Replicative Forms of Human Cytomegalovirus DNA with Joined Termini Are Found in Permissively Infected Human Cells But Not in Non-permissive Balb/c-3T3 Mouse Cells

By ROBERT L. LAFEMINA AND GARY S. HAYWARD*

Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, U.S.A.

(Accepted 10 August 1982)

SUMMARY

Balb/c-3T3 mouse cells were found to be highly restricted non-permissive hosts for human cytomegalovirus (HCMV) strain Towne. These cells did not produce infectious progeny virions, did not permit virus DNA replication, and allowed expression of only a single, major, virus-specific, immediate-early polypeptide. Virus DNA synthesis was examined by three different experimental approaches. In infected Balb/c-3T3 cells, no $^{32}$P-labelled newly synthesized DNA was found at the virus density in CsCl gradients and no virus-specific fragments were detected after cleavage with restriction enzymes. Similarly, hybridization experiments revealed no net increase in total virus DNA over the amount of input virus-specific DNA sequences. In contrast, infected permissive human fibroblast cells synthesized $^{32}$P-labelled virus-specific DNA fragments and accumulated greatly increased amounts of total hybridizing virus DNA. Experiments with a cloned BamHI L–S joint fragment probe provided evidence for the formation of either circular or concatemeric replicative forms of HCMV DNA in which half-molar terminal fragments were missing and the proportion of quarter-molar joint fragments increased. These forms were abundant in the first 48 h after infection of permissive human cells and mature linear monomeric forms accumulated thereafter. No detectable joining of the termini of input virus DNA occurred in either non-permissive Balb/c-3T3 cells or in human fibroblast cells in the presence of phosphonoacetic acid. In the infected Balb/c-3T3 cells a single major protein corresponding to the 68K immediate-early polypeptide could be detected within 2 h after cycloheximide reversal. Few, if any, other virus proteins were synthesized at later times or in the absence of inhibitors. The 68K protein was overproduced in Balb/c-3T3 cells to such an extent that it became a major component of the nuclear fraction and could be readily detected by direct staining procedures in polyacrylamide gels.

INTRODUCTION

We have previously examined the structure of human cytomegalovirus (HCMV) strain Towne DNA molecules and have prepared detailed restriction enzyme cleavage maps (LaFemina & Hayward, 1980; R. L. LaFemina & G. S. Hayward, unpublished results). The 240 kilobase (kb) linear, double-stranded DNA genome (Kilpatrick & Huang, 1977; DeMarchi et al., 1978; Geelen et al., 1978; LaFemina & Hayward, 1980) can be considered to be organized into two segments: a long unique component ($U_L$) bounded by 11 kb inverted repetitions and a short component ($U_S$) bounded by 2 kb inverted repeats (Kilpatrick & Huang, 1977; LaFemina & Hayward, 1980). The presence of submolar L and S terminal and L–S joint fragments in restriction enzyme profiles indicates that the genome of HCMV (Towne), like those of the herpes simplex viruses (Hayward et al., 1975a, b), occurs in four structural isomers created by inversions of the long and short components of the genome relative to each other. In herpes simplex virus, a 250 bp direct terminal redundancy (the ‘a’ sequence) has been implicated in the isomerization of the genome (Mocarski et al., 1980; Smiley et al., 1981). One or more extra copies...
of this 'a' sequence occur in up to 40% of the L-ends and joint fragments in most HSV strains. Similar heterogeneity in the form of additional steps of 750 bp (at the S end) and occasionally also of 300 bp (at the L end) gives rise to even more complicated terminal and joint fragment patterns in HCMV strains (LaFemina & Hayward, 1980; R. L. LaFemina & G. S. Hayward, unpublished results).

Electron microscopic evidence for the joining of termini during replication has been obtained for pseudorabies virus (Ben Porat et al., 1976), HSV (Jacob & Roizman, 1977) and HCMV (Jean et al., 1978). Unfortunately, the HCMV studies were performed before the nature of defective HCMV populations became known and these studies apparently involved the 150 kb 'defective' population of virus DNA molecules (Kilpatrick & Huang, 1977; Stinski et al., 1979). Restriction enzyme cleavage analysis of replicative forms of wild-type and tandem repeat defective HSV DNA has been performed and in both cases there is an apparent loss of terminal restriction fragments accompanied by an increase in the molarity of the L-S joint fragment (Jacob et al., 1979; Ciufio & Hayward, 1981; Jongeneel & Bachenheimer, 1981).

Cytomegalovirus infection in humans has been associated with diseases of various organ systems and is especially serious in newborns and immunosuppressed patients. Like other herpesviruses, HCMV can establish a latent state (Weller, 1970) but the target tissue in latent infections has not been conclusively identified. In infected tissue culture cells, high titre permissive infection is established only in diploid human fibroblasts. Vero cells (Waner & Weller, 1974) and human epithelial cells (Knowles, 1976; Vonka et al., 1976) have been shown to produce extremely low titres of infectious virus. Although macromolecular synthesis was stimulated in infected guinea-pig cells, the cultures did not produce progeny virus and virus DNA synthesis was not detected (Furukawa et al., 1975). At the level of virus gene expression, Stinski (1978) compared permissive infection of human fibroblasts (HF) and non-permissive infection of guinea-pig fibroblasts. Infected guinea-pig cells expressed proteins with mol. wt. of 75K, 72K, 68K, 56K, 53K, 39K, 27K and 19K. Prior to DNA synthesis the permissive human cell system expressed all of these same immediate-early and delayed-early proteins plus additional polypeptides of 59K and 21K. Late viral protein expression was not detected in non-permissive cells. No information is available about the fate of input virus DNA in non-permissive systems.

We have been interested in studying gene regulation prior to replication in permissive HCMV-infected cells, and in examining the limited virus gene expression in non-permissive systems, because these events may be relevant to latency and transformation. In the present studies, we have investigated the use of Balb/c-3T3 mouse cells as the potentially non-permissive host system and describe the results of experiments designed to ask whether any progeny virus is produced, how much virus DNA replication occurs and at what level virus gene expression is restricted. In the course of these studies we also examined novel intracellular forms of HCMV DNA that appeared after infection of permissive human fibroblast cells.

METHODS

Cells and virus. Human foreskin fibroblasts (HF) cell cultures, that were shown to be free from mycoplasma contamination, were used as our permissive system in all experiments. The HF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% foetal calf serum (FCS; Gibco) without the addition of antibiotics. A non-defective, mycoplasma-free sample of HCMV (Towne) was obtained originally from M. Stinski, University of Iowa. Master virus cultures were passed on HF cells at multiplicities of infection (m.o.i.) ranging from 0.2 to 0.0002 p.f.u./ml in 75 cm² tissue culture dishes. These stocks were given a media change every 5 days with a final change made when the cells showed 100% c.p.e. Maximum titres, approaching 5 × 10⁸ p.f.u./ml, were found 4 days later. At this time, new master dishes were infected and large scale cultures were set up in 32 oz. bottles for virion DNA preparations. At 95% confluence the cells were given a complete media change and harvested 5 days following 100% c.p.e. Maximum titres, approaching 5 × 10⁸ p.f.u./ml, were found 4 days later. At this time, new master dishes were infected and large scale cultures were set up in 32 oz bottles for virion DNA preparations. At 95% confluence the cells were given a complete media change with 10% FCS and infected 1 day later at an m.o.i. of 2.5. Released virus was harvested from the medium at 4 to 5 days following 100% c.p.e. For 32P-labelling of replicating virus DNA, cultures were infected with HCMV (Towne) at various multiplicities. Following a 1 h adsorption step and removal of the inoculum the infected cells were incubated at 36 °C in phosphate-free DMEM, containing 0.5 mCi/ml [32P]orthophosphate and 10% foetal calf serum that had been dialysed against isotonic saline, then harvested at appropriate times.

Purification of intracellular and virion DNA. Virion DNA was purified from the supernatant medium after
removal of cell debris by a low-speed centrifugation at 5000 rev/min for 10 min. Released enveloped particles were pelleted by centrifugation at 12000 rev/min for 90 min in a Sorvall GSA rotor. Alternatively, for intracellular DNA preparations the infected cells were harvested by scraping or trypsinization, then pelleted by centrifugation. All pellets from either source were resuspended in a solution of 0.4 M-NaCl, 5 mM-Tris-HCl pH 8 and 100 mM-EDTA and lysed by the addition of Sarkosyl to 2% and SDS to 0.5%. The lysed cells or virions were then incubated with Pronase (1 mg/ml) overnight at 37°C and emulsified with neutralized redistilled phenol followed by chloroform/isomyl alcohol (98:2, v/v) to extract protein. The aqueous phase was subsequently given a preliminary dialysis against 10 mM-Tris-HCl pH 8-4 containing 1 mM-EDTA and 300 mM-NaCl followed by extensive dialysis against 10 mM-Tris-HCl, 1 mM-EDTA, pH 8-4. After treatment with RNase (20 μg/ml) and further phenol extraction and dialysis, this procedure produced DNA suitable for restriction enzyme digestion. DNA that was to be used for in vitro 32P-labelling was isolated from virions that had been resuspended in 10 mM-Tris-HCl, 10 mM-MgCl₂, pH 8-4 and treated with 200 μg/ml DNase (Sigma) at 37°C for 2 h prior to detergent lysis. Further purification was usually accomplished by banding to equilibrium in CsCl buoyant density gradients (7.5 M). Following centrifugation for 44 h at 36000 rev/min at 20°C in a Beckman Type 40 rotor, the HCMV DNA band at 1.716 g/ml (determined relative to an internal 3H-labelled HSV reference DNA) was dialysed initially as above and finally against glass-distilled water containing beads of Dowex Ag-50W-X8 and Chelex.

**RESULTS**

**HCMV DNA replication does not occur in infected Balb/c-3T3 cells**

Our first experiments with the Balb/c-3T3 cell system showed that these cells do not serve as permissive hosts for HCMV (Towne). For example, at 6 and 14 days following a high multiplicity infection in mouse cells, we were unable to detect infectious HCMV by inoculation of either supernatant medium, sonicated cells or intact cells from the infected Balb/c-3T3 cultures on to indicator HF cells. We should have been able to detect a titre as low as 2 × 10³ p.f.u./ml after infection of the non-permissive Balb/c-3T3 cells. By comparison, infection of...
permissive human fibroblast cells results in titres as high as $5 \times 10^8$ p.f.u./ml. As shown below, we were also unable to detect any virus DNA replication following infection of Balb/c-3T3 cells using three different experimental protocols. These methods included CsCl density gradient analysis of newly synthesized DNA, restriction enzyme cleavage and gel electrophoresis of radiolabelled nascent DNA, and hybridization studies with nick-translated cloned virus DNA probes.

To examine and compare progeny virus DNA following infection of Balb/c-3T3 and HF cells, cultures were infected with HCMV (Towne) and newly synthesized DNA was labelled by the incorporation of $^{32}$P orthophosphate. CsCl gradient profiles of infected Balb/c-3T3 DNA did not reveal any significant incorporation of $^{32}$P into virus DNA (density 1.716 g/ml). In contrast, newly synthesized DNA appeared at the virus density in CsCl gradient profiles of all infected HF cell DNA samples (not shown). Because extensive incorporation of label into cellular DNA occurred in these experiments, and also an additional shoulder appeared at intermediate density in the HF samples, these results did not exclude the possibility of virus DNA synthesis in Balb/c-3T3 cells occurring at levels of up to 5 to 10% of that in the HF cells. Therefore, for better resolution, isolated intracellular DNA harvested at various times after infection was cleaved with appropriate restriction enzymes and fractionated by agarose gel electrophoresis. Examples of the results obtained with EcoRI digestion of 48 h samples are presented in Fig. 1. Under our regular infection conditions (95 to 100% confluence, 10% FCS), typical virus-specific bands were detected in HF DNA samples but not in those from Balb/c-3T3 cells. To eliminate the possibility that the contact-inhibited nature of Balb/c-3T3 confluent monolayers was responsible for the lack of incorporation of label into virus DNA, we compared the results of infections carried out in the following cultures: (i) subconfluent cells growing in 10% FCS (lane 2); (ii) 95% confluent

Fig. 1. Restriction enzyme cleavage patterns of newly synthesized DNA from HCMV-infected Balb/c-3T3 and HF cells. The cultures were infected at an m.o.i. of 10 and labelled with $^{32}$P phosphate for 48 h. Extracted DNA samples were digested with EcoRI and fractionated by agarose gel electrophoresis. Subsequently, the gel was dried for autoradiography. Lanes 1 and 2, DNA from mock-infected and infected subconfluent Balb/c-3T3 cells; lane 3, infected confluent Balb/c-3T3 cell DNA; lane 4, DNA from infected Balb/c-3T3 cells kept at confluence in 1% FCS for 4 days prior to virus addition; lane 5, DNA from an infected non-contact-inhibited Balb/c ‘spontaneously transformed’ cell line; lanes 6 and 7, DNA from mock-infected and infected HF cell cultures at 95% confluence; lanes 8 and 9, DNA from mock-infected and infected HF cells that had been kept at confluence in 1% FCS for 4 days prior to infection. The most intensely labelled bands, denoted by solid circles in both mock-infected and virus-infected cell DNA, proved to be mitochondrial DNA.
CMV DNA in non-permissive infections

Cells growing in 10% FCS (lane 3); (iii) contact-inhibited cells held for 4 days in media containing 1% FCS (lane 4); (iv) a spontaneously transformed Balb/c-3T3-derived cell line that did not display the characteristic 'cobblestone' appearance (lane 5). Similarly, we infected permissive HF cultures that were either subconfluent growing in 10% FCS (lane 7) or fully confluent and held for 4 days in 1% FCS (lane 9). Although there were quantitative differences in 32P incorporation between the various Balb/c-3T3 cultures, none of these variations resulted in the appearance of newly synthesized virus DNA fragments. It was evident from both the restriction enzyme cleavage patterns (Fig. 1) and the CsCl profiles (not shown) that the greatly decreased levels of HF cellular DNA synthesis in the confluent cells did not significantly affect the amount of 32P incorporated into virus DNA. Although there was an approximately twofold higher incorporation of 32P into newly synthesized virus DNA in highly metabolizing subconfluent HF cells, the overall ratio of virus DNA synthesis to cell DNA synthesis was greatly increased in infected, slowly metabolizing, HF cultures (Fig. 1, compare lanes 7 and 9). Note that the prominent 'non-viral' bands seen in all autoradiographs of infected Balb/c-3T3 and HF cells (indicated by solid circles) were also found in mock-infected cells. These strongly labelled DNA fragments probably accounted for the intermediate density shoulder in the CsCl profiles.

Fig. 2. Comparison by hybridization of the levels of total virus DNA accumulated in infected Balb/c-3T3 and HF cells. Unlabelled DNA samples harvested at the times indicated were digested with BamHI and fractionated by agarose gel electrophoresis. The DNA was subsequently transferred to nitrocellulose for hybridization with an in vitro labelled total HCMV DNA probe followed by autoradiography. Lanes 1 to 4, Balb/c-3T3 DNA at 2, 24, 48 and 96 h after infection; lanes 5 to 8, HF cell DNA at 2, 24, 48 and 96 h after infection. Equivalent amounts of total intracellular DNA (4 µg) were loaded on to each lane. Virus-specific fragments were visible directly in the gel by ethidium bromide staining in lanes 7 and 8. Lane 9, reconstruction with reference HCMV virion DNA (0.1 µg, equivalent to approx. 1200 copies/cell).
Fig. 3. Demonstration of an increased proportion of L-S joint fragments and the loss of termini in replicating HCMV DNA. Unlabelled DNA samples were extracted from infected HF cells, then digested with BamHI, fractionated by agarose gel electrophoresis and transferred to nitrocellulose. The filters were hybridized with nick-translated 32P-labelled probes of either total HCMV DNA (lanes 1 to 4) or the pRL8 plasmid clone containing the BamHI-H joint fragment (lanes 5 to 9) followed by autoradiography. Lanes 1 and 5, control of input HCMV DNA from an HF infection at high m.o.i. (DNA was harvested from the cells directly after virus adsorption); lanes 2 and 6, DNA from HF cells at 48 h after infection; lanes 3 and 7, DNA from HF cells at 96 h after infection; lanes 4 and 8, HCMV DNA extracted from purified virions recovered from a parallel infected culture; lane 9, longer exposure of the autoradiograph shown in lane 6 to reveal trace amounts of the BamHI-L, -N and -Z end fragments. The relevant virus DNA fragments labelled in Fig. 3, 4, 5, 6 and 7 are named according to our standard HCMV (Towne) restriction enzyme cleavage maps (R. L. LaFemina & G. S. Hayward, unpublished results).

and displayed the typical restriction enzyme cleavage patterns expected for mouse and human mitochondrial DNAs (Brown & Vinograd, 1974; see below).

The best evidence for the lack of virus DNA replication in Balb/c-3T3 cells came from hybridization experiments with infected cell DNA after cleavage with restriction enzymes. At the low level of sensitivity displayed in the experiment shown in Fig. 2 (approx. 20 copies/cell), intracellular HCMV DNA could not be detected at any time after infection of Balb/c-3T3 cells. This result should be contrasted with the enormous increase in the intensity of hybridizing virus DNA fragments observed in the lanes containing infected HF cell DNA at 24, 48, and 96 h after infection. Based on reconstruction experiments and also on the amount of viral and cellular DNA visible in this gel by ethidium bromide staining (not shown) we estimated that the copy numbers of virus DNA in the last three HF cell DNA samples were 200, 4000 and 20000 molecules/cell.

Lack of terminal fragments in replicative forms of HCMV DNA

Analysis of the autoradiographs in Fig. 2 indicated that the pattern of total intracellular virus DNA fragments differed slightly from that of the standard patterns for packaged virion DNA. To examine this difference in more detail, parallel infected HF cell DNA samples were extracted at 48 and 96 h after infection, cleaved with BamHI and electrophoresed alongside a
Fig. 4. Physical map illustrating the location and size of all terminal and joint fragments (solid bars) in XbaI, HindIII and BamHI digests of HCMW (Towne) DNA. The structural arrangement of inverted repeats (open bars) and the prototype orientation of the L and S segments are based on those proposed originally by LaFemina & Hayward (1980) but the fragment nomenclature has been revised to accommodate new information (R. L. LaFemina & G. S. Hayward, unpublished results). Only two of the four equimolar DNA isomers found in virions are drawn. Those fragments from the S-ends and joint regions which display typical tripartite terminal heterogeneity as described by LaFemina & Hayward (1980) are denoted by asterisks. Only the sizes of submolar fragments with the fewest 800 bp terminal repeats are given in the standardized maps.

released virion DNA sample and an input (0 h) infected cell DNA sample. Two sets of these gel-fractionated DNAs were transferred to nitrocellulose and hybridized with probes consisting of either total HCMV DNA or a cloned L-S joint fragment. The HCMV total DNA probe revealed that the normally submolar BamHI-(H, I) L-S joint fragment complex increased in intensity relative to neighbouring molar fragments in the 48 and 96 h DNA samples (Fig. 3), whereas it remained submolar in the virion and input DNA samples. In accordance with this observation, the relative proportion of the BamHI-Z fragment from the L-terminus was greatly reduced at 96 h in comparison with the standard pattern for input or packaged progeny virus DNA and this species was almost undetectable in the 48 h sample. The complex patterns of submolar BamHI-N and -L fragments from the S-terminus were partly hidden behind similar-sized full-molar fragments; however, the proportion of the BamHI-N fragment was clearly less at 96 h. Hybridization to the second set of filters using the cloned BamHI-H fragment (pRL8 plasmid) probe permitted unambiguous identification of terminal fragments, especially those from the S-segment. Only traces of the L- and S-termini were seen at 48 h even in long exposures of the autoradiograph (lane 9), and these fragments were still considerably under-represented at 96 h compared with the level of termini detected in the input and packaged virion DNA samples. The observed maturation of replicating virus genomes, containing essentially molar L-S joint fragments and lacking the normal L- and S-segment terminal fragments, to forms containing submolar L-S joint fragments, two identical submolar L-ends and 'split' submolar S-ends is consistent with similar observations on the replicative forms of other herpesviruses (Jacob et al., 1979; Jongeneel & Bachenheimer, 1981). Molar L-S joint fragments and a deficiency of terminal fragments predict the existence of either circular or concatemeric forms during replication but prior to maturation. The map locations of relevant terminal and submolar restriction fragments in the HCMV (Towne) genome is illustrated in Fig. 4.

Discrimination between input and progeny virus DNA

To examine the state of the input virus DNA at very early stages of infection, infected HF cell DNA was labelled with [32P]phosphate from 0 to 48 h following infection at low multiplicity in low-phosphate medium. A single DNA sample was used to compare the fragment profiles of both newly synthesized molecules and total virus DNA from the same infected culture. The
Fig. 5. Comparison of the structure of newly synthesized and input virus DNA molecules after CsCl fractionation of DNA extracted from infected HF cells at early stages of the replication process. (a) Reference BamHI digest of virion DNA hybridized with the pRL8 joint probe to illustrate the standard pattern of terminal and joint fragments. (b) Cell DNA labelled with $^{32}$P-phosphate for 48 h following infection of HF cells at an m.o.i. of 2 was centrifuged to equilibrium in a CsCl density gradient and DNA samples recovered from individual fractions across the DNA peak (lanes 1 to 10) were cleaved with BamHI. The digests were then fractionated by agarose gel electrophoresis and transferred on to a nitrocellulose filter followed by autoradiography to reveal the pattern of newly synthesized DNA. (c) After 10 half-lives, tracks 1 to 7 from the same filter were hybridized with the pRL8-cloned BamHI L–S joint fragment probe and exposed for further autoradiography to examine the state of the input DNA. The direction of increasing density is indicated by arrows. The positions of $^{32}$P-labelled mitochondrial DNA fragments are denoted by solid circles in Fig. 5, 6 and 7.

Intracellular DNA was first fractionated by CsCl density gradient centrifugation and fractions from across the viral and cellular DNA peaks were individually ethanol-precipitated to remove CsCl. Subsequently, the DNA was cleaved with restriction endonucleases and electrophoresed through 1% agarose slab gels then transferred on to a nitrocellulose filter for autoradiography. An example of the BamHI cleavage pattern of individual fractions of infected $^{32}$P-labelled HF cell DNA is shown in Fig. 5(b). The DNA across the virus peak (density 1.716 g/ml) contained characteristic virus-specific fragments and, as expected, the BamHI-Z fragment from the L-terminus was not detected in newly synthesized DNA (arrowed). Because the submolar S-terminal fragments in a BamHI digest were difficult to detect, additional experiments with XbaI (and also HindIII; not shown) were performed, which revealed that the normal S-end fragments $XbaI$-H and -I were also missing (Fig. 6). After the newly synthesized $^{32}$P-labelled DNA on the filters had been stored for 10 half-lives, these filters were hybridized with the in vitro labelled BamHI-H joint fragment probe (see Fig. 5c). Surprisingly, the experiment revealed that, although no BamHI-Z terminal fragments were detected as newly synthesized $^{32}$P-labelled DNA in this sample, the bulk of the virus DNA in the cell contains almost normal amounts of both BamHI-Z and the S-end fragments relative to joint fragments.

These results indicate that not only have we detected the input parental DNA molecules but also that most of the input DNA remains in a linear monomeric form. The low and nearly
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Fig. 6. Lack of S-end fragments in $^{32}$P-labelled newly synthesized virus DNA from infected HF cells. Lane 1, reference ethidium bromide-stained gel of $XbaI$-digested virion DNA. Lanes 2 to 5, CsCl density gradient fractions corresponding to lanes 3 to 6 in Fig. 5, after digestion with $XbaI$, followed by gel electrophoresis and autoradiography. Lanes 4 and 5 were underexposed to reveal the virus DNA bands over background labelled cell DNA.

Fig. 7. Detection of input virus DNA molecules in intracellular DNA from infected non-permissive Balb/c-3T3 cells. (a) Reference HindIII digest of virion DNA hybridized with the in vitro labelled cloned pRL8 probe to illustrate the standard pattern of heterogeneous terminal and joint fragments. (b) Newly synthesized $^{32}$P-labelled DNA extracted at 48 h after infection of Balb/c-3T3 cells, followed by separation of viral and cellular DNA by CsCl density gradient centrifugation. Individual fractions (lanes 1 to 9) were digested with HindIII and fractionated by agarose gel electrophoresis. The DNA was transferred on to nitrocellulose prior to autoradiography to detect $^{32}$P-labelled newly synthesized molecules. (c) Following 10 half-lives, lanes 1 to 9 from the same filter were examined for the presence of input virus DNA using the pRL8 BamHI-H joint fragment probe.
Fig. 8. Loss of termini from input molecules does not occur in the absence of virus DNA replication. Unlabelled DNA samples from infected HF cells cultured in the presence of PAA or from infected Balb/c-3T3 cells cultured with or without PAA were cleaved with BamHI then fractionated by agarose gel electrophoresis. The DNA was stained with ethidium bromide (not shown) then transferred to nitrocellulose and hybridized with the pRL8 joint fragment probe. Lanes 1, 2, and 3, infected Balb/c-3T3 cells cultured in the presence of PAA for 12, 72 and 120 h; lanes 4, 5, 6 and 7, infected Balb/c-3T3 cells incubated without drugs for 6, 24, 48 and 96 h; lanes 8, 9 and 10, infected HF cells cultured in the presence of PAA for 12, 72 and 120 h; lanes 11 and 12, infected HF cells cultured without drug for 6 and 24 h; lane 13, control of released virion DNA. Net DNA synthesis occurred only in sample 12 (compare with sample 11), which shows a loss of terminal fragments (arrow) and an increase in the molarity of the L-S joint fragments. In all non-replicating samples the termini remain visible and the apparent molarities of the L-S joint fragments do not increase. The experiments without PAA were performed with different virus stocks than those in the presence of PAA and consequently received different (lower) amounts of input DNA.

normal ratio of joint fragments to end fragments in the total intracellular virus DNA in this experiment implies that very little net DNA synthesis (less than twofold) had occurred by 48 h under these less than optimal infection conditions (low m.o.i., preincubation at confluence in 1% FCS, use of low-phosphate medium with dialysed FCS after infection) in comparison to the at least 100-fold increase at 48 h in the experiment shown in Fig. 2. Therefore, we can conclude that progeny DNA molecules from the very first few rounds of virus DNA replication contain covalently linked termini. These experiments also confirmed that the intensely labelled fragments thought to be 'mitochondrial' DNA, banded at the appropriate densities of 1.705 g/ml in HF cell DNA and 1.698 g/ml in Balb/c-3T3 DNA (Fig. 5 and 6) compared to HCMV DNA at 1.716 g/ml, and that they failed to hybridize with total HCMV DNA probes (not shown).

Fate of input virus genomes in the absence of virus DNA synthesis

In a similar experiment with non-permissive Balb/c-3T3 cells, CsCl-fractionated infected cell DNA was cleaved with HindIII and transferred on to nitrocellulose for autoradiography and subsequent hybridization. As expected, there was no incorporation of 32P label into virus-specific fragments that could be detected following restriction enzyme cleavage and gel electrophoresis (Fig. 7b). However, after 10 half-lives, when this filter was hybridized with the BamHI-H L-S joint and termini patterns were observed (Fig. 7c). This experiment confirmed that no new progeny virus DNA was synthesized and that no detectable formation of circular or concatemeric forms of input molecules occurred in Balb/c-3T3 cells.
Fig. 9. Synthesis of the major viral 68K immediate-early protein in infected non-permissive mouse cells. [35S]methionine-labelled proteins were extracted from virus-infected (m.o.i. 5) or mock-infected Balb/c-3T3 cells, primary mouse embryo fibroblasts (MEF) and HF cells, then fractionated into cytoplasmic and nuclear samples by Nonidet P40 treatment. Mock-infected cells and one set of virus-infected cells (immediate-early) were treated with cycloheximide (50 μg/ml) for 2 h, then subsequently washed three times with phosphate-buffered saline, once with methionine-free media and finally incubated for 2 h in methionine-free media supplemented with 5 μCi/ml [35S]methionine. The other set of virus-infected cells (delayed-early) was incubated for 2 h in DMEM prior to radiolabelling from 2 to 4 h following virus adsorption. Electrophoresis was carried out in a 10% polyacrylamide slab gel at 30 mA for 3.5 h. Lanes 1, 2 and 3, Balb/c-3T3 cytoplasmic fractions from mock-infected, immediate-early, and delayed-early samples; lanes 4, 5 and 6, Balb/c-3T3 nuclear fractions from mock-infected, immediate-early and delayed-early samples; lanes 7, 8 and 9, HF cytoplasmic fractions from mock-infected, immediate-early, and delayed-early samples; lanes 10, 11 and 12, HF nuclear fractions from mock-infected, immediate-early and delayed-early samples; lanes 13 and 14, MEF cytoplasmic fractions from mock-infected and immediate-early samples; lanes 15 and 16, MEF nuclear fractions from mock-infected and immediate-early samples. The positions of co-migrating protein mol. wt. (× 10⁻³) standards are given on the right and minor viral polypeptide species (other than 68K) are indicated by solid circles adjacent to lane 12 (39K, 50K, 52K, 54K, 75K and 130K).

We also asked whether the input virus DNA molecules formed linked termini in permissive HF cells in the absence of virus DNA synthesis. Unlabelled infected Balb/c-3T3 and HF cell DNA preparations were extracted at various times after infection and cleaved with restriction endonucleases. In some cultures PAA (100 μg/ml) was added to inhibit the virus DNA polymerase and presumably prevent virus DNA replication (Stinski, 1978). Following electrophoresis and transfer on to nitrocellulose, the DNA was hybridized with nick-translated total HCMV DNA or with the cloned joint fragment probe (Fig. 8). Virus DNA was visible in all infected cell DNA samples, but in the Balb/c-3T3 cells infected in the presence or absence of
Fig. 10. Detection of over-produced 68K by direct staining in nuclei of infected Balb/c-3T3 cells. Unlabelled polypeptides were extracted from the nuclei of mock-infected and HCMV-infected Balb/c-3T3 cells (high m.o.i., approx. 50) following cycloheximide treatment for 2 h plus a 2 h reversal. Coomassie Brilliant Blue-stained polypeptides are shown after electrophoresis through a 10% acrylamide slab gel at 30 mA for 4 h. Lane 1, set of reference polypeptides of known molecular weight; lane 2, mock-infected nuclear fraction after cycloheximide reversal; lane 3, HCMV-infected nuclear fraction after cycloheximide reversal. Under the electrophoresis conditions described in Fig. 9, the 68K polypeptide does not resolve from bovine serum albumin (68K), but under the longer electrophoresis times shown here the 68K immediate-early HCMV polypeptide (arrow) migrates slightly ahead of bovine serum albumin which is the 68K marker in lane 1.

PAA and also in the HF cells infected in the presence of PAA, the termini remain unlinked in the majority of the input molecules. In contrast, less than 10% of the normal proportion of terminal fragments were present in the control infected HF cell DNA sample at 24 h in the absence of PAA (Fig. 8, lane 12). Additional hybridization experiments using total HCMV DNA probes (not shown) gave normal restriction patterns for input virus DNA in Balb/c-3T3 cells and in HF cells in the presence of PAA, providing evidence that there was no site-specific integration, genome rearrangements or loss of specific virus sequences in non-permissive cells.

Virus gene expression in Balb/c-3T3 and HF cells

Having demonstrated the total absence of virus DNA replication in the Balb/c-3T3 cultures the question arose as to whether the input virion DNA actually enters the nucleus and, if so, how much virus gene expression occurs. Infectious virus was added to cultures of both Balb/c-3T3 and HF cells in the presence of $[35S]_{\text{methionine}}$ to assay virus gene expression at various times and under various antibiotic blockage protocols. As shown in Fig. 9, the infected Balb/c-3T3 cells and primary Balb embryo fibroblasts (MEF) synthesized a major protein of 68K that was
Table 1. Comparison of HCMV proteins synthesized at early times in permissive and non-permissive cells

<table>
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<tr>
<th>Mol. wt. (× 10⁻³)</th>
<th>Balb/c-3T3 cells</th>
<th>HF cells</th>
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<tr>
<td></td>
<td>4 h* + CH</td>
<td>4 h + CH + CH + PAA + CH + PAA</td>
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* Labelled from 2 to 4 h during cycloheximide or mock reversal.
† Labelled from 0 to 20 h after infection in the presence or absence of phosphonoacetate.
‡ +, ++, ++++, +++++++, Relative abundance: ±, trace amounts only.

also found in infected HF cells but not found in mock-infected Balb/c-3T3 or HF cells. Synthesis of this protein was enhanced by treatment with cycloheximide followed by brief reversal. In both permissive and non-permissive cells more of the 68K protein fractionated with the nucleus than with the cytoplasm. This experiment also appears to show very minor synthesis of two other virus-specific polypeptides of 38K and 50K. The 50K protein was synthesized within 4 h following infection in both HF and Balb/c-3T3 cells, but was not found within 2 h following cycloheximide reversal in either cell type, although large amounts of the 68K protein and a trace of a 39K protein were synthesized within this time period. Additional delayed-early virus proteins were detected at 4 h in the HF cells (indicated by solid circles in Fig. 9) but not in Balb/c-3T3 cells. These observations suggest that the 68K protein is the first and major immediate-early protein synthesized following HCMV infection. Although we surmise that synthesis of this polypeptide may be necessary for subsequent virus gene expression (by analogy with HSV; Watson & Clements, 1980), the function of the 68K protein is not known. In the experiment shown in Fig. 9, the 68K protein could not be detected by Coomassie Brilliant Blue staining. However, altering the experimental protocol to increase the m.o.i. from 5 to 50 allowed the synthesis of the 68K protein to be increased to such high levels that it could be observed directly in the nuclear fraction in a Coomassie Brilliant Blue-stained gel (Fig. 10).

Additional experiments were performed in which Balb/c-3T3 and HF cells were either mock-infected or infected with virus for 20 h in the presence or absence of PAA (not shown). Under these conditions progeny virus DNA was not synthesized in HF cells and therefore late viral genes presumably were not expressed. At 20 h after infection, some trace of the 68K protein remained in Balb/c-3T3 cells but the 50K protein was no longer detectable. In contrast, the HF cells treated with PAA synthesized virus-specific polypeptides with sizes of 52K and 54K in addition to the 68K, 50K and 39K polypeptides. The 68K, 54K, 52K, 50K and 39K polypeptides and also new polypeptides of 75K and 130K were observed in HF cells incubated for 20 h without PAA. At 48 h following infection the major capsid protein (145K) was observed only in infected HF cells without PAA (not shown). Table 1 summarizes the virus-specific polypeptides that we have detected in Balb/c-3T3 and HF cells at immediate-early and delayed-early time periods.

DISCUSSION

The source of the block or defect in HCMV gene expression in the non-permissive Balb/c-3T3 cell system is not known. Virus DNA must have entered the nucleus but no detectable alterations of the virus genome occurred. All terminal fragments remained visible in hybridization experiments with unlabelled infected Balb/c-3T3 cell DNA, indicating that most of the input genomes failed to circularize. Therefore, not only did the input virus DNA molecules not go on to produce replicative forms in Balb/c-3T3 cells but they did not set up a
latent state of the kind seen with supercoiled EBV DNA in Raji cells (Lindahl, 1976). Since the infected Balb/c-3T3 cells also tended to lose the input virus DNA beyond 48 to 96 h they did not appear to be entering the same kind of association seen in the persistently HCMV-infected human cell cultures described by Mocarski & Stinski (1979). Methylation at CpG dinucleotides in the resident virus genomes of Herpesvirus saimiri-transformed cell lines has been associated with a decrease in virus gene expression in these cells (Desrosiers et al., 1979). However, we have found that encapsidated HCMV DNA is not methylated at CpG sites because HpaI andMspI give identical restriction endonuclease patterns, and we have also observed that at no time after HCMV infection in either permissive or non-permissive cell systems was there any evidence for CpG methylation of input or progeny virus DNA (not shown).

By analogy with HSV, the formation of circular or head-to-tail concatemeric structures in replicating HCMV DNA strongly suggests the existence of a terminally repeated region similar to the ‘a’ sequence that has also been implicated in recombination leading to isomerization in HSV. Preliminary electron microscope evidence for such a sequence has been reported by Geelen & Weststrate (1981) who noticed circular forms following brief exonuclease digestion in vitro. Limited polymerase-associated exonuclease activity on the ‘a’ sequence of input virion DNA early in infection would provide ‘sticky ends’ which could anneal intramolecularly to form circles, resulting in the loss of terminal restriction fragments. This process could be a prerequisite for replication by either the Cairn’s circle or rolling circle mechanisms which would then perpetuate the covalently linked state of terminal fragments in all newly synthesized DNA. Our studies also provide definitive evidence for the maturation of virus genomes from these replicative ‘endless’ forms to a linear monomeric form displaying mature terminal fragments. Whether or not this maturation is also coupled with encapsidation as has been suggested for pseudorabies virus remains to be determined (Ladin et al., 1980).

The linkage of terminal virus DNA fragments in input DNA was not detectable at any time after infection of non-permissive cells or in permissive cells in the presence of PAA, suggesting that active virus DNA polymerase or the replication process itself may be necessary for this event. Furthermore, as shown in Fig. 5, even in a permissive replicative system the majority of the input DNA molecules did not show linked termini at the time of initiation of DNA replication. However, the significance of this latter observation depends upon what percentage of these input molecules enter the replicative pool, something that we do not know at present. If, as Jacob et al. (1979) suggested for HSV, only 5% of the input DNA goes on to enter the replicative pool in permissive systems, then we could not have expected to detect joined ends in Balb/c-3T3 cells either. However, if circularization of a small percentage of input DNA molecules does occur in Balb/c-3T3 cells, it is obviously not sufficient for replication in the non-permissive system.

An alternative explanation for the loss of termini during replication of HCMV in permissive cells could be ‘linear’ integration into the cellular DNA at the termini of the virus DNA molecules. Gadler & Wahren (1981), for example, have suggested that HCMV DNA is associated with host cell DNA when virus DNA replication is blocked by the addition of phosphonoformate. Whilst we cannot eliminate this possibility entirely we can certainly rule out integration at a single unique site within the cellular genome (two new novel fragments would have been detected in the hybridization experiments). The increased molarity of the joint fragments also argues strongly against ‘linear’ integration on a large scale even at random locations, unless multiple tandem insertions occur at the same site(s) (which would be formally little different than concatemers anyway). Our results do not tend to support significant amounts of integration of input molecules in non-permissive Balb/c-3T3 cells either. Although both the joint and L-end fragments (but not S-end fragments) of parental DNA in both HF and Balb/c-3T3 cells are skewed towards light density positions (Fig. 5 and 7), presumably because of their relatively low G + C content and the fragmentation of some molecules, we cannot construct any sensible integration models in which the L-terminal fragments remain free. Nevertheless, the question of whether or not integration into host cell DNA occurs at any stage during virus replication needs to be further examined.

Others have suggested that HCMV infection stimulates cells to enter S-phase (St. Jeor et al.,
1974; Tanaka et al., 1975) and that maximum replication of HCMV is dependent upon S-phase functions in rapidly growing cells (DeMarchi & Kaplan, 1977). However, neither of these factors appear to play a role in Balb/c-3T3 infections. For example, as shown in Fig. 1, both rapidly growing and contact-inhibited Balb/c-3T3 cells were non-permissive for virus DNA synthesis and, furthermore, the metabolic state of HF cells had little effect on the yields of virus in permissive infections. Protein labelling studies (Fig. 10) showed that there was net synthesis of host cell polypeptides in both infected Balb/c-3T3 and HF cells. Similarly, all infected cells continued to synthesize host-specific DNA (Fig. 1, 5, 6 and 7) and RNA (data not shown), indicating that, unlike HSV, cytomegalovirus infection does not rapidly 'shut off' host cell macromolecular synthesis. On the other hand, since the very low incorporation of $^{32}$P phosphate into cell DNA in confluent HF cell cultures maintained in 1% FCS medium did not increase after infection (see Fig. 1, lanes 8 and 9) we suggest that little or no stimulation of S-phase-dependent cell DNA synthesis occurred either. Therefore, our results indicate that non-permissiveness in Balb/c-3T3 cells is probably not related to S-phase effects or contact inhibition. Infection of synchronized cells at defined stages of the cell cycle and an examination of cell cycle-dependent functions after infection may be necessary to fully resolve these questions.

Our Balb/c-3T3 system appears to show more restricted virus gene expression than has been described for other non-permissive rodent cell types such as guinea-pig embryo fibroblasts (Stinski, 1978) and therefore we suggest that there may be several different levels of non-permissiveness in HCMV infected tissue culture systems. The major HCMV polypeptide found in either Balb/c-3T3 or Balb/c embryo fibroblasts is a 68K polypeptide which appears to be an equivalent protein to the major 68K species that weas labelled under immediate-early conditions in infected permissive HF cells. More of the 68K protein fractionated in the nuclear than in the cytoplasmic fractions in both our permissive and non-permissive cells and we presume that this protein is a component of the intranuclear, complement-fixing, immediate-early antigen (Geder, 1976; Gergely et al., 1980; Reynolds, 1978). The nuclear antigen is detected by certain HCMV-positive patient sera when reacted against infected tissue culture cells and has been reported to contain a polypeptide whose size varies between 68K and 86K, depending upon the virus strain (Michelson et al., 1979; Tanaka et al., 1979). Gibson (1981) has also shown that the major phosphorylated, immediate-early protein of HCMV displays strain-dependent size variation, and is distributed approximately equally between the cytoplasmic and nuclear fractions. The major templates for immediate-early RNA transcripts coding for a similar-sized protein in permissive infections have been localized to coordinates 0-66 to 0-77 in the HCMV (Towne) genome (Wathen et al., 1981; Wathen & Stinski, 1982).

Since our mouse non-permissive cell system shows a highly restricted level of HCMV gene expression, the Balb/c-3T3 cells or primary mouse embryo fibroblasts appear to offer considerable advantages for studying the functional role of the 68K protein in gene regulation. They should also serve as a good source of antigen for the production of monospecific or hybridoma monoclonal antibodies directed against the viral immediate-early gene product(s), especially after cycloheximide reversal which leads to overproduction of the 68K immediate-early protein. The production of monospecific antibodies, coupled with transfection studies in mouse cell lines should help to elucidate the function and importance of the HCMV immediate-early gene products.

A sample of the HCMV (Towne) isolate was obtained initially from Mark Stinski of the University of Iowa. We are grateful to Wade Gibson, Clint Roby and Alice Irmiere for assistance in protein polyacrylamide gel electrophoresis procedures and thank Nancy Standish for typing the manuscript and Michael McLane for photographic work. This research was sponsored by Grant Number 2R01 CA22130, awarded by The National Cancer Institute, NIH. R.L.L. is a postdoctoral fellow supported by National Research Service Award F32 CA0633.

REFERENCES


CMV DNA in non-permissive infections


(Received 27 May 1982)