JOURNAL OF VIROLOGY, Jan. 2005, p. 225–233 Vol. 79, No. 1
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.1.225–233.2005

The Carboxyl-Terminal Region of Human Cytomegalovirus $IE1_{491aa}$ Contains an Acidic Domain That Plays a Regulatory Role and a Chromatin-Tethering Domain That Is Dispensable during Viral Replication

Jens Reinhardt,[1]† Geoffrey B. Smith,[1] Christopher T. Himmelheber,[2] Jane Azizkhan-Clifford,[2] and Edward S. Mocarski[1]*

Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford, California,[1] and Department of Biochemistry, Drexel University College of Medicine, Philadelphia, Pennsylvania[2]

Received 4 June 2004/Accepted 30 August 2004

The human cytomegalovirus major immediate-early (α) protein $IE1_{491aa}$ plays an important role in controlling viral gene expression at low multiplicities of infection. With a transient complementation assay, full-length $IE1_{491aa}$ enhanced the growth of *ie*1 mutant virus CR208 20-fold better than a deletion mutant lacking 71 carboxyl-terminal amino acids ($IE1_{1-420aa}$). A 16-amino-acid domain between amino acids 476 and 491 was both necessary and sufficient for chromatin-tethering activity; however, this domain was completely dispensable for complementation of CR208 replication. The proximal 55-amino-acid acidic domain (amino acids 421 to 475) was found to be most important for function. A deletion mutant lacking only this domain retained chromatin-tethering activity but failed to complement mutant virus. Interestingly, serine phosphorylation (at amino acids 399, 402, 406, 423, 428, 431, 448, 451, and 455) was not required for complementation. These results show that $IE1_{491aa}$ is composed of at least two domains that support replication, a region located between amino acids 1 and 399 that complements *ie*1 mutant virus replication to low levels and an acidic domain between amino acids 421 and 479 that dramatically enhances complementation.

Human cytomegalovirus (HCMV), the prototype of the betaherpesviruses, is a widespread opportunistic pathogen (40) causing neurological damage as a result of congenital infection as well as significant disease in organ transplant recipients and AIDS patients. Like all herpesviruses, the replication cycle of HCMV is temporally regulated by viral functions. The α, or immediate-early (IE), phase immediately following viral entry is activated by virion-associated regulators independent of viral gene expression; the β, or delayed-early, phase is activated by α gene products but is independent of viral DNA replication; and the γ, or late, phase is activated by α gene products as well as functions associated with viral DNA replication (33).

Infection has a dramatic impact on cellular gene expression as well (5, 19). The timing and intensity of these phases are also influenced by host cell type, differentiation state, and cell cycle status (6, 7, 11, 33). Undifferentiated human cells and cells from other animal species except chimpanzees (41) are nonpermissive for HCMV replication and are generally restricted at a step following entry, uncoating, and α gene expression (28). The two major α gene products, $IE1_{491aa}$ and $IE2_{579aa}$, control the progression of HCMV replication in permissive cells by regulating later classes of viral gene expression (33). Ribozymes targeted to an *ie*1/*ie*2 common exon inhibit replication (55). Viral mutants that lack $IE1_{491aa}$ but express $IE2_{579aa}$ show a multiplicity of infection (MOI)-dependent block to replication associated with a failure to express critical viral gene products (12, 15, 35). Though less abundant than $IE1_{491aa}$, $IE2_{579aa}$ is widely believed to be the key viral regulatory gene product based on several lines of evidence: (i) strong transactivation of TATA box-containing promoters from viral or cellular sources in transient assays (33), (ii) association with a wide range of cellular transcription factors and cell cycle-related proteins similar to adenovirus E1A (6, 7, 33), and (iii) failure of viral mutants in this gene or its murine CMV homologue (*ie*3) to replicate or to express viral β or γ gene products (3, 30, 50).

$IE1_{491aa}$ (also called IE1 p72, IE72, and IE1), the major α (immediate-early) gene product of CMV, is a 72- to 75-kDa phosphoprotein that accumulates in the nucleus throughout HCMV infection. Early evidence from transient assays revealed an adjunct role for $IE1_{491aa}$ in cooperation with $IE2_{579aa}$ to transactivate viral and cellular promoters (33). In the absence of $IE2_{579aa}$, $IE1_{491aa}$ transactivates the *ie*1/*ie*2 promoter-enhancer (9) as well as promoters from selected cellular genes, including heat shock protein 70 (16), interleukin (IL)-1β (20), IL-6 (13), IL-8 (37), DNA polymerase α (18), origin recognition complex I protein (47), and dihydrofolate reductase (32). In addition to its possible role in activation of host cell gene expression, $IE1_{491aa}$ represses expression of the IL-1 receptor type 1 gene in transient assays (24). $IE1_{491aa}$ induces transcription factors, including NF-κB (9, 44), activator protein 1 (23), and E2F1 (32), and may participate in stimulation of the host cell toward an S-phase-like state (6, 11, 33).

$IE1_{491aa}$ is sumoylated on Lys_{450} (49, 52) and disrupts nu-

* Corresponding author. Mailing address: Department of Microbiology & Immunology, Fairchild Science Building, 299 Campus Dr., Stanford University School of Medicine, Stanford, CA 94305-5124. Phone: (650) 723-6435. Fax: (650) 723-1606. E-mail: mocarski@stanford.edu.

† Present address: Station Biologique, Roscoff Cedex, France.

clear domain 10 (PML bodies) (2, 26), which is dependent on Leu_{174} (36, 51). $IE1_{491aa}$-mediated disruption of nuclear domain 10 plays no apparent role in viral replication because *ie*1 mutant viruses fail to disrupt nuclear bodies (1) at high MOIs, where replication proceeds unimpeded. $IE1_{491aa}$ is a phosphoprotein (38) and is phosphorylated in the presence or absence of other viral proteins (7, 39) at a number of sites (Ser_{399}, Ser_{402}, Ser_{406}, Ser_{423}, Ser_{428}, Ser_{431}, Ser_{448}, Ser_{451}, and Ser_{455}) near or within a carboxyl-terminal acidic domain (AD) (Himmelheber et al., 28th International Herpesvirus Workshop, abstract 5.32). $IE1_{491aa}$ may undergo autophosphorylation and may influence the phosphorylation state of other proteins, such as E2Fs and retinoblastoma protein family members (7, 39). The contribution of individual functional domains to these various $IE1_{491aa}$ functions has not yet been fully evaluated, with the exception that sumoylation (at Lys_{450}) has been shown to be dispensable for viral growth (49).

$IE1_{491aa}$ may alter host cell behavior (46) such as susceptibility to apoptosis (54), suppress p107-mediated block in cellular proliferation (22), and activate cyclin E/cdk2 kinase activity (53), although these phenomena have only been observed in transient assays. The available *ie*1 mutant viruses show no obvious signs of altered host cell behavior during infection in permissive human fibroblasts (HFs) (12, 15; Dittmer and Mocarski, unpublished observations). Furthermore, constitutive expression of $IE1_{491aa}$ in normal or immortalized HFs has little overall impact on cell viability, growth properties, cell cycle control, or susceptibility to apoptosis (16; Dittmer and Mocarski, unpublished observations).

Unambiguous evidence supporting a role of $IE1_{491aa}$ in viral replication emerged from studies on *ie*1-deficient viruses (15, 35), which are replication deficient at low MOIs. The defect has been ascribed to poor expression of viral β and γ genes, including UL44, UL57, UL69, UL98, and UL112 (12, 15). Although the mechanism underlying the MOI-dependent growth of this mutant is not understood, the results are consistent with an accessory role of $IE1_{491aa}$ acting together with $IE2_{579aa}$ to enhance the expression of essential viral β and γ genes. Constitutive (15) or transient (this study) expression of $IE1_{491aa}$ fully complements mutant viral replication.

$IE1_{491aa}$ tethers to host metaphase chromatin during mitosis via an acidic carboxyl-terminal region (29, 51); however, no role for tethering in viral replication or in modifying host cell behavior has been reported. Two well-known gammaherpesvirus proteins tether as a part of their natural function, Epstein-Barr virus nuclear antigen 1 (EBNA-1) (31) and Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen (LANA) (42). Tethering to metaphase chromatin is part of the genome maintenance function of these proteins during latency and collaborates with latent origin-specific binding to control replication of the viral genome. $IE1_{491aa}$ has no known role in HCMV latency and has not been found to bind directly to viral or host DNA. $IE1_{491aa}$ expression has not been detected during natural latency and becomes undetectable within a few days in experimental models of latency in myeloid progenitors (17, 25). Thus, any role of this regulatory protein in latent genome maintenance remains enigmatic.

We used the MOI-dependent growth properties of *ie*1 mutant virus CR208 as an assay system to study the role of $IE1_{491aa}$ domains during infection. A portion of $IE1_{491aa}$ (amino acids 1 to 420) was found to retain partial function, with the carboxyl-terminal acidic region (amino acids 420 to 479) necessary for full *trans*-complementation of the growth of CR208. This assay provided a simple and direct means to separate the roles of the chromatin-tethering domain (CTD; amino acids 476 to 491) and the adjacent AD (amino acids 421 to 479) without introducing the adventitious mutations that result in growth defects independent of the targeted gene that are found in HCMV mutants made by transfection of plasmids, cosmids, or bacmids (34). Our results also indicate that the acidic character of rather than serine phosphorylation in the AD region is important for complementation.

1. Oligonucleotides used to generate IE1 deletion mutants

No.	Sequence
1	5′GGCAAGCTTACGATGGAGTCCTCTGCC3′
2	5′CCGCTCGAGCTGGTCAGCCTTGCTTCTAG3′
3	5′CCGCTCGAGAGCCACAATTACTGAGGAC3′
4	5′GCCCTCGAGGCTCTTGCCTCCAGAG3′
5	5′TCGAGACGATGGGAGGCAAGAGCACCCACCC TATGGTGACTAGAAGCAAGGCTGACCAGT3′
6	5′CTAGACTGGTCAGCCTTGCTTCTAGTCACCAT AGGGTGGGTGCTCTTGCCTCCCATCGTC3′
7	5′GGC GCTAGCACGATGGAGTCCTCTGCC3′
8	5′GGCCTCGAGCTCCTCCTGAGCACCCTCCTC3′
9	5′GCC CTCGAGCAGAGGGATAGTCGCGGG3′
10	5′GCC CTCGAGATCAGAGGAGCTGACACC3′
11	5′GCTGATGAGGAAGAGGAGGAGGGTGCTCAG GAGGAGCGGGAGGACACTGTGGCTGTCAAG GC-TGAGCCAGTGGCTGAG3′
12	5′ATCAGCCTGCTCAGCTTCTTCCTGATCAACGT TCTCAGCCACAATTACTGCGGCCAGAGGGAT A-GTCGCGGGTACAGG3′
13	5′GGCGAATTCGAGGAGATCTGCATGAAG3′
14	5′GGCGAATTCGGCCAGCATCACACTAGTC3′

MATERIALS AND METHODS

Plasmids. All *ie*1 mutants were constructed by PCR-driven mutagenesis with plasmid pON2202 (21) as a template. pCMV $IE1_{full\text{-}length}$ and pCMV $IE1_{full\text{-}length}$GFP were constructed from a cDNA generated by PCR with oligonucleotides 1 and 2 (Table 1). pCMV $IE1_{1\text{-}420}$ and pCMV $IE1_{1\text{-}420}$GFP were generated by PCR with oligonucleotides 1 and 3. pCMV $IE1_{1\text{-}479}$ and pCMV $IE1_{1\text{-}479}$GFP were generated by PCR with oligonucleotides 1 and 4. The resulting PCR products for these nontagged cDNAs were cloned into HindIII- and XhoI-digested pcDNA3.1 (Invitrogen) and subsequently cloned into pCMV(4x)GFP after digestion with NheI and XbaI as green fluorescent protein (GFP)-tagged derivatives.

Plasmid pCMV(4x)GFP, a gift of Irina Conboy, expresses GFP under the control of an HCMV promoter in which the four NF-κB sites were destroyed with point mutations (Conboy and Jones, personal communication). pCMV $IE1_{\Delta 421\text{-}475}$GFP was generated by ligating annealed oligonucleotides 5 and 6 to XhoI- and XbaI-digested pCMV $IE1_{1\text{-}420}$GFP, thereby adding two amino acids (threonine and methionine) between codons 420 and 476. To construct pCMV $IE1_{476\text{-}491}$GFP, annealed oligonucleotides 5 and 6 were ligated to Xhol- and Xbal-digested pCMV(4x)GFP, thereby placing codons 476 to 491 immediately downstream of the natural $IE1_{491aa}$ initiation codon. To construct pCMV $IE1_{1\text{-}442}$GFP, pCMV $IE1_{1\text{-}414}$GFP, and pCMV $IE1_{1\text{-}399}$GFP, PCR products generated with oligonucleotides 2 and 8, 2 and 9, 2 and 10, respectively, were cloned into NheI- and XhoI-digested pCMV(4x)GFP. The pSG5-IE1 plasmid (provided by John Sinclair) was used as a template to make *ie*1 mutants with serine modifications at amino acids 399, 402, 406, 423, 428, 431, 448, 451, and 455. These serines were exchanged in groups of three to either alanine (pSG5-$IE1_{S\rightarrow A}$) or glutamate (pSG5-$IE1_{S\rightarrow E}$) with the QuikChange site-directed mutagenesis kit (Strategene) according to the manufacturer's protocol. The sequences of all primers are shown in Table 2 (48).

To generate the construct in which all nine serines were mutated, mutation

TABLE 2. Oligonucleotides used to generate IE1 serine substitution mutants

Sites mutated	Sequence[a]
399, 402, 406A	5′GATGCTCTGGTGGCACCCCCAGAGGCCC CT3′
399, 402, 406E	5′CCTCTGATGAGCTGGTGGAGCCCCCAGAGGAGCCTGTACCCGCG3′
423, 428, 431A	5′GAGAACGCTGATCAGGAAGAAGCTGAGCAGGCTGATGAG3′
423, 428, 431E	5′GAGAACGAAGATCAGGAAGAAGAAGAGCAGGAAGATGAG3′
448, 451, 455A	5′ACTGTGGCTGTCAAGGCTGAGCCAGTGGCTGAGATA3′
448, 451, 455E	5′GGACACTGTGGAGGTCAAGGAGGAGCCAGTGGAGGAGATAGAGG3′

[a] Mutated codons are underlined.

reactions were performed sequentially with single and then double group mutations as templates. The original constructs were transferred into the pCMV-GFP backbone for comparison with the deletion mutants by PCR to amplify the region representing amino acids 350 to 491 followed by BglII and XhoI digestion and ligation into BglII- and XhoI-digested pCMV-$IE1_{491}$GFP.

Cell and virus culture. All cells were cultivated at 37°C in a 5% CO_2 atmosphere. HFs prepared locally from pooled newborn foreskins and immortalized ihfie1.3 cells stably expressing $IE1_{491aa}$ (15) were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% NuSerum I (Collaborative Research Inc.), 100 U of penicillin/ml, 100 U of streptomycin sulfate/ml, 0.66 mM arginine, 1.48 mM glutamine, and 0.24 mM asparagine (complete medium). The *ie*1-deficient HCMV strain CR208 and the repaired strain CRQ208 (15) were cultured in ihfie1.3 or in HF cells, respectively, as described (15). Cell supernatants were clarified by sedimentation at 1,500 x *g* for 20 min, disrupted with a probe type sonicator, and stored in aliquots at −80°C as virus stock. Infections were carried out with virus diluted in DMEM-0.2% NuSerum, and cells were rinsed once and incubated in complete medium supplemented with 0.16% pooled human gamma globulin (Baxter Corp.) to prevent viral spread through the medium. Virus titrations were performed in six-well dishes incubated for 10 days postinfection, at which time cells were methanol fixed and stained with Giemsa.

Cell transfection. Transfection of HFs was carried out with calcium phosphate precipitation (12, 15) modified as follows. HFs were split at a ratio of 1:2, incubated for 1 day, and split so that 2.2×10^5 cells were seeded into each well of a six-well dish. One day later, the medium was replaced with 2 ml of complete medium. To 15 μg of plasmid DNA and 31 μl of 2 M $CaCl_2$, sterile H_2O was added to a volume of 219 μl. Subsequently, 250 μl of 2x HBS (280 mM NaCl, 1.4 mM Na_2HPO_4, 10 mM KCl, 5.6 mM glucose, 20 mM HEPES, pH 7.05) was added dropwise, and this mixture was added to cells. After incubation for 4 to 6 h, the medium was removed and the cells were incubated for 90 s with 15% (vol/vol) glycerol in 1× HBS followed by four washes and addition of complete medium. GFP-based fluorescence 24 h later was used to assess transfection efficiency. For transfection-infection assays, the transfected cells were infected at 2 days posttransfection.

Immunoblot. Cells were harvested at 48 h posttransfection, lysed in 2x Laemmli buffer, and separated on a 10% polyacrylamide–sodium dodecyl sulfate gel (27). Proteins transferred to a nitrocellulose membrane were detected with anti-IE1/IE2 monoclonal antibody 810 (Chemicon) and anti-β-actin antibody A5441 (Sigma) with the enhanced chemiluminescence kit (Amersham Corp.) following the manufacturers' instructions.

Sequence analysis. The sequence of HCMV Towne strain $IE1_{491aa}$ (accession number gi:73633) amino acids 421 to 491 was aligned to that of full-length chimpanzee CMV IE1 (gi:19881132), rhesus CMV IE1 (gi:332085), and simian CMV IE1 (gi:1616980) with ClustalW of MacVector 7.1.1 (Accelrys, San Diego, Calif.).

RESULTS

Complementation of CR208 by transiently expressed $IE1_{491aa}$ and $IE1_{1-420}$. Replication of *ie*1-deficient CR208 is severely compromised on normal HFs at MOIs of <1, and this defect is complemented on $IE1_{491aa}$-expressing ihfie1.3 cells (12, 15). We took advantage of this MOI-dependent growth phenotype to investigate the ability of full-length and mutant forms of $IE1_{491aa}$ to complement CR208 plaque formation following transient transfection. We first evaluated the relationship between MOI and plaque formation by plating serial threefold dilutions of CR208 on either normal HFs or complementing ihfie1.3 cells (Fig. 1A). Maximal differences in efficiency of plaque formation on 2.2×10^5 HFs were observed at MOIs below 0.01, where CR208 yielded few or no plaques

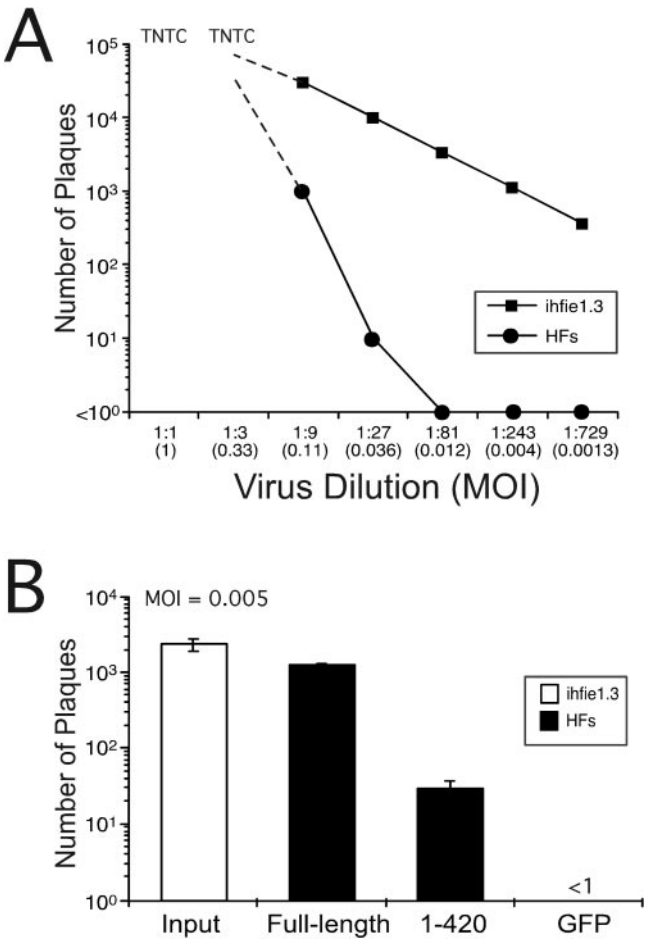


FIG. 1. Complementation of CR208 by transient transfection. (A) Titration of the *ie*1-deficient HCMV mutant CR208 by serial threefold dilution on complementing (ihfie1.3) and noncomplementing (HF) cells. TNTC, too numerous to count. (B) CR208 complementation by wild-type and mutant *ie*1 expression plasmids transfected into HFs. HFs were transfected with pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1-420}$GFP, or pCMV-GFP alone and infected with CR208 at an MOI of 0.005. The graph shows the mean plaque counts from three parallel assays, with the standard deviation of the mean indicated by bars. The input titer of CR208 was determined on ihfie1.3 cells (open bar). Expression constructs are named in the figure by the portion of IE1 that is expressed.

and transfection of full-length $IE1_{491aa}$ into 10% of the cells resulted in the formation of up to 10^3 plaques (Fig. 1B). Therefore, MOIs in this range were employed in experiments to compare $IE1_{491aa}$ and mutants by a transient complementation assay.

We compared complementation of CR208 by full-length $IE1_{491aa}$ and a mutant lacking the carboxyl-terminal region (amino acids 421 to 491). This region has a dense concentration of 26 acidic amino acids (29), a chromatin localization domain (51), and nine sites of potential serine phosphorylation (Himmelheber et al., 28th International Herpesvirus Workshop, abstract 5.32). We transiently expressed pCMV $IE1_{full\text{-}length}$GFP or mutant pCMV $IE1_{1\text{-}420}$GFP in HFs and subsequently infected these cells with CR208 at an MOI of 0.005 (determined by plaque assay on ihfie1.3 cells and depicted by the open bar in Fig. 1). Transient expression of the mutant resulted in 30-fold fewer plaques than with the full-length construct (Fig. 1B), even though the transfection efficiency of the plasmids was similar, based on the number of GFP-positive cells (data not shown). HFs transfected with the full-length construct complemented CR208 at levels that were comparable to infection of ihfie1.3 cells (Fig. 1B), so long as the transfection efficiency was taken into consideration. Plaquing efficiency following transfection by the method used here was consistently 10 to 20% of that observed in stably expressing cells.

As expected, the failure of CR208 to form plaques at this MOI did not change when a control GFP expression vector was transfected into the cells (Fig. 1B). The pCMV $IE1_{full\text{-}length}$GFP construct showed a transfection efficiency and complementation pattern similar to that of the nontagged full-length $IE1_{full\text{-}length}$ construct (data not shown). These results identified at least one potentially important functional domain in the carboxyl-terminal region of $IE1_{491aa}$ and revealed a low-level complementation activity of the truncated $IE1_{1\text{-}420}$ that was greater than that of negative controls. Furthermore, plaques formed with either full-length IE1 or the $IE1_{1\text{-}420}$ mutant were large, comparable in size to those formed by CR208 in ihfie1.3 monolayers or by parental virus on HFs (data not shown), suggesting that both retained full ability to complement cell-to-cell spread (12, 16) despite the fact that the transient transfection procedure did not deliver IE1 to all cells.

Conservation of two distinct domains within the carboxyl terminus of primate CMV homologs of $IE1_{491aa}$. The carboxy-terminal 71 amino acids of $IE1_{491aa}$ include 26 acidic amino acids distributed in clusters containing as many as six consecutive acidic amino acids (Fig. 2A). The region is therefore strongly acidic (38%) and contains only one basic amino acid except for a short region located at the extreme carboxyl terminus. This 16-amino-acid region has a greater proportion of basic amino acids (Fig. 2A). The estimated isoelectric point of the extreme carboxyl-terminal 16 amino acids (476–491) is 9.81, compared with 3.64 for amino acids 420 to 491 and 4.54 for full-length $IE1_{491aa}$. A comparison of this 71-amino-acid region from HCMV with homologues from chimpanzee CMV, simian CMV, and rhesus CMV revealed an AD with an adjacent highly conserved basic region towards the extreme carboxyl terminus in all (Fig. 2A). For example, the carboxyl terminus of HCMV and chimpanzee CMV IE1 exhibit 87% identity, compared to an overall 67% identity for the full-length proteins and 50% identity for the AD (data not shown). Parenthetically, neither rat nor murine CMV *ie*1 proteins have a domain that is homologous to this short carboxyl-terminal basic region (data not shown).

Chromatin tethering by the carboxyl-terminal 16 amino acids of $IE1_{491aa}$. Previous studies showed that the $IE1_{491aa}$ protein tethers to metaphase chromosomes in transiently transfected cells (29, 51), although the importance of this activity to virus replication has not been addressed. A deletion affecting a large portion of the carboxyl-terminal 71 amino acids (amino acids 421 to 485), removing the entire acidic region along with part of the basic, highly conserved extreme carboxyl terminus, disrupted the ability of $IE1_{491aa}$ to tether (51). In order to more precisely identify the region responsible for chromatin tethering by $IE1_{491aa}$, we constructed a series of deletion mutants, leaving amino acids 1 to 420 (pCMV $IE1_{1\text{-}420}$GFP), 1 to 479 (pCMV $IE1_{1\text{-}479}$), or 1 to 420 together with 476 to 491 (pCMV $IE1_{\Delta 421\text{-}475}$GFP) all fused to the amino terminus of GFP. In parallel, the extreme carboxyl-terminal $IE1_{491aa}$ amino acids 476 to 491 were placed downstream of the natural $IE1_{491aa}$ start codon and expressed as an amino-terminal fusion to GFP (pCMV $IE1_{476\text{-}491}$GFP). The constructs were transiently transfected into HFs to assess chromatin-tethering activity in mitotic cells with either $IE1_{491aa}$ monoclonal antibody or GFP monoclonal antibody in immunofluorescence analysis (Fig. 2B) together with a DNA counterstain (Hoechst 33342 or propidium iodide; Fig. 2B). For comparison, the full-length $IE1_{491aa}$ protein was also visualized in stably expressing ihfie1.3 cells with monoclonal antibody.

While full-length protein associated with chromatin in dividing cells as expected (30, 51) (Fig. 2B), $IE1_{1\text{-}479}$ failed to bind to metaphase chromatin (Fig. 2B) (51). To directly assess the capacity of the different regions to mediate tethering, deletion of the 55-amino-acid acidic domain (pCMV $IE1_{\Delta 421\text{-}475}$GFP) did not disrupt the ability to associate with metaphase chromatin, and addition of the extreme carboxyl-terminal 16 amino acids (pCMV $IE1_{476\text{-}491}$GFP) to GFP was sufficient for association (Fig. 2B). GFP alone stained the entire metaphase cell and was excluded from chromatin (data not shown). Thus, a CTD is located in a highly conserved, extremely carboxyl-terminal 16-amino-acid region of $IE1_{491aa}$. This domain is both necessary and sufficient for binding to metaphase chromatin in transfected cells.

Role of the AD and CTD in complementation of CR208. In order to evaluate the role(s) of the 55-amino-acid AD and 16-amino-acid CTD in complementation of *ie*1 mutant virus, HFs were transfected with pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}479}$, pCMV $IE1_{1\text{-}420}$GFP, pCMV $IE1_{\Delta 421\text{-}475}$GFP, and control pCMV-GFP. At 48 h posttransfection, cells were infected with CR208 at low MOIs, and plaque counts were performed 10 days later. Figure 3A shows complementation experiments at MOIs of 0.0004 and 0.002, which revealed the importance of the AD in complementation of *ie*1 mutant virus during replication at low MOIs. As expected, $IE1_{1\text{-}420}$ was defective in complementation, $IE1_{491aa}$ fully complemented plaque formation, and the GFP control plasmid failed to complement virus growth (Fig. 3A and data not shown). $IE1_{1\text{-}479}$ complemented CR208 as well as full-length IE1 at all MOIs

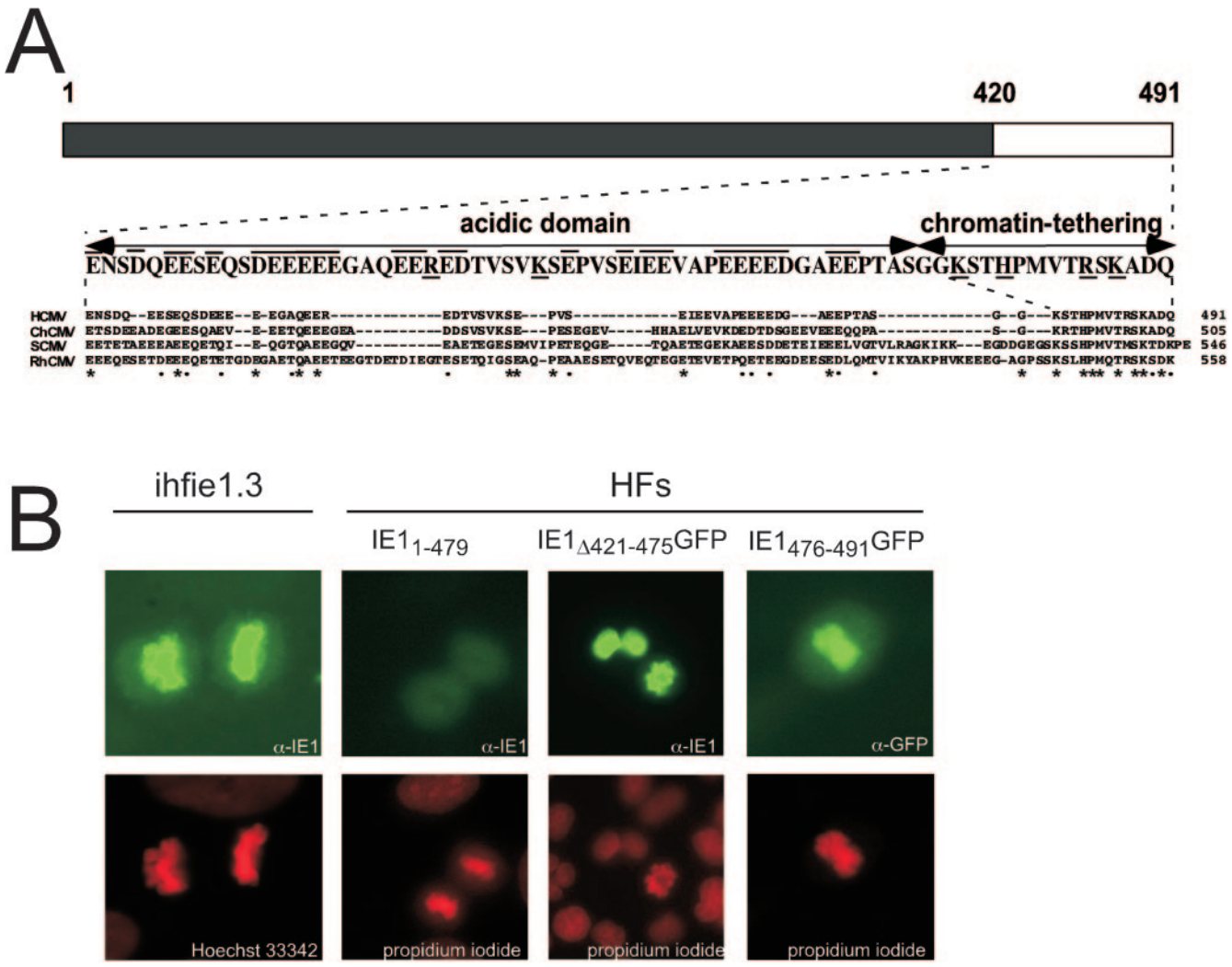


FIG. 2. Localization of the chromatin-tethering domain in $IE1_{491aa}$. (A) Sequence comparison of the acidic regions of primate CMV *ie*1 proteins. HCMV $IE1_{491aa}$ is depicted with amino acids 421 to 491 expanded and divided into a 55-amino-acid acidic domain (amino acids 421 to 475) and a 16-amino-acid chromatin-tethering domain (amino acids 476 to 491). Basic amino acids are underlined, and acidic amino acids are overlined. An alignment of the acidic region of HCMV $IE1_{491aa}$ and homologues from chimpanzee CMV (ChCMV), simian CMV (SCMV), and rhesus CMV (RhCMV) is displayed below the expanded sequence. Conserved amino acids are marked with asterisks; positions with conservative substitutions are indicated by a dot. Dashes indicate a gap in the alignment. (B) Metaphase chromatin tethering by wild-type and and mutant *ie*1 constructs. ihfie1.3 cells and HFs transfected with pCMV $IE1_{1-479}$, pCMV $IE1_{\Delta421-475}$GFP, or pCMV $IE1_{476-491}$GFP were stained with anti-GFP and fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin antibody (ihfie1.3 and $IE1_{476-491}$GFP) to visualize GFP expression or IE1- and IE2-specific antibody and fluorescein isothiocyanate-labeled anti-mouse immunoglobulin ($IE1_{1-479}$ and $IE1_{\Delta421-475}$GFP) to visualize *ie*1 expression. Chromatin was stained with Hoechst 33342 or propidium iodide as indicated on the individual panels.

tested (Fig. 3A and data not shown), consistent with a role for the AD in supporting replication.

Transfection of pCMV $IE1_{\Delta421-475}$GFP, which lacked the 55-amino-acid AD but contained the 16-amino-acid CTD (Fig. 2B), complemented at about the same poor level as $IE1_{1-420}$. These results, which employed $IE1_{1-479}$ without a GFP fusion, were confirmed with pCMV $IE1_{1-479}$GFP (data not shown). Thus, the ability to tether chromatin did not contribute to $IE1_{491aa}$ function in viral replication in HFs, suggesting that this activity may be important in other cell types or, as suggested by the function of the other characterized herpesvirus tethering proteins LANA (42) and EBNA-1 (31), in latency. The AD is critical to the activity of $IE1_{491aa}$ during viral replication.

When the ratios of plaques obtained with these three *ie*1 mutants were calculated as a percentage of the plaques obtained with the full-length construct, differences were readily appreciated at MOIs of 0.0004, 0.002, and 0.01 (Fig. 3B). First, as expected, background replication in the absence of complementation was detected at the highest MOI, consistent with the established growth properties of CR208 (12, 15) and as shown in Fig. 1. Second, complementation appeared to be highly dependent on the AD at all MOIs tested, so that amino acids 1 to 420 complemented to consistently low levels. Finally, complementation by the 1 to 479 construct, which lacked the ability to tether chromatin, ranged from 180% of that of the full-length construct (MOI of 0.0004) to 130% (at an MOI of 0.002) to 60% (at an MOI of 0.01), all consistent with our interpretation that the CTD is dispensable for replication.

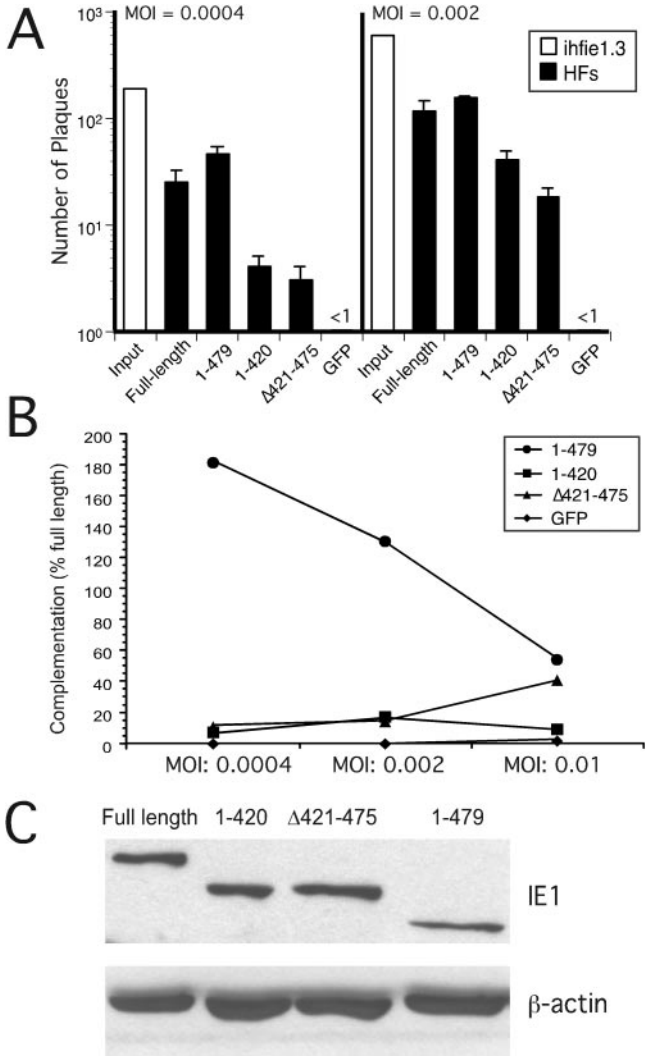


FIG. 3. Role of the acidic domain (AD) and the chromatin-tethering domain (CTD) for complementation by $IE1_{491aa}$. (A) Complementation of CR208 by different $IE1_{491aa}$ constructs at various MOIs. HFs transfected with pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}479}$, pCMV $IE1_{1\text{-}420}$GFP, pCMV $IE1_{\Delta421\text{-}475}$GFP, or pCMV-GFP alone were infected with CR208 at the indicated MOIs. The black bars represent the mean plaque count of three replicate assays, with the standard deviation of the mean indicated by error bars. The input titer of CR208 was determined on ihfie1.3 cells (open bars). (B) MOI dependence of complementation of CR208 by *ie*1 constructs. The graph shows the percentage of plaques relative to the wild-type level achieved in HFs transfected with pCMV $IE1_{1\text{-}420}$GFP, pCMV $IE1_{1\text{-}479}$, pCMV $IE1_{\Delta421\text{-}475}$ GFP, or pCMV-GFP alone and infected with CR208 at MOIs of 0.0004, 0.002, and 0.01. (C) Immunoblot detection of transfected IE1 constructs. Lysates of HFs transfected with pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}420}$GFP, pCMV $IE1_{\Delta421\text{-}475}$GFP, and pCMV $IE1_{1\text{-}479}$. The $IE1_{1\text{-}479}$ migrates faster because it is not GFP tagged. The proteins were detected with IE1- and IE2-specific monoclonal antibody. β-Actin was detected on the same blot as a loading control. Expression constructs are named in the figure by the portion of IE1 that is expressed or deleted.

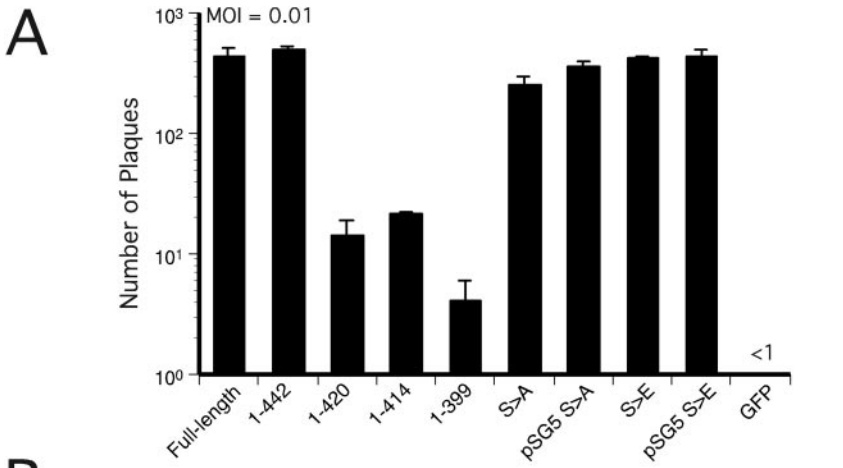
The behavior of the mutant with amino acids 1 to 420 fused to the CTD at amino acids 476 to 491 but lacking the AD (pCMV $IE1_{\Delta421\text{-}475}$GFP) showed complementation similar to the region with amino acids 1 to 420 alone at low MOI (0.0004); however, complementation improved as the MOI was increased to 0.01, suggesting that the addition of the CTD may have had a modest positive impact on amino acids 1 to 420 in the absence of the 55-amino-acid AD. Taken together, these data emphasize the importance of this region of high negative charge as a critical functional domain for optimal viral replication.

To exclude any possibility that the differences in complementation resulted from different expression levels, we performed an immunoblot analysis of lysates of HFs transfected with $IE1_{full\text{-}length}$GFP, $IE1_{1\text{-}420}$GFP, $IE1_{\Delta421\text{-}475}$GFP, and $IE1_{1\text{-}479}$ (Fig. 3C). These four IE1 constructs did not differ in their expression levels. Therefore, the carboxyl-terminal 71 amino acids contribute to replication primarily through the presence of an AD and not because of variation in expression levels.

Role of phosphorylated serines in the acidic domain for complementation of CR208. $IE1_{491aa}$ was subjected to additional deletions and point mutations to investigate the role of acidic amino acids as well as other sequences in this region. pCMV $IE1_{1\text{-}442}$GFP lacks 37 amino acids of the AD, retaining only 13 of the 26 acidic amino acids; pCMV $IE1_{1\text{-}414}$GFP and pCMV $IE1_{1\text{-}399}$GFP lack the AD plus additional amino acids. When these three mutants were compared to pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}420}$GFP, and pCMV GFP, the $IE1_{1\text{-}414}$ and $IE1_{1\text{-}399}$ constructs exhibited poor complementation of CR208, similar to $IE1_{1\text{-}420}$.

In order to summarize the complementation efficiency of the mutants, we related the ability of each to complement across a range of MOIs (0.004 to 0.02, based on the titer of CR208 on ihfie1.3 cells) in multiple experiments. From this depiction (Fig. 4B), the $IE1_{1\text{-}442}$ construct behaved like the wild-type and $IE1_{1\text{-}479}$ constructs, with complementation ranging from 50% to 110% of that with the full-length construct, indicating that the 22 amino acids from 421 to 442, including two clusters of acidic amino acids, retained relatively full activity. Complementation by $IE1_{1\text{-}414}$, ranging from 4 to 14%, and $IE1_{1\text{-}399}$, ranging from 1 to 8% of that of the full-length construct, was similar to that with $IE1_{1\text{-}420}$ (Fig. 4A), suggesting that deletion of amino acids adjacent to the AD did not further decrease function. This analysis clearly reinforces the importance of the AD as a functional domain supporting replication. This domain appears to act together with at least one additional functional domain in the 399 amino-terminal amino acids of $IE1_{491aa}$ because this region retains basal complementation activity above that of the GFP control.

The 55-amino-acid AD contains a number of hallmark components. A sumoylation site (that has been mapped to Lys_{450}) is dispensable for viral growth in assays similar to those described here (49). The recent mapping of the phosphorylation sites on IE1 to serine residues 399, 402, 406, 423, 428, 431, 448, 451, and 455 (Himmelheber et al., 28th International Herpesvirus Workshop, abstract 5.32) prompted a final series of experiments. These nine serines were mutated to alanine (S→A) to prevent phosphorylation and were changed to glutamate (S→E) to mimic phosphorylation as well as add negative charge. Six of these serines were located within the 55-amino-acid AD that we determined to be important, so both the $IE1_{S\rightarrow A}$ and $IE1_{S\rightarrow E}$ mutant constructs were transiently expressed in HFs that were subsequently infected with CR208. The resulting plaque counts were compared to that of controls



B

Construct	Acidic aa (#)	Phosphorylation sites (#)	Complementation (% of full-length)
Full-length	26	9	100
1-479	26	9	60-180
1-442	13	6	50-110
1-420	-	3	3-17
1-414	-	3	4-14
1-399	-	1	1-8
S > A	26	0	60-130
S > E	32	9 (simulated)	90-140

FIG. 4. Impact of carboxyl-terminal mutations in $IE1_{491aa}$ on complementation of CR208. (A) Complementation of CR208 by $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}442}$GFP, pCMV $IE1_{1\text{-}420}$GFP, pCMV $IE1_{1\text{-}414}$, pCMV $IE1_{1\text{-}399}$, pCMV $IE1_{S\rightarrow A}$GFP, pSG5 $IE1_{S\rightarrow A}$, pCMV $IE1_{S\rightarrow E}$GFP, pSG5 $IE1_{S\rightarrow E}$ and pCMV-GFP alone. HFs transfected with pCMV were infected with CR208 at an MOI of 0.01. S→A and S→E mutants were assayed as GFP fusion proteins in the same expression plasmid as other mutants (pCMV-GFP) and as native proteins in the expression plasmid in which they were constructed (pSG5). The black bars represent mean plaque counts of three replicate assays of each construct, with the standard deviation of the mean indicated by error bars. (B) Summary of complementation results. A diagram of each construct with the number of acidic residues in the 55-amino-acid AD, the number of putative phosphorylation sites, and range of complementation as a percentage of that of the full-length construct. The positions of the acidic (AD) and the chromatin-tethering (CT) domains are indicated on the diagram of the full-length $IE1_{1\text{-}491}$ construct. Expression constructs are identified in the column by the portion of IE1 that is expressed or by the type of mutations introduced. The S→A mutant has alanines replacing serines at positions 399, 402, 406, 423, 428, 431, 448, 451, and 455 (positions denoted by gray circles); the mutant S→E has glutamates replacing these serines (position denoted by black circles).

transfected with pCMV $IE1_{full\text{-}length}$GFP, pCMV $IE1_{1\text{-}420}$GFP, or pCMV GFP and infected with CR208 in parallel. Both the $IE1_{S\rightarrow A}$ and $IE1_{S\rightarrow E}$ mutants complemented the growth of CR208 as well as the full-length, wild-type protein did (Fig. 4A). Similar results were obtained with both native and GFP-tagged expression constructs. These data further reinforce the importance of the acidic character of the AD and, curiously, demonstrate that phosphorylation of these serines plays little role in IE1 function during viral replication.

DISCUSSION

$IE1_{491aa}$ is the most abundant gene product made by HCMV immediately following entry into cells. The importance of this protein in the transition from the α (immediate early) to the β (delayed early) and γ (late) phases of infection has been well established from studies on the IE1-deficient virus CR208 (12, 15). CR208 displays impaired replication that correlates with poor expression of β and γ viral gene products and failure to initiate DNA replication under low-MOI conditions. While the exact mechanism of action has not yet emerged from these studies, the primary role does not rely on disruption of nuclear domain 10 (1) and is independent of $IE1_{491aa}$ sumoylation (49).

To get a better understanding of the function of this protein within the context of the replication cycle, we evaluated *ie*1 mutants for their ability to complement the growth of CR208 at low MOIs and found a dominant role for the AD located between amino acids 421 and 475 acting in concert with a separate functional domain located in the amino-terminal 399 amino acids. Further analysis showed that independent expression of amino acids 420 to 479 was unable to complement CR208 replication (data not shown), indicating that the amino-terminal 399 amino acids provide a basal ability that is en-

hanced by the AD. The amino-terminal 85 amino acids of $IE1_{491aa}$ are identical to a region of $IE2_{579aa}$ and contain a mapped transactivation domain (43) that merits further analysis in the context of the $IE1_{491aa}$ replication function that we have described here. Thus, our study supports a role for $IE1_{491aa}$ in regulation of gene expression dependent on an AD in combination with at least one other region of the protein.

When the expression of the β gene product ppUL44 was evaluated by immunofluorescence analysis, CR208-infected cells complemented with $IE1_{1-420}$ appeared to be less intensely stained than CR208-infected cells complemented with full-length protein (data not shown), consistent with previous analyses that focused $IE2_{579aa}$ function on the transition from the α to β phase of infection (12, 15). Interestingly, higher levels of expression of $IE1_{1-420}$ correlated with higher ppUL44 levels, suggesting that one component of AD-independent complementation depended on the level of expression of the mutant protein in individual cells. This threshold was apparently lowered during complementation with full-length $IE1_{491aa}$, because ppUL44 expression could be readily detected in cells even with low levels of $IE1_{491aa}$ expression (data not shown). These two independent $IE1_{491aa}$ domains, defined by amino acids 1 to 399 and amino acids 421 to 475, appear to influence the transition from α to β gene expression. The mechanism of control should become better understood through the evaluation of mutant viruses that express mutant forms of $IE1_{491aa}$.

We showed that the AD was more critical at low MOIs and that the acidic character appears to be responsible for this activity. The mechanism of $IE1_{491aa}$ AD function is unknown, although acidic domains play well-established roles in transactivation by many cellular and viral regulatory proteins (4). In some settings, such as herpes simplex virus VP16 (10) and *Saccharomyces cerevisiae* Gal4 (14), the transactivation activity may be conferred by the acidic quality of the domain rather than the primary amino acid sequence. The AD of $IE1_{491aa}$ appears substantially more acidic than either of these classic acidic transactivators. Consistent with this, our experiments showed that transactivation was only modestly affected when 13 of the 26 acidic amino acids were removed or when the acidic character was increased. Thus, the critical role of the AD was not entirely dependent on very high acidity, even though acidic amino acids make up a very high proportion of the domain (amino acids 421 to 475).

The structure of this region is predicted to be different from that of classical acidic transactivation domains, where acidic residues are exposed on the surface of an amphipathic helix and contact the transcription initiation machinery (4). Although preliminary attempts to substitute the *S. cerevisiae* transcription factor Gal4 acidic transactivation domain in place of the $IE1_{491aa}$ AD failed to reconstitute function in the assays described here (data not shown), additional experiments in this direction are warranted.

Comparison of the HCMV $IE1_{491aa}$ carboxyl-terminal region with the homologous regions from three other primate CMVs reveals similarities in the AD as well as a striking level of conservation in a 16-amino-acid domain that we define as a CTD. This conservation predicts that $IE1_{491aa}$ homologues will likely promote tethering to metaphase chromatin, as has already been observed for the homologue encoded by simian CMV (8). Although our experiments failed to detect any role for chromatin tethering during productive replication, conservation of the CTD suggests an important role in CMV biology, pathogenesis, or, particularly, latency. The $IE1_{491aa}$ CTD does not appear to exhibit sequence similarity with the tethering domains of gammaherpesvirus latency proteins EBNA-1 or Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen LANA, except in that all of these domains are small and contain basic amino acids. Also, the CTD maps to an amino-terminal region in both as a 14-amino-acid domain (amino acids 72 to 84) in EBNA-1 (31) and as an 18-amino-acid domain (amino acids 5 to 22) in LANA (42).

Chromatin tethering is important for latent genome maintenance in gammaherpesviruses and does not appear to be involved in transactivation functions in these systems (42, 45). How or whether $IE1_{491aa}$ plays a role in HCMV genome maintenance during latency is not known. In addition to their ability to tether to chromatin, both EBNA-1 and LANA bind specifically to viral DNA replication origins that control latent DNA replication (31, 42). Despite many attempts, $IE1_{491aa}$ has not shown the ability to directly bind viral or cellular DNA.

$IE1_{491aa}$ is phosphorylated on serines between amino acids 399 and 455 and is sumoylated at Lys_{450} (49, 52); however, substitution of glutamate or alanine at the nine known phosphorylated serines yielded a protein that retained full complementation ability. A mutation of Lys_{450} to arginine was previously shown to destroy the only sumoylation site but not the ability to complement CR208 (49). Therefore, it is not likely that sumoylation underlies AD function. Modifications such as sumoylation and phosphorylation may be important for protein stability or cell type-specific activity rather than the regulatory activity that we have studied here.

ACKNOWLEDGMENTS

We appreciate cell culture assistance by Yin Dong and discussions with Richard Greaves that led to this approach for analysis of CR208 defects.

This work was supported by grants from the PHS (AI20211 and AI30363 to E.S.M., CA71019 to J.A.-C.), and Medical Scientist Training Grant GM007365 supported G.B.S.

REFERENCES

1. **Ahn, J. H., and G. S. Hayward.** 2000. Disruption of PML-associated nuclear bodies by IE1 correlates with efficient early stages of viral gene expression and DNA replication in human cytomegalovirus infection. Virology **274:**39–55.
2. **Ahn, J. H., and G. S. Hayward.** 1997. The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. J. Virol. **71:**4599–4613.
3. **Angulo, A., P. Ghazal, and M. Messerle.** 2000. The major immediate-early gene ie3 of mouse cytomegalovirus is essential for viral growth J. Virol. **74:**11129–11136.
4. **Berk, A. J., T. G. Boyer, A. N. Kapanidis, R. H. Ebright, N. N. Kobayashi, P. J. Horn, S. M. Sullivan, R. Koop, M. A. Surby, and S. J. Triezenberg.** 1998. Mechanisms of viral activators. Cold Spring Harb. Symp. Quant. Biol. **63:** 243–252.
5. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J. Virol. **75:**12319–12330.
6. **Castillo, J. P., and T. F. Kowalik.** 2004. HCMV infection: modulating the cell cycle and cell death. Int. Rev. Immunol. **23:**113–139.
7. **Castillo, J. P., and T. F. Kowalik.** 2002. Human cytomegalovirus immediate early proteins and cell growth control. Gene **290:**19–34.
8. **Chang, Y. N., K. T. Jeang, T. Lietman, and G. S. Hayward.** 1995. Structural organization of the spliced immediate early gene complex that encodes the major acidic nuclear (IE1) and transactivator (IE2) proteins or African green monkey cytomegalovirus. J. Biomed. Sci. **2:**105–130.
9. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus ie1

transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63:**1435–1440.
10. **Cress, W. D., and S. J. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251:**87–90.
11. **Fortunato, E. A., V. Sanchez, J. Y. Yen, and D. H. Spector.** 2002. Infection of cells with human cytomegalovirus during S phase results in a blockade to immediate-early gene expression that can be overcome by inhibition of the proteasome. J. Virol. **76:**5369–5379.
12. **Gawn, J. M., and R. F. Greaves.** 2002. Absence of IE1 p72 protein function during low-multiplicity infection by human cytomegalovirus results in a broad block to viral delayed-early gene expression. J. Virol. **76:**4441–4455.
13. **Geist, L. J., and L. Y. Dai.** 1996. Cytomegalovirus modulates interleukin-6 gene expression. Transplantation **62:**653–658.
14. **Gill, G., and M. Ptashne.** 1987. Mutants of GAL4 protein altered in an activation function. Cell **51:**121–126.
15. **Greaves, R. F., and E. S. Mocarski.** 1998. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low multiplicity infection by a human cytomegalovirus *ie1* mutant. J. Virol. **72:**366–379.
16. **Hagemeier, C., S. M. Walker, P. J. Sissons, and J. H. Sinclair.** 1992. The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently transactivate the c-fos, c-myc and hsp70 promoters via basal promoter elements. J. Gen. Virol. **73:**2385–2393.
17. **Hahn, G., R. Jores, and E. S. Mocarski.** 1998. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc. Natl. Acad. Sci. USA **95:**3937–3942.
18. **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.
19. **Hertel, L., and E. S. Mocarski.** Global analysis of host cell gene expression late during cytomegalovirus infection reveals extensive dysregulation of cell cycle gene expression and induction of pseudomitosis independent of US28 function. J. Virol. **78:**11988–12011.
20. **Hunninghake, G. W., B. G. Monks, L. J. Geist, M. M. Monick, M. A. Monroy, M. F. Stinski, A. C. Webb, J. M. Dayer, P. E. Auron, and M. J. Fenton.** 1992. The functional importance of a cap site-proximal region of the human prointerleukin 1β gene is defined by viral protein trans-activation. Mol. Cell. Biol. **12:**3439–3448.
21. **Jenkins, D. E., C. L. Martens, and E. S. Mocarski.** 1994. Human cytomegalovirus late protein encoded by ie2: a trans-activator as well as a repressor of gene expression. J. Gen. Virol. **75:**2337–2348.
22. **Johnson, R. A., A. D. Yurochko, E. E. Poma, L. Zhu, and E. S. Huang.** 1999. Domain mapping of the human cytomegalovirus IE1–72 and cellular p107 protein-protein interaction and the possible functional consequences. J. Gen. Virol. **80:**1293–1303.
23. **Kim, S., S. S. Yu, I. S. Lee, S. Ohno, J. Yim, and H. S. Kang.** 1999. Human cytomegalovirus IE1 protein activates AP-1 through a cellular protein kinase(s). J. Gen. Virol. **80:**961–969.
24. **Kline, J. N., L. J. Geist, M. M. Monick, M. F. Stinski, and G. W. Hunninghake.** 1994. Regulation of expression of the IL-1 receptor antagonist (IL-1ra) gene by products of the human cytomegalovirus immediate early genes. J. Immunol. **152:**2351–2357.
25. **Kondo, K., J. Xu, and E. S. Mocarski.** 1996. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proc. Natl. Acad. Sci. USA **93:**11137–11142.
26. **Korioth, F., G. G. Maul, B. Plachter, T. Stamminger, and J. Frey.** 1996. The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. Exp. Cell Res. **229:**155–158.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
28. **LaFemina, R. L., and G. S. Hayward.** 1988. Differences in cell-type-specific blocks to immediate early gene expression and DNA replication of human, simian and murine cytomegalovirus. J. Gen. Virol. **69:**355–374.
29. **LaFemina, R. L., M. C. Pizzorno, J. D. Mosca, and G. S. Hayward.** 1989. Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. Virology **172:**584–600.
30. **Marchini, A., H. Liu, and H. Zhu.** 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J. Virol. **75:**1870–1878.
31. **Marechal, V., A. Dehee, R. Chikhi-Brachet, T. Piolot, M. Coppey-Moisan, and J. C. Nicolas.** 1999. Mapping EBNA-1 domains involved in binding to metaphase chromosomes. J. Virol. **73:**4385–4392.
32. **Margolis, M. J., S. Pajovic, E. L. Wong, M. Wade, R. Jupp, J. A. Nelson, and J. C. Azizkhan.** 1995. Interaction of the 72-kilodalton human cytomegalovirus IE1 gene product with E2F1 coincides with E2F-dependent activation of dihydrofolate reductase transcription. J. Virol. **69:**7759–7767.
33. **Mocarski, E. S., Jr., and C. T. Courcelle.** 2001. Cytomegaloviruses and their replication, p. 2629–2673. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
34. **Mocarski, E. S., and G. W. Kemble.** 1997. Recombinant cytomegaloviruses for study of replication and pathogenesis. Intervirology **39:**320–330.
35. **Mocarski, E. S., G. W. Kemble, J. M. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding $IE1_{491aa}$ is replication defective due to a failure in autoregulation. Proc. Natl. Acad. Sci. USA **93:**11321–11326.
36. **Muller, S., and A. Dejean.** 1999. Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. J. Virol. **73:**5137–5143.
37. **Murayama, T., N. Mukaida, H. Sadanari, N. Yamaguchi, K. S. Khabar, J. Tanaka, K. Matsushima, S. Mori, and Y. Eizuru.** 2000. The immediate early gene 1 product of human cytomegalovirus is sufficient for up-regulation of interleukin-8 gene expression. Biochem. Biophys. Res. Commun. **279:**298–304.
38. **Otto, S. M., T. G. Sullivan, C. L. Malone, and M. F. Stinski.** 1988. Subcellular localization of the major immediate early protein (IE1) of human cytomegalovirus at early times after infection. Virology **162:**478–482.
39. **Pajovic, S., E. L. Wong, A. R. Black, and J. C. Azizkhan.** 1997. Identification of a viral kinase that phosphorylates specific E2Fs and pocket proteins. Mol. Cell. Biol. **17:**6459–6464.
40. **Pass, R. F.** 2001. Cytomegalovirus, p. 2675–2705. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
41. **Perot, K., C. M. Walker, and R. R. Spaete.** 1992. Primary chimpanzee skin fibroblast cells are fully permissive for human cytomegalovirus replication. J. Gen. Virol. **73:**3281–3284.
42. **Piolot, T., M. Tramier, M. Coppey, J. C. Nicolas, and V. Marechal.** 2001. Close but distinct regions of human herpesvirus 8 latency-associated nuclear antigen 1 are responsible for nuclear targeting and binding to human mitotic chromosomes. J. Virol. **75:**3948–3959.
43. **Pizzorno, M. C., M. A. Mullen, Y. N. Chang, and G. S. Hayward.** 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. J. Virol. **65:**3839–3852.
44. **Sambucetti, L. C., J. M. Cherrington, G. W. G. Wilkinson, and E. S. Mocarski.** 1989. NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8:**4251–4258.
45. **Sears, J., J. Kolman, G. M. Wahl, and A. Aiyar.** 2003. Metaphase chromosome tethering is necessary for the DNA synthesis and maintenance of oriP plasmids but is insufficient for transcription activation by Epstein-Barr nuclear antigen 1. J. Virol. **77:**11767–11780.
46. **Shen, Y., H. Zhu, and T. Shenk.** 1997. Hum. cytomegalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA **94:**3341–3345.
47. **Shirakata, M., M. Terauchi, M. Ablikim, K. Imadome, K. Hirai, T. Aso, and Y. Yamanashi.** 2002. Novel immediate-early protein IE19 of human cytomegalovirus activates the origin recognition complex I promoter in a cooperative manner with IE72. J. Virol. **76:**3158–3167.
48. **Sinclair, J. H., J. Baillie, L. A. Bryant, T.-W. J. A., and J. G. Sissons.** 1992. Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line. J. Gen. Virol. **73:**433–435.
49. **Spengler, M. L., K. Kurapatwinski, A. R. Black, and J. Azizkhan-Clifford.** 2002. SUMO-1 modification of human cytomegalovirus IE1/IE72. J. Virol. **76:**2990–2996.
50. **White, E. A., C. L. Clark, V. Sanchez, and D. H. Spector.** 2004. Small internal deletions in the human cytomegalovirus IE2 gene result in nonviable recombinant viruses with differential defects in viral gene expression. J. Virol. **78:**1817–1830.
51. **Wilkinson, G. W., C. Kelly, J. H. Sinclair, and C. Rickards.** 1998. Disruption of PML-associated nuclear bodies mediated by the human cytomegalovirus major immediate early gene product. J. Gen. Virol. **79:**1233–1245.
52. **Xu, Y., J. H. Ahn, M. Cheng, C. M. apRhys, C. J. Chiou, J. Zong, M. J. Matunis, and G. S. Hayward.** 2001. Proteasome-independent disruption of PML oncogenic domains but not covalent modification by SUMO-1 is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. J. Virol. **75:**10683–10695.
53. **Zhang, Z., S. M. Huong, X. Wang, D. Y. Huang, and E. S. Huang.** 2003. Interactions between human cytomegalovirus IE1–72 and cellular p107: functional domains and mechanisms of up-regulation of cyclin E/cdk2 kinase activity. J. Virol. **77:**12660–12670.
54. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.
55. **Zou, H., J. Lee, S. Umamoto, A. F. Kilani, J. Kim, P. Trang, T. Zhou, and F. Liu.** 2003. Engineered RNase P ribozymes are efficient in cleaving a human cytomegalovirus mRNA in vitro and are effective in inhibiting viral gene expression and growth in human cells. J. Biol. Chem. **278:**37265–37274.