; Fig. 1) were digested with *Xba*I and *Hin*dIII and subjected to electrophoresis in parallel. (Note: the construction of pKpX2 or pKX2-βG3 results in the loss of the *Xho*I recognition sequence; however, the equivalent fragment could be excised with *Xba*I and *Hin*dIII, which recognize sites within the multicloning region of these plasmids.) Duplicate filters were hybridized to either ^{32}P-labeled HSV-1 *Hin*dIII K fragment or ^{32}P-labeled *lacZ* fragment. The filter shown in the left-hand portion of Fig. 3 (HSV-1 probe) shows that pKpX2 and KOS contain the wild-type 2.3-kb version of this *Xho*I fragment, whereas *hr*R3 and pKX2-βG3 contain a 5.3-kb version expected if the *lacZ* gene was inserted. When the duplicate filter was hybridized with a *lacZ* probe, only the 5.3-kb fragment of pKX2-βG3 and *hr*R3 was detected (right-hand portion of Fig. 3). These results demonstrate that the *lacZ* fragment is inserted into the appropriate site in the genome.

Ribonucleotide reductase activity of *hr*R3. Figure 4 shows a time course of ribonucleotide reductase activity in *hr*R3- and KOS-infected BHK-21 C13 cell extracts. Peak activity from KOS-infected cell extracts was obtained within 8 h postinfection with 1.73 nmol of CDP converted with 1 mg of total protein in 1 h at 37°C. In contrast, *hr*R3 was incapable of inducing activity above levels obtained from mock-infected cell extracts at all times assayed. Activity from mock-infected cell extracts was consistently between 2,500 and 4,000 cpm/1 mg of protein in 1 h at 37°C (data not shown).

Growth properties of *hr*R3. The ability of *hr*R3 to form plaques was initially analyzed in exponentially growing Vero and D14 cells. Surprisingly, *hr*R3 was capable of forming plaques in Vero cells, although plaque size was reduced to about one-third that of the wild type (Table 1). The mutant, however, did form wild-type-size plaques in D14 cells. Table

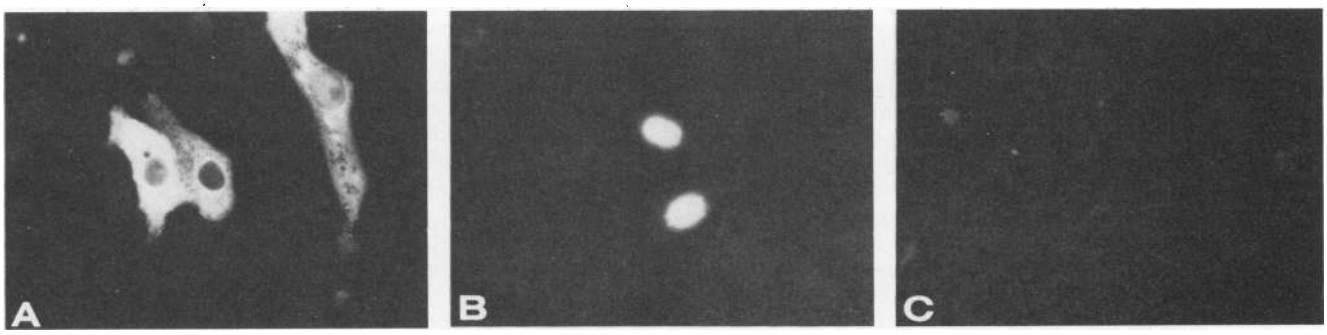

FIG. 2. Indirect IF of transfected D14 cells. (A) Cells transfected with a plasmid containing both ICP4 and ICP0 genes (pSG1) and stained with anti-ICP6 antibody (48S) as described in Materials and Methods. (B) Cells transfected with pK1-2 and stained with anti-ICP4 antibody (58S). (C) Cells transfected with pK1-2 and stained with 48S antibody.

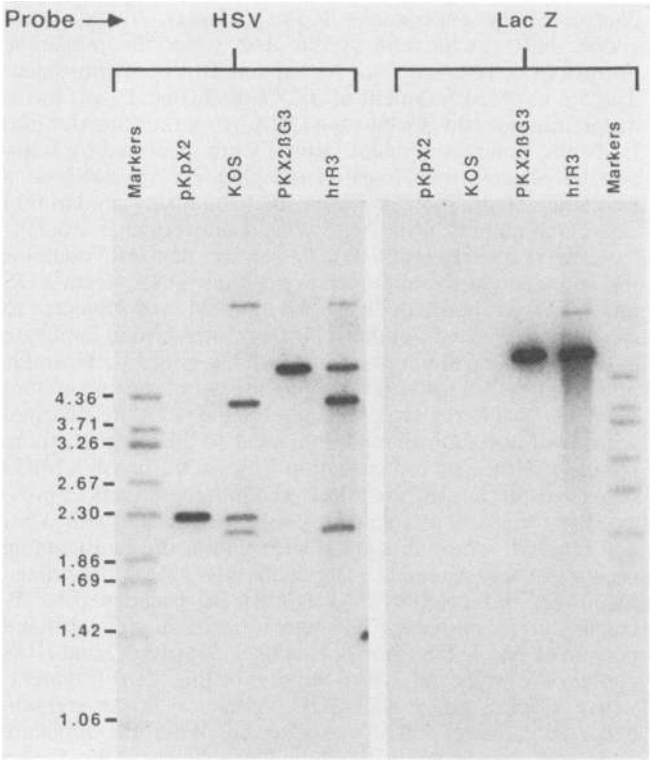

FIG. 3. Southern analysis of KOS and *hr*R3 DNAs. KOS and *hr*R3 DNAs were digested with *Xho*I and electrophoresed in parallel with *Xba*I- and *Hin*dIII-digested pKpX2 and pKX2-βG3 (digestion of these plasmids with *Xba*I and *Hin*dIII removes the equivalent *Xho*I fragments; see Results). Digested DNAs were separated on a 0.8% agarose gel, blotted onto Gene Screen Plus, and hybridized to ^{32}P-labeled *Hin*dIII fragment K (HSV probe; left-hand blot) or *lacZ* fragment (right-hand blot). Locations of molecular size markers (in kilobases) are shown at the left.

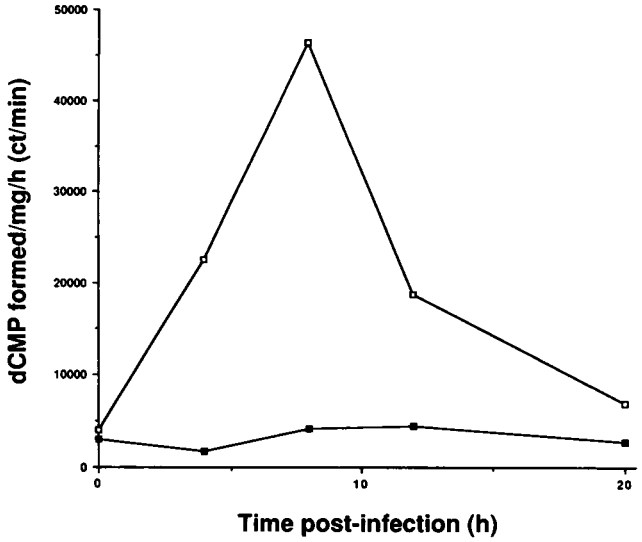

FIG. 4. Time course of ribonucleotide reductase activity in KOS- and *hr*R3-infected BHK cell extracts. Cells were infected at a multiplicity of 5 PFU per cell. Crude extracts containing between 1 and 2 mg of protein were assayed as described in Materials and Methods. 25,000 cpm = 1 nmol of dCMP. Symbols: □, KOS activity; ■, *hr*R3 activity.

1 shows that, although *hr*R3 plaque size is reduced in Vero cells as compared with that in D14 cells, the titer of *hr*R3 is similar in both.

Since it is known that the activity of the cellular ribonucleotide reductase is strongly correlated to ongoing cellular DNA synthesis with little activity occurring during the G1 phase (12, 50), it is likely that rapidly dividing cells in culture contain substantial levels of deoxyribonucleotides. Hence, it is possible that the presence of these precursor pools is able to compensate for the defect in *hr*R3. To determine whether the growth state of the cell has an effect on plaquing ability, we determined *hr*R3 titers in serum-starved Vero and D14 cells (Table 1). Although the titer of *hr*R3 decreased only fourfold in growth-restricted Vero cells as compared with that in exponentially growing Vero cells, plaque size was significantly reduced (to about one-tenth of wild type on Vero or D14 cells). Mutant plaque size in serum-starved D14 cells was substantially larger than that in serum-starved Vero cells, suggesting that the presence of the viral reductase gene in D14 cells can compensate for the growth defect of the mutant. KOS formed plaques efficiently in both growing and serum-starved Vero and D14 cells.

To further test the growth properties of *hr*R3 in growing and serum-starved Vero cells, we measured the efficiency of virus production by single-step growth analysis (Fig. 5). Cells were infected at a multiplicity of 5 PFU per cell and harvested at various times up to 24 h postinfection. Yields of progeny virus were titrated in permissive D14 cells. Final mutant virus yield in exponentially growing Vero cells (Fig. 5, solid triangles) was reduced only four- to fivefold compared with that of the wild type grown under these conditions (Fig. 5, solid squares). Growth curves of *hr*R3 and KOS determined in serum-starved Vero cells indicated that the final yield of mutant virus (Fig. 5, open triangles) was reduced approximately 30- to 35-fold compared with that of the wild type in serum-starved cells (Fig. 5, open squares). Although wild-type virus production in serum-starved cells was delayed relative to wild-type virus production in exponentially growing cells, the same final wild-type virus yields were obtained under both conditions by 24 h postinfection. Similarly, there was a delay in mutant virus production under serum-starved conditions. However, if the infection was allowed to proceed beyond 24 h, the mutant virus yield in serum-starved cells did not reach the levels obtained from mutant-infected, exponentially dividing cells until 44 h postinfection (data not shown), indicating a further delay of virus production compared with that of the wild type in serum-

TABLE 1. Plaquing of KOS and *hr*R3 on serum-starved and exponentially growing Vero and D14 cells[a]

Virus	Cell	Condition	Titer	Plaque size[b]
KOS	Vero	Exponential	2.0×10^9	Large
*hr*R3	Vero	Exponential	1.4×10^8	Small
KOS	Vero	Serum starved	5.0×10^8	Medium
*hr*R3	Vero	Serum starved	4.8×10^7	Tiny
KOS	D14	Exponential	9.5×10^8	Large
*hr*R3	D14	Exponential	2.0×10^8	Large
KOS	D14	Serum starved	7.3×10^8	Large
*hr*R3	D14	Serum starved	1.8×10^8	Medium

[a] Plaquing efficiencies were determined by titrating virus stocks in monolayers of Vero or D14 cells under the condition indicated.

[b] Large is defined as 66 to 100% as compared with KOS in exponentially growing Vero cells; medium = 33 to 66%; small = 10 to 33%; tiny = 0 to 10%.

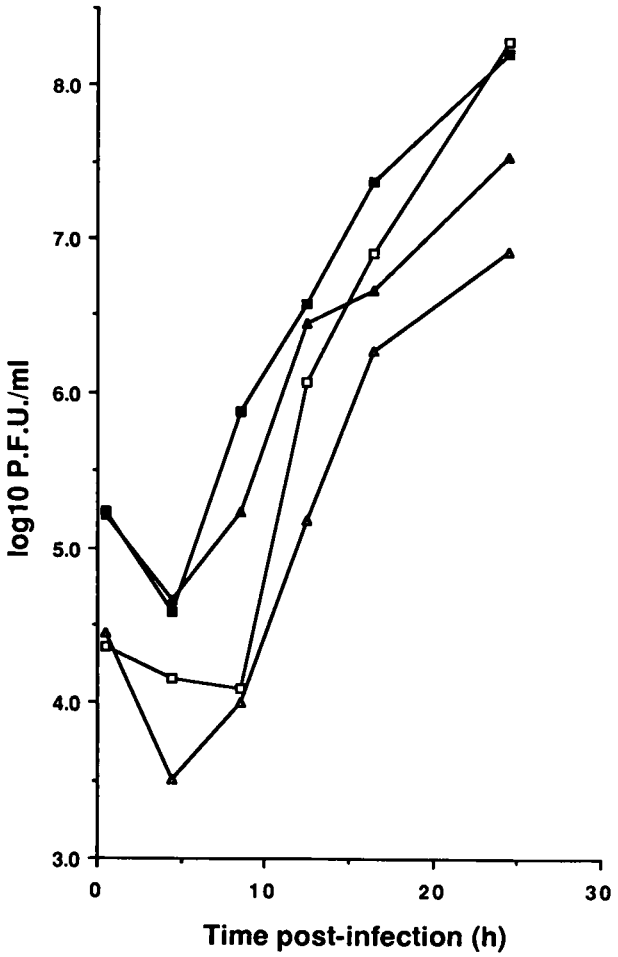

FIG. 5. Single-step growth curves of wild-type KOS and mutant *hr*R3. Monolayers of exponentially growing or resting Vero cells were infected at a multiplicity of 5 PFU per cell with either KOS or *hr*R3. At various time points after infection up to 24 h, virus progeny were harvested and titrated on monolayers of D14 cells. Symbols: ■, KOS yields in growing Vero cells; □, KOS yields in resting Vero cells; ▲, *hr*R3 yields in growing Vero cells; △, *hr*R3 yields in resting Vero cells.

starved cells. The final mutant virus yield at 44 h was 35-fold lower than that of the wild type grown under both conditions (data not shown). Thus, these data suggest that the mutant is more severely compromised in growth-restricted cells than in exponentially dividing cells.

To investigate the possibility that the growth properties of this mutant are altered in different cell types, we determined the ability of *hr*R3 to produce virus in BHK, Vero, and D14 cells after infection at low multiplicity. These cells were infected with *hr*R3 or KOS at a multiplicity of 0.01 PFU per cell. Virus progeny were harvested 44 h postinfection and titrated in D14 cells. *hr*R3 virus production was somewhat compromised in Vero and BHK cells (5-fold and 10-fold reductions, respectively) compared with production in D14 cells (Table 2). On the other hand, KOS virus production was similar in all cells tested.

Ability of *hr*R3 to induce viral polypeptides. To determine the ability of *hr*R3 to express viral proteins, we analyzed extracts of ^{35}S-labeled mutant- and KOS-infected D14 and Vero cells by polyacrylamide gel electrophoresis. The autoradiogram in Fig. 6 shows protein profiles of KOS-infected Vero and D14 cells and demonstrates the normal appearance of ICP6. When Vero cells were infected with *hr*R3, no wild-type form of ICP6 was detected; however, a new band of the molecular weight predicted for the fusion protein (~155,000) was apparent. Both the wild-type form of ICP6 and the putative fusion protein were evident when *hr*R3 was grown on D14 cells. Other viral proteins appeared to be synthesized normally in *hr*R3-infected Vero cells, suggesting that no block to late protein synthesis exists. This is not surprising since the mutant produces infectious virus in Vero cells.

TABLE 2. Efficiency of virus production in Vero, BHK, and D14 cells[a]

Virus	Cell	Titer
KOS	Vero	9.1×10^6
KOS	BHK	8.4×10^6
KOS	D14	1.3×10^7
*hr*R3	Vero	2.0×10^6
*hr*R3	BHK	9.7×10^5
*hr*R3	D14	9.8×10^6

[a] Viruses were grown in the indicated cells at 0.01 PFU per cell, harvested after 44 h, and titrated in D14 cells.

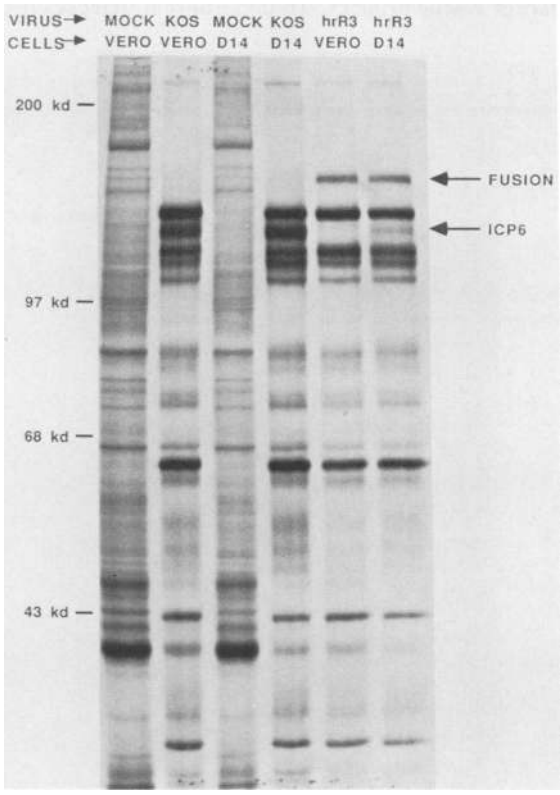

FIG. 6. Polypeptide profiles of *hr*R3- and KOS-infected D14 and Vero cells. Cells were infected at a multiplicity of 10 PFU per cell, labeled with [^{35}S]methionine from 5 to 16 h postinfection, and harvested at 16 h postinfection. Samples were electrophoretically separated on a denaturing 9.0% polyacrylamide gel. Arrows to right of gel indicate positions of fusion protein (~155 kDa) and wild-type-size ICP6 (140 kDa). To the left of the gel are indicated positions of molecular size markers (kd, kilodaltons).

To confirm that the new 155-kilodalton (kDa) band was the fusion protein, we used antibodies to β-gal and ICP6 in immunoprecipitation experiments (Fig. 7). The 155-kDa species could be immunoprecipitated with antibodies directed against β-gal and 48S (Fig. 7). This clearly demonstrated that the 155-kDa polypeptide is the fusion protein and showed that the antigenic determinant for the 48S antibody resides in the N-terminal 434 amino acids of the polypeptide. Furthermore, 48S antibody precipitated both fusion protein and wild-type ICP6 from extracts of *hr*R3-infected D14 cells. This indicated that D14 cells are induced to synthesize wild-type ICP6 upon infection with *hr*R3.

Viral DNA synthesis of *hr*R3. The ability of *hr*R3 to synthesize viral DNA was analyzed by isopycnic density gradient centrifugation of ^{3}H-labeled DNA from infected exponentially growing or serum-starved cells. *hr*R3 was capable of synthesizing substantial amounts of viral DNA in exponentially growing Vero cells (74% as compared with wild-type levels) (Table 3). However, viral DNA synthesis of the mutant was more compromised when it was grown in serum-starved Vero cells (29% as compared with wild-type levels in serum-starved Vero cells). This defect was complemented by serum-starved D14 cells (83% as compared with wild-type levels in serum-starved D14 cells). No difference existed in the relative amounts of DNA synthesis when wild-type virus was grown under either condition (data not shown). Based on these results, it is likely that the cell cycle has an effect on the ability of *hr*R3 to synthesize viral DNA.

Marker rescue of *hr*R3. To confirm that defects observed in the growth properties of *hr*R3 compared with the wild type were due to the insertion mutation within the ICP6 gene, we performed marker rescue experiments using the cloned 2.3-kb *Xho*I fragment from plasmid pKpX2 (coordinates 0.564 to 0.580; Fig. 1). Vero cells were cotransfected with this clone and infectious *hr*R3 DNA. White plaques appeared at a frequency of about 1%, while no white plaques appeared when the wild-type *Xho*I fragment was excluded from marker rescue transfections. Five white plaques were picked and analyzed for their ability to produce wild-type plaque size and virus yields on resting Vero cells. All five recombinants had restored wild-type plaque size and yields in resting Vero cells (data not shown), indicating that the mutation in *hr*R3 is responsible for the defects observed. Moreover, several blue plaques were picked from the original marker transfer experiment, and all were shown to have growth characteristics identical to those of *hr*R3. Thus, the existence of a secondary mutation affecting the growth properties of the mutant is extremely unlikely.

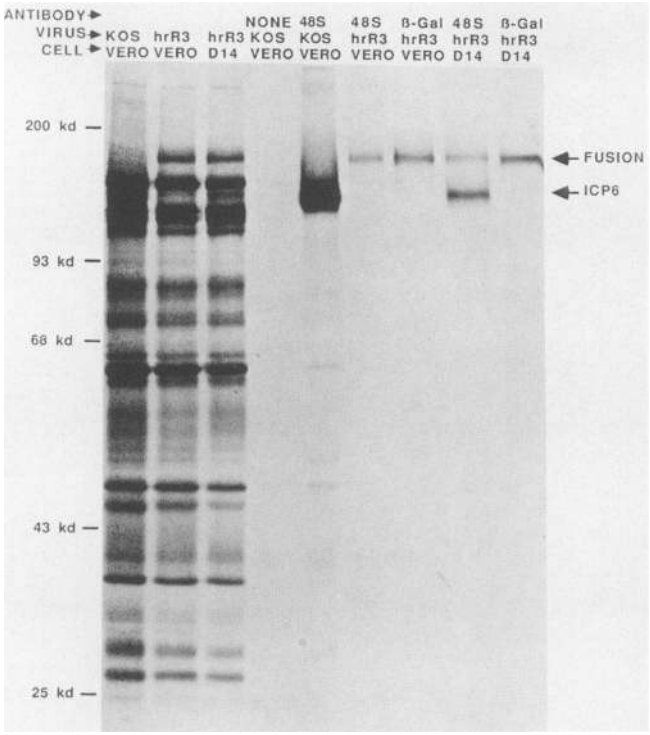

FIG. 7. Autoradiogram of immunoprecipitates from extracts of KOS- and *hr*R3-infected Vero and D14 cells. The first three lanes are total extracts from the indicated virus-infected cells. The remaining six lanes are immunoprecipitation pellets of the indicated virus-infected cell extracts incubated with the indicated primary antibody. Arrows to right of gel show positions of fusion protein and wild-type ICP6. To the left of gel are indicated positions of molecular size markers (kd, kilodaltons).

TABLE 3. Viral DNA synthesis induced by HSV-1 KOS and *hr*R3[a]

Virus	Cell	Condition	DNA synthesis (% HSV-1 wild-type synthesis)
KOS	Vero	Exponential	100
*hr*R3	Vero	Exponential	74
KOS	Vero	Serum starved	100
*hr*R3	Vero	Serum starved	29

[a] Incorporation of [^{3}H]thymidine into viral DNA as performed by Aron et al. (2).

DISCUSSION

To address the question of whether viral ribonucleotide reductase is essential for viral DNA synthesis and to circumvent the difficulties posed in the study of potentially leaky temperature-sensitive mutants, we isolated a host range mutation in the ICP6 gene. Host cell lines (D cells) containing the genes for both subunits of the ribonucleotide reductase were constructed and used for the isolation and propagation of a *lacZ* insertion mutant, *hr*R3.

Regulation of ICP6 expression. The induction of reductase activity has been shown to have kinetics similar to the induction described for other HSV enzymes involved in DNA metabolism, such as thymidine kinase, DNA polymerase, and alkaline nuclease (29). Ribonucleotide reductase has therefore been classified as a β function. The 38-kDa subunit does appear to be expressed as a true β polypeptide (52). ICP6, on the other hand, appears to have an unusual pattern of expression. Substantial levels of the 5.0-kb ICP6 transcript are expressed within 2 h postinfection (22), and small amounts of this mRNA are synthesized in the presence of cycloheximide (53). These experiments suggest that no immediate-early gene products are required for ICP6 expression. Furthermore, the ICP6 message is expressed by an ICP4 mutant at the restrictive temperature (22). Honess and Roizman (23, 24) have shown that ICP6 is expressed very early in a lytic infection. Others have shown that the ICP6 polypeptide is expressed by ICP4 and ICP0 mutants under nonpermissive growth conditions, as was suggested by the mRNA studies (8, 33, 34, 42). Our studies are consistent with the notion that ICP6 has an unusual pattern of expression. Transfection and IF experiments in D14 cells described herein demonstrated that ICP6 is not constitutively ex-

pressed and identified ICP0 and not ICP4 as a positive regulator of ICP6 expression. Taken together, these results suggest that, while ICP0 can induce the expression of ICP6, it is not the only positive regulator.

Characterization of *lacZ* insertion mutant. The *lacZ* gene was inserted into a clone containing coding sequences for ICP6 such that a protein consisting of the N-terminal 38% of ICP6 would be fused in frame to β-gal. This construct was introduced into infectious KOS DNA by marker transfer in D14 cells, and a recombinant virus that formed blue plaques was isolated.

*hr*R3, which is incapable of inducing viral ribonucleotide reductase activity in untransformed cells, was used to assess the ability of the virus to replicate in the absence of the viral enzyme. Studies on the growth properties of this mutant in exponentially growing Vero and BHK cells indicated that viral ribonucleotide reductase activity is not absolutely required for growth and DNA synthesis during lytic infection in culture. These results are consistent with data of M. Challberg (personal communication), who has demonstrated in a recently described transient transfection assay (6) that the viral reductase subunit genes are required only for optimal HSV origin-directed plasmid amplification.

Since the expression of the mammalian ribonucleotide reductase is cell cycle dependent (12, 50), it is possible that the growth of a viral reductase mutant depends on the cell type and stage of the cell cycle. To address the question of the cell cycle, we analyzed growth of the mutant on growth-restricted, serum-starved Vero cells which were likely to have reduced cellular reductase activity. The most striking characteristic of *hr*R3 in serum-starved cells was the extremely small plaque size compared with that of the wild type grown in serum-starved cells. Single-step growth curves indicated a 30- to 40-fold reduction of mutant virus yield in these cells compared with that of the wild type grown in serum-starved cells. Furthermore, there was a further reduction in the ability of the mutant to synthesize viral DNA in serum-starved cells. These results suggest that the viral ribonucleotide reductase activity is not absolutely essential for growth in tissue culture and that the cellular enzyme, when active, can support virus growth.

Role of ICP6. According to sequence information provided by McLauchlan and Clements (personal communication), insertion of the *lacZ* gene into the *Bam*HI site at coordinate 0.574 (Fig. 1) would disrupt the ICP6 polypeptide at amino acid 434. Thus, the portion of ICP6 which contains homology to reductases of other systems (Epstein-Barr virus, *E. coli*, clam, and mouse [47]) is not expected to be expressed by this construct. The portion of the ICP6 molecule which is expected to be expressed contains the 300- to 400-amino-acid domain which has been shown to be unique to HSV-1 and HSV-2 (47). The fact that *hr*R3 contains the N-terminal 434 amino acids of ICP6 raises the interesting possibility that this region represents a domain required either for an additional function or for structural interaction with other replication functions (47). Since we and others (43, 56) have demonstrated that ICP6 is localized to the cytoplasm, we consider it unlikely that the reductase is part of a replication complex in the nucleus. However, to exclude the possibility that the N-terminal domain performs some other function, it will be necessary to construct a mutant which does not synthesize any portion of ICP6.

The results presented for mutant *hr*R3 are in apparent contradiction to studies with the strain 17 mutant, *ts*1207, which suggest that viral reductase is essential for virus replication (40). At the NPT, this mutant fails to induce reductase activity, with a concomitant decrease in virus production in BHK cells (2 orders of magnitude decrease in virus yield at NPT with respect to that at the permissive temperature). Several possibilities exist for the apparent discrepancy. One explanation may be that the altered, temperature-sensitive form of the ribonucleotide reductase in *ts*1207-infected cells inhibits virus growth at the NPT. Another possibility may be that the N terminus of ICP6 provides an essential function for the virus and that the mutation in *ts*1207 disrupts this region at the restrictive temperature. *hr*R3, on the other hand, synthesizes 434 amino acids of the wild-type N terminus of ICP6 whose presence may permit virus growth and DNA synthesis. A third and perhaps more likely possibility is that the extent to which *ts*1207 is compromised at the NPT may reflect the level of cellular reductase present at the time of assay. In other words, it is possible that the confluent BHK cells at 39.5°C contain significantly less cellular reductase activity than Vero cells under the assay conditions used in the present study. As a result, *hr*R3 may appear less compromised than *ts*1207. We are presently testing the ability of *hr*R3 to grow in BHK cells under various growth conditions.

Since HSV-1 and HSV-2 encode a ribonucleotide reductase which is biochemically distinct from the mammalian counterpart, it follows that this enzyme may play a critical role in the regulation of HSV DNA replication and can furthermore be exploited as a target for antiviral drugs. There have been conflicting reports concerning the sensitivity of HSV to various drugs specific for viral and cellular ribonucleotide reductases (31, 35, 51). As mentioned above, these differences may also be explained by various levels of cellular reductase activity present under conditions used in each study. Questions therefore remain as to whether the viral enzyme is a suitable target for antiviral agents and whether the cellular enzyme can substitute for viral reductase. Additional experiments will be necessary to resolve this issue.

Our results suggest that the viral ribonucleotide reductase activity is not essential for growth in tissue culture and that the cellular enzyme, when active, can support virus growth. This finding, in considering the biology of the virus, is of particular interest. It has been found that the HSV thymidine kinase (TK) is not essential for DNA replication in exponentially growing tissue culture cells (9) but that TK^- viruses become more severely compromised in serum-starved cells (27). Several researchers have shown that TK^- viruses have greatly diminished abilities to establish latent infections (16, 17, 48, 49). Since the mammalian reductase is present only in actively dividing cells (50) and appears to be absent from terminally differentiated cells which have stopped synthesizing DNA (11), it would be of particular interest to use *hr*R3 and other reductase mutants to assess the role of viral ribonucleotide reductase in animal models of both acute and latent infections.

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