Detection of human cytomegalovirus DNA replication in non-permissive Vero and 293 cells

Victoria Ellsmore, G. Gordon Reid and Nigel D. Stow

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

Human cytomegalovirus (HCMV) displays an exceptionally restricted host range in tissue culture with human fibroblasts being the principal fully permissive system. Nevertheless, immediate early (IE) proteins are expressed following infection of many non-permissive cell types of human, simian and murine origin, and viral origin-dependent DNA synthesis has been reconstituted by transfection of plasmids into Vero cells, a non-permissive line from African green monkey. We have examined the accumulation of HCMV strain AD169 DNA, and the replication of transfected HCMV origin-containing plasmids, in infected Vero and human embryonic kidney 293 cells, which were previously reported to express the major IE protein in a small proportion of infected cells but to be non-permissive for viral DNA synthesis. In Vero cells accumulation of origin-containing plasmid but not viral DNA occurred, whilst in 293 cells both DNAs accumulated. Immunofluorescence experiments indicated that following infection with 3 p.f.u. per cell, a small fraction of both cell types expressed the UL44 DNA replication protein. Neither cell line, however, supported the generation of infectious progeny virus. These results suggest that IE proteins expressed in Vero and 293 cells can induce the synthesis of early proteins capable of functioning in viral DNA replication, but there is a failure in later events on the pathway to infectious virus production. This provides further support for transfected Vero cells being a valid system in which to study HCMV DNA synthesis, and suggests that 293 cells may also prove useful in similar experiments.

INTRODUCTION

Human cytomegalovirus (HCMV), like other members of subfamily Betaherpesvirinae of the Herpesviridae, is extremely host-cell restricted in tissue culture (for a review see Mocarski & Tan Courcelle, 2001). Only a limited range of cell types support viral DNA replication, the most commonly employed permissive cells being human fibroblasts (HF) from foreskin or lung. LaFemina & Hayward (1986, 1988) reported that of 21 cell types tested only four, all of which were of human origin, supported replication of HCMV strain Towne DNA as determined by incorporation of 32P-labelled orthophosphate into restriction enzyme fragments of viral DNA. In several cell lines of simian or murine origin efficient expression of immediate early (IE) protein, IE1, occurred in the absence of viral DNA synthesis, but in other lines IE1 was expressed at greatly reduced levels. Immunofluorescence studies revealed that following infection with 20 p.f.u. per cell HCMV, 1–2 % of Vero cells (an established line from African green monkey kidney) and approximately 0·2 % of 293 cells (human embryonic kidney cells transformed with sheared adenovirus 5 DNA) expressed IE1, but in neither line was viral DNA replication detectable. Input HCMV genomes were detected in the nuclei of infected Vero cells, and IE proteins were strongly expressed from transfected plasmid DNAs in both cell types. Virus replication was proposed to be blocked at the level of synthesis or stability of IE mRNA transcribed from input viral genomes (LaFemina & Hayward, 1988).

The requirements for HCMV DNA replication were identified through the use of transient transfection assays in permissive HF cells (Anders et al., 1992; Pari & Anders, 1993; Anders & McCue, 1996). These assays defined an origin of lytic DNA synthesis (oriLyt) and 11 protein-encoding loci. OriLyt-dependent DNA synthesis was subsequently demonstrated in transfection assays with non-permissive Vero cells, although in this case expression of the viral proteins was driven by strong constitutive promoters (Sarisky & Hayward, 1996). These results define an origin of lytic DNA synthesis (oriLyt) and 11 protein-encoding loci. The required proteins comprise six that constitute the replication fork machinery, plus a set of auxiliary proteins involved in their expression and possible origin-specific functions (Anders & McCue, 1996; Iskenderian et al., 1996; Fortunato & Spector, 1999). OriLyt-dependent DNA synthesis was subsequently demonstrated in transfection assays with non-permissive Vero cells, although in this case expression of the viral proteins was driven by strong constitutive promoters (Sarisky & Hayward, 1996). These results reveal that there is no intrinsic block to the functioning of the HCMV replicative machinery in Vero cells.

Several different laboratory strains of HCMV are in common use and differences between strains have been reported both in tissue culture and the SCID-hu mouse model system (Brown et al., 1995; Sinzger et al., 1999, 2000). The first complete genome sequence was determined for
HCMV strain AD169 (Chee et al., 1990), and the majority of the plasmids utilized in investigations of viral origin-dependent DNA replication contain inserts of strain AD169 DNA (Anders et al., 1992; Pari & Anders, 1993; Sarisky & Hayward, 1996). It was reported that up to 10% of Vero cells infected with strain AD169 expressed IE1 protein, a proportion slightly higher than that previously observed with strain Towne virus (Bystreyskaya et al., 1997; LaFemina & Hayward, 1988).

In view of the above observations it was interesting to determine whether the limited expression of IE genes in infected Vero and 293 cells might be sufficient to activate the lytic cycle and allow viral DNA synthesis. We therefore employed a sensitive DNA blot hybridization approach to analyse replication of genomic DNA and a transfected oriLyt-containing plasmid in HF, Vero and 293 cells infected or superinfected with HCMV strain AD169.

**METHODS**

**Cells and virus.** Human foetal foreskin fibroblast (HFFF2) and Vero cells were obtained from ECACC (European Collection of Cell Cultures, Porton Down, UK). 293 cells were provided by Vivien Mautner (University of Birmingham) and human foetal lung (HFL; Flow 2002) cells were obtained from Flow Laboratories. All four cell types were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (EFC10). Following infection or transfection, Vero and 293 cells were maintained in medium containing 5% foetal calf serum (EFC5). HCMV strain AD169 was obtained from Derrick Dargan (MRC Virology Unit, Glasgow) and grown in HFFF2 or HFL cells. Unconcentrated supernatant medium from 175 cm² flasks of infected cells was routinely used as virus stock. Titrations were performed on monolayers of HFFF2 or HFL cells.

**Plasmids.** The HCMV oriLyt-containing plasmid, pSP50 (Anders et al., 1992) was kindly provided by David Anders. Plasmid pADori was constructed by cloning an Aartl plus BsrEI oriLyt-containing fragment spanning nucleotides 91321–94168 of HCMV strain AD169 (Chee et al., 1990; accession no. X17403), into pGEM7Zf(−). Plasmid pADoriΔXhol contains a 1160 bp deletion between the two Xhol sites in pADori which removes sequences essential for origin function (Zhu et al., 1998).

**Infection with HCMV.** Monolayers of cells in 35 mm diameter Petri dishes were infected with 3 p.f.u. per cell HCMV strain AD169. After 1 h at 37°C the inoculum was removed and the cells washed with ‘acid glycine’ to inactivate residual virus (Stow, 2001), prior to continuation of incubation in EFC10 or EFC5.

**Transfections.** Cell monolayers in 35 mm diameter Petri dishes were transfected using the calcium phosphate precipitation technique followed by brief exposure at 4 h post-transfection to 25% DMSO (Stow & Wilkie, 1976). Each plate of cells received 0·5 ml of precipitate containing 12 µg calf thymus carrier DNA and 1·6 µg originating plasmid. Six hours post-transfection the cells were infected with HCMV as described above except that the ‘acid glycine’ treatment was omitted. Transfection efficiencies were routinely monitored using the plasmid pElasZ, which comprises the Escherichia coli β-galactosidase gene inserted into the expression vector pCMV10. Uninfected plates were fixed, stained for β-galactosidase expression and positive cells counted in representative microscopic fields (Stow et al., 1993).

**DNA preparation and analysis.** Monolayers of cells were harvested at the indicated times post-infection (p.i.) and total cellular DNA was prepared and analysed as previously described (Hodge & Stow, 2001). One-tenth of the DNA recovered from each plate was digested with EcoRI (for the analysis of HCMV genome replication) or EcoRI plus DpnI (for the analysis of oriLyt-containing plasmid replication). DNA fragments were resolved by electrophoresis through a 0·8% agarose gel, transferred to nitrocellulose and detected by blot hybridization. 32P-labelled pSP50 and pAT153 DNAs were used to detect viral and plasmid fragments, respectively. The plasmid vector pAT153 includes the majority of the vector sequences present in the HCMV oriLyt-containing plasmids. Following hybridization, the washed filters were exposed to a phosphorimager screen and data collected and analysed with the personal Molecular Imager and Quantity One software (Bio-Rad).

**Immunofluorescence.** Cells were seeded onto glass coverslips in Linbro wells (1·5 × 10⁶ cells per 13 mm diameter coverslip) 1 day prior to infection with 3 p.f.u. per cell HCMV. At 48 h p.i. the cells were fixed and permeabilized as previously described (Abbotts et al., 2000). The coverslips were reacted with FITC-conjugated mouse monoclonal antibody CCH-2 against HCMV UL44 (Dako) for 1 h at room temperature, extensively washed, treated with 0·5 µg Hoechst 33258 ml⁻¹ in PBS for 10 min to enable visualization of cell nuclei, washed again and mounted. Slides were examined under a UV fluorescence microscope (Nikon Microphot SA) and the images captured on a digital camera.

**RESULTS AND DISCUSSION**

**HCMV genome replication in HFFF, Vero and 293 cells**

In order to compare the levels of HCMV DNA replication in non-permissive Vero and 293 cells with that in permissive HFFF cells, monolayers of each cell type were either mock-infected or infected with 3 p.f.u. per cell HCMV. Total cell DNA was prepared at various times after infection and the accumulation of viral DNA analysed by Southern blot hybridization (Fig. 1). In all three cell types input viral genomes were detected immediately after adsorption and in reduced amounts, at 1 day p.i. This indicates that a significant proportion of the input DNA is not stably taken up by the cells, although it is not possible to conclude whether loss of particles from the cell surface or the intracellular degradation of DNA is responsible.

In agreement with previous observations (Mocarski & Tan Courrecle, 2001) the viral DNA content of infected HFFF cells increased dramatically between 2 and 4 days p.i. In contrast, no significant change in the amount of HCMV DNA in Vero cells was observed between 1 and 6 days p.i., suggesting either that viral DNA replication is blocked or that synthesis and degradation are occurring at comparable rates. In 293 cells there was a modest accumulation of HCMV DNA between 1 and 4 days p.i., demonstrating that viral DNA replication had occurred, although the final yield was not significantly greater than the level detected immediately after adsorption. Quantification of this and similar experiments indicated that the amount of viral DNA in 293 cells at 4 days p.i. was 1–2% of that in permissive HFFF cells.
Amplification of HCMV oriLyt-containing plasmids in HFFF, Vero and 293 cells

The ability of the HCMV DNA replication machinery to function in the three cell types was also assessed by superinfecting cells that had been transfected with oriLyt-containing plasmids. This approach has the advantage that unreplicated input plasmid DNA can be distinguished from molecules which have been replicated because of its susceptibility to digestion with DpnI. The plasmids used are shown in Fig. 2. Plasmid pSP50 contains the complete core and auxiliary regions required for maximum origin activity, whilst pADori more closely resembles a minimum functional origin. Replicate monolayers transfected with pADori were also mock-infected or following superinfection were incubated in the presence of 200 μg phosphonoacetic acid (PAA) ml⁻¹, which specifically blocks the activity of the viral DNA polymerase (Huang, 1975).

Fig. 3 shows the results of an assay in which Vero and HFFF cell monolayers received samples of the same calcium phosphate precipitates. The behaviour of the two cell types was very similar. Replication of pADori occurred in superinfected but not mock-infected cells, and was blocked by the addition of PAA. Neither the plasmid vector, pGEM7Zf(−), nor the deletion mutant, pADoriΔXhol, was replicated. Plasmid pSP50 accumulated to slightly higher levels than pADori, in agreement with the presence of more extensive auxiliary sequences flanking the core origin region in this plasmid (Anders et al., 1992). A separate experiment in 293 cells (Fig. 3) yielded similar results, consistent with the limited accumulation of HCMV DNA observed in Fig. 1.

Fig. 2. Structure of HCMV oriLyt-containing plasmids. The positions of EcoRI fragments surrounding the HCMV oriLyt locus are shown in the top line, with an expanded version of the PvuII plus KpnI insert of pSP50 immediately below (nucleotide coordinates taken from Chee et al., 1990; accession no. X17403). The thickest line represents the essential core region and the line of intermediate thickness indicates flanking auxiliary elements. At the bottom are linear representations of plasmids pSP50, pADori and pADoriΔXhol with the EcoRI sites in the vector sequences at their left-hand ends. HCMV and vector DNA are shown as continuous and dashed lines, respectively. The EcoRI sites in the HCMV inserts are shown as arrows and the sizes of the EcoRI fragments (kbp) are indicated. Fragments which do not hybridize to the plasmid vector are shown in parentheses.
Thus, although HCMV DNA replication had previously been undetectable in non-permissive Vero and 293 cells (St Jeor et al., 1974; LaFemina & Hayward, 1986, 1988), the use of a sensitive DNA blotting approach and plasmid replication assay convincingly demonstrate that HCMV mediated origin-dependent DNA synthesis can occur in both cell lines. The accumulation of origin-containing plasmid DNA but not HCMV genomes in infected Vero cells suggests that the two DNA species may behave differently. These DNAs are delivered as circular and linear molecules, respectively, and a defect in circularization of input genomes could lead to a situation in which gene expression occurs but DNA synthesis is either blocked or the products are unstable. However, we are unable to exclude the possibilities that very low level synthesis of viral DNA might remain undetectable against the background signal resulting from unreplicated input DNA, or that synthesis and degradation of HCMV DNA are in equilibrium.

Over a large number of experiments we observed that, in marked contrast to genomic DNA, the accumulation of orILyt-containing plasmids was of the same order in non-permissive Vero and 293 cells as in permissive HFFFs. This is probably because the significantly smaller proportion of infected Vero and 293 cells which express DNA replication proteins is counterbalanced by the much greater efficiency with which these cells can be transfected. Comparisons of transfection efficiencies throughout the course of these studies using a beta-galactosidase expressing plasmid, pElacZ, yielded values in the range 1.5–10% and 5–20% positive Vero and 293 cells, respectively. In contrast, the proportion of positive HFFF cells was generally approximately 0.1%.

**Expression of the UL44 protein**

The above experiments provide evidence that proteins required for HCMV DNA synthesis are expressed after infection of non-permissive Vero and 293 cells. Immunofluorescence experiments were performed to assess the proportion of cells that expressed one particular DNA replication protein, the DNA polymerase processivity subunit, UL44.

Monolayers of HFFF, Vero and 293 cells grown on glass coverslips were infected with 3 p.f.u. per cell HCMV and examined for UL44 expression 48 h.p.i. by UV fluorescence microscopy. The number of cells per field was determined by counting Hoechst-stained nuclei. Fig. 4 shows that, as expected, almost all HFFF cells expressed UL44. In contrast, UL44 expression was detectable in only a very small
proportion of infected Vero or 293 cells. In repeat experiments the fraction of Vero and 293 cells which expressed UL44 ranged between 0\%-1\% and 0\%-5\%, respectively. Although UL44 was expressed in many fewer Vero and 293 than HFFF cells, the fluorescence intensities suggested that similar amounts were present in positive cells of the three different cell types. The expression of the early HCMV DNA replication protein UL44 in only a fraction of Vero cells agrees with an earlier report for viral early antigen in these cells (Einhorn et al., 1982).

Previous examination of HCMV gene expression in Vero and 293 cells had similarly indicated that only a minority of infected cells was able to express IE1 protein (LaFemina & Hayward, 1986, 1988; Bystrevkaya et al., 1997). The data for UL44 and early antigen therefore suggest that IE proteins expressed from the HCMV genome are able to transactivate the expression of the full set of early proteins necessary for oriLyt-dependent DNA synthesis, and that this allows DNA replication to take place in a small number of cells. This is consistent with experiments demonstrating that the IE proteins can activate a number of HCMV early and non-HCMV promoters in non-permissive Vero, HeLa and murine cells (Everett, 1984; Pizzorno et al., 1988; García-Ramírez et al., 2001). Comparison of our data with published results (St Jeor et al., 1974; Einhorn et al., 1982; LaFemina & Hayward, 1986, 1988; Bystrevkaya et al., 1997) also indicates that HCMV strains AD169 and Towne exhibit qualitatively similar behaviour in Vero and 293 cells.

LaFemina & Hayward (1986, 1988) previously demonstrated that HCMV infection of non-permissive cells may be blocked at two distinct stages. In several rodent and simian fibroblastic cell types the IE1 protein is abundantly expressed but viral DNA synthesis is undetectable, suggesting that in such cells the viral IE (or possibly early) proteins are functionally defective. In contrast, in cell lines such as Vero and 293, in which only a small fraction of infected cells express IE1 protein, the block was suggested to operate at the level of transcription initiation or mRNA stability. This block to expression from the major IE promoter was not evident in the context of transfected plasmid DNAs, again suggestive of some form of discrimination between plasmid and genomic DNA. More recently, it has been observed that fibroblast-adapted laboratory strains of HCMV exhibit 100- to 1000-fold lower infectivity in human endothelial cells than low passage isolates grown in these cells and that this appears to correlate with defects in transport of capsids towards the nucleus and nuclear import of the viral genome (Slobbe van Drunen et al., 1998; Sinzger et al., 2000).

**Fig. 4.** Expression of UL44 in infected HFFF, 293 and Vero cells. Infected cells were fixed, permeabilized and reacted sequentially with FITC-conjugated anti-UL44 antibody and Hoechst 33258 stain. Slides were visualized by UV fluorescence microscopy with a 40 \( \times \) objective lens and appropriate filters. The top panels (293 F, HFFF F and Vero F) show FITC-staining of representative fields of 293, HFFF and Vero cells, respectively, and the bottom three panels (293 H, HFFF H and Vero H) show Hoechst staining of the same fields.
Although the proportion of pre-labelled input viral DNA recovered from Vero cell nuclei was somewhat less than from the nuclei of permissive cells, the reduction did not appear sufficient to account for the low level expression of IE1 protein (LaFemina & Hayward, 1988). Nevertheless it remains possible that inefficient delivery of genomes to the nucleus may contribute to the observed block in protein expression and DNA replication, particularly in 293 cells in which nuclear DNA uptake has not been examined.

### Table 1. HCMV growth in HFFF, 293 and Vero cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Titre at 1 h p.i.</th>
<th>Titre at 4 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFFF</td>
<td>20</td>
<td>8.4 × 10^3</td>
</tr>
<tr>
<td>293</td>
<td>60</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Vero</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

In summary, our data demonstrate that DNA replication proteins expressed from infecting HCMV genomes in Vero and 293 cells can drive oriLyt-dependent DNA synthesis. This provides support for transfected Vero cells being a valid system in which to study this process, and suggests that 293 cells may also prove useful in similar experiments.

### ACKNOWLEDGEMENTS

We thank David Anders, Vivien Mautner and Derrick Dargan for providing reagents, and are grateful to Andrew Davison for helpful comments on the manuscript. V.E. was supported by a GlaxoSmithKline studentship.

### REFERENCES


HCMV DNA synthesis in Vero and 293 cells