Differences in Cell Type-specific Blocks to Immediate Early Gene Expression and DNA Replication of Human, Simian and Murine Cytomegalovirus

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SUMMARY

We have previously described blocks to the viral lytic cycle at two different levels in cytomegalovirus (CMV)-infected non-permissive cells. BALB/c-3T3 cells express only the predominant immediate early (IE) nuclear phosphoproteins (IE68 or IE94) of human CMV (HCMV) or simian CMV (SCMV) and do not replicate the input viral genomes. However, in human teratocarcinoma stem cells and 293 cells, expression of the HCMV IE68 gene (but not the SCMV IE94 gene) is blocked at the transcriptional level. Here we report the results of an extensive comparison of the level of permissiveness for HCMV, SCMV and murine CMV (MCMV) in a variety of additional cell types of human, monkey and mouse origin. We also describe a subtle change in the tryptic peptide pattern of the IE68 polypeptide produced in BALB/c-3T3 cells compared to permissive human foreskin fibroblasts. Neither the IE68 nor IE94 proteins could be detected by biochemical labelling procedures in infected mouse Ltk− or F9 teratocarcinoma stem cells, although IE94 was synthesized after retinoic acid-induced differentiation of the F9 cells. Synthesis of [35S]methionine-labelled IE94 protein, but not that of HCMV IE68, was detected in infected Vero cells and in human peripheral blood leukocyte cultures. The failure to synthesize detectable IE68 protein in infected Vero cells appeared to be unrelated to a lack of entry of viral DNA and to a lack of appropriate transcription factors. Indeed, immunofluorescence assays showed that the IE68 antigen was expressed efficiently in DNA-transfected Vero cells and in a small fraction of infected Vero cells. Overall, two clear host range trends emerged. First, whilst all three viruses showed a tendency for repression of IE expression in transformed cell lines, the effect was severe for HCMV and only minimal for SCMV. Secondly, progression of infection to the viral DNA synthesis level in non-transformed fibroblast cell types occurred in a much wider range of host species cell types for SCMV and MCMV than for HCMV.

INTRODUCTION

Human cytomegalovirus (HCMV) displays a strict host cell type and species specificity such that in tissue culture systems at least, high titres of progeny virions are produced only in human diploid fibroblasts (Rowe et al., 1956; Smith, 1956; Weller et al., 1957). Other human cell types tested such as epithelial cells are, at best, only moderately permissive (Knowles, 1976; Vonka et al., 1976). However, efficient growth even in fibroblast cultures apparently requires adaptation by serial passaging in cell culture. Fresh clinical isolates of HCMV have been reported to produce some degree of viral gene expression and phenotypic change in peripheral blood

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lymphocyte, T cell and macrophage cell cultures, whereas fibroblast-adapted laboratory isolates usually do not (Rice et al., 1984; Schrier et al., 1985). Since HCMV is believed to persist in a latent state in cells of the monocyte/macrophage lineage in the human host, an understanding of the specific features of different isolates and adapted strains which control host-range, and the interactions of viral and cellular regulatory elements in general is likely to be of great importance. Because of the great difficulties at present in carrying out biochemical analyses with fresh clinical isolates, we have opted to explore and compare further the host range properties of different mammalian CMV strains that have been adapted for growth in fibroblast culture.

The HCMV genome is organized into long and short unique regions, each bounded by inverted repeated sequences, and the packaged virion DNA molecules exist in four different isomeric forms (Kilpatrick & Huang, 1977; Fleckenstein et al., 1982; LaFemina & Hayward, 1980). In permissive cells, HCMV gene expression is regulated such that under immediate early (IE) conditions, the bulk of transcription is limited to within the major IE locus between 0-71 and 0-75 map units (DeMarchi, 1981; Wathen & Stinski, 1982; Stenberg et al., 1984; Stinski et al., 1983). At delayed early (DE) times, only a trace amount of the HCMV IE68 protein continues to be detectable and the remainder of the viral genome becomes transcriptionally active (Stinski, 1978; DeMarchi, 1981; Wathen et al., 1981; LaFemina & Hayward, 1983). Following DNA replication, the late structural proteins are synthesized, capsid assembly and genome packaging occur and progeny virions are released. The well defined major IE promoter/regulatory region contains several series of repetitive elements between positions -50 and -550 (Thomsen et al., 1984) and can act as a strong transcriptional enhancer (Boshart et al., 1985). The gene encoding the major IE68 nuclear phosphoprotein consists of four exons and gives rise to a spliced mRNA species of 1-95 kb (Stinski et al., 1983; Stenberg et al., 1984; Akrigg et al., 1985). In all CMV species the IE polypeptides probably act as transcriptional transactivators of subsequent viral gene expression (Everett, 1984; O'Hare & Hayward, 1984; Spaete & Mocarski, 1985; Koszinowski et al., 1986; M. Pizzorno, P. O'Hare, L. Sha, R. L. LaFemina & G. S. Hayward, unpublished results).

In non-permissive cells, blocks to progression of the lytic cycle could occur at any of the steps between adsorption and virion production, although restrictions at the IE transcriptional level and DE post-transcriptional level are the only ones that have been described in any detail so far. In rodent fibroblasts, both HCMV (Towne) and simian CMV (SCMV) (Colburn) IE proteins are overproduced and DE transcripts accumulate in the nucleus, but no significant amounts of DE and late polypeptides are produced and no viral DNA synthesis occurs (Jeang et al., 1982; DeMarchi, 1983; Jeang, 1984; Geballe et al., 1986). A second type of block occurs in human teratocarcinoma stem cells where HCMV (Towne) IE transcripts fail to accumulate (LaFemina & Hayward, 1986; Nelson & Groudine, 1986), but subsequent retinoic acid-induced cell differentiation results in complete IE viral gene expression, DNA replication and release of progeny virions (Gönczöl et al., 1984, 1985; LaFemina & Hayward, 1986). In contrast, SCMV (Colburn) infection of human teratocarcinoma cell cultures is fully permissive for both IE gene expression and DNA replication even in the undifferentiated stem cells (LaFemina & Hayward, 1986). The change in status of the teratocarcinoma cells for HCMV after differentiation bears at least a superficial resemblance to the situation in vivo with murine CMV (MCMV) in which differentiation of monocytes to macrophages leads to reactivation of latent infection (Dutko & Oldstone, 1981).

DNA hybridization studies show that the genomes of HCMV (Towne) and the Colburn strain of African green monkey SCMV have several colinear homologous loci (Jeang, 1984) and this similarity is extended to the protein level in that the two species express many counterpart polypeptides, some of which are antigenically related (Gibson, 1983). However, SCMV and MCMV DNA molecules do not display the same typical inverting L/S type of genome structure as HCMV, but possess a single isomer, linear genomic organization (LaFemina & Hayward, 1980; Jeang & Hayward, 1983; Eberling et al., 1983; Mercer et al., 1983). Although the structural genome arrangement of SCMV and MCMV differs from that of HCMV, IE gene expression is also confined to a single major colinear locus at 0-71 to 0-73 map units for SCMV.
CMV expression in non-permissive cells

and 0.77 to 0.81 map units for MCMV (Jeang et al., 1982, 1984; Marks et al., 1985; Keil et al., 1984). The apparent Mr of both the SCMV IE94 and MCMV IE98 polypeptides are significantly larger than that of HCMV IE68 (Jeang & Gibson, 1980; Keil et al., 1985), but the genes encoding the HCMV IE68 polypeptide and the SCMV IE94 polypeptide are remarkably similar in their intron/exon structure and maintain homologous protein domains despite the size differences (Jeang et al., 1984; Jeang, 1984; Stenberg et al., 1984).

The present studies were directed at further understanding the interactions of mammalian CMV species with cultured cells. To examine whether or not repression of CMV major IE gene expression is a generalized property of transformed cells and to ask in what other cell types the differences between HCMV and SCMV IE gene expression might also be manifested, we have carried out additional analyses of the level of HCMV, SCMV and MCMV expression in infected cultures over a large range of cell types from a variety of host species. We show that HCMV infection of non-permissive BALB/c-3T3 cells results in expression of an altered HCMV IE68 polypeptide, and that infection of F9, Ltk⁻, Vero, NBE and HL60 cells even at high m.o.i. fails to yield significant levels of IE68 synthesis. In contrast, synthesis of the SCMV IE94 polypeptide occurs in many cell lines that do not express HCMV IE68. Both SCMV and MCMV also display a much wider permissive host cell range than HCMV, with viral genome replication being detectable in some transformed cell lines and across species barriers.

METHODS

Cells and virus. The sources of the cell lines used in these studies were as follows: 293 human embryonic kidney cell line expressing adenovirus E1A and E1B genes, Dr G. Ketter, Department of Biology, Johns Hopkins University; NBE human fibroblasts transformed by simian virus 40 (SV40), Dr S. Rhode III, Eppley Institute, University of Nebraska, Omaha; NT2/D1 human teratocarcinoma cells, Dr P. Andrews, Wistar Institute, Philadelphia, Pa.; HL60 human promyelocytic leukaemia cells, Dr C. Civan, Johns Hopkins University Oncology Center; 142 tk⁻ human osteosarcoma tumour-derived cell line, Dr F. Graham, University of Ontario, Hamilton, Canada; MG63, human osteosarcoma tumour cell-derived cell line, American Type Culture Collection; FOS human megakaryocyte-like cells, Dr D. Morgan, Hahnemann University, Philadelphia, Pa.; PBL primary lymphocytes from HCMV seronegative donors, Johns Hopkins University Hemapheresis Center; BALB/c-3T3 established murine cells, Dr T. Kelly Jr, Department of Molecular Genetics, Johns Hopkins University School of Medicine; Ltk⁻ transformed murine cells, Dr S. Silverstein, Columbia University School of Medicine, N.Y.; P9 murine teratocarcinoma cells and PYS murine transformed parietal yolk sac cells, Dr J. Shaper, Johns Hopkins University Oncology Center; MEF murine embryonic kidney fibroblasts, Drs W. Burns and S. Sanford, Johns Hopkins University Oncology Center; AGMKF African green monkey kidney fibroblasts, Whittaker, M.A. Bioproducts, Walkersville, Md.; Vero-established AGMK cells, American Type Culture Collection. HF cells are early passage secondary cultures prepared from diploid human foetal skin fibroblasts.

Cells cultured as monolayers were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum (FCS, Hyclone). Suspension cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS for HL60 and primary lymphocyte cultures. Stock cultures of HCMV Towne strain, SCMV Colburn strain and MCMV Smith strain, (provided by Drs K. Burns and S. Sanford) were passaged at low m.o.i, to preclude the appearance of defective viruses. HCMV and SCMV were grown on HF cells and MCMV at high titres, fresh stocks of supernatant virions were used in each experiment.

Extraction of intracellular and virion DNA. The infected cell DNA samples used in this study (25 cm² dishes) were harvested by scraping, and processed following cell lysis, Pronase digestion and phenol/chloroform extraction as described by LaFemina & Hayward (1983). The m.o.i. for these infections ranged from approximately 20 p.f.u./cell for DNA labelling to 50 p.f.u./cell for protein labelling. Because of the difficulties in maintaining frozen CMV stocks at high titres, fresh stocks of supernatant virions were used in each experiment.

All experiments which required either ³²P or [³⁵S]methionine were conducted in phosphate-free or methionine-free DMEM (Gibco) containing 10% dialysed FCS (DFCS) added at the time of addition of the radiolabel as previously described (LaFemina & Hayward, 1983). The m.o.i. for these infections ranged from approximately 20 p.f.u./cell for DNA labelling to 50 p.f.u./cell for protein labelling. Because of the difficulties in maintaining frozen CMV stocks at high titres, fresh stocks of supernatant virions were used in each experiment.

Under these conditions, infected HF cells processed before 24 h post-infection did not contain detectable levels of...
newly synthesized viral DNA. Samples collected from 48 to 96 h contained replicative forms lacking terminal restriction fragments and samples extracted beyond 96 h contained L and S terminal restriction fragments, demonstrating that viral DNA maturation had occurred (LaFemina & Hayward, 1983). SCMV and MCMV displayed a slightly faster replicative cycle than HCMV such that mature genomes could be detected by 96 h after infection.

**Protein labelling, SDS–PAGE and tryptic peptide analysis.** For the detection of the CMV IE polypeptides, cells were infected in the presence of 50 μg/ml cycloheximide (Sigma, C 6255) for 12 to 18 h followed by incubation for 3 h in methionine-free DMEM supplemented with 10% DFCS and 25 μCi/ml [³⁵S]methionine. Infected cell samples were processed and SDS–PAGE was performed as described in LaFemina & Hayward (1983). The procedure used for the analysis of tryptic peptide cleavage products of the IE68 protein was that of Weiner & Gibson (1983). Cell extracts were electrophoresed through a 10% SDS–polyacrylamide gel, after which the gel was dried for autoradiography. After locating the radiolabelled IE68 band, the appropriate region was excised from the gel and rehydrated, then the polypeptide was eluted and digested to completion with trypsin ( Worthington). The resulting tryptic peptides were spotted on to pre-coated cellulose plates (EM Reagents) and were separated initially by electrophoresis (acetic acid 78 : formic acid 25 : water 897 pH 1.9) and subsequently by chromatography (butanol 75 : pyridine 50 : acetic acid 15 : water 60).

**Retinoic acid-induced cell differentiation.** The F9 murine teratocarcinoma stem cell line was cultured in DMEM containing 10% FCS and confluent monolayers were subcultured by trypsinization and dilution (1:50). For retinoic acid-induced cell differentiation, cells were trypsinized, diluted (1:25) and cultured in DMEM containing 10% FCS and 1 μM-retinoic acid (Sigma, R 2625) for 4 days prior to infection. In some experiments F9 cells were differentiated further by the addition of 1 mM-dibutyryl cyclic AMP (dbcAMP, Sigma, D 0627; Strickland et al., 1980). Retinoic acid and dbcAMP were maintained in the media throughout the course of the infection and radiolabelling.

**RNA dot blot analysis.** Cells cultured in 75 cm² dishes were infected with HCMV (Towne) under similar conditions to those used in the polypeptide labelling experiments, using an m.o.i. of 50 p.f.u./cell in the presence of cycloheximide (50 μg/ml for 16 h). RNA was isolated following cell lysis with guanidinium isothiocyanate and centrifugation through 5-7 M-CsCl as described by Chirgwin et al. (1979). Infected cell RNA was transferred to nitrocellulose by filtration through a 96-well dot blot apparatus. The procedures described by Thomas (1980) were used for prehybridization, hybridization and filter washing. The hybridization probe was prepared by nick translation of pRL42 plasmid DNA, which contains a BamHI (0-740 map unit) to HindIII (0-766 map unit) subfragment of HCMV (Towne) BamHI J and includes the coding region for the IE68 polypeptide. Confirmation that equivalent amounts of RNA (2 to 4 μg) from the various cell types used in these assays was obtained by methylene blue staining of the nitrocellulose filters after hybridization.

**Immunofluorescence.** Confluent monolayers of 2 × 10⁵ cells cultured in two-well slide culture chambers were transfected with 2.0 μg of supercoiled pRL103 plasmid DNA containing HCMV (Towne) HindIII C (0-675 to 0-766 map units) by the standard calcium phosphate technique (Middleton et al., 1982). At 48 h after transfection or 18 h after infection, cells were fixed in absolute methanol at -20 °C for 5 min. Cells were stained at 37 °C for 30 min, with either the E3 monoclonal antibody (Goldstein et al., 1982; Stinski et al., 1983) or a commercial anti-CMV IE monoclonal antibody (Biotec), followed by incubation with fluorescein isothiocyanate-labelled goat anti-mouse antibody, and were then examined under oil immersion with a Leitz epifluorescence u.v. microscope. Photographs were taken on 125 ASA PLUS-X film using a × 40 objective and narrow band fluorescein isothiocyanate filters.

Chloramphenicol acetyltransferase (CAT) assays. Confluent monolayers of 5 × 10⁵ cells grown in six-well dishes were transfected with 24 μg of supercoiled DNA by the calcium phosphate precipitation procedure as described by O'Hare & Hayward (1984). After 4 h the cells were treated with 1 ml of 15% glycerol in DMEM without serum for 1 min. The cultures were then washed with calcium- and magnesium-free phosphate-buffered saline (PBS-A) and further incubated for an additional 44 h in DMEM with 10% FCS. Cell extracts were prepared and CAT assays were performed as described by Gorman et al. (1982) and modified by O'Hare & Hayward (1984).

**RESULTS**

**Characterization of the IE68 polypeptide synthesized in non-permissive BALB/c-3T3 cells**

Previous studies showed that following HCMV (Towne) infection in the presence of cycloheximide and subsequent reversal (i.e. under IE conditions) non-permissive BALB/c-3T3 cells overproduced a novel polypeptide of Mr 68K. To determine whether or not the 68K polypeptide produced in mouse cells was phosphorylated, BALB/c-3T3 and HF cells were infected under IE cycloheximide reversal conditions in the presence of [³⁵S]methionine or ³²P. Analysis of extracts of both infected cell types revealed an abundant methionine-labelled 68K
Fig. 1. The major HCMV IE68 polypeptide is phosphorylated in non-permissive mouse cells. (a) Non-permissive BALB/c-3T3 and (b) permissive HF cell cultures were both infected at an m.o.i. of 20 in the presence of 50 μg/ml cycloheximide for 12 h. The cells were then washed with phosphate-buffered saline (reversal) and the IE polypeptides were labelled with either [35S]methionine (lanes 1 and 2) or [32P]i (lanes 3 and 4) for 3 h. Mock-infected (lanes 1 and 3) and HCMV-infected (lanes 2 and 4) IE polypeptides were separated by electrophoresis through 10% SDS-polyacrylamide gels. The position of the HCMV IE68 polypeptide is indicated by an arrow in the autoradiographs.

polypeptide that was not detected in mock-infected cells and this species also proved to be the major phosphate-labelled protein present in both the permissive and non-permissive cell extracts (Fig. 1).

Further evidence that the 68K polypeptide synthesized in infected mouse cells was indeed equivalent to the authentic viral IE68 protein from HF cells was obtained by two-dimensional analysis of tryptic peptide cleavage products. The 35S-labelled infected cell polypeptides were overproduced by cycloheximide reversal and the major 68K bands were isolated by preparative gel electrophoresis (Fig. 2). Both 68K bands gave similar tryptic peptide cleavage patterns, with
Fig. 2. Two-dimensional separation of [35S]methionine-labelled HCMV IE68 tryptic peptide cleavage products shows the presence of a novel peptide in IE68 synthesized in mouse cells. (a) The [35S]methionine-labelled HCMV polypeptides synthesized in infected BALB/c-3T3 or HF cells after cycloheximide reversal were separated from other viral and cellular proteins by preparative SDS-polyacrylamide gel electrophoresis. (b) The 68K polypeptide bands were extracted from the gel as described by Weiner & Gibson (1983) and digested to completion with trypsin. The resulting tryptic peptides were separated by electrophoresis (direction indicated by − and +) and chromatography (direction indicated by the thin arrow). The major [35S]methionine-containing peptide that was detected in the mouse cell IE68 protein but was barely visible in the human cell IE68 protein is indicated by the thick arrow.
the exception that the mouse cell protein band produced at least one major tryptic peptide (arrowed) that was underrepresented by approximately 20-fold in the control IE68 band extracted from HCMV-infected human cells. A very similar result was obtained in a second experiment of this type and all of the more intensely labelled tryptic peptides (including the variable one) were absent following similar processing of protein extracted from the 68K region in the gel lanes containing mock-infected cell polypeptides (not shown). Similar separation of tryptic peptides from the isolated 32P-labelled 68K proteins of infected BALB/c-3T3 and HF cells resulted in identical but much simpler patterns from both host cell types (not shown). Therefore, the variable peptide was not included amongst the phosphorylated tryptic peptides of IE68 and there was no evidence that the phosphorylation patterns differed in permissive and non-permissive cells.

**Differences between HCMV and SCMV IE protein expression in transformed mammalian cell lines**

We have previously demonstrated that synthesis of the HCMV IE68 protein is not detectable by our assays following infection of the 293 adenovirus DNA-transformed human cell line or the NT2/D1 human teratocarcinoma stem cell line, although both are permissive for SCMV IE94 protein expression and viral DNA replication (LaFemina & Hayward, 1986). Similar results were obtained in the current studies after infection of the SV40-transformed, large T antigen positive NBE human cell line (not shown) and in Vero cells (Fig. 3). In contrast, we observed that transformed mouse F9 stem cells and the PYS derivative did not synthesize detectable amounts of either the HCMV IE68 or SCMV IE94 proteins after high multiplicity infection (Fig. 4). Like the human teratocarcinoma cells, the permissiveness of F9 stem cells could be altered after differentiation by retinoic acid treatment, but only for synthesis of the SCMV IE94 protein and not HCMV IE68 (Fig. 4). Further differentiation induced by dbcAMP treatment of F9 cells still did not result in HCMV IE68 synthesis (Fig. 4). We were unable to detect replication of either the HCMV or SCMV genomes following infection of any of these cell lines in the presence or absence of retinoic acid (not shown).

We have previously been unable to detect accumulation of steady-state IE68 mRNA following infection of human teratocarcinoma stem cells with HCMV in the presence of cycloheximide (LaFemina & Hayward, 1986), which implied that the block to IE68 expression occurred at the level of mRNA transcription or stabilization. Several of the non-permissive transformed cell lines used in these present studies were similarly examined by an RNA dot blot hybridization procedure for their ability to express IE68 mRNA. Using nick-translated plasmid pRL42 DNA (which contains the coding region for the HCMV IE68 protein) as probe, little if any IE68-specific RNA was detected in either 293, Vero or F9 cells (Fig. 5). Therefore, apparently these cells were also blocked at the mRNA level. In contrast, abundant HCMV IE68 RNA was produced in infected permissive HF cells.

**Differences between HCMV and SCMV IE protein expression in human lymphocyte cell types**

Infection of primary human lymphocytes in culture with clinical isolates of HCMV has been reported to yield a low fraction of positive nuclei by immunofluorescence assay with monoclonal antibody against the IE68 protein (Rice et al., 1984). However, laboratory-adapted strains of HCMV which grow to high titres in fibroblasts are generally negative in these assays. We were unable to detect HCMV (Towne) IE68 polypeptide synthesis by [35S]methionine radiolabelling and SDS-PAGE in either PBL (Fig. 6a), or in the FOS cell line (Morgan & Brodsky, 1985) or the human HL60 cell line (Reitsma et al., 1983) either before or after 12-O-tetradecanoylphorbol 13-acetate- or retinoic acid-induced differentiation. Nevertheless, low level synthesis of the SCMV (Colburn) IE94 polypeptide was detected following infection of both PBL (Fig. 6a) and the FOS cell line (not shown), although not in HL60 cells (not shown). Despite the reports that fresh clinical isolates of HCMV express the IE68 protein in a small proportion of PBL as assayed by immunofluorescence, we were also unable to detect a 35S-labelled IE68 protein band under IE conditions after infection of the PBL culture with a recent clinical HCMV isolate (JHH 431) at fourth passage in HF cells (lanes 3 in Fig. 6a). We also asked whether SCMV DNA replication
could be detected in the PBL cultures which permitted synthesis of the IE94 protein. Parallel HF and PBL cultures were infected, and newly synthesized $^{32}\text{P}$-labelled DNA was examined for the presence of virus-specific restriction enzyme fragments. However, we were unable to detect replication of the SCMV genome following infection of PBL cultures (Fig. 6b), even though the cells remained viable as evidenced by $^{32}\text{P}$ incorporation into host cell DNA. Not surprisingly the lymphocytes were also non-permissive for HCMV DNA replication.

Detection of input HCMV genomes in the nuclei of infected Vero cells

Since the type of non-permissiveness found in transformed cell lines could potentially be associated with a lack of virion receptors on the cell surface, it was important that we address this question for HCMV infection in a cell line that did not permit synthesis of the HCMV IE68 protein. Therefore, we infected BALB/c-3T3, HF and Vero cells with purified virions the DNA of which had been radiolabelled during replication in HF cells in the presence of $^{32}\text{P}$. As a measure of virus entry, cell nuclei were isolated at 16 h after infection by NP40 lysis and centrifugation and found to contain the parental $^{32}\text{P}$-labelled HCMV DNA (Fig. 7a).

Fig. 3. HCMV IE68 is not synthesized in infected Vero cells. BALB/c-3T3 (a), human diploid fibroblasts (b) and Vero (c) cells were mock-infected (lanes 1) or infected with SCMV (lanes 2) or HCMV (lanes 3). The locations of the IE68 and IE94 polypeptides are indicated by arrows.
**CMV expression in non-permissive cells**

(a) CMV expression in non-permissive cells

(b) 1 2 3 1 2 RA
dbcAMP

(c) 3 1 2 3 1 2 3

Fig. 4. Differentiation of murine F9 teratocarcinoma cells. Infections of untreated HF cells (a) and the transformed PYS cells (c) are shown with F9 cells (b) before and after treatment with retinoic acid (RA) alone or in conjunction with dbcAMP. Polypeptides from uninfected (lanes 1), HCMV (Towne)-infected (lanes 2) and SCMV (Colburn)-infected (lanes 3) cells were labelled with [35S]methionine and analysed after SDS–PAGE and fluorography. The locations of SCMV IE94 and HCMV IE68 polypeptides are indicated.

Furthermore, although the proportion of input 32P-labelled DNA recovered in the nuclear fraction of infected Vero cells was somewhat less than that in the permissive HF cells it was equal to that of the input SCMV genomes in all three cases. Thus, although Vero cells do not express IE68, an adequate amount of HCMV DNA does enter the nuclei. This result is consistent with the observations of Einhorn et al. (1982) who demonstrated the presence of HCMV receptors on the surface of various non-permissive cell types including Vero cells.
Fig. 5. The block to HCMV IE68 expression in transformed cells occurs at the transcriptional level. RNA produced in mock-infected HF- and HCMV-infected HF, 293, F9 and Vero cells in the presence of a cycloheximide block was extracted and fixed onto nitrocellulose filters. Duplicate samples of 2 to 4 μg of RNA were hybridized with a 32P-labelled pRL42 DNA probe which contains the coding region for HCMV IE68 as a BamHI to HindIII fragment (0-740 to 0-766 map units) within this plasmid.

Analysis of the BamHI restriction digest pattern of input 32P-labelled HCMV DNA at 16 h after infection of permissive HF cells revealed that the L terminus represented by the BamHI Z fragment was decreased in intensity relative to the similarly sized BamHI Y fragment (Fig. 7a). This result suggested that a significant fraction of the input genomes had generated covalently linked termini prior to DNA replication, similar to an observation reported for MCMV (Marks & Spector, 1984). In contrast, the parental BamHI Z fragment present in the digest from infected BALB/c-3T3 cells was barely reduced in intensity implying that few if any molecules displayed linked L/S termini. The formation of a small percentage of joined termini in HCMV is difficult to document clearly because the resulting circular or concatemeric structures merely increase the molarity of already existing L/S joint restriction fragments. However, this problem was avoided with SCMV, where linkage of the left and right termini generates a completely new restriction fragment which was easily detected in the infected HF sample (Fig. 7b, A*). Therefore, the total absence of this novel fragment in the recovered input DNA from SCMV-infected BALB/c-3T3 cells provided further support for our earlier conclusion that prereplicative intermediates with joined termini do not form in non-permissive mouse cells (LaFemina & Hayward, 1983).

SCMV and MCMV have a broader host-range than HCMV

An overall summary of IE gene expression and viral DNA replication in a variety of primary, established and transformed rodent, simian and human cell cultures infected with HCMV, SCMV or MCMV is given in Table 1. Sufficient HCMV IE68 synthesis to give a 35S-labelled band by PAGE after reversal of a cycloheximide block was never observed in transformed cell lines of human, monkey or mouse origin (except in the 143 and MG63 human osteosarcoma cell lines). Although IE68 expression did occur in all diploid fibroblasts tested, evidence for HCMV DNA replication obtained by 32P incorporation into the characteristic newly synthesized viral DNA fragments was not detected in secondary diploid kidney fibroblast cell cultures of African green, cynomolgus or rhesus monkey origin, nor in MEF. In contrast, SCMV displayed a much wider host cell range, with IE94 expression being undetectable only in transformed mouse cells such as the Ltk- and F9 stem cell lines and in human HL60 cells. Furthermore, SCMV DNA replication was observed even in transformed human 293 cells, the NT2/D1 teratocarcinoma stem cell line and in Vero cells, although not in any of the mouse cell cultures.

Extension of these studies to MCMV revealed that it also displayed an expanded host cell range in contrast to the tightly restricted host cell range of HCMV. Although we routinely
CMV expression in non-permissive cells

Fig. 6. PBL express SCMV IE94 but are non-permissive for SCMV DNA replication. (a) Human PBL and HF cells were infected with either the 431 clinical isolate of HCMV (lanes 1), the established Towne strain of HCMV (lanes 2) or SCMV Colburn (lanes 3) or mock-infected (M) in the presence of cycloheximide, followed by reversal in the presence of [35S]methionine. Newly synthesized polypeptides were separated by electrophoresis in 10% SDS-polyacrylamide gels and visualized by fluorography. The location of HCMV IE68 and SCMV IE94 polypeptides are indicated by arrows. (b) Human PBL and HF cells were infected for 16 h followed by 32P radiolabelling of newly synthesized DNA as described in Methods. The autoradiograph shows the agarose gel electrophoresis profiles of EcoRI restriction enzyme cleavage products from newly synthesized total cellular DNA from these cultures. The arrows denote the locations of mitochondrial DNA fragments.

passaged MCMV (Smith) in secondary cultures of MEF cells, infection of the established BALB/c-3T3 cell line proved to result in easily detectable synthesis of 32P-labelled MCMV DNA and expression of the MCMV major IE98 protein (which under our electrophoresis conditions appeared slightly larger than that reported by Keil et al., 1985). Surprisingly, the transformed mouse Ltk− cell line, which failed to permit expression of either IE68 or IE94, also proved to contain a block to MCMV IE98 (Fig. 8a). The F9 teratocarcinoma cells proved not to be directly informative for study of MCMV IE gene expression because they expressed a cellular 98K polypeptide. However, infection of the F9 cells resulted in low but detectable levels of viral DNA replication against a high background of host cell DNA synthesis (data not shown) and we
Fig. 7. Entry and terminal joining of viral DNA in permissive compared to non-permissive cells. (a) Parental input HCMV (Towne) DNA does enter the nucleus in infected non-permissive Vero cells. BALB/c-3T3, HF and Vero cells were infected with $^{32}$P-labelled parental HCMV (lanes 1) or SCMV (lanes 2) for 16 h at which time nuclei were collected by NP40 lysis at 0 °C followed by centrifugation. Nuclear DNA was extracted and examined for the presence of input parental radiolabelled virion DNA by BamHI digestion and electrophoresis through 1% agarose gels. The presence of the SCMV BamHI joint restriction fragment is indicated (closed arrows A*) and also the HCMV L terminus BamHI-Z fragment (closed arrows) which displays decreased relative molarity presumably related to its joining with the terminal BamHI-L and -N fragments from the S segment. (b) Unambiguous formation of novel joint fragments during SCMV replication. Newly synthesized $^{32}$P-labelled DNA was extracted from HF cells infected with SCMV for 72 (lane 1) or 120 (lane 2) h and compared with SCMV DNA extracted from supernatant progeny virions (lane 3). The infected cell samples contain BamHI-A* species whereas released virions do not. The sizes of the SCMV BamHI terminal fragments are given. The open arrows denote high $M_r$, cellular DNA.

presume that IE98 expression must have occurred. Treatment of F9 cells with retinoic acid reduced the amount of host DNA synthesis such that replication of the input MCMV genomes was readily detectable (Fig. 8b). Infected secondary cultures of diploid AGMKF cells (Fig. 8b)
### Table 1. Summary of cytomegalovirus IE expression and DNA replication in various cell types

<table>
<thead>
<tr>
<th>Host cell infected</th>
<th>HCMV (Towne)</th>
<th>SCMV (Colburn)</th>
<th>MCMV (Smith)</th>
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<tr>
<td></td>
<td>IE*</td>
<td>DNA†</td>
<td>IE*</td>
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<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>+++++</td>
<td>+++++</td>
<td>+++++</td>
</tr>
<tr>
<td>NT2/D1†</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NT2/D1 + RA†</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>143</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
<td>MG63</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>PBL</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>F05</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>HL60</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>NBE</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>293‡</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Simian</strong></td>
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<td></td>
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<tr>
<td>AGMKF</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CyMF</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>RhMF</td>
<td>++</td>
<td>ND</td>
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</tr>
<tr>
<td>Vero</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CVI</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>ND</td>
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<tr>
<td><strong>Murine</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MEF</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BALB/c-3T3</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F41A1</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F9</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>F9 + RA</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>PYS</td>
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<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Ltk-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
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</table>

* Relative level of synthesis of [35S]methionine-labelled HCMV IE68, SCMV IE94, or MCMV IE98 proteins after cycloheximide reversal. HCMV or SCMV infection in fully permissive HF cells and MCMV in MEF cells defines the +++++ level; 30% to 70% of that level of synthesis is indicated by ++++; 10% to 30% by +++; 3% to 10% by + and less than 3% by -. 
† Relative level of newly synthesized 32P-labelled viral DNA. The symbols used represent the same range of values used in *
‡ Data from LaFemina & Hayward (1986).
§ ND, Not done.

and HF cells (data not shown) both expressed IE98 and gave low level synthesis of MCMV DNA.

In summary, HF cells lay at one end of the spectrum, permitting both IE gene expression and viral DNA replication for all three virus species (HCMV, SCMV and MCMV). At the other extreme, mouse Ltk- cells were not permissive for IE gene expression with any of these viruses (and hence also failed to synthesize viral DNA). Diploid fibroblasts from all three host species groups were permissive for IE gene expression for all three CMV types, but showed a clear hierarchy for DNA replication, with MCMV DNA synthesis occurring in mouse, monkey and human cells, whereas SCMV DNA synthesis was limited to monkey and human cells, and HCMV DNA synthesis was restricted to human cells only.

**HCMV IE gene expression in non-permissive cells following DNA transfection**

Considering both the structural similarities and strong constitutive promoters of IE68 and IE94, it was somewhat surprising that we were unable to detect IE68 mRNA or polypeptide synthesis in infected NT2/D1, 293 or NBE cells, whereas these transformed human cell lines did not display any blocks to IE94 gene expression. Therefore, to ask whether these types of cells, or Vero cells, might show an intrinsic and selective block to HCMV IE68 transcription, we...
examined transient expression of hybrid genes under the control of the HCMV IE68 promoter/enhancer sequences after DNA transfection. The plasmids pWT760-CAT (Stinski & Roehr, 1985) and pSV2-CAT contain a bacterial CAT gene under the transcriptional control of either the HCMV IE68 gene promoter or the early promoter/enhancer region of SV40. These plasmids were introduced into several representative HCMV permissive and non-permissive cells by the calcium precipitation procedure and cell extracts were examined for CAT activity.
CMV expression in non-permissive cells

(a) CMV expression in non-permissive cells

(b) 369

760 SV2 760 SV2 760 SV2

HF 293 Vero

Fig. 9. DNA transfection assays demonstrate that there is no intrinsic block to HCMV IE gene expression in transformed cells. (a) Expression of CAT enzyme from a hybrid HCMV IE promoter/enhancer construct. Supercoiled pWT760-CAT or pSV2-CAT DNA (2 μg) was precipitated by calcium phosphate and added directly to permissive HF and non-permissive 293 or Vero cells. Cell extracts were made at 48 h and were used in standard CAT assays. (b) Expression of the HCMV intact IE68 gene in 293 cells following transfection of pRL103 plasmid DNA containing the HCMV (Towne), HindIII-C fragment (0.675 to 0.766 map units). At 48 h cells were fixed in absolute methanol and synthesis of the IE68 protein was detected in the cell nuclei by immunofluorescence using a monoclonal antibody against the HCMV IE nuclear antigen.

Table 2. Comparison of HCMV IE68 gene expression after infection or DNA transfection in different cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Virus infection (m.o.i. 20)</th>
<th>DNA transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCMV DNA synthesis</td>
<td>Input DNA in nucleus</td>
</tr>
<tr>
<td></td>
<td>CHX/Rev*</td>
<td>IE68 protein</td>
</tr>
<tr>
<td>HF</td>
<td>++++†‡</td>
<td>+++</td>
</tr>
<tr>
<td>MG63</td>
<td>++</td>
<td>ND§</td>
</tr>
<tr>
<td>BALB-c/3T3</td>
<td>++++</td>
<td>ND</td>
</tr>
<tr>
<td>293</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vero</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Cycloheximide and reversal.
† IF, immunofluorescence.
‡ IF, ++++, ++++, ++*, relative levels of efficiency of protein, RNA and DNA synthesis or DNA entry.
§ ND, Not done.

after 48 h. The pWT760-CAT DNA gave high level basal expression in 293 and Vero cells but only low level expression in HF cells (Fig. 9a). All three cell types displayed more abundant CAT expression following transfection with pWT760-CAT DNA than with pSV2-CAT DNA. The known low efficiency of DNA transfection into HF cells presumably accounts for the low CAT levels obtained in the permissive control. In contrast, 293 cells in particular are known to be highly efficient for DNA transfection (Alwine, 1985).

We next asked whether we could detect expression of the intact IE68 polypeptide itself in non-permissive transformed cells following short-term DNA transfection. For these studies we used the 20 kb plasmid pRL103 (HindIII C) which includes the complete major IE gene region from HCMV (Towne). The IE68 nuclear antigen proved to be readily detected in the nucleus of 5% to 15% of transfected 293 cells, in 1% to 2% of Vero cells and in approximately 0.2% of HF, BALB/c-3T3 and MG63 cells by immunofluorescence using the E3 monoclonal antibody (Fig. 9b and Table 2). Again, this result appears to reflect only the DNA transfection efficiency properties of the cells and was independent of their permissive or non-permissive character for
expression of the IE68 protein after virus infection. Thus the block to HCMV IE68 gene expression in transformed cells was not evident following the introduction of plasmid DNA containing either the intact HCMV IE68 gene or hybrid CAT genes under the control of the HCMV IE promoter.

A small proportion of cells in the non-permissive transformed cell cultures escape the block to IE expression

When the E3 monoclonal antibody assay was employed to monitor IE68 expression at 18 h after HCMV infection in the absence of cycloheximide treatment (at our standard high m.o.i. of 20 p.f.u./cell), strong nuclear fluorescence was observed in 1% to 2% of Vero cells and in approximately 0.2% of the 293 cells, compared to 20% in MG63 cells and greater than 95% in HF cells (Table 2). At an even higher m.o.i. (greater than 100 p.f.u./cell) the proportion of positive nuclei was increased to 5% in Vero cells and 1% in 293 cells, whereas at a lower m.o.i. (i.e. 1 p.f.u./cell), which was still sufficient to give 30% to 50% positive nuclei in HF cells, less than 0.2% positives were obtained in Vero cells. Therefore, these results further support the idea that there is no absolute block to IE68 expression in the transformed non-permissive cells and that apparently once some threshold is reached the level of IE polypeptide produced in an individual cell may be just as high as in a standard permissive cell. However, at a given m.o.i. the proportion of cells that reach this threshold is extremely small in Vero or 293 cell cultures compared to HF cells.

DISCUSSION

The additional [35S]methionine-labelled tryptic peptide present in the HCMV IE68 protein made in BALB/c-3T3 cells was almost totally absent from the equivalent protein synthesized in permissive human fibroblasts. This result could be explained by an additional protein processing event that occurs only in permissive cells. Alternatively, the 1.95 kb IE68 mRNA transcript may be spliced differently in mouse cells compared to human fibroblasts, with the altered mRNA encoding the additional or substituting tryptic peptide. A trace of the extra peptide was also found in the HF cell IE68 protein sample and therefore we presume that a low level of the aberrant protein processing or mRNA splicing must also occur in permissive cells. Since the apparent lack of proper functioning of IE68 in non-permissive BALB/c-3T3 cells might potentially be related to the presence of this novel form of the IE68 protein, the origin of the extra peptide and its role in affecting the function of IE68 in non-permissive cells warrants further investigation.

The outcome of HCMV infection is extremely host cell-dependent. Both rodent and simian fibroblasts allow abundant expression of the HCMV IE68 polypeptide, but we have been unable to detect any HCMV DNA replication in these cells. In contrast, IE68 mRNA expression was blocked at either the transcription initiation or mRNA stabilization level in several established transformed cell lines including those of human origin (Fig. 6; LaFemina & Hayward, 1986; Nelson & Groudine, 1986). This tight block was noted only following virus infections and not after introduction of plasmid DNA containing the intact IE68 gene or hybrid IE68 promoter/enhancer CAT constructs by DNA transfection protocols. Therefore, there is no intrinsic block to transcription of the IE68 gene in the non-permissive transformed cells. Since we know that HCMV DNA enters these cells efficiently after infection (Fig. 7), several questions arise regarding the nature of the virus-cell interaction. The repression of the HCMV IE68 gene in transformed cells following virus infection may be the result of autoregulation or of the introduction of a negative virion factor acting directly or indirectly on the IE promoter itself. Evidence has been presented for the existence of positive HCMV virion factors (Spaete & Mocarski, 1985; Stinski & Roehr, 1985) and it may be that these function properly only in permissive cells with aberrant regulation occurring in non-permissive cells. Alternatively, the repression may result from a more generalized host cell response to HCMV infections. Clearly, transcription factors in the host cell are of considerable importance and these may vary from cell type to cell type. We cannot conclude as yet whether infected HF cells have positive factors necessary for HCMV IE gene expression or are lacking in some repressor-like activities found in the non-permissive transformed cells.
In F9 cells, CAT target constructions containing the polyomavirus or simian virus 40 early promoter/enhancer region or the Moloney murine leukaemia virus long terminal repeat are expressed poorly even in short-term DNA transfection protocols (Linney et al., 1984; Amati, 1985; Sleigh & Lockett, 1985). However, we observed substantial basal IE68-CAT expression in both Vero and 293 cells in transient assays. Potentially, the DNA transfection protocols may introduce such high concentrations of DNA that limiting amounts of cellular repressor molecules are titrated out, allowing the HCMV IE gene to be expressed. In addition, other considerations such as the high efficiency of 293 cells for stabilizing input plasmid DNA may also be important (Alwine, 1985). Our preliminary experiments suggest that SCMV superinfection of non-permissive Ltk- cells that had previously received an IE94-CAT plasmid by transient DNA transfection results in shut-off of the high basal expression of IE94-CAT, indicating that a negative virion factor may indeed be a necessary component of the non-permissive interaction in infected cells (D. Gay & G. S. Hayward, unpublished data).

Two quite different trends emerged with regard to the cell types that allowed expression of CMV IE gene products compared to those that permitted viral DNA replication. At both levels, the highest degree of restriction was exhibited by HCMV, with SCMV displaying the broadest host range with regard to IE gene expression and MCMV the broadest with regard to DNA synthesis. In terms of IE gene expression, some unknown feature of transformed cell lines and teratocarcinoma stem cells compared to diploid fibroblasts or differentiated teratocarcinoma cells appeared to predominate over species-specific effects, whereas for viral DNA replication the species type of the host cell was the most important feature.

The significance of the very small percentage of cells in HCMV-infected non-permissive Vero and 293 cell cultures that did express IE68 detectable by the more sensitive immunofluorescence assay is not clear at present. Zerbini et al. (1985) have shown that the level of HCMV early antigen expression can be increased fourfold after heat shock treatment in infected Vero cells. It is neither unreasonable nor unprecedented for limiting cellular ‘repressor’ factors to be overcome at high m.o.i. or for a small proportion of defective virus particles (that may lack shut-off factors) to be present. We presume that additional criteria, including surface receptor and entry or adsorption problems, may hinder infection in other cell types, especially those of lymphoid origin (Einhorn et al., 1982).

We have previously suggested that differences in the organization of the upstream regulatory elements of the HCMV and SCMV IE promoters may influence the cell type-specific expression of IE genes (LaFemina & Hayward, 1986). Considerable DNA sequence homology occurs in the upstream promoter/regulatory sequences and 5' introns of the HCMV IE68 and SCMV IE94 genes (Thomsen et al., 1984; Jeang et al., 1987), although otherwise the protein products have little amino acid homology. Both HCMV and SCMV IE genes possess several nuclear factor I (NFI)-binding sites just beyond the enhancer region, but only SCMV contains an additional cluster of 20 tandemly repeated NFI-binding sites (at −550 to −1300 bp) (Rawlins et al., 1986; Hennighausen & Fleckenstein, 1986). Potentially, the presence of these tandemly repeated NFI-binding sites, together with multiple consensus CCAAT factor binding sites in the far upstream 5'-regulatory region may help to overcome some form of enhancer repression that occurs in transformed and teratocarcinoma stem cell lines, thus permitting SCMV IE94 protein synthesis in a variety of infected cell types in which HCMV IE68 is not expressed.

HCMV and MCMV are both believed to establish latent infections in vivo in lymphoid cells, most probably of the monocyte/macrophage lineage. MCMV can be reactivated after stimulation of monocyte differentiation in infected mice (Dutko & Oldstone, 1981) and infection with clinical isolates has been reported to alter the phenotypic characteristics of monocyte subpopulations from human PBL in culture (Rice et al., 1984). Our eventual goal is to be able to characterize these interactions of HCMV biochemically in cell culture. However, until high titre virus stocks of HCMV isolates with different target cell specificity than the ‘fibroblast-adapted’ laboratory strains or cell lines of appropriate susceptibility become available, the ability of SCMV (Colburn) to express the major IE gene product in human PBL cultures and the megakaryocyte-like FOS cell line may offer some promise for useful investigations.
We would like to thank the individuals listed in Methods for their gifts of cell lines and virus stocks. We thank Wade Gibson for advice and the use of equipment to perform the tryptic peptide cleavage studies, Alice Irmieri for assistance with the tryptic peptide studies, Jane Gimigliano for assistance with the immunofluorescence studies, Pamela Wright for photographic expertise and Phyllis Broughton for assistance in the preparation of this manuscript. These studies were funded by grants to G.S.H. from the DHEW (CA28473 and CA37314).

REFERENCES


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