Cloning and identification of regulatory gene UL76 of human cytomegalovirus

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The major immediate-early promoter/enhancer (MIEP, N1139 to M52) of human cytomegalovirus (HCMV) is regulated by cell type-specific transcriptional factors, its own MIE proteins (IE2p40, IE1p55, IE1p72 and IE2p86) as well as viral proteins pUL69, pUL82 and pUL84. To investigate the hypothesis that the regulation of HCMV MIEP is modulated by additional viral genes, HCMV (AD169) genomic sublibraries were constructed and in vitro transient co-transfection assays were performed to assess the ability of these sublibraries to modulate MIEP expression. In this study, enhancement of MIEP expression was exhibited by a number of sublibraries, from one of which a genomic clone was selected for augmentation of expression. Subcloning the insert fragment led to the identification of the responsible locus, UL76. To generate a UL76-specific antibody for immunodetection, the UL76 ORF was constructed as a histidine-tagged fusion protein that was produced in prokaryotic cells. A polyclonal antibody raised against the UL76 fusion protein immunoreacts with a protein of 38 kDa (pUL76) in UL76 ORF-transfected cells. Additionally, pUL76 is present in HCMV-infected cells at the immediate-early to late stages of the reproductive cycle. Characterized by its highly basic composition (predicted pl 11.6), a free form of pUL76 tagged with green fluorescent protein was found to localize exclusively to the nucleus. In this report, pUL76 is defined as a novel regulatory protein that modulates both activation and repression of gene expression, depending on the promoter context and the ratio of transfected effector DNA.

Introduction

Human cytomegalovirus (HCMV), a betaherpesvirus, is a ubiquitous pathogen known to cause severe foetal abnormalities and life-threatening infection in immunocompromised patients. Like other herpesviruses, HCMV assumes a lifelong latent but asymptomatic infection in normal healthy people once they acquire the virus. Reactivation of latent virus is generally attributed to the compromised immune system. Still, the detailed mechanism of HCMV reactivation remains poorly understood (reviewed in Britt & Alford, 1996; Sinclair & Sissons, 1996).

The mature HCMV virion is composed of a lipid bilayer envelope, a tegument and a capsid (Gibson, 1996). The tegument layer comprises a large number of proteins, particularly phosphoproteins, which have been demonstrated to be important for virus infectivity and gene transactivation (Liu & Stinski, 1992; Winkler et al., 1994; Baldick & Shenk, 1996). The capsid of the intact virion contains a 229 kbp dsDNA encoding at least 200 gene products (Chee et al., 1990; Cha et al., 1996). Expressed in a cascade mode during the reproductive cycle, HCMV genes are mainly classified into three groups, immediate-early (IE), early (E) and late (L) (reviewed in Mocarski, 1996). The major IE proteins (MIEs), encoded by the major immediate-early gene region (UL122–123), are the most abundant proteins produced at the IE stage. Recognized as promiscuous regulatory proteins, MIEs engage in the regulation of expression of a wide range of cellular genes and viral IE, E and L genes. They not only play essential roles in progression from the early to late stages of productive virus replication, but the MIEs may also be important in the initiation of virus reactivation (reviewed in Stenberg, 1996; Sinclair & Sissons, 1996).

The MIE regulatory region (N1139 to +52), designated MIEP in this study, encompasses complex arrangements including a basal promoter, a strong enhancer and a modulator (Meier & Stinski, 1996). An array of cellular transcriptional
facultators are responsible for either activation or repression through the MIEP. At present, seven HCMV-encoded proteins have been demonstrated to be involved in MIEP regulation during the reproductive HCMV life-cycle. The viral tegument proteins pUL69 (encoded by UL69) and pUL82 (also called the upper matrix phosphoprotein, pp71, encoded by UL82) have been shown to augment MIEP expression while accompanying the virus particle into the nucleus at the initial stage of infection (Liu & Stinski, 1992; Winkler et al., 1994; Winkler & Stamminger, 1996). The production of MIEs (IE1p55, IE1p72, IE2p86 and IE2p40) is manifest in positive or negative autoregulation (Baracchini et al., 1992; Lang & Stamminger, 1993; Macias & Stinski, 1993; Huang et al., 1994; Jenkins et al., 1994). Furthermore, an early–late-expressed protein, pUL84 (encoded by UL84), specifically enhances IE2p86-mediated repression of MIEP (Gebert et al., 1997). Conclusions summarized from previous reports indicate that the HCMV MIEP is modulated by proteins present at all stages, including virus components and IE, E and L proteins. Understanding MIEP regulation may be important for designing a therapeutic strategy to control HCMV infection.

A gene regulation paradigm is generally established by the assembly of basal transcriptional proteins, transcriptional factors, coactivators and corepressors, as well as DNA architectural proteins that alter chromatin structure (Kingston et al., 1996; Carey, 1998). Thus, we postulated the possibility that novel HCMV regulatory genes target the MIEP. In this report, we provide evidence that the MIEP is activated synergistically by certain combinatorial genomic clones in a transient cell culture co-transfection expression system. As we carried out the subcloning and evaluated promoter activity, a regulatory gene, UL76, and its encoded protein (pUL76) were identified. Furthermore, we demonstrated that pUL76 is a nucleus-bound protein and acts as a gene regulator with dual functions in activation and repression of the MIEP and other HCMV genes.

Methods

■ Virus and cells. A human embryonic lung cell line (HEL 299) and HCMV strain AD169 (VR-538) were obtained from the ATCC. HEL cells were maintained as recommended by the supplier. Cell passages 11–20 were used throughout the experiments.

■ Nucleotide and protein sequence analysis. DNA sequencing was performed to confirm the plasmid constructs whenever necessary. The GCG sequence analysis software (University of Wisconsin, USA) was used in the analysis of nucleotide and amino acid sequences. HCMV DNA sequence comparison was made to the complete genome of HCMV strain AD169 deposited in GenBank (accession number X17403; Chee et al., 1990).

■ Construction of an HCMV genomic library. Purification of HCMV DNA was based on a modification of an enzymatic digestion protocol. In brief, released HCMV particles were harvested from the supernatant of infected HEL cells 10 days after virus inoculation at an m.o.i. of 2–3. The supernatant containing extracellular virus was concentrated by centrifugation and the virus pellets were then resuspended in Tris-buffered saline. To eliminate cellular nucleic acid contamination, the virus suspension was digested with DNase I and RNase A (100 µg/ml). The virus particles were then ruptured in a lysis buffer containing 1% SDS, 0.5 mM EDTA and 300 µg/ml proteinase K. High molecular mass HCMV DNA was further purified from the lysate solution by repetitive phenol–chloroform extractions followed by several changes of dialysis against TE buffer. To prepare insert fragments for genomic library construction, HCMV DNA was partially digested with SstIII before being subjected to low-melting-point agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA). DNA fragments corresponding to 5–15 kb were excised from the gel. The trapped DNA was recovered by digesting the agarose with agarase and sized DNA inserts were ligated with BamHI-digested vector pBCK-CMV (Stratagene). Twelve clones were selected randomly from E. coli (XL-Blue MRF) culture transformants by the genomic ligation to make up one sublibrary combination.

■ Southern blot hybridization. One µg HCMV genomic DNA was digested with the indicated restriction endonuclease for 1 h before being separated electrophoretically in a 0.6% agarose gel in 1x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) running buffer at a constant voltage of 25 V for 18 h. The separated DNA fragments were transferred onto Hybond-N nylon membrane. Prehybridization and hybridization were carried out as described by Ausubel et al. (1987). The membrane was probed with a 32P-labelled, random primer-generated DNA fragment isolated from pCL77, as depicted in Fig. 3(a).

■ Reporter plasmid construction. Reporter plasmid pMIEP-CAT was created by ligating an HCMV MIEP –1139–+52 PvuII–Mspl fragment (nt 173670–174472) into PvuII-predigested pCAT-Basic. Beforehand, both insert and vector were blunt-ended by T4 DNA polymerase (Fig. 1a). Plasmid pMIEP-Luc was constructed by ligating MIEP into pGL3-Basic vector (Promega). Plasmid pGL3-promoter, containing the simian virus 40 (SV40) early gene promoter upstream of the luciferase gene, was obtained from Promega and is designated pSV40-EP in this paper.

Promoter fragments for UL54 and UL112 were generated by PCR amplification of HCMV AD169 genomic DNA catalysed by Pfu polymerase (Stratagene). Primers 5’ ATCGCTCGAGATCGAAGGCACGACGGTGTGTC and 5’ ATCGGAGCTCGATTCACAAGTTCAACCTGTTACAAGCA were used in PCR cycling to generate a UL54 promoter sequence of 445 bp (nt 80996–81440). The amplified product was cleaved by ScaI and XhoI, recognition sequences of which were incorporated into the primers, and the fragment was then cloned into pGL3-Basic. The resulting plasmid was designated pPrUL54. Primers 5’ ATCGGAAGCTTGTGATCCGACGAAAGGTTAACAAC and 5’ ATCGGCTAGACACGGCAACA CGCCTCGTCGGG were used in PCR amplification to create a UL112 promoter sequence of 413 bp (nt 160155–160567). The amplified product was cleaved by ScaI and XhoI, recognition sequences of which were incorporated in the primers, and the fragment was then cloned into pGL3-Basic. The resulting plasmid was designated pPrUL112.

■ Subclones of pCL77. Before constructing subclones from pCL77, the promoter sequences within the inserts were analysed by using the Baylor College of Medicine (BCM) Search Launcher gene feature programs. The 14.4 kbp insert of plasmid pCL77, which covers nt 99455–113838 of the AD169 genomic sequence, contains eight ORFs, UL70–UL77. Plasmid pTA1-E was constructed by deleting a 3.6 kbp EozRI fragment from nt 103020 to the MCS (multiple cloning site), containing UL70, and religating the remaining 10.8 kbp insert extending from nt 103020 to 113838, containing ORFs UL71–UL77. Plasmid pTA1-B was generated by deleting a BamHI fragment from pCL77 from nt 108086 to the MCS, and then religating the remaining insert,
extending from nt 99455 to 108088 and containing complete ORFs UL70 to UL74. Plasmid pTA1-H was created by religating pCL77 after deleting the HindIII fragment from nt 107677 to 113838, retained intact coding sequences of UL75 to UL77. Plasmid pTA1-HS containing ORFs UL75 and UL76 was obtained by subcloning a 4.5-kb HindIII–StII fragment from pTA1-H extending from nt 107677 to 111219, and ligating it into pBK CMV. To express the UL76 ORF, pTA1-Sm was made by subcloning a 1.2-kb Smal fragment extending from nt 110228 to 111437 from pTA1-HS and ligating it into Smal-digested pBK-CMV. Plasmid pTA1-SmFS, with a frame-shift mutation within UL76, was created by linearizing the plasmid with PstI and subsequently blunt-ending and religating it. To express the UL76 ORF under the control of the SV40 promoter featured in the cloning vector pHK-3, plasmid was designated pSV-UL76.

**Transfections and reporter enzyme assays.** The activities of sublibraries were assessed by co-transfection with reporter plasmid pMIEP-CAT. HCMV-permissive HEL cells were plated at approximately 5 × 10^4 cells per 60-mm dish 16 h prior to DNA transfection, which was mediated by lipofectamine. Ten μl (2 μg/ml) lipofectamine was added to the DNA mixture containing 6 μg sublibrary DNA and 0.2 μg reporter plasmid pMIEP-CAT. To test the CAT activation of individual subclones in this study, 3 μg effector DNA and 0.2 μg reporter DNA were used for each transfection experiment. The liposome–DNA complexes were left on cells for 16 h. Afterwards, the cells were covered with fresh culture medium. The transfected cells were harvested for CAT assay 48 h post-transfection. Protein extracts were prepared by washing cells with PBS before lysing in an ice-cold lysis solution (0.25 M Tris–HCl, pH 7.5, 0.5% Triton X-100). After centrifugation to pellet the insoluble portion, the supernatant was saved for enzyme assay. CAT activity was assessed either by TLC (Gorman et al., 1982) or by phase extraction assay (Seed & Sheen, 1984) as described previously.

In luciferase reporter assays, DNA transfection was performed as described above, except where the indicated reporter plasmid was replaced. Cell extract preparation and assay conditions were according to the manufacturer’s guidelines (Promega). Fold activation for effector DNA was normalized by dividing by the values for the control experiment, in which vector DNA was used instead of the test clones. Each data point was a statistical value derived from at least three independent experiments.

**Purification of His-tagged UL76 protein, generation of antiserum and immunodetection.** The fragment containing ORF UL76 was excised from pSV-UL76 and inserted into the prokaryotic expression vector pET15b (Novagen) at the Xhol site. The resulting plasmid was designated pHis-UL76. The coding sequence of ORF UL76 was tagged in-frame with six histidine codons from the vector at the N terminus. To produce the fusion protein, E. coli BL21(DE3) transformed with pHis-UL76 was cultured and expression was induced with 0.5 mM IPTG. After sonication, the induced cell lysates were digested with 200 μg/ml DNase and RNase A for 1 h. The fusion protein was purified from induced culture via metal-chelation affinity chromatography (Arnold, 1991). To generate antiserum, rabbits were injected three times at 14-day intervals with the purified protein and bled at the time of the second boost. Afterwards, blood clots were removed and polyclonal antiserum was collected. HCMV-infected HEL cells were harvested at the indicated time. Equal amounts of protein were separated by 0.4–15% SDS–PAGE and transferred electrophoretically to PVDF membranes in 25 mM CABS buffer (4-cyclohexylamino)-1-butanesulphonic acid, pH 11.4. The PVDF blots were blocked before being probed with the anti-UL76 antiserum. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody. Antigen detection was accomplished by incubation with enhanced ECL-plus reagent.
Enhanced green fluorescent protein (EGFP)-tagged UL76.
The Xho–EcoRI fragment containing the UL76 ORF was excised from pHis-UL76 and cloned into vector pEGFP-C3 (Clontech) (Yang et al., 1996). The resulting construct was designated pEGFP-UL76. After 24 h transient expression in HEL and COS-1 cells, the transfected cells were fixed and visualized under a fluorescence microscope.

Results

HCMV selective sublibraries activate MIEP expression

In order to identify HCMV genes that participate in MIEP regulation, we first constructed a set of genomic sublibraries. Each sublibrary was combined from twelve randomly selected HCMV genomic clones made by partial Sau3AI digestion of inserts. As estimated from the insertion sizes, ranging from 5 to 15 kbp, each sublibrary consisted of at least 60 kbp of HCMV DNA. Reporter plasmid pMIEP-CAT (−1139 to +52), used in this experiment, includes the basal promoter, an enhancer, NF-1 and modulator regions (Fig. 1a). Transient expression assays were performed by co-transfection with the viral genomic sublibrary and target reporter plasmid into HEL cells. Twenty sets of sublibraries were analysed for their effects on CAT production. We observed that five sublibraries, SL-F, SL-M, SL-N, SL-O and SL-Q, exhibited significant stimulation, more than five-fold, of MIEP expression (Fig. 1b). Furthermore, the augmentation by all five sublibraries displayed effector DNA dose-dependence (data not shown). The results obtained from these transient co-transfection experiments indicated that the enhancement of MIEP was due to the genomic content within each sublibrary.

Mapping a clone that encodes a potential activator(s)
in the HCMV AD169 genome

Subsequently, we proceeded to identify the clone(s) responsible for MIEP activation from one (SL-F) of the effective sublibraries in this report. In order to quantify the DNA precisely in the following transfection experiments, sublibrary SL-F DNA was mixed with equal amounts of DNA from each of the twelve clones. The reconstituted sublibrary still retained the ability to increase MIEP expression (Fig. 2). CAT activity was determined on subtracted sublibraries, in which each clone was replaced by an equal amount of cloning vector. The results of clone subtraction showed that sublibrary SL-F activity dropped greatly when pCL77 was omitted (Fig. 2, bar 5). Furthermore, we confirmed the HCMV origin and integrity of the insert by genomic Southern hybridization. This information matched those predicted by MAPSORT (data not shown). We also observed that the CAT activity increased slightly when co-transfection was conducted with effector pCL77 in the absence of the other 11 genomic clones. Apparently, pCL77 alone failed to regain the effect imposed by sublibrary SL-F (Fig. 2, bar 3). As the amount of pCL77 was increased, the relative CAT activity increased (Fig. 2, bars 3–5). These results suggested that pCL77 encodes an autonomous activator. Therefore, pCL77 was selected for further study.

In order to resolve the coding content of the pCL77 insert, nucleotide sequencing data were compared with the complete genomic sequence of HCMV AD169 (accession number X17403; Chee et al., 1990). Nucleotide sequencing data revealed that the two ends of the pCL77 insert match the sequence at positions 99455 and 113838, and that this particular fragment contains a total of eight complete ORFs, from UL70 to UL77 (Fig. 3a). Next, we wanted to confirm the integrity of the insert and to ensure that there were no major aberrant rearrangements, deletions or additions within the insert. To achieve this, two alternative strategies were employed. During the following experiments, nucleotide and protein sequences were analysed by using the GCG software. For the first step, the insert DNA was digested with a number of restriction endonucleases and the DNA fragments generated were resolved by either agarose electrophoresis or PAGE. The mass of each fragment was then estimated by computational analysis. We found that the sizes of the digested fragments matched those predicted by MAPSORT (data not shown). Furthermore, we confirmed the HCMV origin and integrity of the insert by genomic Southern hybridization. This information also provided a clear location of the insert in the restriction endonuclease maps of the AD169 genome. Viral genomic DNA was digested with HindIII (Fig. 3b, lanes 1 and 3) and EcoRI (Fig. 3b, lanes 2 and 4) before carrying out agarose gel electrophoresis. The Southern blots were probed with a radiolabelled 1.3 kbp internal fragment of pCL77 (shown in a shaded box in Fig. 3a). The apparent sizes of the bands that hybridized with the probe were in agreement with the molecular masses of the predicted HindIII fragment and also corresponded to EcoRI A fragment. In summary, we conclude that the insert of pCL77 is located in the D–L fragment of the HCMV AD169 genome.

Fig. 2. Evidence that pCL77 is essential for modulation of HCMV MIEP expression in the SL-F sublibrary combination. The liquid-phase extraction protocol was used to determine the CAT activity. Sublibrary effector DNA (6 µg) for this transfection experiment was combined from equal amounts (0.5 µg) of each of twelve purified clones. Bars: 1, the effect on MIEP reporter enzyme production of the complete SL-F combination; 2, the effect on MIEP activation of SL-F with pCL77 omitted. Bars 3–5: target pMIEP-CAT (0.2 µg) was co-expressed with 0.5 (bar 3), 2.0 (bar 4) or 6 (bar 5) µg effector plasmid pCL77. The total mass of effector DNA was made up to 6 µg with cloning vector pBK-CMV for each transfection.
Characterization of HCMV regulatory gene UL76

Fig. 3. (a) Physical maps for HindIII and EcoRI in the HCMV AD169 genome and the location of the insert from pCL77, which encodes a potential gene regulator. The scale indicates the genomic position. TRL, TRs, IRL, IRs denote the terminal (TR) and internal (IR) repeats flanking the long (L) and short (S) components of the HCMV genome. The numbers shown at the ends of the plasmid insert are the coordinates in the HCMV sequence (accession X17403). Intact ORFs contained in the insert are indicated by arrows. (b) Southern blot analysis of the pCL77 insert to confirm its integrity and HCMV origin. Genomic DNA of HCMV digested with HindIII (lanes 1 and 3) or EcoRI (lanes 2 and 4) was separated electrophoretically in a 0–6% agarose gel. The blot was probed with a 1–3 kbp BamHI fragment (nt 112520–113838) of pCL77 (lanes 3 and 4), depicted by the hatched box in (a). Sizes of molecular mass markers in lane M are indicated on the left.

Fig. 4. The UL76 locus contained in pCL77 regulates HCMV MIEP expression. (a) Physical location and ORFs of the pCL77 insert. The numbers at the ends indicate the coordinates within the HCMV AD169 genomic sequence. Restriction sites are marked as: B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SphI; Sa, SalI and Xm, XmaI. The diagram shows identified loci and predicted ORFs contained within the insert of pCL77, based on the protein sequence analysis of Chee et al. (1990). Subclones derived from pCL77 were used as effector DNA for transient co-transfection expression into HEL cells. SV40pr denotes that gene expression is under the control of the SV40 early gene promoter. The rightward arrow above pTA1-Sm denotes a predicted transcriptional initiation site. (b) Evidence that MIEP reporter enzyme activities respond to co-transfection of UL76. Two reporter constructs, pMIEP-CAT and pMIEP-Luc, were used to evaluate the regulatory ability of effector DNA. Individual effector DNAs (3 µg), shown in (a), were co-transfected into HCMV-permissive cells (HEL) with 0.2 µg reporter plasmid. Cell extracts were harvested for enzyme assays.
UL76 contained in pCL77 modulates MIEP activity

We reviewed previous reports in an attempt to reveal candidate regulatory loci. Some proteins encoded between UL70 and UL77 have been identified and characterized previously (Fig. 4a). ORF UL70 has been demonstrated to be a component of the primase–helicase complex required for oriLyt-dependent virus DNA replication (Pari & Anders, 1993; Smith & Pari, 1995). ORF UL72 is a dUTPase homologue of other herpesviruses (Preston & Fisher, 1984; Messerle et al., 1995). A putative membrane protein is encoded by ORF UL73, with an N-terminal signal peptide and a C-terminal membrane anchor region (Barnett et al., 1992). The protein products of UL74 (glycoprotein O, gO) and UL75 (gH) constitute parts of the gCIII envelope complex (Cranage et al., 1988; Kaye et al., 1992; Huber & Compton, 1998, 1999). An enzymatic active site of a putative pyruvyl decarboxylase is encoded by ORF UL77 (Yoakum, 1993). Protein sequence analysis revealed that ORFs UL71, UL73 and UL76 share positionally conserved regions amongst herpesvirus members. The genetic content of these three loci has not been identified or characterized previously.

Since none of the loci residing in the pCL77 insert has been reported to encode a gene regulator, a series of subclones were used to construct an effort to identify the gene(s) responsible (Fig. 4a). Two reporter plasmids, pMIEP-CAT and pMIEP-Luc, were used to conduct the following co-transfection experiments. Plasmid pTA1-E, encoding UL71 to UL77 without UL70, retained the ability to activate MIEP expression (Fig. 4b, bars 1–4). The observation that activation was not retained by pTA1-B, encoding UL70 to UL74, suggested that the candidate was not located between UL71 and UL74 (Fig. 4b, bars 5 and 6). To verify whether UL75 to UL77 encoded an activator that conferred regulation on MIEP, pTA1-H was constructed. Transient expression analysis indicated that a locus between UL75 and UL77 was responsible for the activation (Fig. 4b, bars 7 and 8). Both constructs pTA1-HS and pTA1-SP, carrying UL76 and either UL75 or UL77, induced MIEP in the transient cell culture system (Fig. 4b, bars 9, 10, 13 and 14). Before we cloned the single ORFs UL75, UL76 or UL77, cloned fragments were analysed by several programs to locate potential promoters by using the BCM Search Launcher gene feature programs. Because no promoter was predicted for the cloned UL75 or UL77 fragments, expression of both ORFs was driven by the SV40 early gene promoter. The resulting plasmids, pTA1-PH and pTA1-P, were used to assess the gene regulatory activities of UL75 and UL77, respectively (Fig. 4b, bars 11, 12, 19 and 20). Both UL75 and UL77 showed little effect on MIEP. When we compiled the cloned UL76 sequence with the BCM eukaryotic promoter NNPP neural network program, a putative basal promoter motif was predicted at nt 110238–110287, upstream of the ORF. Further analysis showed that the UL76 cloning insert was able to activate the target promoter in either the sense (pTA1-Sm; Fig. 4b, bars 15 and 16) or antisense (not shown) orientation. Therefore, expression of UL76 was presumably driven by this endogenous promoter. Moreover, a frame-shift mutation within the UL76 ORF (pTA1-SmFS) abolished the activity (Fig. 4b, bars 17 and 18). Taking these results together, we concluded that UL76 was the candidate gene regulator. We also noticed that ORF UL76 was composed of more than 20% basic residues, i.e. Arg and Lys, giving a predicted pI of 11.6.

Prokaryotic production of the protein encoded by ORF UL76 and the generation of antiserum

Our next goal was to produce a UL76-specific antiserum and to investigate protein expression from the UL76 region. In order to generate enough protein for immunization, the ORF UL76 product was synthesized in E. coli strain BL21(DE3). The fragment containing ORF UL76 was inserted into prokaryotic vector pET15b. The resulting plasmid, pHis-UL76, expressed a UL76 fusion protein tagged in-frame with 19 aa at the N terminus, including six histidines. The transformed E. coli cells were cultured in the presence of IPTG to induce synthesis of the UL76 fusion protein. Meanwhile, the uninduced culture grown without the addition of IPTG served as a negative control. Protein extracts prepared from both cultures were resolved by SDS–PAGE and Coomassie blue staining. As shown in Fig. 5 (lanes 1 and 2), one extra protein band, migrating at the estimated position of the fusion protein, was synthesized in bacteria cultured in the presence of IPTG,

HindIII genomic map of HCMV and in the EcoRI physical genomic map, the pCL77 insert is mapped within the I–A fragment (Fig. 3a).
Characterization of HCMV regulatory gene UL76

Fig. 6. Transient expression of UL76-encoded protein in eukaryotic cells and its production kinetics in HCMV-infected cells. (a) Western blot analysis of COS-1 cells transfected with cloning vector pHK-3 (lanes 1 and 3) or pSV-UL76 (lanes 2 and 4). Protein lysates were recovered from transfected cells and separated by SDS–PAGE. The blots were incubated with anti-UL76 antiserum (lanes 1 and 2) or preimmune serum (lanes 3 and 4). Sizes of molecular mass markers are shown on the left. (b) Western blot analysis of UL76-encoded protein during the HCMV replicative cycle. Cell extracts were harvested at different times after infection of HEL cells with HCMV AD169. The blot was probed with the anti-UL76 antiserum. Lanes: 1, mock-infected cells (M); 2–6, extracts harvested at 2 (lane 2), 12 (3), 24 (4), 48 (5) or 72 (6) h p.i.

whereas the specific band was not observed in the culture lacking IPTG induction. We next tried to purify the fusion protein via metal-affinity chromatography. During the purification process, we noticed that the induced protein could not be eluted without the inclusion of DNase and RNase in the IPTG-induced cell extract. Protein elution fractions collected from a Ni²⁺ resin column were analysed by SDS–PAGE and Coomassie blue staining. This revealed a single purified protein with a molecular mass of 41 kDa (Fig. 5, lane 3), which corresponded approximately to the predicted mass of the fusion protein. The purified protein was used to immunize rabbits. Western blot analysis was performed to test the specificity of the antiserum. As shown in Fig. 5 (lanes 4 and 5), the antiserum reacted with the protein purified from induced bacterial cells, whereas the preimmune serum displayed no reaction at all. Due to its high pI, we found that the purified fusion protein could only be transferred electrophoretically to PVDF membrane in CABS buffer adjusted to pH 11.4. Subsequent blotting experiments were carried out with the same buffer system.

Expression of UL76 protein in eukaryotic cells and in the HCMV infectious cycle and its potential localization within the cell

We attempted to identify UL76 protein in COS-1 cells transfected with pCL77, but were not able to demonstrate any protein expression. To investigate further the expression of UL76 protein, a eukaryotic expression plasmid pSV-UL76 was constructed to drive ORF UL76 expression under the SV40 early gene promoter. Cell extracts prepared from COS-1 cells transfected with cloning vector pHK-3 or pSV-UL76 were subjected to Western blot analysis. A protein of 38 kDa, corresponding to the predicted molecular mass of the ORF UL76 product, was detected in cells transiently expressing pSV-UL76 (Fig. 6a, lane 2). The band was not observed in cells transfected with cloning vector pHK-3 (Fig. 6a, lane 1). Again, the preimmune serum showed no reaction with any cell extract (Fig. 6a, lanes 3 and 4).

Subsequently, the production kinetics of the UL76-encoded protein during the HCMV reproductive life-cycle were investigated. Cells infected with HCMV were taken at various
time intervals and lysed for Western blot analysis, which revealed the same 38 kDa protein appearing at 2 h post-infection (p.i.). Production of this protein reached the maximum at 24 h p.i. and the level remained the same through the late phase of the virus life-cycle (Fig. 6b, lanes 1–6). Because the protein could only be immunodetected under the transferring conditions, we believe that the 38 kDa protein (pUL76) produced from pSV-UL76-transfected cells was the same as that from HCMV-infected cells. The signal with a molecular mass of 25 kDa was a non-specific cellular protein that reacted with the antiserum.

The protein sequence of pUL76 (Fig. 7a) contains three putative nuclear localization signals (NLS). NLS2, at aa 28–31, matches the NLS consensus sequence for the SV40 large T antigen (Kalderon et al., 1984). NLS1 and NLS3, with bipartite consensus sequences, are predicted at aa 24–40 and 191–207, respectively (Robbins et al., 1991). To obtain further confirmation that pUL76 is a potential gene regulator, its subcellular localization was examined to elucidate whether pUL76 was targeted to the nucleus. In this experiment, EGFP was used as a reporter to locate pUL76. Cells transfected with cloning vector pEGFP-C3 served as a negative control. Fluorescence was distributed evenly in the cytoplasm of both HEL and COS-1 cells (Fig. 7b). Plasmid pEGFP-UL76, which synthesizes an EGFP-tagged pUL76, was constructed. In contrast to the control experiment, fluorescence was seen exclusively in nuclei in cells transfected with pEGFP-UL76 (Fig. 7b). Within these nuclei, the fluorescence was predominantly aggregated in spots and globular foci against a diffuse background. Our results showed that, in the absence of other viral proteins, pUL76 is located in the nucleus, and this fact agrees with the site of a regulatory protein. Furthermore, these images indicate an uneven association of pUL76 with nuclear domains.

**pUL76 functions in activation and repression of gene expression**

As the results described in the previous section show that pUL76 is associated preferentially with certain nuclear domains, we subsequently investigated the promoter specificity of pUL76. Four target promoters were used in this experiment, the HCMV major immediate-early gene (pMIEP-Luc), the UL112–113 regulatory gene (pPrUL112), UL54 viral DNA polymerase (pPrUL54) and SV40 early gene promoter (pSV40-EP). HEL cells were co-transfected with the target promoter and increasing ratios of effector DNA. The SV40 early gene promoter, which drives pUL76 expression within the construct pSV-UL76, was examined first. Results shown in Fig. 8 (bars 1–4) indicate that pSV-UL76 had minimal effect on the activity of the SV40 early gene promoter. Therefore, the construct was considered as a better choice to assess the gene-regulatory activities exerted by pUL76. The promoter for UL54, encoding an early-expressed DNA polymerase, was only moderately affected by pUL76 (Fig. 8, lanes 13–16). Our results indicate that pUL76 has dual functions in activation and repression of gene expression.
repression of gene expression. Additionally, the regulatory activities of pUL76 depend on the specificity of the target promoter.

Discussion

In this study, we hypothesized that the HCMV major immediate-early gene promoter/enhancer (MIEP) is regulated by multiple viral gene products. By adopting a target promoter-orientated screening strategy, we provided evidence that the MIEP is affected by a number of combinatorial genomic clones (Fig. 1b). From the screening process, we identified a novel regulatory gene, UL76, which modulates gene expression in a dual activating and repressing manner.

The UL76 gene product is classified in a family of conserved proteins found in all herpesviruses, e.g. UL24 of herpes simplex virus type 1 (HSV-1), BXR1F of Epstein–Barr virus, ZVZ35 of varicella-zoster virus and U49 of human herpesvirus types 6 and 7 (Dezèlée et al., 1996). This group of proteins contains Arg- and Lys-rich sequences, giving theoretical pl values ranging from 10 to 11/6. Previously, UL24 of HSV-1, a gene non-essential for replication, has been studied and much of our understanding has been derived from the phenotypic behaviour of virus mutants (Roizman & Sears, 1996). HSV-1 deficient in UL24 displays reduced replication efficiency in cultured cells (Jacobson et al., 1989). Cells infected with a number of UL24 mutant viruses form syncytial plaques (Sanders et al., 1982; Tognon et al., 1991). In an animal model, certain UL24-defective mutant viruses had reduced abilities for acute replication in trigeminal ganglia and for reactivation from latency (Jacobson et al., 1998). It remains to be examined whether these two homologues, UL76 of HCMV and UL24 of HSV-1, display any biological similarities.

In an attempt to find protein motifs of pUL76 shared with other gene regulators, we compared its protein sequence to the latest version of the Prosite database (release 16). None of the motifs commonly found in transcription factors, histone or non-histone, matched the sequence of pUL76 perfectly. However, two lines of evidence demonstrated in this paper indicate that pUL76 is a novel regulatory protein with gene context specificity. Firstly, EGFP-tagged pUL76 distributes predominantly in globular foci within the nuclei of transfected cells. This may imply that pUL76 has some preferential association with nuclear domains. This finding agrees with the result that pUL76 exerts differential activities on various virus promoters. Although the mechanism of action is unclear, pUL76 does not seem to repress expression through toxicity, because it does not affect the activity of the SV40 early gene. The growth rate and morphology of transfected cells were not changed visibly under our assay conditions.

In this paper, we have identified and described the in vitro properties of pUL76 in the absence of other viral proteins. There are some interesting and unresolved aspects regarding pUL76 expression in HCMV-infected cells. Because of the detection of pUL76 at 2 h p.i., we speculate that pUL76 is a virus component or an IE-expressed protein. Work is in progress to elucidate these characteristics.

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References


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