Multiple Regulatory Events Influence Human Cytomegalovirus DNA Polymerase (UL54) Expression during Viral Infection

JULIE A. KERRY,¹ MORRIS A. PRIDDY,¹ TABMITHA Y. JERVEY,¹ CATHERINE P. KOHLER,¹
TERRI L. STALEY,¹ CYNTHIA D. VANSOON,¹ THOMAS R. JONES,²
ANDREA C. ISKENDERIAN,³ DAVID G. ANDERS,³
AND RICHARD M. STENBERG^{1*}

Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501¹; Molecular Biology Section, Medical Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, New York 10965²; and David Axelrod Institute, Wadsworth Center for Laboratories and Research, and Department of Biomedical Sciences, University at Albany School of Public Health, Albany, New York 12201³

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The human cytomegalovirus (HCMV) DNA polymerase gene (UL54; also called pol) is a prototypical early gene in that expression is mandatory for viral DNA replication. Recently, we have identified the major regulatory element in the UL54 promoter responsive to the major immediate early (MIE) proteins (UL122 and UL123) (J. A. Kerry, M. A. Priddy, and R. M. Stenberg, *J. Virol.* 68:4167–4176, 1994). Mutation of this element, inverted repeat sequence 1 (IR1), abrogates binding of cellular proteins to the UL54 promoter and reduces promoter activity in response to viral proteins in transient-transfection assays. To extend our studies on the UL54 promoter, we aimed to examine the role of IR1 in UL54 regulation throughout the course of infection. These studies show that viral proteins in addition to the MIE proteins can activate the UL54 promoter. Proteins from UL112-113 and IRS1/TRS1, recently identified as essential loci for transient complementation of HCMV *ori*_{Lyt}-dependent DNA replication, were found to function as transactivators of the UL54 promoter in association with MIE proteins. UL112-113 enhanced UL54 promoter activation by MIE proteins three- to fourfold. Constitutive expression of UL112-113 demonstrated that the MIE protein dependence of UL112-113 transactivational activity was not related to activation of cognate promoter sequences, suggesting that UL112-113 proteins function in cooperation with the MIE proteins. Mutation of IR1 was found to abrogate stimulation of the UL54 promoter by UL112-113, suggesting that this element is also involved in UL112-113 stimulatory activity. These results demonstrate that additional viral proteins influence UL54 promoter expression in transient-transfection assays via the IR1 element. To confirm the biological relevance of IR1 in regulating UL54 promoter activity during viral infection, a recombinant virus construct containing the UL54 promoter with a mutated IR1 element regulating expression of the chloramphenicol acetyltransferase (CAT) reporter gene (RVIRmCAT) was generated. Analysis of RVIRmCAT revealed that mutation of IR1 dramatically reduces UL54 promoter activity at early times after infection. However, at late times after infection CAT expression by RVIRmCAT, as assessed by RNA and protein levels, was approximately equivalent to expression by wild-type RVpolCAT. These data demonstrate IR1-independent regulation of the UL54 promoter at late times after infection. Together these results show that multiple regulatory events affect UL54 promoter expression during the course of infection.

Human cytomegalovirus (HCMV) is a common infectious agent that can cause significant morbidity and mortality (reviewed in reference 1). Newborn infants infected in utero with HCMV develop severe complications such as mental retardation and hearing loss. Reactivation of HCMV from a latent state in immunosuppressed or immunocompromised individuals can also result in severe disease. HCMV is a major opportunistic pathogen in patients with AIDS and patients undergoing immunosuppressive therapy following organ transplantation. The virus-host cell interactions which regulate HCMV reactivation in the immunocompromised host remain unidentified. Three sequential phases of HCMV gene expression have been defined: immediate early (IE), early, and late (6, 32, 56). The transition from IE gene expression to early-gene expression is

likely to be a pivotal determinant of HCMV replication. For example, in undifferentiated peripheral blood macrophages, HCMV infection is limited to IE events (9, 36, 38, 40). These cells are thought to be a source of HCMV persistence (54). The inability of HCMV to enter the early phase of viral replication may therefore be involved in the establishment of latency in this cell type. When monocytes are activated to differentiate they can be productively infected with HCMV (15, 29, 57). It is possible that monocyte differentiation alters the expression of cellular factors important in HCMV early-gene regulation, allowing for virus replication. Analysis of early-gene regulation in the HCMV-infected cell is therefore crucial for understanding HCMV replication and pathogenesis.

Various cellular transcription factors such as USF, AP-1, E2F, and NF-Y are involved in the regulation of individual HCMV early-gene promoters in transient-transfection assays (14, 24, 26, 45, 55). We have also identified other as-yet-uncharacterized cellular proteins involved in regulating the promoter for the viral DNA polymerase gene (UL54; also called pol) (22). In addition to requiring cellular transcription factors,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, 700 W. Olney Rd., Norfolk, VA 23501. Phone: (804) 446-5684. Fax: (804) 624-2255. Electronic mail address: RMS@BORG.EVMS.EDU.

activation of early-gene promoters exhibits an absolute requirement for viral proteins, in particular the major IE proteins (48). It is generally thought that proteins from the major IE-gene region interact with cellular factors to activate early-gene promoters, possibly by acting as coactivators of transcription. In fact, recent evidence has shown that IE72 and IE86 can interact directly with some cellular transcription factors (12, 21, 28, 41, 42). In addition, IE86 can bind directly to sequence elements within the early UL112-113 promoter to activate transcription (2, 39). Other IE HCMV transactivators such as US3, UL36-38, and IRS1/TRS1 have also been identified (5, 47). In addition, HCMV early- and late-gene products can also function as transactivators (18, 30, 58). Collectively, these studies suggest that a wide range of interactions occur between viral transactivators and those cellular transcription factors involved in regulating HCMV early-gene expression within an HCMV-infected cell.

In an effort to understand how HCMV early genes are regulated, we have undertaken an in-depth analysis of the UL54 promoter (22, 48). The UL54 gene is a prototypical early gene in that expression is mandatory for virus DNA replication. Previous studies determined that binding of cellular proteins to the UL54 promoter was associated with an 18-bp region of the promoter containing a single copy of a novel inverted repeat, IR1 (22). Mutation of IR1 abrogated binding of cellular factors to the UL54 promoter and significantly reduced the activation of the promoter by viral major IE (MIE) proteins or superinfecting virus. Thus, IR1 was determined to be the major regulatory element influencing UL54 promoter regulation in transient-transfection assays. The aim of the present study was to determine how IR1 influences UL54 promoter regulation during the course of HCMV infection. We have determined that the UL112-113 open reading frames (ORFs) stimulate MIE-mediated activation of the UL54 promoter, either directly or indirectly, via the IR1 element. These studies demonstrate that additional viral proteins act in association with the MIE proteins to regulate UL54 promoter activity. To test the biological relevance of IR1, a recombinant virus containing a mutation in the IR1 element of the UL54 promoter was generated. Analysis of this recombinant virus revealed that IR1 is crucial for UL54 promoter activity at early times after infection. However, at late times after infection, alternative pathways of promoter activation are employed by this essential early gene. Together, these studies demonstrate that multiple regulatory events influence UL54 expression within an HCMV-infected cell.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HF) cells were grown and infected with HCMV Towne and AD169 strains as previously described (51, 53). The AD169 recombinant virus derivative RV134, containing the β -glucuronidase gene between the US9 and US10 open reading frames, has been previously described (20). RVpolCAT has been described previously (27).

Plasmids. The plasmid constructs pPolCAT and pdHPolCAT, which both contain the UL54 promoter fragment from -425 to +20, have been described elsewhere (48, 49). pdHPolCAT differs from pPolCAT by the absence of a *HindIII* site at the 3' end of the UL54 promoter (48). The deletion mutant constructs pd2PolCAT and pd177CAT were derived from pdHPolCAT and contain UL54 promoter sequences from -128 to +20 and -52 to +20, respectively (48). The pZP24, pZP13, pZP8, pTRS1, and pZP3 plasmids, which contain, respectively, the UL112-113, UL84, UL36-38, TRS1, and IRS1 ORFs expressed under the control of the cognate promoters in pGEM7Zf(-) (Promega, Madison, Wis.), have been described previously (16, 34, 35). The pZP24 construct was modified by the insertion of a 12-bp *HindIII* linker into the *ThaI* site 4 nucleotides upstream of the UL112-113 open reading frames to generate pZP24C. The MIE promoter from p760CAT (52) was then cloned into the *HindIII* site of pZP24C to generate pZP24CMIEP. The pON2334 plasmid expressing the TRS1 open reading frame has been described elsewhere (47). The pPolCAT derivatives containing mutations in the putative regulatory elements DR1, ATF, CAAT, and IR1 of the UL54 promoter have been described previ-

ously (22). Plasmids expressing the cDNAs of IE72 (pIE72kd) and IE86 (pIE86kd) under the control of the MIE promoter have also been previously described (49). Luciferase reporter plasmids were generated by cloning the UL54 promoter and the UL54 derivative containing the IRm mutation from pPolCAT and pIRmCAT into the *HindIII* site of the vector pGL2-Basic to generate pUL54Luc and pIRmLuc, respectively (Promega Corp.).

Transient expression of the UL54 promoter. pPolCAT or its mutated derivatives (5 μ g) were assessed by transient cotransfection with pSVH (2.5 μ g), a plasmid expressing the MIE proteins, and plasmids containing putative transactivators (2.5 μ g) as previously described (7, 49). Briefly, HF cells were cotransfected by the DEAE-dextran method, harvested at 48 h after transfection, and subsequently assayed for chloramphenicol acetyltransferase (CAT) activity (7). Total DNA levels in transient-transfection experiments were standardized to 10 μ g by the addition of the control plasmid pSV0d (33) or pGEM7Zf(-). Superinfection experiments performed with pPolCAT and its mutated derivatives using the Towne strain of HCMV were as previously described (7). In brief, HF cells were transfected with the appropriate reporter plasmid by the DEAE-dextran method. At 18 h after transfection, cells were superinfected with HCMV strain Towne by using 5 to 20 PFU per cell and incubating for 2 h. At this time, the inoculum was removed and the cells were overlaid with fresh media. Infections in the presence of phosphonoacetic acid (PAA) were done as previously described (7). At various times after infection, the cells were harvested and assessed for CAT activity (7). Cotransfection experiments with the luciferase reporter plasmids and various combinations of effector plasmids were carried out exactly as described previously (16).

Oligonucleotide-directed mutagenesis of the UL54 promoter. The LIR3, -4, -5, -6, and -7 mutations were introduced into the UL54 promoter by using the Altered Sites in vitro mutagenesis system (Promega) as previously described (22). These mutations were designed to introduce a unique *EcoRV* restriction site into the UL54 promoter at the indicated sequences. The oligonucleotides used to generate the LIR mutants are as follows (mutated nucleotides are underlined): LIR3, 5' GGAGCCTGTAACGAGATATCTTTGGTGTCTGC; LIR4, 5' GGAA GCGGAGCCGATATCGACGTGTCTTTG; LIR5, 5' TTCCTCGTAGGAT ATCGGAGCCTGTAAC; LIR6, 5' ATAAACATCTTCGGATATCGAAGG GGAGCCTG; LIR7, 5' CAGAATTATAACGATATCTCTCGTAGGAA.

Gel mobility shift analysis. Gel mobility shift assays were performed exactly as described previously (22). In brief, nuclear extracts (10 μ g) were incubated with poly(dI-dC) · poly(dI-dC) (5 μ g) and competitor DNAs for 15 min at room temperature in 50% buffer D (8). Radiolabeled activator probe (22) was added (20,000 cpm), and the incubation was continued for 30 min. Samples were subjected to polyacrylamide gel electrophoresis in standard 1× Tris-borate-EDTA (22). Gels were then dried and subjected to autoradiography.

Generation and isolation of recombinant virus. The virus RVd2PolCAT was generated from pd2PolCAT exactly as previously described (27). The virus RVIRmCAT was generated and isolated in the same manner with the following modifications. The *ApaI* site (nucleotide 199021 of the HCMV sequence [4]) of recombination vector pXSB was modified by the addition of an oligonucleotide linker containing *HindIII* and *BamHI* restriction enzyme sites to generate pRV3. The 1.02-kb *HindIII*-to-*BamHI* fragment from pSV0CATd (7) containing the CAT gene was then cloned into pRV3 to generate pRC2. The UL54 promoter fragment containing a 4-nucleotide mutation in the IR1 element (22) was then inserted into the *HindIII* site upstream of the CAT gene in pRC2 to generate pRCIRm. This construct was linearized at the unique *XmnI* restriction site within the pT7-1 sequence and was subsequently cotransfected with RV134 DNA as previously described (19). Purified recombinant viral isolates were then selected from the transfectant population exactly as previously described (27).

Southern blot analysis. HF cells infected with recombinant viruses were harvested for total-cell DNA at 100% cytopathic effect as previously described (27). The infected-cell DNAs were digested with the appropriate restriction enzymes and subjected to electrophoresis on 0.8 or 1.2% agarose gels in 1× Tris-borate-EDTA buffer. The gels were either subjected to Southern blot analysis exactly as previously described (27) or transferred to positively charged nylon membranes (Biohynde B; Gibco Life Technologies, Grand Island, N.Y.) and hybridized to a probe for CAT or the *HindIII* X fragment of AD169 by using the Genius detection system (Boehringer-Mannheim Corporation, Indianapolis, Ind.).

Northern (RNA) blot analysis. Total-cell RNA was isolated from infected cells with the RNeasy system (Qiagen, Chatsworth, Calif.). Equal quantities of RNA were subjected to Northern blot analysis as previously described (27). Blots were hybridized to ³²P-radiolabeled probe for CAT as previously described (27). RNA levels were quantitated by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, Calif.). Multiplicity of infection was assessed by stripping the Northern blots and reprobing with a ³²P-radiolabeled probe specific for the late pp28 gene (UL99).

RESULTS

UL54 promoter regulatory elements. Our previous analyses of the UL54 promoter revealed that the interaction between cellular factors and the IR1 element played the major role in UL54 promoter regulation in transient-transfection assays

FIG. 1. (A) Scanning mutations within the IR1 binding domain of the UL54 promoter. The top line shows the sequence of the UL54 promoter region mutated in this analysis. The CAAT box, TATA element, and 8-bp IR1 repeat sequence are indicated by black boxes. Mutations were introduced at the indicated sites with the replacement sequences shown in lowercase letters. (B) Gel shift analysis of UL54 promoter mutants. Radiolabeled activation domain fragments (-128 to +20) from the wild-type (Act) or mutated UL54 promoter regions as indicated in panel A were incubated with nuclear extracts (lanes 2 to 8) isolated at 72 h after infection. Lane 1, no extract. A, B, and C, specific protein-DNA complexes; P, probe. (C) Effect of UL54 promoter mutations on activation by viral proteins. The indicated UL54 promoter-CAT plasmids were transfected into human fibroblasts and superinfected with virus in the presence or absence of PAA (200 μ g/ml). Cell extracts were prepared at the indicated times after infection, diluted 1:30, and examined for CAT activity. Activity was quantitated by Phosphorimager analysis. Data from a single experiment of duplicate experiments are shown.

(22). These studies demonstrated that the IR1 element from -55 to -48 was important for activation of the UL54 promoter by MIE proteins. However, a second copy of the 8-bp IR1 inverted repeat element located at -225 appeared to play no role in UL54 promoter activity (48). As part of our strategy to further define the requirements for UL54 promoter regulation, additional mutants within the UL54 activation domain were generated (Fig. 1A). Consecutive scanning mutations of the sequences surrounding the IR1 element between the CAAT element and the TATA box of the UL54 promoter were introduced into the full-length UL54 promoter. These pro-

motor mutants were tested for their ability to bind IR1-binding proteins (Fig. 1B) as well as function in transient-transfection assays after viral superinfection (Fig. 1C).

Gel shift analysis of the wild-type activation domain fragment (-128 to +20; Act) revealed the presence of three cellular-protein-DNA complexes (A, B, and C) as previously described (22, 48). Analysis of the various activation domain mutants indicated that substitutions within IR1 or the adjacent LIR5 region dramatically affect the ability of Act to bind cellular proteins (Fig. 1B). These mutations therefore define the limits of the IR1 element from -53 to -45 (GCTCCGCCT)

and confirm that sequences outside the 8-bp inverted repeat are important for IR1-binding protein activity (Fig. 1A). A faint band comigrating with cellular-protein-DNA complex B was observed with the IRm and LIR5 probes. Preliminary analysis indicate that this protein-DNA complex, termed complex B', appears only in nuclear extracts isolated at late times after infection (23). Assessment of Act fragments containing mutations in the LIR3, LIR4, LIR6, or LIR7 region by gel shift analysis indicated that they bound cellular proteins with an affinity approximately equal to that of the wild-type Act fragment (Fig. 1B).

The UL54 promoter mutants were then tested for their ability to be activated by viral proteins (Fig. 1C). This experiment shows that UL54 promoter activity in response to the HCMV proteins correlated with the ability to bind proteins via the IR1 element. For example, mutation of the LIR3 and LIR7 regions had no effect on UL54 promoter activity. These mutants also retained the ability of the activation domain fragments to bind cellular proteins. Mutation of the IR1 element and the LIR5 region, which result in the abrogation of protein binding by the UL54 promoter, also resulted in a ca. threefold drop in promoter activity. The mutation of regions immediately surrounding the IR1 element (LIR4 and LIR6) appeared to have an intermediate effect on UL54 promoter activity, although these mutants bound IR1 proteins with an affinity approximate equal to that of the wild-type promoter (Fig. 1B). It is possible that the mutation of these regions had a more subtle effect on IR1 binding that results in reduced promoter activity within the context of an infected cell. However, it is clear that sequences surrounding the IR1 element can influence UL54 promoter activity.

Effect of viral proteins on UL54 promoter activity. While the MIE proteins are likely to play a major role in HCMV early gene activation, it is possible that other HCMV transactivators could be involved in UL54 promoter regulation. Recently, 11 loci required for transient complementation of HCMV DNA replication were identified (34). These loci include open reading frames that encode the MIE proteins, other known transactivators (UL36-38 and IRS1), and two ORFs (UL84 and UL112-113) that encode proteins of unknown function. We reasoned that these proteins may be required to regulate the promoters of genes involved in DNA replication. In fact, the products of four of these ORFs (MIE, UL112-113, UL36-38, and IRS1/TRS1) have recently been shown to act cooperatively to enhance the promoter activity of DNA replication genes (16, 50). Therefore, we examined the ability of these potential viral transactivators to individually stimulate UL54 promoter activity in association with the MIE proteins (Fig. 2). In these experiments, HF cells were cotransfected with the UL54 reporter plasmid (pPolCAT) in the presence of IE1 and IE2 (pSVH) and plasmids expressing potential activators of HCMV early gene transcription. The inclusion of pZP24, a plasmid which contains the UL112-113 open reading frames (34), results in a three- to fourfold stimulation of UL54 promoter activity. This is similar to the level of stimulation observed with the IRS1 gene, while TRS1 was found to stimulate UL54 promoter activity approximately fivefold. This finding is consistent with a recent study indicating that TRS1 activates the UL44 promoter to higher levels than IRS1 (16). In contrast, UL84 was unable to stimulate UL54 promoter activity above that observed with the MIE proteins alone. Analysis of the UL36-38 gene gave variable results which were construct dependent (data not shown). The role of UL36-38 in UL54 promoter activation is currently under investigation.

These data show for the first time that UL112-113 functions as a transactivator which can stimulate early UL54 promoter

FIG. 2. Augmentation of MIE protein activation of the UL54 promoter. HF cells were cotransfected with pPolCAT (5 μ g), pSVH (2.5 μ g), and the indicated plasmids expressing various HCMV ORFs (2.5 μ g) or the equivalent amount of control plasmid [pSV0d or pGem7Zf(-); 2.5 μ g] as described in Materials and Methods and subsequently assayed for CAT activity. Values from a typical experiment of numerous replicate experiments are shown, with data expressed as percent acetylation assessed by Phosphorimager analysis. pZP24, plasmid expressing the UL112-113 open reading frame; pZP3, plasmid expressing the IRS1 ORF; pON2334, plasmid expressing the TRS1 ORF; pZP13, plasmid expressing the UL84 ORF.

activity in transient-transfection assays. This stimulation required the presence of the viral MIE proteins, as cotransfection of pZP24 with the pPolCAT reporter plasmid alone did not result in activation of the UL54 promoter (Fig. 3B and data not shown). However, in the pZP24 construct, UL112-113 gene expression is regulated by its cognate promoter (34). This promoter has been extensively characterized and is known to require MIE proteins, in particular IE86, for activation (39, 45). Therefore, the presence of MIE proteins in these experiments may simply be required to switch on UL112-113 gene expression. To test if the proteins encoded by the UL112-113 ORFs contained inherent transactivational activity, the UL112-113 gene was expressed under the control of the MIE promoter (pZP24CMIEP [Fig. 3A]). Cotransfection of pZP24 CMIEP with the pPolCAT reporter plasmid did not result in significant stimulation of UL54 promoter activity (Fig. 3B). This result suggests that UL112-113 by itself is insufficient for activation of the UL54 promoter and that activation requires the presence of the MIE proteins. This was confirmed by cotransfection of pZP24CMIEP with IE1 and IE2. In this case, stimulation of MIE protein-mediated UL54 promoter activity was again observed, confirming that pZP24CMIEP functions equivalently to pZP24 in this assay. These data suggest that the proteins encoded by UL112-113 cooperate in some manner with the MIE proteins to stimulate UL54 promoter activity. Lastly, cotransfection of pZP24 with plasmids expressing cDNAs of IE72 or IE86 alone did not result in UL54 promoter stimulation (Fig. 3B). This demonstrates that maximal augmentation of UL54 promoter activity by UL112-113 required the presence of both IE1 and IE2.

UL54 promoter requirements for pZP24 activation. Extensive analyses of UL54 promoter regulation in transient assays have revealed that IE1 and IE2 proteins act via the IR1 element (22). If the transactivational activity of UL112-113 was dependent on the MIE proteins, it would be expected that deletions or mutations of the UL54 promoter that affect IR1 activation by IE proteins would also affect UL112-113 stimulation. To assess this, well-defined deletions in the UL54 promoter region (48) were assessed for their ability to be stimulated by UL112-113 in the presence of MIE proteins (Fig. 4). Deletion of UL54 promoter sequences from -425 to -128

FIG. 3. (A) Schematic representation of the construction of pZP24CMIEP as described in Materials and Methods. Pr, promoter; MIEP, MIE promoter cloned from p760CAT. *Hind*III refers to a 12-bp oligonucleotide linker inserted into a *Tha*I site 4 nucleotides 5' of the UL112-113 ORF. (B) Activity of the UL112-113 ORFs in the presence or absence of the IE proteins. HF cells were cotransfected with pPolCAT (5 μ g), plasmids expressing MIE proteins (2.5 μ g), and plasmids expressing the UL112-113 ORF (2.5 μ g), and cell extracts were subsequently assayed for CAT activity. The total amount of DNA in each transfection was standardized to 10 μ g by the addition of pSV0d or pGEM7Zf(-). Values from a typical experiment of three replicate experiments are shown, with data expressed as percent acetylation assessed by Phosphorimager analysis. pIE72kd and pIE86kd indicate transfection with the IE72 and IE86 cDNAs, respectively, expressed under the control of the MIE promoter. pZP24CMIEP, plasmid which expresses the UL112-113 ORFs under the control of the MIE promoter; pZP24C, plasmid which contains the UL112-113 ORFs expressed under the control of the UL112-113 promoter with a *Hind*III linker inserted 5' of the ORFs; pZP24, plasmid expressing the UL112-113 ORFs under the control of the UL112-113 promoter.

(pd2polCAT) resulted in increased activation of the UL54 promoter in the presence of MIE proteins and UL112-113 compared with that of the full-length promoter. This is consistent with previous data indicating that this region may contain a repressor of UL54 promoter activity (48). Further deletion of the UL54 promoter to -52 (pd177CAT) resulted in a loss of promoter activity in response to IE1 and IE2 as well as a loss of stimulation by the UL112-113 construct. Deletion from -128 to -52 resulted in the removal of the activation domain of the UL54 promoter, including the IR1 element required for MIE-mediated activation of this promoter.

These data suggest that UL112-113 stimulation of UL54 promoter activity may be dependent on MIE protein-mediated activation of this promoter. To address this possibility, we assessed the ability of the UL112-113 construct to stimulate gene expression from a comprehensive set of UL54 promoter

FIG. 4. Augmentation of UL54 promoter activity by UL112-113 is affected by promoter deletions. The top section is a schematic representation of the pPolCAT plasmid and derivatives containing deletions within the UL54 promoter sequences. dHpolCAT differs from pPolCAT by the elimination of a *Hind*III site downstream of the promoter sequences. HF cells were cotransfected with pPolCAT or derivatives as indicated (5 μ g) and pSVH (2.5 μ g) in the presence or absence of pZP24 (2.5 μ g). The total amount of DNA in each transfection was standardized to 10 μ g by the addition of pSV0d or pGEM7Zf(-). Values from a single experiment of two replicate experiments are shown, with data expressed as percent acetylation assessed by Phosphorimager analysis.

variants containing mutations in putative regulatory motifs within the activation domain (Fig. 5). Previous studies have shown that mutation of the IR1 element (IRm) results in a decrease in activation by the MIE proteins (22). Figure 5 demonstrates that the addition of UL112-113 to the IRm promoter in the presence of the MIE proteins did not stimulate UL54 promoter activity. Thus, the MIE proteins and pZP24 appear to regulate UL54 promoter activity via the same sequence element. Mutation of other potential UL54 promoter elements in the activation domain had no effect on the ability of UL112-113 to stimulate UL54 promoter activity. These data suggest that UL112-113 acts in cooperation with MIE proteins

FIG. 5. Augmentation of UL54 promoter activity is affected by promoter mutations. The top section is a schematic representation of the UL54 activation domain indicating potential regulatory elements within the UL54 promoter region. HF cells were cotransfected with pPolCAT or the indicated mutated derivative (5 μ g) and pSVH (2.5 μ g) in the presence or absence of pZP24 (2.5 μ g). The total amount of DNA in each transfection was standardized to 10 μ g by the addition of pSV0d or pGEM7Zf(-). Values from a typical experiment of three replicate experiments are shown, with data expressed as percent acetylation assessed by Phosphorimager analysis.

FIG. 6. Cooperative activation of the UL54 promoter in cotransfection experiments with either pUL54Luc or pIRmLuc reporter plasmids and various combinations of putative transactivators. Transfections and luciferase assays were performed exactly as previously described (16). None, no effector plasmids; all, a mixture of pSVH and plasmids expressing TRS1 (pTRS1), UL33-38 (pZP8), UL84 (pZP13), and UL112-112 (pZP24). The remaining transfections contained all effector plasmids except for the indicated plasmid. The results of two independent experiments are shown (shaded bars, experiment 1; solid bars, experiment 2).

and cellular IR1-binding proteins either directly or indirectly to stimulate UL54 gene expression.

Role of IR1 in the cooperative activation by viral transactivators. The IR1 element in the UL54 promoter appears to be essential for UL112-113 stimulation of promoter activity. To determine if the IR1 element is necessary for complete activation of the UL54 promoter, we assessed the ability of the potential transactivators to cooperatively stimulate gene expression from the UL54 promoter (Fig. 6). The results demonstrate that the combination of UL36-38, UL112-113, TRS1, and the MIE proteins acts cooperatively to stimulate the wild-type UL54 promoter up to 11-fold over MIE proteins alone (Fig. 6). Omission of either the TRS1-expressing plasmid (pTRS1), the UL36-38-expressing plasmid (pZP8), pSVH, or pZP24 results in a dramatic decrease in UL54 promoter activity (Fig. 6). Previous studies demonstrated that mutation of the IR1 element within the UL54 promoter (IRm) results in a loss of protein binding to this promoter as well as a threefold reduction in the response of this promoter to IE proteins (22). In the presence of UL36-38, UL112-113, TRS1, and the MIE proteins, a four- to sevenfold decrease in promoter activity was observed with the IRm promoter relative to the wild-type promoter (Fig. 6). These results suggest that these viral transactivators act cooperatively with the MIE proteins to stimulate UL54 promoter activity via the IR1 element.

Assessment of UL54 promoter regulation in infected cells. Collectively, these experiments reveal that viral proteins, in addition to the MIE proteins, can influence UL54 promoter regulation via the IR1 element. To assess how these factors may influence UL54 promoter regulation within an HCMV-infected cell, two recombinant virus constructs were generated (Fig. 7). The first, RVd2polCAT, contains the region of the UL54 promoter from -128 to +20 upstream of the CAT reporter gene and thus contains the minimal activation domain of the UL54 promoter (48). The second recombinant virus construct, RVIRmCAT, contains the full-length UL54 promoter with the IRm mutation (Fig. 7).

RVd2polCAT and RVIRmCAT were generated by inserting the respective UL54 promoter-CAT constructs between the US9 and US10 ORFs of RV134 by using our previously described strategy (27). The integrity of the recombinant virus

constructs were assessed by Southern blot analysis (Fig. 7). The RVd2polCAT construct was identical to RVpolCAT except for the absence of UL54 promoter sequences from -425 to -128 (Fig. 7B). Appropriate integration of the UL54 IRm promoter-CAT construct was demonstrated by the presence of the CAT gene within a 4.2-kb subfragment of the *Hind*III X fragment of RVIRmCAT (Fig. 7C). This construct differs slightly from the original RVpolCAT construct by the presence of an intact *Hind*III site at the 5' and 3' ends of the UL54 promoter. Therefore, to confirm the presence of the UL54 promoter within RVIRmCAT, further Southern blot analysis was carried out (Fig. 7D). *Sall*-*Bam*HI digests of RVIRmCAT show that the UL54 promoter-CAT construct is essentially identical to RVpolCAT. Digestion of these viruses with *Eco*RV confirms the presence of the 4-nucleotide mutation within the IR1 element of RVIRmCAT.

CAT expression in RVd2polCAT- and RVIRmCAT-infected cells was then compared with that of the wild-type UL54 promoter recombinant virus construct (RVpolCAT), which has been previously described (27). HF cells were infected with the recombinant viruses under study at an equivalent multiplicity of infection and harvested at various times after infection. Mutation of the IR1 element of UL54 resulted in a three- to fourfold drop in the activity of this promoter at early times after infection (at 15 and 24 h and with PAA) (Fig. 8A). This is in agreement with our previous data which demonstrated that mutation of the IR1 element resulted in a threefold drop in UL54 promoter activity in response to viral proteins (22). A slightly higher level of CAT activity was observed with the RVd2polCAT-infected cells at the early time points. These data are consistent with the role of the -425 to -128 region of UL54 as a potential repressor of gene expression in transient-transfection assays. However, the magnitude of this effect was minimal compared with the fivefold difference observed in transient-transfection assays (48).

At late times after infection (72 and 96 h), similar levels of CAT activity were observed in RVpolCAT-, RVd2polCAT-, and RVIRmCAT-infected cells. To correlate protein levels with RNA levels over the course of the experiment, Northern blot analysis was performed (Fig. 8B). Consistent with CAT enzyme activity, CAT RNA levels from RVIRmCAT-infected cells were reduced significantly (threefold) relative to levels from RVpolCAT-infected cells at early times after infection. In particular, no CAT mRNA could be detected within HF cells infected with RVIRmCAT in the presence of PAA, a DNA synthesis inhibitor. In contrast, CAT mRNA was readily detectable in PAA-treated RVpolCAT and RVd2polCAT cells. Equivalent levels of CAT RNA were detected in RVpolCAT- and RVIRmCAT-infected cells at 72 h after infection. Collectively, these results suggest that the IR1 element is important for UL54 promoter activation at early times after infection but that at late times, alternative mechanisms of UL54 promoter regulation are employed.

DISCUSSION

The regulation of early-gene expression within an HCMV-infected cell is a very complex process. To date, studies analyzing early-gene regulation have relied on transient-transfection assays to study promoter regulatory elements (3, 7, 16, 22, 25, 26, 31, 39, 41, 43, 45, 46, 50, 54, 55). Studies of this type have revealed that the activation of early-gene promoters is dependent on the cooperation of cellular transcription factors with viral MIE proteins. However, this simple model cannot account for the wide range of temporal variation of this class of genes observed within an HCMV-infected cell. Recent studies

FIG. 7. (A) Schematic representation of recombinant virus constructs. The top line represents the HCMV genomic sequence with the 5-kb *HindIII* X fragment shown underneath. The indicated UL54 promoter-CAT constructs were inserted into the *ApaI* site in the US9-US10 intergenic region as described in Materials and Methods. Relevant restriction sites utilized in Southern blot analysis are indicated. A, *ApaI*; B, *BamHI*; H, *HindIII*; E, *EcoRV*; S, *SmaI*. The *BamHI* site at the 3' end of UL54-CAT in RVpolCAT was fortuitously regenerated by blunt-end cloning of the *HindIII*-*BamHI* fragment into the *ApaI* site as described previously (23). (B) Southern blot hybridization analysis of recombinant viruses RVpolCAT and RVd2polCAT. Total cellular DNA was isolated from cells infected with the indicated virus for 72 h, digested with *HindIII*, and subjected to Southern blot analysis. The blots were hybridized with ³²P-labeled probes, generated from the *HindIII* X fragment (HX) or CAT. The molecular sizes (in kilobase pairs) of the relevant bands are indicated, exhibiting the expected 297-bp difference in the *HindIII* X fragment of RVpolCAT and RVd2polCAT. (C and D) Southern blot analyses of RVIRmCAT. (C) *HindIII* digests of two independent isolates of RVIRmCAT probed with either a *HindIII*-X (HX) or a CAT digoxigenin-labeled probe. As shown in panel A, RVIRmCAT differs from the original RVpolCAT construct by the presence of intact *HindIII* sites at the 5' and 3' ends of the UL54 promoter, resulting in fragments of 4.2, 1.8, and 0.45 kb upon *HindIII* digestion. The 0.45-kb UL54 promoter fragment resulting from *HindIII* digestion does not hybridize with either probe. (D) Southern blot analyses of *EcoRV* digests of RVIRmCAT and RVpolCAT hybridized with the *HindIII* X probe (EcoRV) and *SmaI*-*BamHI* digests of RVIRmCAT, RVpolCAT, and the recombination vector used to generate RVIRmCAT probed with CAT (*SmaI*-*BamHI*). In the first gel the presence of the additional *EcoRV* site in RVIRmCAT is demonstrated by the 4.2- and 2.3-kb *EcoRV* fragments as opposed to the 6.5-kb fragment of RVpolCAT. In the second gel, the 1.4-kb *SmaI*-*BamHI* UL54 promoter-CAT fragment is indicated. Abbreviations (B to D): RV134, parental DNA utilized for homologous recombination; AD169, wild-type HCMV strain; RVpol, RVpolCAT; IRm1 and -2, two independent isolates of RVIRmCAT; Vector, recombination vector utilized to generate RVIRmCAT; RVd2pol, RVd2polCAT; pol, RVpolCAT.

have begun to identify additional viral transactivators expressed at various times after infection which are likely to play a role in early-gene regulation (5, 16, 18, 30, 47, 58). These transactivators typically act in cooperation with the MIE proteins but exhibit promoter specificity in transient-transfection assays. In the present study, we have begun to define the complex mechanisms involved in regulating a single HCMV early gene encoding the viral DNA polymerase (UL54). In addition to IE1 and IE2, other viral regulatory proteins that were capable of activating UL54 promoter expression in transient-transfection assays were identified. The analysis of this promoter in recombinant virus constructs indicates that different pathways of promoter activation may be employed by this gene at early and late times after infection.

Previous studies analyzing UL54 promoter regulation revealed that MIE protein activation of this promoter in transient-transfection assays was dependent upon a novel inverted

repeat element, IR1 (22). Mutation of this element abrogated binding of cellular proteins to the UL54 promoter and reduced activation of this promoter in response to both MIE proteins and viral superinfection. In this study, we showed that the IR1 element extends from -53 to -45 and includes sequences outside the 8-bp inverted repeat sequence. These results help to explain why deletion of the second copy of the IR1 repeat element located at -225 has no effect on UL54 promoter activity in transient-transfection assays (22, 48).

UL112-113 open reading frames are also involved in regulating the UL54 promoter. The UL112-113 gene produces a series of multiply spliced mRNAs regulated by a single promoter expressed at early times after infection (46). These RNAs encode four nuclear phosphoproteins of 34, 43, 50, and 84 kDa that have a common amino terminus (59). The mechanism of UL112-113 stimulation of promoter activity is unknown. It has been reported that the UL112-113 proteins can

FIG. 8. Kinetics of CAT expression from recombinant viruses. HF cells were infected with 5 PFU of the indicated viruses per cell in the presence or absence of PAA (200 μ g/ml). Cells were harvested at the indicated times (in hours) and assessed for CAT enzyme activity (A) or CAT RNA levels detected by Northern blot analysis utilizing a ^{32} P-labeled probe specific to the CAT gene (B). Percent acetylation and RNA levels were quantitated by Phosphorimager analysis. Equivalent multiplicities of infection for the viruses tested were verified by stripping the blot in panel B and reprobing with a probe to the pp28 gene (data not shown). Values represent typical results from two replicate experiments.

bind with a low affinity to DNA (17), suggesting that they may function by binding to the UL54 promoter. Alternatively, the fact that UL112-113 stimulatory activity requires IE1 and IE2 as well as IR1-binding proteins suggests that UL112-113 may affect gene expression via protein-protein interactions. One possibility is that UL112-113 acts to stabilize the interaction between cellular transcription factors and the IE proteins (Fig. 9). However, we cannot completely discount the possibility that UL112-113 affects HCMV early-gene expression via an indirect mechanism. These possibilities will be examined in future experiments by determining if the UL112-113 proteins can associate directly with IE proteins, cellular transcription factors, or both.

In addition to the UL112-113 ORF, the IRS1 and TRS1 genes were shown to activate UL54 promoter activity. IRS1 and TRS1 are highly related genes located within the *c* repeats of the HCMV genome and are expressed under IE conditions (4, 47). The proteins encoded by these two genes have previously been shown to activate the UL44 early-gene promoter in association with the MIE proteins (47). Mixing experiments demonstrate that the combination of IRS1 and UL112-113 together with the MIE proteins can stimulate UL54 promoter activity to even higher levels, suggesting that these proteins act cooperatively to regulate HCMV early-gene expression (Fig. 6) (16).

The UL84 open reading frame has been shown to be an essential gene for transient complementation of *ori*Lyt-dependent DNA replication (34, 35). The function of this gene is unknown, although it has recently been shown to associate with IE86 in HCMV-infected cells (44). This ability to interact with IE86 suggests a possible regulatory role for this protein. However, our experiments indicate that UL84 is unable to activate the UL54 promoter in our transient-transfection assays. These results are consistent with other studies showing that removal of UL84 from a mixture of transactivators had minimal effects on the activity of a variety of HCMV promoters (16). It is still possible that UL84 acts as a promoter-specific regulatory protein for other HCMV genes. Further experiments will be required to determine the true function of UL84 in HCMV replication.

Assessment of the IR1 element within the context of the viral genome confirmed the importance of this element in promoter regulation at early times after infection. However, at late times after infection a dramatic increase in the activity of this promoter was observed independent of IR1 mutation, such that CAT RNA levels were similar to those driven by the wild-type promoter. Essentially, mutation of the IR1 element in the UL54 promoter results in kinetics which mimic those of a true late promoter. It is possible that UL54 promoter activation late in infection is a result of nonspecific stimulation of this promoter via the TATA box. Although the requirements for late-gene expression in HCMV have not been extensively analyzed, one such study has shown that sequences from -40 to +106 are sufficient for late-gene promoter regulation within the context of the viral genome (27). Analysis of late-gene promoters of herpes simplex virus has revealed that the sequence elements important for late-gene activation consist of the TATA element and downstream sequences (10, 11, 13). In addition, the presence of a TATA box was found to be sufficient for activation of the herpes simplex virus IE3 promoter at late times after infection (37). We are currently inserting the d177 deletion mutant into the viral genome to assess if the UL54 TATA box is sufficient for late-phase activation of this promoter.

Our data suggest that there is a specific mechanism of UL54 promoter activation at late times after infection which is IR1 independent. We have evidence for other proteins which may be involved in late activation of this promoter. First, we have demonstrated the presence of an additional UL54 promoter-binding protein (B') at later stages of infection (Fig. 1). Second, preliminary results indicate that viral infection enhances

FIG. 9. Model for UL54 promoter activation at the early and late phases of infection as described in the text. Viral and cellular proteins that may influence UL54 promoter activity at the indicated times after infection are indicated. B' represents an additional protein that appears only late in infection.

the level of proteins binding to the ATF site within the UL54 promoter (23). We are presently screening for recombinant viruses containing a mutation in the ATF site to determine if this element is required for UL54 promoter activation at late times.

Together, these studies suggest a model for UL54 promoter activation in HCMV-infected cells (Fig. 9). The initial activation of this promoter requires viral MIE proteins which are the first viral proteins expressed at significant levels. As the infection proceeds, additional viral transactivators, including IRS1/TRS1, UL112-113, and UL36-38, are expressed, which results in additional stimulation of the UL54 promoter. These events are dependent primarily upon the integrity of the IR1 binding site, which is known to bind cellular proteins. At late times after infection, IR1-binding-protein levels are increased, which would be predicted to further increase UL54 gene expression as viral genome production reaches maximal levels. However, an alternative pathway of UL54 promoter regulation that is independent of IR1 binding to this promoter exists within HCMV-infected cells at the later stages of infection. It is intriguing that an IR1-independent pathway of UL54 promoter activation exists within HCMV-infected cells. Currently it is unknown if this pathway only functions in the absence of IR1-mediated activation or if these two pathways cooperate to maximize UL54 expression late in infection. We speculate that these two mechanisms of UL54 promoter regulation may have evolved to ensure expression of this essential early gene in different cell types which can vary in their expression of cellular transcription factors. It will be of interest to subsequently determine if this model is applicable to all permissive cell types and to determine how this model relates to UL54 promoter regulation in semipermissive and nonpermissive cells. These types of studies may reveal the role of early gene regulation in viral latency, reactivation, and pathogenesis.

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REFERENCES

- Alford, C. A., and W. J. Britt. 1990. Cytomegalovirus, p. 1981–2010. In B. N. Fields and D. M. Knipe (ed.), *Virology*, 2nd ed. Raven Press, New York.
- Artl, H., D. Lang, S. Gebert, and T. Stamminger. 1994. Identification of binding sites for the 86-kilodalton IE2 protein of human cytomegalovirus within an IE2-responsive viral early promoter. *J. Virol.* **68**:4117–4125.
- Chang, C.-P., C. L. Malone, and M. F. Stinski. 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. *J. Virol.* **63**:281–290.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Hersnell, C. A. Hutchinson III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154**:125–170.
- Colberg-Poley, A. M., L. D. Santomenna, P. P. Harlow, P. A. Benfield, and D. J. Tenney. 1992. Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. *J. Virol.* **66**:95–105.
- DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan. 1980. Patterns of transcription of human cytomegalovirus in permissively infected cells. *J. Virol.* **63**:1232–1238.
- Depto, A. S., and R. M. Stenberg. 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. *J. Virol.* **63**:1232–1238.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475–1489.
- Einhorn, L., and A. Ost. 1984. Cytomegalovirus infection of human blood cells. *J. Infect. Dis.* **149**:207–214.
- Guzowski, J. F., J. Singh, and E. K. Wagner. 1994. Transcriptional activation of the herpes simplex virus type 1 U_L38 promoter conferred by the *cis*-acting downstream activation sequence is mediated by a cellular transcription factor. *J. Virol.* **68**:7774–7789.
- Guzowski, J. F., and E. K. Wagner. 1993. Mutational analysis of the herpes simplex virus type 1 strict late U_L38 promoter/leader reveals two regions critical in transcriptional regulation. *J. Virol.* **67**:5098–5108.
- Hayhurst, G. P., L. A. Bryant, R. C. Caswell, S. M. Walker, and J. H. Sinclair. 1995. CCAAT box-dependent activation of the TATA-less human DNA polymerase alpha promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. *J. Virol.* **69**:182–188.
- Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine. 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ_2) gene lie within bases –34 to +124 relative to the 5' terminus of the mRNA. *Mol. Cell. Biol.* **6**:3652–3666.
- Huang, L., C. L. Malone, and M. F. Stinski. 1994. A human cytomegalovirus early promoter with upstream negative and positive *cis*-acting elements: IE2 negates the effect of the negative element, and NF-Y binds to the positive element. *J. Virol.* **68**:2108–2117.
- Ibanez, C. E., R. Schrier, P. Ghazal, C. Wiley, and J. A. Nelson. 1991. Human cytomegalovirus productively infects primary differentiated macrophages. *J. Virol.* **65**:6581–6588.
- Iskenderian, A. C., L. Huang, A. Reilly, R. M. Stenberg, and D. G. Anders. 1996. Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. *J. Virol.* **70**:383–392.
- Iwayama, S., T. Yamamoto, T. Furuya, R. Kobayashi, K. Ikuta, and K. Hirai. 1994. Intracellular localization and DNA-binding activity of a class of viral early phosphoproteins in human fibroblasts infected with human cytomegalovirus (Towne strain). *J. Gen. Virol.* **75**:3309–3318.
- Jenkins, D. E., C. L. Martens, and E. S. Mocarski. 1994. Human cytomegalovirus late protein encoded by ie2: a transactivator as well as a repressor of gene expression. *J. Gen. Virol.* **75**:2337–2348.
- Jones, T. R., and V. P. Muzithras. 1992. A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66**:2541–2546.
- Jones, T. R., V. P. Muzithras, and Y. Gluzman. 1991. Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. *J. Virol.* **65**:5860–5872.
- Jupp, R., S. Hoffmann, R. M. Stenberg, J. A. Nelson, and P. Ghazal. 1993. Human cytomegalovirus IE86 protein interacts with promoter-bound TATA-binding protein via a specific region distinct from the autorepression domain. *J. Virol.* **67**:7539–7546.
- Kerry, J. A., M. A. Priddy, and R. M. Stenberg. 1994. Identification of sequence elements in the human cytomegalovirus DNA polymerase gene promoter required for activation by viral gene products. *J. Virol.* **68**:4167–4176.
- Kerry, J. A., and R. M. Stenberg. Unpublished data.
- Klucher, K. M., D. K. Rabert, and D. H. Spector. 1989. Sequences in the human cytomegalovirus 2.7-kilobase RNA promoter which mediate its regulation as an early gene. *J. Virol.* **63**:5334–5343.
- Klucher, K. M., M. Sommer, J. T. Kadonaga, and D. H. Spector. 1993. In vivo and in vitro analysis of transcriptional activation mediated by the human cytomegalovirus major immediate-early proteins. *Mol. Cell. Biol.* **13**:1238–1250.
- Klucher, K. M., and D. H. Spector. 1990. The human cytomegalovirus 2.7-kilobase RNA promoter contains a functional binding site for the adenovirus major late transcription factor. *J. Virol.* **64**:4189–4198.
- Kohler, C. P., J. A. Kerry, M. Carter, V. P. Muzithras, T. R. Jones, and R. M. Stenberg. 1994. Use of recombinant virus to assess human cytomegalovirus early and late promoters in the context of the viral genome. *J. Virol.* **68**:6589–6597.
- Lang, D., S. Gebert, H. Artl, and T. Stamminger. 1995. Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. *J. Virol.* **69**:6030–6037.
- Lathey, J. L., and S. A. Spector. 1991. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. *J. Virol.* **65**:6371–6375.
- Liu, B., and M. F. Stinski. 1992. Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 *cis*-acting elements. *J. Virol.* **66**:4434–4444.
- Malone, C. L., D. H. Vesole, and M. F. Stinski. 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. *J. Virol.* **64**:1498–1506.
- McDonough, S. H., and D. H. Spector. 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. *Virology* **125**:31–46.
- Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis. 1981. Identification of DNA sequences required for transcription of the human $\alpha 1$ globin gene in a new SV40-host-vector system. *Cell* **27**:279–288.
- Pari, G. S., and D. G. Anders. 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus *ori-Lyt*-dependent DNA replication. *J. Virol.* **67**:6979–6988.

35. **Pari, G. S., M. A. Kacica, and D. G. Anders.** 1993. Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus *oriLyt*-dependent DNA synthesis. *J. Virol.* **67**:2575-2582.
36. **Rice, G. P. A., R. D. Schrier, and M. B. A. Oldstone.** 1984. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. *Proc. Natl. Acad. Sci. USA* **81**:5134-5143.
37. **Rivera-Gonzalez, R., A. N. Imbalzano, B. Gu, and N. A. Deluca.** 1994. The role of ICP4 repressor activity in temporal expression of the IE-3 and latency-associated transcript promoters during HSV-1 infection. *Virology* **202**:550-564.
38. **Schrier, R. D., J. A. Nelson, and M. B. A. Oldstone.** 1985. Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. *Science* **230**:1048-1051.
39. **Schwartz, R., M. H. Sommer, A. Scully, and D. H. Spector.** 1994. Site-specific binding of the human cytomegalovirus IE2 86-kilodalton protein to an early gene promoter. *J. Virol.* **68**:5613-5622.
40. **Scott, D. M., B. C. Rodgers, C. Freeke, J. Buiter, and J. G. P. Sissons.** 1989. Human cytomegalovirus and monocytes: limited infection and negligible immunosuppression in normal mononuclear cells infected in vitro with mycoplasma-free virus strains. *J. Gen. Virol.* **70**:685-694.
41. **Scully, A. L., M. H. Sommer, R. Schwartz, and D. H. Spector.** 1995. The human cytomegalovirus IE2 86-kilodalton protein interacts with an early gene promoter via site-specific DNA binding and protein-protein associations. *J. Virol.* **69**:6533-6540.
42. **Sommer, M. H., A. L. Scully, and D. H. Spector.** 1994. Transactivation by the human cytomegalovirus IE2 86-kilodalton protein requires a domain that binds to both the TATA box-binding protein and the retinoblastoma protein. *J. Virol.* **68**:6223-6231.
43. **Spector, D. H., K. M. Klucher, D. K. Rabert, and D. A. Wright.** 1990. Human cytomegalovirus early gene expression. *Curr. Top. Microbiol. Immunol.* **154**: 21-45.
44. **Spector, D. J., and M. J. Tevethia.** 1994. Protein-protein interactions between human cytomegalovirus IE2-580aa and pUL84 in lytically infected cells. *J. Virol.* **68**:7549-7553.
45. **Staprans, S. I., D. K. Rabert, and D. H. Spector.** 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. *J. Virol.* **62**:3463-3473.
46. **Staprans, S. I., and D. H. Spector.** 1986. 2.2-kilobase class of early transcripts encoded by cell-related sequences in human cytomegalovirus strain AD169. *J. Virol.* **57**:591-602.
47. **Stasiak, P. D., and E. S. Mocarski.** 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. *J. Virol.* **66**:1050-1058.
48. **Stenberg, R. M.** 1993. Immediate-early genes of human cytomegalovirus: organization and function, p. 330-359. *In* E.-S. Huang (ed.), *Molecular aspects of human cytomegalovirus diseases*. Springer-Verlag, Heidelberg, Germany.
49. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. *J. Virol.* **64**:1556-1565.
50. **Stenberg, R. M., and J. A. Kerry.** Cytomegalovirus genes: their structure and function. *Scand. J. Infect. Dis.*, in press.
51. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. *J. Virol.* **49**: 190-199.
52. **Stinski, M., and T. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. *J. Virol.* **55**:431-441.
53. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. *J. Virol.* **46**:1-14.
54. **Taylor-Wiedeman, J., J. G. Sissons, L. K. Borysiewicz, and J. H. Sinclair.** 1991. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* **72**:2059-2064.
55. **Wade, E. J., K. M. Klucher, and D. H. Spector.** 1992. An AP-1 binding site is the predominant *cis*-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. *J. Virol.* **66**:2407-2417.
56. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. *J. Virol.* **41**:462-477.
57. **Weinschenker, B. G., S. Wilton, and G. P. A. Rice.** 1988. Phorbol ester-induced differentiation permits productive human cytomegalovirus infection in a monocyte cell line. *J. Immunol.* **140**:1625-1631.
58. **Winkler, M., S. A. Rice, and T. Stamminger.** 1994. UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression. *J. Virol.* **68**: 3943-3954.
59. **Wright, D. A., S. I. Staprans, and D. H. Spector.** 1988. Four phosphoproteins with common amino termini are encoded by human cytomegalovirus AD169. *J. Virol.* **62**:331-340.