Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the Escherichia coli guanosine phosphoribosyl transferase (gpt) gene

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We describe the mutagenesis of the IRS1-US5 region of the human cytomegalovirus genome, demonstrating the potential of the E. coli guanosine phosphoribosyl transferase (gpt) gene as a selectable marker for insertion and deletion mutagenesis of high passage (AD169, Towne) as well as low passage (Toledo) strains of virus. Despite evidence suggesting that the US3 gene product may play a regulatory role, disruption of this gene with a gpt insert had no effect on growth of any of these strains of virus in resting or dividing human fibroblasts, or in human thymus plus liver implants in SCID–hu mice. Transcripts of the gpt gene, under control of the herpes simplex virus thymidine kinase promoter adjacent to the US3 enhancer in the viral genome, accumulated with delayed early (β) kinetics. Mutants with deletions in the IRS1 and US3-US5 regions were isolated by back-selection against gpt with the drug 6-thioguanine by growing virus in human Lesch–Nyhan (hypoxanthine–guanine phosphoribosyl transferase deficient) skin fibroblasts immortalized with human papillomavirus oncogenes. Thus, we demonstrate a dependable method for insertion and deletion mutagenesis that can be applied to any region of the viral genome.

Introduction

Human cytomegalovirus (CMV), an important pathogen, carries over 200 genes whose functions are largely undefined (Mocarski, 1993). Although engineered mutations have provided important information on gene function in other viruses such as herpes simplex virus type 1 (HSV-1; Roizman & Sears, 1993), human CMV has not yet been subjected to such systematic analysis. Comparisons of different strains (Takekoshi et al., 1987), adventitious deletions (Kollert-Jons et al., 1991), and deletion mutagenesis using indicator gene insertions (Spaete & Mocarski, 1987; Ripalti & Mocarski, 1991; Takekoshi et al., 1991; Jones et al., 1991; Jones & Muzithras, 1992; Browne et al., 1992; Kaye et al., 1992 (H. Browne & T. Minson, personal communication; T. R. Jones, personal communication; G. Duke, M. Prichard & E. S. Mocarski, unpublished) have shown that 29 open reading frames (ORFs; UL1–UL10, UL16, UL18, UL20, UL33, UL144, UL128, US1–US13, US27 and US28), and one copy of repeated ORFs (TRL/IRL4–14, IRS1), are dispensable for growth in human fibroblast cells. Most mutagenesis of human CMV has employed indicator genes encoding β-galactosidase (Spaete & Mocarski, 1987; Takekoshi et al., 1991; Browne et al., 1992; Kaye et al., 1992) and β-glucuronidase (Jones et al., 1991; Jones & Muzithras, 1992) in conjunction with laborious co-transfection methods.

Marker genes that confer a growth advantage under selective conditions allow for greater ease in construction of viral mutants. The use of the thymidine kinase (tk) gene as marker for insertion and deletion mutagenesis of HSV-1 (Post & Roizman, 1981) has led to the definition of all essential and dispensable genes in this virus (Roizman & Sears, 1993). The tk gene has not been employed with human CMV, primarily because of the unavailability of tk-deficient CMV-permissive human cells. Any marker gene used for mutagenesis of human CMV must be both dominant and applicable to primary human fibroblast cells. While the neo gene has been used successfully to isolate a CMV insertion mutant (Wolff et al., 1993), the gpt gene of E. coli (Mulligan & Berg, 1981) has been employed in vaccinia virus (Falkner & Moss,
lines, GM02291 or GM02290A cells were cultured overnight with maintenance in medium with 10% FCS. To immortalize primary cell Galloway, Fred Hutchinson Cancer Research Center, and were maintained in the same medium except for 0.66 mM-arginine, 1.48 mM-glutamine and 0–24 mM-asparagine. respectively, from a Lesch-Nyhan patient (Tischfield, 1979) were used that 15% fetal calf serum (FCS, Gibco BRL) was used instead of NuSerum. The PA317 and LXSN16E6E7 amphotropic retroviral construct, which contains the gpt gene in HSV-1 (Post & Roizman, 1991).

We used this mutagenesis approach to evaluate the function of two immediate early (α) genes, US3 and IRS1, previously shown to be dispensable for productive infection of actively growing human fibroblast cells (Kollert-Jons et al., 1991; Jones & Muzithras, 1992). Expression of US3 is under control of an upstream transcriptional enhancer (Weston, 1988), and although the product of the US3 ORF has sequence characteristics of a transmembrane glycoprotein (Weston & Barrell, 1986; Chee et al., 1990), it has been shown to trans-activate certain promoters in epithelioid (HeLa) cells (Colberg-Poley et al., 1992). IRS1 and TRS1 ORFs, which are encoded by the two copies of the repeats flanking the S component, are identical over a region of 490 amino-terminal amino acids (aa) although they differ in their carboxy-terminal sequence (Weston & Barrell, 1986; Chee et al., 1990). Both are expressed immediately after infection and can cooperate with the principle regulatory functions (IE1491aa and IE2<q>79aa</q>) to trans-activate the UL44 early promoter (Stasiak & Mocarski, 1992). Either IRS1 or TRS1 is required for viral origin-dependent DNA replication (Pari et al., 1993). In this paper, we describe the use of the gpt gene to produce insertion and deletion mutants of human CMV. Our studies extend the biological settings in which suspected regulatory genes in the S component of the viral genome are dispensable for viral growth and demonstrate the potential of gpt as a marker gene for systematic mutagenesis of the human CMV genome.

**Methods**

**Cell and virus culture.** Human foreskin fibroblasts (HFFs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) supplemented with 10% NuSerum 1 (Collaborative Research Inc.) 100 units penicillin/ml, 100 mg streptomycin sulphate/ml, 0.66 mm-arginine, 1.48 mm-glutamine and 0.24 mm-asparagine. GM02291 and GM02290A primary lung and skin fibroblast cells, respectively, from a Lesch–Nyhan patient (Tischfield, 1979) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ, USA, and were maintained in the same medium except that 15% fetal calf serum (FCS, Gibco BRL) was used instead of NuSerum. The PA317 and LXSN16E6E7 amphotropic retroviral packaging lines (Halbert et al., 1991) were the kind gift of Denise Galloway, Fred Hutchinson Cancer Research Center, and were maintained in medium with 10% FCS. To immortalize primary cell lines, GM02291 or GM02290A cells were cultured overnight with undiluted filtered (0.45 mm filter) supernatant medium from the LXSN16E6E7 cell line. The infected cells were plated at low density in medium with 15% FCS and 400 μg Genticin/ml (Gibco BRL). Genticin-resistant cells were expanded to produce the polyclonal cell lines LNL1 (GM02291 lung fibroblast derived) and LNL7 (GM02290A skin fibroblast derived), which have been propagated 30 passages beyond the point when the parental primary cells senesced. Supernatant medium from these two cell lines was determined to be free of amphotropic retrovirus by functional assay (ability to transfer Genticin resistance). The genetically immunodeficient CB-17 (scid/scid) mouse strain was used at 6 to 8 weeks post-implantation with human fetal thymus plus liver (thy/liv) as previously described (McCune et al., 1988). These SCID–hu (thy/liv) mice, kindly provided by Systemix Inc., were inoculated with virus in a 10–50 μl volume directly into the exposed implant and infection was allowed to proceed for 15 days until virus was harvested as previously described (Mocarski et al., 1993). After harvest, infected implants were sonicated in 2 ml of medium which was subjected to plaque assay on HFF cells.

Human CMV strains were obtained and cultured as previously described (Mocarski et al., 1993; Spaete & Mocarski, 1985). Human CMV Toledo strain was further plaque-purified once (passage level 12), prior to use in these experiments. Viral stocks were made from sonicated infected cells and sedimented virions, and stored at –80 °C in a:1 mixture of medium and autoclaved 9% reconstituted non-fat milk. Where specified, culture medium containing supernatant virus was passed through a 0.45 μm filter to avoid transfer of infected cells. Virus adsorption was carried out for 1 h in serum-free medium. Viral titres were determined by plaque assay on HFF cells, using medium supplemented with human γ-globulin (165 μg/ml) after adsorption. Plaque-purification was carried out under 0.5% agarose (Gibco BRL) using an m.o.i. of 0.00001 p.f.u./cell. For viral growth curves, approximately 5 × 10⁶ HFF cells were seeded into each well of six-well culture plates (Falcon). After 1 day, the cells were infected at an m.o.i of 1 p.f.u./cell infecting a majority of cells or an m.o.i of 0.01 p.f.u./cell (multiple-step growth curves). The input inocula were titrated by plaque assay at the time of use, and this was used as the zero time point. On the specified days, cells and 1/6th of the medium were harvested, mixed with an equal volume of 9% autoclaved, reconstituted non-fat dry milk, and frozen at –80 °C. At the end of the experiment, all samples were thawed, sonicated and subjected to plaque assay on HFF cells.

**Viral nucleic acid isolation and analysis.** Virus was harvested from infected fibroblasts (initial m.o.i was 0.001 p.f.u./cell) and culture medium 5 days after cultures reaches 100 % cytopathic effect (CPE). Virus adsorption was carried out for 1 h in serum-free medium. Virus was harvested from viral particles collected by centrifugation (60 min, 12000 g) from the supernatant medium combined with this cytoplasmonic fraction. Treatment with 1% SDS and 200 μg/ml proteinase K for 1 h at 60 °C was followed either by purification on sodium iodide equilibrium density gradients (Walboomers & ter Schegget, 1976), or by phenol and chloroform extractions followed by isopropanol precipitation.

Viral DNA samples for blot hybridization were digested to completion with restriction enzymes, separated by agarose gel electrophoresis, visualized by ethidium bromide staining, denatured and transferred to nylon membranes (and UV-cross-linked). After 1 h of pre-hybridization without probe, blots were hybridized with 32P-radiolabelled DNA probes overnight at 65°C in 6× SSC, 5× Denhardt’s solution, 20% formamide, 0.1% SDS and 200 μg/ml denatured salmon sperm DNA (Spaete & Mocarski, 1985; Masse et al., 1992). Filters were washed at 65°C in 0.2× SSC, 0.1% SDS. Hybridization with oligonucleotide ROG2024 (’5’ GTTACCAAGCAGA 3’) was carried out at 45 °C, without formamide, and filters were washed at 45°C in 2× SSC, 0.1% SDS (Maniatis et al., 1982). Viral DNA released from cells into the medium was dot-blotted by
denaturation of culture supernatants in 200 mM-NaCl and 600 mM-NaOH, and vacuum transfer to nylon membranes prior to hybridization. Results were visualized by autoradiography on Kodak XAR film.

Total cellular RNA was purified from infected cells by lysis in guanidinium isothiocyanate and sedimentation through a CsCl cushion (Chirgwin et al., 1979). When used, cycloheximide (Sigma; 50 μg/ml) or sodium phosphonofluoridate (Sigma; 200 μg/ml) were added to the culture medium commencing 1 h prior to infection. For RNA blot analysis, 5 μg RNA samples were separated by electrophoresis on denaturing formaldehyde-1% agarose gels, visualized by ethidium bromide, transferred to nylon membranes and UV-cross-linked. Hybridizations of filters to probes were carried out overnight at 55 °C in 6 x SSC, 5 x Denhardt's solution, 15% formamide, 0.1% SDS and 200 μg/ml denatured salmon sperm DNA. Filters were washed at 55 °C in 0.2 x SSC, 0.1% SDS (Maniatis et al., 1982). Results were visualized by autoradiography on Kodak XAR film.

Plasmids, oligonucleotides and probes. Plasmid pON1101 was a mammalian cell expression cassette carrying the E. coli gpt coding sequences under control of the HSV tk gene promoter and the simian virus 40 (SV40) early region polyadenylation signal. An intermediate plasmid, tk-gpt, was constructed by inserting a BgIII-BamHI fragment containing the gpt ORF (a 671 bp BgIII-DraI fragment) and the SV40 polyadenylation signal (a 133 bp HpaI-BamHI fragment representing nucleotides 2666-2533 on the SV40 genome) into MluI-cut pRB103 (Post et al., 1980) after all 5' overhangs were filled in using the Klenow fragment of DNA polymerase I. To construct pON1101, the PstI-BamHI fragment from plasmid tk-gpt containing a 208 bp PstI-MluI fragment from the HSV tk promoter (−221 to −13 relative to the transcription start site) was inserted into PstI/BamHI-cut vector pUC21 (Vieira & Messing, 1991).

Plasmid pON2442 contains a 4.75 kb SacI fragment of human CMV AD169, representing nucleotides 19251-197394 of the published sequence (Chee et al., 1990), cloned into the SacI site of plasmid pGEM-3ZF+. A PvuII-XbaI fragment containing the tk-gpt-SV40 poly(A) cassette from pON1101 was cloned into SacI/XbaI-cut pON2442 to give pON2514. To make pON2515, a PvuII-XbaI fragment from pON2442 was cloned into EcoRI/XbaI-cut pON2514 after the EcoRI site was filled in using Klenow polymerase. pON2515 contains a SacI fragment from pON2442 with the tk-gpt-poly(A) cassette replacing the US3 promoter and coding sequences (nucleotides 194113-194832) as depicted in Fig. 1.

Plasmid pON2520 was constructed by ligation a PvuII-XbaI fragment of pON2442 containing US1-US2 sequences to an XbaI-NruI fragment of pON2442 containing vector and US6/US7 genes. pON2530 contained the same human CMV SacI fragment as pON2442, but with a 1.2 kb deletion (nucleotides 194113-195324) which removed ORFs US3-US5.

Plasmid pON2520 was constructed by cloning a 7-kbp BamHI-KpnI fragment from pCM1052 (Fleckenstein et al., 1982), containing IRL1, IRL2, J1, IRS1, US1 and US2 of strain AD169, into BamHI-KpnI-cut pGEM-3ZF+. pON2527 was derived from pON2520, by cutting with NotI and MmuI and filling in 5' overhanging ends before ligation. The 1.7-kbp NotI-MmuI deletion removes 68% of the IRS1 ORF. Plasmid pON2528 was generated by ligation an EcoRI-Nhel fragment from pON2442 into Nhel/EcoRI-cut pON2527. pON2528 thus contains a BamHI-SacI fragment of strain AD169 (nucleotides 187115-197394), including ORFs IRL2-US7, but has a 1.7-kbp deletion (nucleotides 188911-191532) in the IRS1 gene.

All gpt-positive plasmids (pON1101, pON2514 and pON2515) were propagated in either of two gpt-deficient E. coli strains, WB1 and WB2, which we derived for this purpose. All other plasmids were propagated in E. coli strain DH5α. WB1 was derived from strain NK6051 [96186, from the E. coli Genetic Stock Center at Yale University; genotype Δ(gpt-lac5 purE79::Tn10(ter) relA spoT1 thi-1 Hfr], first by plating under selection for the loss of tetracycline resistance (Maloy & Nunn, 1981) and then by the transduction of transposon Tn10 from phage P1 grown on E. coli JV30 (also called Mvl184; genotype araΔ(lac-proAB) thiA (868lacZAM15) Δ(ter-recA)306 -· Tn10(ter) [F' traD36 proAB lacZAM15]) using selection for resistance to tetracycline. The resulting WB1 genotype was: Δ(gpt-lac5 purE relA spoT1 thi-1 Δ(ter-recA)306 -· Tn10(ter) Hfr. WB2 was derived from WB1 and carries both ampicillin and tetracycline resistance genes. The gpt mutation in these strains blocks the normal purine salvage pathway and purE destroys the first enzyme in the de novo synthesis of purines. Together, these mutations allow selection of plasmids with the gpt gene by growth in M9 minimal medium (Miller, 1972) supplemented with 0.2% glucose, 100 μg/ml xanthine (Sigma), 0.001% thiamin (Sigma) and 0.5% vitamin-free Casamino acids (Difco). Plasmid DNA was purified by the alkaline lysis method followed by equilibrium sedimentation in CsCl density gradients (Maniatis et al., 1982).

Oligonucleotide ROG024 (5' GGTACCAGCGACAGAA 3') was synthesized on an Applied Biosystems 394 synthesizer. Oligonucleotides were end-labelled by the transfer of phosphate from [γ-32P]ATP, using T4 polynucleotide kinase (Maniatis et al., 1982). Double-stranded DNA probes were isolated from low melting point agarose by phenol extraction, and radiolabelled by primer extension from annealed random hexamer oligonucleotides (Feinberg & Vogelstein, 1984), using [α-32P]dCTP and the Klenow fragment of E. coli DNA polymerase I.

**Purification of viral recombinants.** To generate recombinant viruses, 8 μg of plasmid DNA was transfected by the calcium phosphate method (Chen & Okayama, 1987) into a 60 mm tissue culture plate containing approximately 104 HFF cells, plated the previous day. One day after transfection, the cells were infected with 3 p.f.u./cell of human CMV and progeny virus was harvested 5 days after infection. To enrich for gpt-containing viruses, stocks were used to infect fresh HFFs at approximately 0.1 p.f.u./cell. After adsorption for 1 h, medium was replaced with medium supplemented with 10 μg/ml mycophenolic acid (Gibco BRL) and 250 μg/ml xanthine (Sigma). Virus was harvested 5 days after cultures exhibited 100% CPE. After three such rounds of enrichment, the cytoplasmic fraction of infected cells was used to isolate viral DNA for blot hybridization and medium was used as a source of virus for further plaque-purification under agarose overlay in the presence of 10 μg/ml mycophenolic acid and 250 μg/ml xanthine. Recombinant viruses that appeared to be free of parental virus DNA by blot hybridization analysis after these steps were plaque-purified one additional time without selection.

To select against the gpt gene, GM02291 cells were infected at an approximate multiplicity of 0.3 p.f.u./cell, in medium containing 20 μg/ml 6-thioguanine (Sigma). Under these conditions, most of the infected cells rapidly died due to expression of gpt, but cultures were maintained for 2 weeks. The surviving cells were then cocultivated with fresh HFF cells in medium lacking drug. Five days after cultures reached 100% CPE cytoplasmic DNA was subjected to blot hybridization and supernatant virus used as a source of virus for further plaque-purification under agarose overlay. Recombinant viruses that failed to hybridize with a gpt probe by blot hybridization were plaque-purified one additional time prior to preparation of working stocks.

**Results**

**Generation of US3 gpt insertion mutants.** To investigate the potential function of the US3 gene product in the replication of high and low passage virus
strains with different growth properties (Brown et al., 1995), we prepared three parallel human CMV recombinants with an insertion mutation disrupting this ORF. Plasmid pON2515 (Fig. 1), which carried a gpt replacement of the US3 gene from the genome of CMV strain AD169, was linearized with restriction enzymes that cut in sequences flanking the insert (either SacI, for construction of strain Towne or Toledo recombinants, or HindIII and XhoI, for construction of the strain AD169 recombinant) and used for construction of recombinant viruses as described in Methods. Recombinant viruses isolated in the three different strain backgrounds were denoted RC2515AD (AD169 parent), RC2515T (Towne parent) and RC2515Tol (Toledo parent).
DNA blot hybridization with probes specific for gpt and US6/US7 sequences was used to show that the gpt gene was incorporated at the desired location in the viral genome in each of the recombinants. By DNA blot hybridization, a 2-7 kbp EcoRI fragment containing a small portion of the gpt insert as well as the US4–US7 sequences was identified in all three recombinants (Fig. 1). Further analysis of additional digests of viral DNA using gpt and US2 probes (data not shown) suggested that the insertion was precise, placing the gpt gene into the viral genome as depicted in Fig. 1. Adventitious deletions or rearrangements were not detected when HindIII or XbaI digests of recombinant virus DNAs were compared to parental wild-type DNAs following separation of fragments by agarose gel electrophoresis and visualization with ethidium bromide staining (data not shown).

Yields of RC2515AD were approximately 1000-fold higher than parental AD169 when grown in the presence of mycophenolic acid and xanthine (Fig. 2). The presence of gpt does not interfere with virus replication. Wild-type and gpt-positive, US3-deficient viruses grow with similar kinetics in the absence of selection, consistent with previous observations (Kollert-Jons et al., 1991; Jones & Muzithras, 1992).

**Kinetics of gpt expression from a US3 enhancer/tk promoter fusion**

During the construction of RC2515, the HSV tk promoter had been fused downstream of the a region containing 18 bp repeats, previously characterized as the US3 enhancer (Weston, 1988). This placed the gpt ORF into the same context as the US3 ORF within the viral genome. A diagram of the structure of the US2–US6 region from parental AD169 and the predicted structure of RC2515AD is presented in Fig. 3(a). It was predicted that the position of the US3 enhancer would potentially regulate gpt expression and result in expression of the gene product as an α gene.

To assess the temporal pattern of gpt expression by recombinant virus, RNA was prepared from HFF cells infected with at 2, 4, 8, 24 and 72 h post-infection (p.i.) with 3 p.f.u./cell of RC2515T or parental Towne. An RNA blot analysis of resultant whole cell RNAs probed for gpt or US3 transcripts is shown in Fig. 3(b, c) respectively. The US3 transcript was detected by 4 h p.i. and was enriched in RNA from cells treated for 8 h with cycloheximide; however, the chimeric gpt transcript was not detected until 24 or 48 h p.i. Expression was sensitive to treatment with cycloheximide, but resistant to treatment with phosphonoformate suggesting that gpt was regulated with β gene characteristics. The estimated size (0.95 kb) of the gpt transcript was consistent with expression from the tk promoter. Our data suggest that elements in addition to those immediately upstream of the US3 ORF may act to confer α gene characteristics on the natural gene.

**Growth of US3 mutants in confluent fibroblasts and in thymus plus liver implants in SCID–hu mice**

US3 has been shown to trans-activate cellular genes that may play a role supporting viral growth in quiescent cells (Colberg-Poley et al., 1992). We compared the growth characteristics of the parental Towne strain with the US3 gpt insertion mutant, RC2515T, in confluent and actively dividing HFF cells. In preparation for this experiment, HFF cells were held for 20 days without replacing the growth medium, virus (m.o.i of 0.01 pf.u./cell) was adsorbed in serum-free medium, and the original spent growth medium was replaced after infection to avoid stimulation of cells by the addition of fresh serum. There were no differences in the growth potential of Towne or RC2515T in confluent or subconfluent fibroblasts (data not shown).

Although fibroblast cells are used to assay replication of human CMV, epithelial cells are an important target in the infected host (Alford & Britt, 1993). US3 has been implicated as a possible determinant of human CMV...
growth in epithelial cells by its ability to trans-activate gene expression in HeLa cells, which are epithelial in origin (Colberg-Poley et al., 1992). In order to test the ability of US3 mutants to grow in epithelial cells, we employed the recently described SCID-hu (thy/liv) model for human CMV infection, in which viral replication occurs in thymic medullary epithelial cells (Mocarski et al., 1993). Because different CMV strains exhibit marked differences in the ability to replicate in this model (Brown et al., 1995), and the AD169 parent used here fails to grow at all, RC2525T, Towne, RC2515Tol and Toledo were compared.

In these experiments, SCID–hu (thy/liv) implants were inoculated with Towne or RC2515T at $10^6$ p.f.u./implant, or with Toledo or RC2515Tol at $10^5$ p.f.u./implant, and the levels of virus detected by plaque assay of sonicated implants at 15 days post-inoculation, which is the peak time of viral replication in this system (Mocarski et al., 1993). There was no marked difference between either of the US3 mutants compared to their parental viruses (data not shown), although as previously reported (Brown et al., 1995), Towne and its derivative replicated more poorly than Toledo and its derivative in this system. These experiments do not support a role for the US3 gene in epithelial cells.

Selection against the gpt gene in hprt-deficient cells

Dominant selection for gpt in the presence of the normal mammalian hprt enzyme depends upon use of xanthine as a substrate; however, under normal conditions, gpt can use either xanthine or guanine. We
investigated the use of toxic guanine analogues in cells that lack the mammalian hprt enzyme as a means of selecting against gpt-positive viruses. Primary human lung and skin fibroblasts lacking hprt, isolated by biopsy of patients with X-linked Lesch–Nyhan syndrome (Tischfield, 1979), were obtained from the ATCC. These cells were resistant to the toxic compounds 6-thioguanine and 8-azaguanine due to their enzyme defect. Our first plaque assay experiments, using 6-thioguanine selection in primary Lesch–Nyhan fibroblasts, suggested that this strategy might be successful (data not shown); however, the limited lifespan of the biopsy-derived fibroblasts proved to be a problem in this investigation. To obtain a more plentiful supply of cells, primary fibroblasts were exposed to an amphotropic retroviral vector, LXSN16E6E7, that was known to express the human papillomavirus 16 (HPV-16) E6 and E7 oncogene products (Halpert et al., 1991). This strategy was first used on normal human fibroblasts, which continued to be permissive for CMV replication (Compton, 1993). The resultant polyclonal cell lines (ILN1 and ILN7) expressed E6 transcripts (data not shown), exhibited an extended life span in culture, and were fully permissive for human CMV.

A single-step viral growth curve using ILN7 immortalized skin fibroblasts is depicted in Fig. 4.

![Graph showing viral yield over time](image)

**Fig. 4.** One-step growth curves of viruses AD169 and RC2515AD in ILN7 cells, demonstrating selection with 6-thioguanine. AD169 (■) and RC2515AD (○) grown in medium supplemented with 6-thioguanine at 2 μg/ml, and AD169 (■) and RC2515AD (○) grown in medium without drug were compared. Cells (5 × 10⁵) were exposed to an input m.o.i. of 10 p.f.u./cell and time 0 titres represent input inocula. Detection limit (dotted line) was 10 p.f.u./ml.

Fig. 5. Recombinant CMV constructed by back-selection. (a) CMV genome (top line) with the J11 through US8 region expanded to depict the replacements and deletions introduced into recombinant virus RC2515AD to yield viruses RQ2515AD, RC2528AD and RC2530AD. ORFs J11–US8 are represented by open arrows, the tk–gpt–poly(A) cassette is depicted by the shaded arrow, and deletions present in RC2528AD and RC2530AD are depicted by 'Δ'. The predicted sizes of EcoRI S component terminal fragments are given above this region for each virus. (b) Autoradiogram of a DNA blot of EcoRI digests of viral genomic DNA from AD169, RC2515AD, RQ2515AD, RC2528AD and RC2530AD (lanes 1–5). The blot was probed with a 357 bp XhoI–NheI fragment from US2 shown as the filled box in (a). The positions of molecular size markers (kb) are indicated to the left of the autoradiogram.
RC2515AD failed to replicate in the presence of 6-thioguanine at 2 μg/ml, whereas parental AD169 replication was unaffected by the addition of this drug. Both viruses replicated normally in the absence of drug. At 7 days p.i. there was a 1000-fold reduction in yields of RC2515AD compared to AD169 in the presence of drug or to RC1515 in the absence of drug. During treatment, microscopic examination revealed that RC2515AD-infected cells appeared to die under 6-thioguanine treatment without the development of characteristic CPE of CMV infection that was observed in all other cultures (data not shown).

**Generation of RC2515 derivatives lacking gpt**

Derivatives of RC2515 lacking gpt were constructed following transfection of cells with BamHI-linearized pON2528 or SacI-linearized pON2530 and infection with RC2515. Virus from these stocks was subjected to selection using 6-thioguanine at 2 μg/ml in immortalized Lesch–Nyhan fibroblasts and the desired recombinant viruses were isolated using a final dot-blot hybridization step as described in Methods. The structures of three derivatives of RC2515AD made by this approach are shown in Fig. 5(a). Virus RQ2515AD repaired the gpt insertion in RC2515AD and rescued the US3 gene. RC2528AD carried a deletion of 1-7 kbp in the IRS1 gene, introduced into the viral genome by linking the deletion to US3 on pON2528. Virus RC2530AD carried a deletion encompassing ORFs US3–US5, including the US3 enhancer. Fig. 5(b) shows a DNA blot confirming the structures of these viruses. EcoRl digestion of DNA from each virus results in S terminal and L–S junction fragments that contained US2 sequences detected by a specific probe. These fragments exhibited the characteristic ladder pattern of a sequence heterogeneity (Spaete & Mocarski, 1985). In the case of wild-type and RQ2515AD viruses, 8.6 kbp S terminal and 11.9 kbp L–S junction fragments were detected; for RC2515AD, 6.1 kbp S terminal and 9.5 kbp L–S junction fragments were detected; for RC2515AD, 6.9 kbp S terminal and 10.3 kbp L–S junction fragments were detected; and for RC2530AD, 7.4 kbp S terminal and 10.8 kbp L–S junction fragments were detected. A 16-mer oligonucleotide ROG024 (5′ GGTAACCAGCGCAGAA 3′), spanning the NrdI/PvuII deletion in pON2530, was further used to identify RC2530AD (data not shown).

Additional analyses using US6/US7 and gpt probes confirmed these results. Ethidium bromide-stained agarose gels of HindIII- and Xhol-digested recombinant viral DNA indicated that no unexpected, adventitious genomic alterations had occurred. Consistent with previous work in which either ORFs IRS1 and US1–US9 (Jones & Muzithras, 1992) or ORFs US1–US13 (Kollert-

![Fig. 6. Multiple-step growth curves of viruses AD169 (○), RC2515AD (△), RQ2515AD (○), RC2528AD (▼) and RC2530AD (□) in HFF cells. Cells (5 x 10⁵) were exposed to an input m.o.i. of 0.01 p.f.u./cell and time 0 titres were determined on input inocula. Detection limit (dotted line) was 10 p.f.u./ml.

Jons *et al.,* 1991) were deleted, these recombinant viruses exhibited normal growth potential in HFF cells (Fig. 6).

**Discussion**

The method we used, plasmid DNA transfection followed by viral infection, was similar to that recently applied to murine CMV by Vieira *et al.* (1994). The bacterial gpt gene may be inserted into the human CMV genome under selective conditions that are similar to those for selection of cells (Mulligan & Berg, 1981) or other viruses (Falkner & Moss, 1990; Vieira *et al.,* 1994; Marshall *et al.,* 1993). This allows the generation of recombinants by a transfection/infection protocol and does not require preparation of transfected viral DNA. The gpt gene was incorporated into the US3 locus of human CMV without any apparent rearrangements of the viral genome and the resultant gpt-containing recombinant viruses were stable. The gpt gene had no effect on viral growth in either HFF cells or in SCID-hu (thy/liv) implants.

Recombinant viruses were isolated using conditions (250 μg xanthine and 100 μg mycophenolic acid per ml of medium) that we had adopted from the literature (Mulligan & Berg, 1981; Vieira *et al.,* 1994). We evaluated different selection conditions for growth of gpt-positive human CMV, and, as depicted in Fig. 2, a xanthine concentration of 100 μg/ml and a mycophenolic acid concentration of 10 μg/ml in the medium
provided the most consistent selection conditions. Wild-type virus was found to 'break through' when higher concentrations of xanthine were employed (data not shown). Conditions have not yet been found that completely block wild-type growth without also reducing the growth of gpt-containing viruses, and therefore we used conditions that produced at most a 1000-fold growth advantage. Enrichment of gpt-positive viruses was best carried out under low m.o.i. to prevent coinfection by both gpt-positive and parental virus, and the resultant complementation of parental virus. In addition, the optimal selection conditions allow the survival of uninfected HFF cells and plaque formation was observed in cultures undergoing treatment.

An alternative method can be employed to select gpt-positive virus. By propagating the hprt-deficient ILN7 cells in hypoxanthine–aminopterin–thymidine (HAT) medium to prevent purine salvage, virus growth can be made dependent upon gpt. Despite some promise, we found that HAT selection rapidly killed uninfected ILN7 cells so that there was poor amplification of gpt-positive virus in treated cultures (data not shown).

The use of the toxic guanine analogue 6-thioguanine allowed selection against gpt in hprt-deficient cells. Although gpt-positive viruses were genetically stable when propagated in normal medium, both RC2515AD and RC2515T yielded 6-thioguanine-resistant progeny when grown under selective conditions. These spontaneous gpt-deficient revertants had genome structures consistent with the accumulation of deletions or point mutations in the gpt gene. The presence of such revertants did not prevent isolation of the defined mutants so long as isolates were confirmed by dot-blot hybridization for gpt. Thus, the gpt back-selection required a final hybridization step to select desired recombinant viruses and to avoid spontaneous gpt-deficient mutants. The combination of positive and negative selection techniques in conjunction with the generation of immortal cell lines permissive for human CMV (Compton, 1993) will greatly simplify the genetic analysis of essential as well as dispensable functions.

Insertion mutagenesis of CMV with the neomycin phosphotransferase (neo) gene has been reported to be efficient and a neo insertion mutant lacking viral genes TRL13–UL5 was successfully isolated (Wolff et al., 1993). Although neo and gpt can both be used for insertion mutagenesis, only gpt allows 'push and pull' mutagenesis, similarly to that employed with the tk gene for HSV-1 mutagenesis (Post & Roizman, 1981). In contrast to the selective conditions used for the neo marker, selection for gpt did not kill uninfected cells and so allowed plaque formation which simplified virus isolation.

We sought to expand previous observations (Jones & Muzithras, 1992; Kollett-Jons et al., 1991) on the dispensability of US3 by evaluating US3 mutant growth in quiescent fibroblasts and in thy/liv implants in SCID–hu mice. The role of HSV-1 genes in contact-inhibited cells or in animal models has been employed to reveal phenotypes not otherwise obvious in cell culture (Bolovan et al., 1994; Preston et al., 1988; Roizman & Sears, 1993). The normal growth of a US3 mutant in both of these situations suggests that US3 does not play a role that is critical to virus growth, and leaves open the question of how this function may be important to CMV biology or pathogenesis.

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References


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