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Human Cytomegalovirus Contains a Tegument Protein That Enhances Transcription from Promoters with Upstream ATF and AP-1 *cis*-Acting Elements

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The tegument proteins of human cytomegalovirus are introduced into cells as components of infectious virus. The tegument proteins may affect viral and cellular transcription prior to the synthesis of the immediate-early viral regulatory proteins. The phosphorylated tegument protein of 71 kDa (pp71) is reported to be encoded by the UL82 gene. The UL82 gene products transactivated promoters containing upstream ATF or AP-1 binding sites. In contrast, the phosphorylated tegument protein of 65 kDa (pp65), encoded by the UL83 gene, had no detectable effect on these promoters. Enhancement by UL82 of downstream transcription was directly proportional to the number of upstream ATF sites. Response to UL82 transactivation was abolished by mutation of the ATF site. Mutation in the carboxy-terminal region of UL82 also eliminated transactivation. Even though the major immediate-early promoter of human cytomegalovirus is a strong enhancer-containing promoter, UL82 further enhanced its transcription as much as 20-fold. The mechanism of UL82 enhancement of transcription from viral or cellular promoters is not known, but the enhancement may be mediated by triggering one of the protein kinase signaling pathways, increasing the affinity of ATF or AP-1 for the target sequence, or stabilizing the complex between the eucaryotic transcription factor and the target sequence.

Disease induced by human cytomegalovirus (HCMV), a betaherpesvirus, can range from congenital malformation in newborns and pneumonitis in immunocompromised adults to subclinical infection in normal healthy individuals. After infection, the complicated viral genome of 230 kb can either establish latency in certain cell types or undergo a sequential, highly regulated expression of the viral genes, which ultimately leads to replication of progeny virus (reviewed in reference 21).

Transcription of the HCMV immediate-early (IE) genes is regulated by a very strong and complex enhancer located upstream of the major IE promoter (MIEP). Within this regulatory region are elements that modulate expression not only in different cell types (35) but also in undifferentiated and differentiated cells (49). The enhancer region contains several different repeat elements with binding sites for known eucaryotic transcriptional factors (5, 56, 57). The 21-, 18-, and 19-bp repeat elements contain binding sites for Sp1, NF-κB, and ATF, respectively. The 13-bp repeat is a part of a NF-1/CBP site. There are also SRE and AP-1 sites in the enhancer region (31, 39, 55, 59). DNase footprinting and in vitro transcription experiments indicate that both repetitive and nonrepetitive sequences influence the level of downstream transcription (10, 57).

Stimulation of the cellular protein kinase A or C signaling pathways enhances the level of transcription from the MIEP (6, 22, 30, 53). Transcription is also strongly stimulated by a structural component of the virion (52, 56). Infection of cells containing a stably integrated MIEP upstream of a reporter gene increases transcription of this gene approximately 20-fold, even in the presence of an inhibitor of de novo protein synthesis (56). In addition, a rapid increase in the transcription of cellular proto-oncogenes, c-*fos*, c-*jun*, and c-*myc*, occurs after HCMV infection (3, 4). Enhanced transcription of these proto-oncogenes is not affected by either inactivation of the virus with UV irradiation or inhibition of de novo protein synthesis with cycloheximide. Therefore, IE gene expression is not required for this effect. Since digestion of the virions with proteases abolishes the activity, it has been suggested that a virion component may be involved in this transcriptional enhancement. Herpes simplex virus (HSV), an alphaherpesvirus, has a virion-associated transactivator (VP16) that strongly stimulates HSV IE gene transcription (60).

HCMV is an enveloped virus with a tegument surrounding the nucleocapsid. Two phosphorylated tegument proteins have been identified in the virion, and their genes have been mapped on the viral chromosome (41). They are referred to as the upper and lower tegument proteins on the basis of their electrophoretic behavior in denaturing gels (46). These two viral genes were found in tandem on the viral chromosome between 0.510 and 0.530 map units. Two open reading frames (ORF) were identified and designated UL82 and UL83 (7, 47). UL82 and UL83 code for phosphorylated proteins of 71 and 65 kDa and are also referred to as pp71 and pp65, respectively. pp65 is a protein kinase (51). Surprisingly, pp65 is transported to the nucleus of HCMV-infected cells immediately after infection and can be detected in significant quantities before the appearance of IE1 and IE2 (15). Specific antibodies to pp71 should help determine whether this viral protein is also quickly transported to the nucleus of the infected cell or whether it remains in the cytoplasm.

We investigated the effect of the UL82 and UL83 gene products on transcription from the MIEP. Although UL83 had no detectable effect on transcription, UL82 strongly stimulated transcription from the MIEP. Deletion and mutational analyses identified the ATF sites in the enhancer as the *cis*-responsive element to the viral transactivator. The related *cis*-acting site, AP-1, also responded to transactivation by pp71. The effect of this novel virion-associated

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transactivator on transcription from the viral MIEP and on the activation of transcription from cellular proto-oncogenes is discussed.

MATERIALS AND METHODS

Cell culture and virus. Growth of human foreskin fibroblast (HFF) cells and propagation of HCMV (Towne) have been described previously (54).

Plasmid construction. Plasmid pTK760 and pCAT760 have the enhancer containing MIEP of HCMV upstream of the HSV thymidine kinase (TK) gene and bacterial chloramphenicol acetyltransferase (CAT) gene, respectively. Plasmids pCAT*dl*36, pCAT*dl*Nde, and pCAT*dl*14 have deletions within the enhancer region and have been described previously (56). Plasmids pTJ278 and pSV11CAT contain the MIEP of simian CMV or murine CMV upstream of the CAT gene as described previously (8, 23, 34). Plasmid pSomaCAT (kindly provided by R. Maurer, University of Iowa) contains the mouse somatostatin gene promoter upstream of the CAT gene. Plasmid pAP1/1CAT (kindly provided by L. Turek, University of Iowa) contains a single AP-1 binding site and HSV-1 TK promoter upstream of the CAT gene. Plasmids pRSVCAT, pSV2CAT, and pLTRCAT, containing the Rous sarcoma virus (RSV), simian virus 40 (SV40), and human immunodeficiency virus (HIV) enhancer-containing promoters, respectively, upstream of the CAT gene, have been described previously (2, 12, 13).

Plasmid p*dl*760CAT was described previously (22). It contains −68 to +7 of the MIEP and was isolated from plasmid pCAT760 after *Hin*dIII and *Hin*cII digestion. The TATA and CAAT boxes are present, but the upstream enhancer elements are absent. This fragment was blunt ended and inserted into the blunt-ended *Hin*dIII site of plasmid pSV0CAT. A *Nde*I site 59 bp upstream of −68 was converted into a *Xba*I site and used for constructing pIE193CAT, pIE183CAT, pIE213CAT, and pIE163CAT by inserting three copies of either the 19-, 18-, 21-, or 16-bp synthetic repeat elements flanked by *Xba*I sticky ends. The DNA sequence for these synthetic repeat elements are 5′-ctagCCCCATTGACGTCAATGGG-3′ for the 19-bp, 5′-ctag ACTAACGGGACTTTCCAA-3′ for the 18-bp, 5′-ctagGCC AGGCGGGCCATTTACCGT-3′ for the 21-bp, and 5′-ctagC TTGGCAGTACATCAA-3′ for the 16-bp repeat element. Lowercase letters designate nonviral sequence.

Plasmids pIEM1/193CAT, pIEM2/193CAT, and pIEM3/193CAT contain mutated 19-bp repeat elements upstream of the CAT reporter gene. pIEM1/193CAT has a mutation at the ATF site of the 19-bp repeat element. pIEM2/193CAT and pIEM3/193CAT have mutations at either the 5′- or 3′-flanking side of the ATF binding site of the 19-bp repeat element, respectively. These clones were constructed by inserting three copies of each mutant synthetic repeat element at the *Xba*I site of p*dl*760CAT.

Plasmid pTE2CAT contains the 13-bp repeat-NF1 elements upstream of the enhancerless MIEP. To construct this plasmid, we digested pLink760 (20) with restriction endonucleases *Tha*I and *Eco*RI. Two of the 238-bp DNA fragment containing the 13-bp repeat-NF-1 elements from −522 to −760 of MIEP were blunt ended with T4 DNA polymerase and cloned into the blunt-ended *Xba*I site of p*dl*760CAT.

Plasmids pCMV71 and pCMV65, containing the UL82 and UL83 ORFs, respectively, are driven by the MIEP of HCMV. These two genes were cloned from plasmid pMSDT-C (58) by digestion with *Xba*I and partial digestion with *Hin*dIII. A 4.04-kb DNA fragment containing both UL82 and UL83 was isolated. A 950-bp DNA fragment containing the poly(A)$^+$ signal sequence for the two genes was isolated from pMSDT-J (58) by digestion with *Xba*I and *Bam*HI. The 760-bp MIEP was isolated from plasmid pLink760 by digestion with *Bam*HI and *Hin*dIII. The above three DNA fragments were then inserted into the *Bam*HI site of plasmid pSP65. The plasmid was designated as pCMV65-71HB. These two viral genes were also cloned downstream of the procaryotic SP6 promoter of plasmid pSP65 for in vitro transcription and translation. This plasmid is designated as pSP65CMV65/71.

To construct pCMV65, plasmid pCMV65-71HB was partially digested with restriction endonuclease *Hin*dIII and a 2.3-kb DNA fragment containing the UL83 ORF was cloned into the *Hin*dIII site of expression vector pCMVI. This expression vector contains the MIEP of HCMV for high levels of expression of cloned genes and a transcription terminator from the human growth hormone gene (1). To construct pCMV71, plasmid pCMV65-71HB was partially digested with *Hin*dIII to remove most of the coding sequences for pp65. Then the plasmid was religated and digested with *Nco*I. The *Nco*I site was made blunt with mung bean nuclease. The plasmid was religated and digested with *Xba*I to isolate a 2.2-kb DNA fragment containing the UL82 ORF. This fragment was then ligated into the *Xba*I site of the expression vector pCMVI.

Mutants of the UL82 coding sequence were constructed by deletion mutagenesis at specific restriction endonuclease sites of the parental plasmid, pCMV71. Termination codons in all three potential reading frames were introduced with a synthetic *Xba*I nonsense codon linker (5′-CTAGTCTAG ACTAG-3′; Stratagene, La Jolla, Calif.). To construct pCMV71*dl*KpnI, plasmid pCMV71 was digested with restriction enzyme *Kpn*I and the ends were made blunt with T4 DNA polymerase. *Xba*I nonsense linkers were ligated at the blunt ends. This 2.0-kb DNA fragment containing the N-terminal portion of the pp71 gene was ligated into the *Xba*I site of the pCMVI vector. A similar strategy was used to construct mutants pCMV71*dl*BglII, pCMV71*dl*SmaI, and pCMV71*dl*PvuI.

Plasmid pLink760 was used as enhancer control DNA as previously described (20).

In vitro transcription, translation, and immunoprecipitation. For in vitro transcription, plasmid pSP65CMV65/71, containing both UL82 and UL83 genes, was linearized with restriction endonuclease *Xba*I and used as the DNA template for in vitro mRNA synthesis by SP6 RNA polymerase (Promega Biotec, Madison, Wis.). In vitro transcription, translation, and immunoprecipitation were performed as described previously (16, 27).

DNA sequencing. The dideoxy-chain termination method was used in conjunction with primers and the Sequenase Version 2.2 kit (U.S. Biochemical Corp., Cleveland, Ohio). [α-^{35}S]dATP (1,000 Ci/mmol) was purchased from Amersham (Arlington Heights, Ill.).

Transfection. Permissive HFF cells in 100-mm culture dishes were transfected in duplicate with calcium phosphate precipitates as described previously (13, 20). For each transfection measuring CAT activity, 0.1 μg of reporter plasmid and 2 μg of effector plasmid were used. For assays measuring the steady-state level of RNA, 0.4 μg of reporter plasmid plus 4 μg of internal control plasmid and 2 μg of effector plasmid were used.

CAT assays. Cell harvesting and CAT assays were performed as described by Gorman et al. (13). CAT activity was quantitated with a thin-layer chromatography scanner. The

percent conversion to the 3′-acetylated derivative was measured relative to the same number of cells.

RNase protection assays. Construction of the plasmid for the CAT antisense-riboprobe synthesis has been described previously (34). For the TK antisense-riboprobe synthesis, pTK760 (56) was digested with restriction endonucleases *Sst*I (−16) and *Eco*RV (+300) and a 316-bp DNA was isolated and inserted at the *Sst*I and *Hin*cII sites of the vector pGEM-blue (Promega Biotec). The resulting clone, pGEM-TK, was linearized with *Eco*RI and used as a template for the SP6 RNA polymerase. [^{32}P]RNA probe synthesis, hybridization, and RNase digestion conditions were as described by Krieg and Melton (27), with the following modifications. Cytoplasmic RNA was harvested from two 100-mm plates of transfected cells at 48 h after transfection as described previously (34). In the presence of cycloheximide (50 μg/ml), cells were either mock infected or HCMV infected (5 PFU per cell) at 24 h after transfection. Cytoplasmic RNA was isolated 8 h after infection. RNA (40 μg) was hybridized with either the TK riboprobe or both the TK and CAT riboprobes. For detecting TK RNA, 30 U of RNase A plus 50 U of RNase T_1 per sample were used at room temperature for 30 min. Because of internal cleavage of the RNA hybrid by RNase A, only 200 U of RNase T_1 per sample was used at room temperature for 30 min for detecting both CAT and TK RNAs. The protected RNAs were subjected to electrophoresis in 6% polyacrylamide–urea gels. The bands were detected by autoradiography, and the levels of protected RNAs were quantitated by scanning with a DU-8 spectrophotometer containing the DU-8 gel-scanning Compuset module (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Transactivation by a viral tegument protein. Even in the presence of an inhibitor of protein synthesis, infection by HCMV up-regulates MIEP-driven transcription (52, 56). We cloned the region of the HCMV genome that contains the upper and lower tegument genes. This region of the genome located between 0.510 and 0.530 map units contains two ORFs, UL82 and UL83, which code for phosphorylated proteins designated pp71 and pp65, respectively (Fig. 1A). To confirm the protein products of UL82 and UL83, we translated in vitro-transcribed mRNA in a rabbit reticulocyte lysate. The protein products were immunoprecipitated with a monoclonal antibody to pp65 or control antibody 9E10. [^{35}S]Met-labeled virions of HCMV were also lysed and treated with antibody to pp65 as described in Materials and Methods. Figure 1B (lane 2) demonstrates that two proteins with apparent molecular masses of 71 and 65 kDa were synthesized. These viral proteins were not precipitated by the control antibody 9E10 (lane 5). Monoclonal antibody to pp65 immunoprecipitated a protein of 65 kDa from the virion lysate (lane 3) and from the rabbit reticulocyte lysate (lane 4). Antibody to pp71 (41) is no longer available.

The two viral genes were further subcloned into the expression vector pCMV1, and the plasmids were designated pCMV71 and pCMV65, respectively. A reporter plasmid, pTK760, was cotransfected into HFF cells with control DNA (pLink760), pCMV65, or pCMV71. Cells were also transfected with pTK760 and then subjected to either mock infection or HCMV infection in the presence of cycloheximide (50 μg/ml). Cytoplasmic RNA was then isolated and assayed for the steady-state levels of TK RNA by the RNase protection assay as described in Materials and Methods.

Figure 1C shows that the steady-state level of TK RNA increased 22-fold after HCMV infection (Fig. 1C, compare lanes 3 and 4). pp65 had little to no effect on the steady-state level of TK RNA (lane 7). Cotransfection of pCMV65 with DNA concentrations ranging from 0.5 to 4.0 μg also had no effect. A monoclonal antibody to pp65 (95/28) was used to demonstrate pp65 expression in transfected cells (data not shown). In contrast, the steady-state level of TK RNA increased 18-fold in the presence of UL82 (lane 6). These data indicated that transactivation of the MIEP was due either directly or indirectly to the UL82 gene product.

Effect of carboxy-terminal deletion of UL82 on transactivation of the MIEP. To further define the requirement for a UL82 gene product in transactivation of the MIEP, we constructed a series of carboxy-terminal deletions. The deletion endpoints of UL82 are diagramed in Fig. 2A, and the amino acid sequence of the wild-type and mutant proteins is designated in Fig. 2B. The reporter plasmid pCAT760 was cotransfected with either wild-type pp71 or mutant pp71 plasmids and assayed for CAT activity as described in Materials and Methods.

Three mutants, pCMV71*dl*BglII, pCMV71*dl*SmaI, and pCMV71*dl*PvuI, completely lost the ability to transactivate the MIEP. However, one mutant, pCMV71*dl*KpnI, significantly transactivated the MIEP (Fig. 2A). These data indicated that most of the UL82 ORF was required for the protein product to transactivate the enhancer-containing MIEP. Additional studies are needed to define the regions of the UL82 ORF that are required for transactivation of the MIEP.

Transactivation by UL82 is promoter specific. To determine whether transactivation by UL82 is promoter specific, reporter plasmids that express the bacterial CAT gene driven by enhancer-containing promoters of the HCMV IE gene (pCAT760), simian CMV IE gene (pTJ278CAT), murine CMV IE gene (pSVIICAT), RSV long terminal repeat (LTR) (pRSVCAT), HIV LTR (pLTRCAT), or SV40 (pSV2CAT) were cotransfected into HFF cells with either control DNA (pLink760) or pCMV71 (pp71). The CAT genes driven by the enhancerless promoters from the MIEP of HCMV (p*dl*760CAT), HSV TK gene (pTKCAT), and HCMV early gene E1.7 (pE1.7CAT) were also tested in the transfection assay. To establish that the effect of UL82 was at the level of transcription, we determined the steady-state level of CAT RNA synthesis at 48 h after transfection. In this assay, a third plasmid, pRSVTK, was included as an internal control for transfection efficiency, RNA recovery, and RNase protection, as described in Materials and Methods. Figure 3A demonstrates that the RSV and HIV LTR promoters did not respond to UL82. However, UL82 had an effect on the steady-state level of CAT RNA when transcription was driven by the MIEP of either HCMV (17-fold), simian CMV (12-fold), or murine CMV (3-fold) (Fig. 3A, compare lanes 4 and 5, 6 and 7, and 8 and 9, respectively). UL82 also had a minor effect on the steady-state level of RNA from the SV40 promoter (Fig. 3A, compare lanes 14 and 15). In contrast, UL82 had little to no effect on the steady-state level of CAT RNA from the promoters of enhancerless MIEP, HSV TK, and HCMV early gene E1.7 (data not shown).

After 48 h, the cells were also lysed and assayed for CAT activity as described in Materials and Methods. The MIEP from both HCMV and simian CMV responded strongly to transactivation by UL82 (18- and 15-fold, respectively) (Fig. 3B). The MIEP from murine CMV also responded, although to a lesser extent (fivefold). UL82 activated expression from the SV40 promoter approximately threefold. However, none

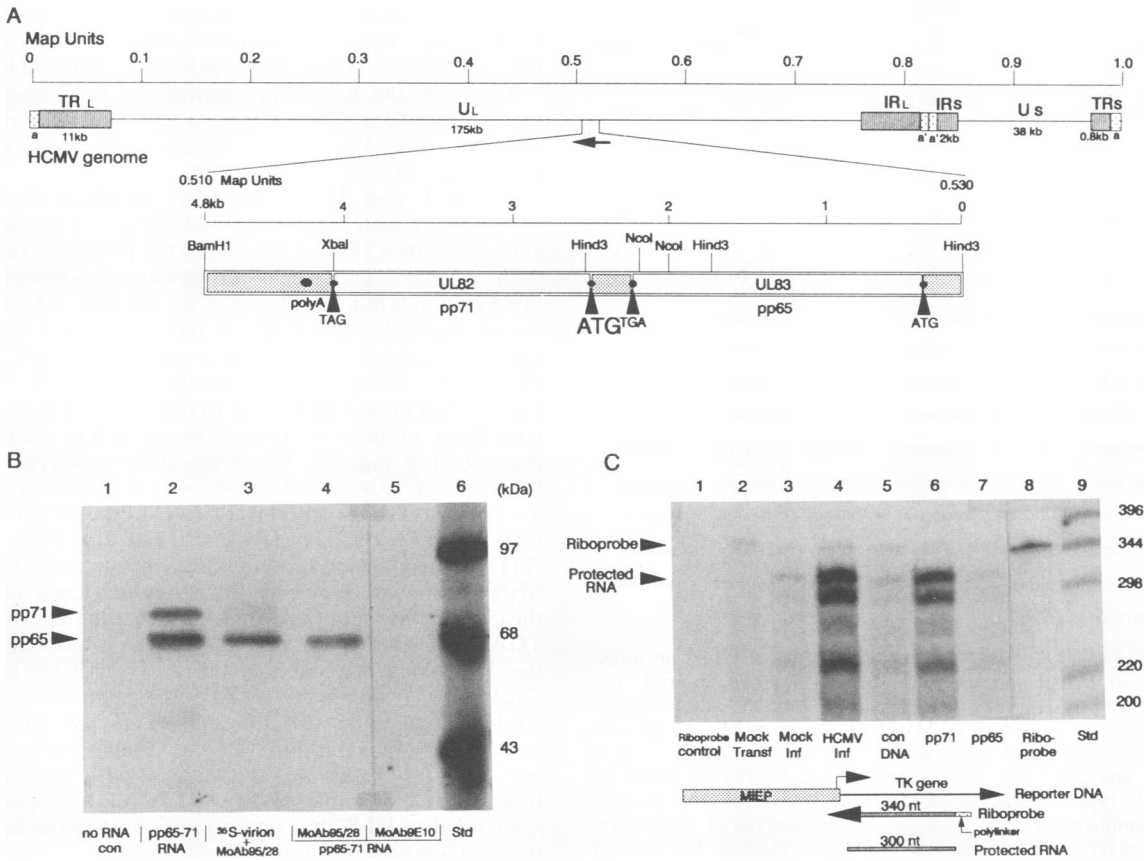


FIG. 1. Location of the genes that encode pp65 and pp71 and their effects on the steady-state level of TK RNA synthesis from the MIEP. (A) Location of UL82 and UL83 genes in the HCMV genome. The prototype organization of the HCMV genome is designated as described previously (55). The direction of transcription is indicated by an arrow. Restriction endonuclease sites, the UL82 and UL83 ORFs, translation initiation, and translation termination signals are indicated. The 3′ end of the transcription unit is designated by polyA. (B) In vitro translation and immunoprecipitation of UL82 and UL83 gene products. Lanes: 1, no RNA control translation; 2, translation of bicistronic pp65 and pp71 mRNA derived from *Xba*I-linearized pSP65CMV65/71 DNA; 3, immunoprecipitation of [^{35}S]Met-labeled virion purified from HCMV-infected cells with monoclonal antibody 95/28 (MoAb95/28) specific to pp65; 4, immunoprecipitation of in vitro-translated protein with monoclonal antibody 95/28; 5, immunoprecipitation of the in vitro-translated protein with control monoclonal antibody 9E10 (MoAb9E10); 6, ^{14}C-labeled protein standard size marker (Std). The positions of pp65 and pp71 are indicated by arrowheads. (C) Effect of UL82 or UL83 on the steady-state level of TK RNA synthesis. pTK760 DNA was cotransfected with either control DNA (pLink760), pCMV71, or pCMV65 into HFF cells. At 24 h after transfection, the cells were either mock infected or HCMV infected in the presence of cycloheximide at 24 h after transfection. RNA isolation and RNase protection assays were done as described in Materials and Methods. Lanes (lanes 1 through 7 have been treated with RNase): 1, riboprobe; 2, mock-transfected cells; 3, pTK760-transfected and mock-infected cells; 4, pTK760-transfected and HCMV-infected cells; 5, pTK760 cotransfected with control DNA (pLink760); 6, pTK760 cotransfected with pCMV71 (pp71); 7, pTK760 cotransfected with pCMV65 (pp65); 8, riboprobe not treated with RNase; 9, standard size DNA markers (Std). The riboprobe and the predicted RNase-protected fragment are indicated.

of the promoters from RSV (Fig. 3B), HIV (Fig. 3B), HSV TK, HCMV E1.7, or the enhancerless MIEP responded to UL82 (data not shown). The potential *cis*-acting elements associated with the various enhancer-containing promoters are designated in Fig. 3B. These results indicate that activation by UL82 enhances gene expression at the level of transcription. Since the enhancerless MIEP (p*dl*760CAT) did not respond to UL82, the upstream enhancer regulatory region may contain a *cis*-acting element that responds to UL82.

UL82-responsive *cis*-acting element located within the 19-bp repeat. To locate a UL82-responsive *cis*-acting element, we used the wild-type enhancer-containing MIEP and its deletion-containing derivatives. The enhancer deletions were pCAT*dl*36, pCAT*dl*Nde, and pCAT*dl*14; they are diagramed in Fig. 4A. We also reconstructed elements of the enhancer by inserting synthetic sequences of the 19-, 18-, 21-, and 16-bp repeat elements upstream of the enhancerless MIEP. In addition, the region containing the 13-bp repeat and NF-1 elements was inserted upstream of the enhancerless MIEP. These plasmid constructs with the upstream *cis*-acting elements are diagramed in Fig. 4A.

The wild-type reporter plasmid pCAT760 and the various constructs described above were cotransfected with either control DNA (pLink760) or effector DNA (pCMV71) into duplicate 100-mm culture dishes of HFF cells. RSV TK DNA was used as an internal control. The steady-state levels of CAT RNA and internal control TK RNA were determined as described in Materials and Methods.

The wild-type MIEP had the strongest response to UL82 (17-fold) (Fig. 4B, compare lanes 2 and 3). Plasmids pCAT*dl*36, pCAT*dl*Nde, and pCAT*dl*14 gave 10-, 8-, and

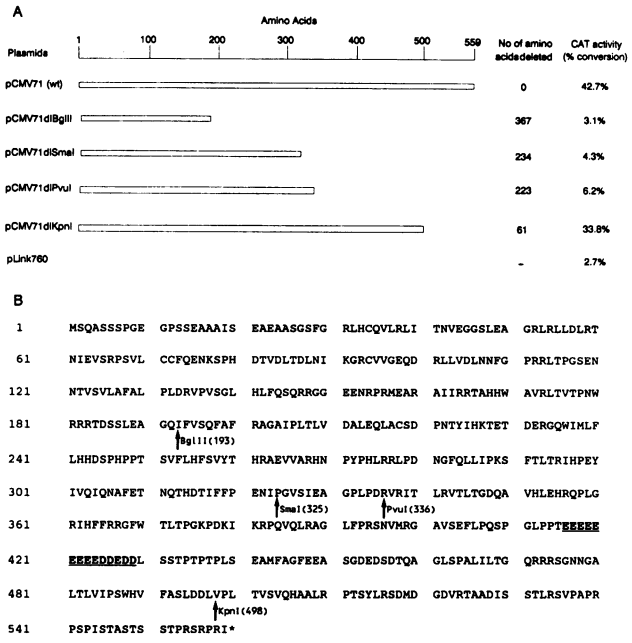


FIG. 2. Effect of carboxy-terminal deletions of UL82 on transactivation of MIEP and the amino acid sequence of pp71. (A) Carboxy-terminal deletions of UL82. pCAT760 DNA was cotransfected with either wild-type pCMV71 or mutant pCMV71. After 48 h, cells were lysed and assayed for CAT activity as described in Materials and Methods. Transactivation of CAT expression by either wild-type or mutant pCMV71 is shown by the percent ^{14}C-chloramphenicol converted to the 3′-acetylated derivative. Open boxes represent pp71 coding sequences retained. The number of amino acid residues deleted is designated. (B) Amino acid sequence of pp71. Single-letter abbreviations for each amino acid were used. The carboxyl deletion endpoint for each of the mutants is designated below the amino acid sequence adjacent to the restriction endonuclease site used. Underlined boldface letters designate a region rich in acidic residues.

2-fold responses, respectively, to UL82 (Fig. 4B, compare lanes 4 and 5, 6 and 7, and 8 and 9, respectively). These data indicated that a responsive element was being removed by deletion. CAT gene constructs with multiple copies of a single element upstream of enhancerless MIEP were tested for a response to UL82. The CAT gene driven by the MIEP with the 19-bp repeat upstream responded strongly to UL82 (8-fold) (compare lanes 12 and 13). In contrast, the enhancerless plasmid (p*dl*760CAT) and the reconstructed plasmids containing the 18-, 21-, or 16-bp repeat did not respond to UL82 (compare lanes 10 and 11, 14 and 15, 16 and 17, and 18 and 19).

This experiment was repeated and assayed for the CAT translation product. The reporter plasmids were cotransfected with effector DNA into HFF cells. The CAT activities were quantitated with a thin-layer chromatography scanner; the results are shown graphically in Fig. 4C. The wild-type MIEP responded strongly to UL82 (18-fold). With the constructs containing deletions in the enhancer-containing MIEP, there was a direct correlation between percent CAT conversion and the number of 19-bp repeat elements upstream of the MIEP. The CAT gene driven by the enhancerless MIEP with the 18-, 21-, or 16-bp repeats and the 13-bp repeat–NF-1 elements upstream had little to no response to UL82. The CAT gene with 19-bp repeat elements upstream of the enhancerless MIEP responded strongly to UL82 (sevenfold). The potential *cis*-acting elements associated with the various reporter plasmids are designated in Fig. 4C.

Together, the RNase protection and CAT assay experiments indicate that the response to UL82 is related to the presence of the 19-bp repeat. This repeat element contains a consensus ATF *cis*-acting site.

The ATF and AP-1 motifs are *cis*-acting elements that respond to UL82. The HCMV MIEP has potential binding sites for both ATF and AP-1, and the promoter responds to both cAMP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation (6, 22, 30, 31, 39, 53). Therefore, we tested plasmids with ATF or AP-1 *cis*-acting sites in the upstream regulatory region for a response to UL82. We also mutated the 19-bp repeat sequence either to the 5′ side, in the middle of the ATF site, or to the 3′ side. These plasmids, with their number of respective *cis*-acting elements, are diagramed in Fig. 5A. When the 19-bp wild-type sequence CCCCATTGACGTCAATGGG was mutated to CCCCAT TactaTCAATGGG (pIEM1/193CAT), the MIEP failed to respond to UL82. This DNA also failed to bind a purified ATF-bacterial fusion protein (kindly provided by R. Maurer) (data not shown). However, M2/19, which has a mutation on the 5′-flanking sequence of the ATF binding site (CtttgT TGACGTCAATGGG), and M3/19, which has a mutation on the 3′-flanking sequence of the ATF (CCCCATTGACGT CAAcaaa), responded to UL82 to the same extent as did the wild-type sequence (pIE193CAT) (Fig. 5B). These DNAs complexed with a purified ATF-bacterial fusion protein (data not shown). Likewise, promoters with a single ATF (pSomaCAT) or single AP-1 (pAP1/1CAT) site upstream also responded to UL82 but only to one-third of the level of the plasmid containing three ATF sites.

These results suggest that UL82, which encodes a phosphorylated tegument protein of 71 kDa, either directly or indirectly, mediates transcriptional activation of the MIEP through ATF or AP-1 sites and consequently has a major effect on transcription downstream of the MIEP.

DISCUSSION

Regulation of HCMV IE gene transcription presumably plays a central role in both latent and productive infection. Transcription of IE genes defines the earliest stage of infection that influences the entire replication cycle of the virus. Transcription of these viral regulatory genes is controlled by a very complex enhancer-containing promoter, MIEP. We have identified a novel virion-associated transactivator that strongly stimulates transcription from the MIEP of HCMV. The viral protein designated pp71 is located in the tegument between the viral membrane and the capsid structure. The transactivator is the UL82 gene product, which is reported to be a 71-kDa phosphorylated tegument protein (7, 41, 46, 47). It presumably stimulates transcription from both viral and cellular genes.

Comparison of the predicted UL82 protein sequence with sequence data bases revealed little homology with other known proteins. A 344-amino-acid N-terminal region of UL82 shares about 25% homology with the N-terminal region of the pp65 protein, which is encoded by the adjacent gene, UL83 (7, 47). The C-terminal portion of UL82 has a stretch of acidic amino acids, EEEEEEEEEDDEDD, located between residues 416 and 429 (Fig. 2B) that shares significant similarity with the acidic tail of the homeo-domain transcriptional factors (45, 61). The N-terminal flanking side of this negatively charged region between residues 415 and

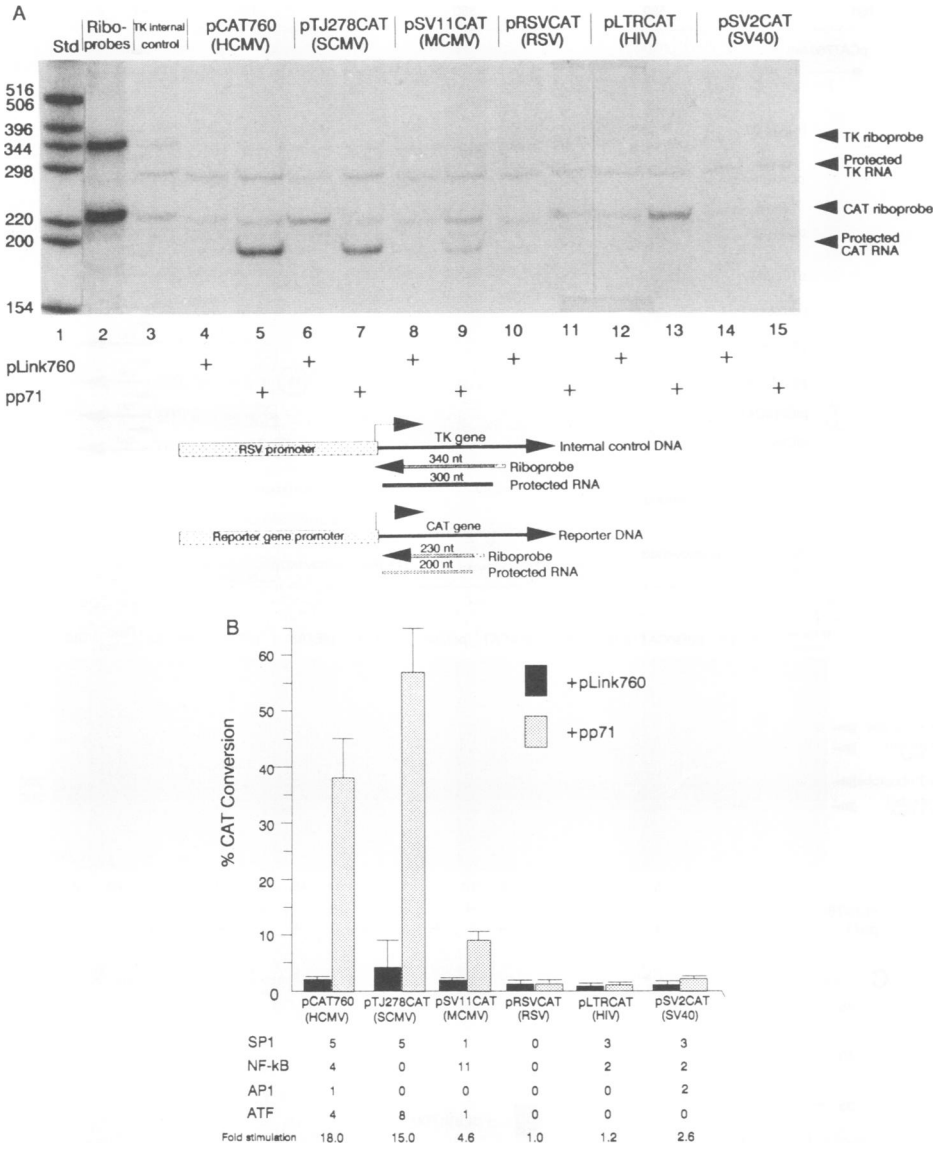
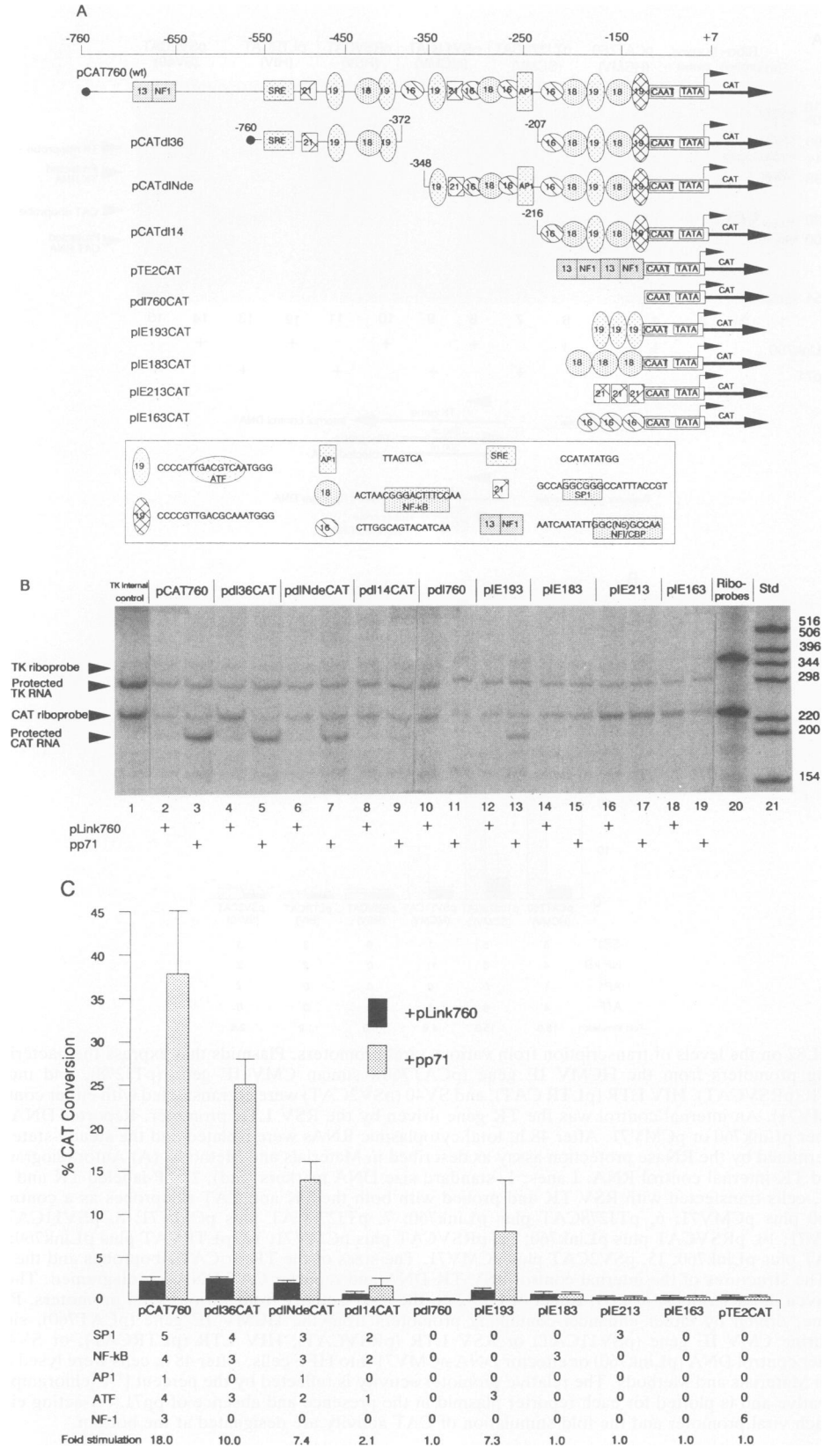


FIG. 3. Effect of UL82 on the levels of transcription from various viral promoters. Plasmids that express the bacterial CAT gene, driven by enhancer-containing promoters from the HCMV IE gene (pCAT760), simian CMV IE gene (pTJ278), and murine CMV IE gene (pSV11CAT), RSV LTR (pRSVCAT), HIV LTR (pLTR CAT), and SV40 (pSV2CAT) were cotransfected with either control DNA (pLink760) or effector DNA (pCMV71). An internal control was the TK gene driven by the RSV LTR promoter. Reporter DNA plus RSV TK were cotransfected with either pLink760 or pCMV71. After 48 h, total cytoplasmic RNAs were isolated and the steady-state levels of CAT RNA and TK RNA was determined by the RNase protection assay as described in Materials and Methods. (A) Autoradiogram of the steady-state level of CAT RNA and TK internal control RNA. Lanes: 1, standard size DNA markers (Std); 2, ^{32}P-labeled TK and CAT riboprobes not treated with RNase; 3, cells transfected with RSV TK and probed with both the TK and CAT riboprobes as a control; 4, pCAT760 plus pLink760; 5, pCAT760 plus pCMV71; 6, pTJ278CAT plus pLink760; 7, pTJ278CAT plus pCMV71; 8, pSV11CAT plus pLink760; 9, pSV11CAT plus pCMV71; 10, pRSVCAT plus pLink760; 11, pRSVCAT plus pCMV71; 12, pLTRCAT plus pLink760; 13, pLTRCAT plus pCMV71; 14, pSV2CAT plus pLink760; 15, pSV2CAT plus pCMV71. The sizes of the TK or CAT riboprobes and the protected RNAs are indicated by arrows. The structures of the internal control RSV TK DNA and reporter CAT DNA are diagramed. The riboprobes and the protected RNAs are given in nucleotides (nt). (B) Effect of UL82 on the CAT activity from various viral promoters. Plasmids that express the bacterial CAT gene, driven by either enhancer-containing promoters from the HCMV IE gene (pCAT760), simian CMV IE gene (pTJ278CAT), and murine CMV IE gene (pSV11CAT) or RSV LTR (pRSVCAT), HIV LTR (pLTRCAT), or SV40 (pSV2CAT) were cotransfected with either control DNA (pLink760) or effector DNA pCMV71 into HFF cells. After 48 h, cells were lysed and assayed for CAT activity as described in Materials and Methods. The relative promoter activity is reflected by the percent [^{14}C]chloramphenicol converted to the 3′-acetylated derivative and is plotted for each reporter plasmid in the presence and absence of pp71. *cis*-acting elements known to be located upstream of each viral promoter and the fold stimulation of CAT activity are designated at the bottom.


A
-760
-650
-550
-450
-350
-250
-150
+7
pCAT760 (wt)
pCATdl36
pCATdlNde
pCATdl14
pTE2CAT
pdl760CAT
pIE193CAT
pIE183CAT
pIE213CAT
pIE163CAT
CAT
CAAT
TATA
SRE
AP1
NF1
-372
-207
-348
-216
CCCCATTGACGTCAATGGG
ATF
CCCCGTTGACGCAAATGGG
TTAGTCA
ACTAACGGGACTTTCCAA
NF-kB
CTTGGCAGTACATCAA
CCATATATGG
GCCAGGCGGGCCATTTACCGT
SP1
AATCAATATTGGC(N5)GCCAA
NFI/CBP
B
TK internal control
pCAT760
pdl36CAT
pdlNdeCAT
pdl14CAT
pdl760
pIE193
pIE183
pIE213
pIE163
Ribo-probes
Std
TK riboprobe
Protected TK RNA
CAT riboprobe
Protected CAT RNA
516
506
396
344
298
220
200
154
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
pLink760
pp71
C
% CAT Conversion
+pLink760
+pp71
pCAT760
pdl36CAT
pdlNdeCAT
pdl14CAT
pdl760
pIE193
pIE183
pIE213
pIE163
pTE2CAT
SP1 5 4 3 2 0 0 0 3 0 0
NF-kB 4 3 3 2 0 0 3 0 0 0
AP1 1 0 1 0 0 0 0 0 0 0
ATF 4 3 2 1 0 3 0 0 0 0
NF-1 3 0 0 0 0 0 0 0 0 6
Fold stimulation 18.0 10.0 7.4 2.1 1.0 7.3 1.0 1.0 1.0 1.0

419, TEEEE, has a potential casein kinase II recognition site, S*/T*-[D/E/S(P)$_{1-3}$ X$_{2-0}$], in which the phosphoacceptor group is denoted by an asterisk or by the letter P in parentheses and X denotes sequence positions judged to be recognition neutral (26). The C-terminal flanking side between residues 425 and 433, DDEDDLSST, resembles a casein kinase I recognition site, D/E$_{2-4}$,X$_{2-0}$-S*/T*. Therefore, the net charge of this acidic region may be regulated by phosphorylation of the protein. When the region containing the acidic amino acids and the potential phosphoacceptor amino acids was deleted, the viral protein lost the ability to transactivate the MIEP. Further studies are necessary to define the functional domains of the UL82 gene product. It is interesting that pp65, the lower tegument protein, is a protein kinase (51). Whether pp71 is a substrate for the pp65 protein kinase requires further investigation.

As a virion-associated transactivator, the predicted protein of UL82, pp71, has features that are different from those of the well-known virion-associated transactivator VP16 of HSV. First, there is no sequence homology between VP16 and pp71. Although both proteins have a negatively charged region near their carboxyl termini, the organization of these acidic amino acid residues is very different. The acidic residues of VP16 are periodic (60). In contrast, the acidic residues of pp71 are consecutive. Second, transcriptional activation by pp71 is through the ATF and AP-1 *cis*-acting sequences. In contrast, VP16 interacts with Oct-1, and this protein, complexed with other cellular factors, can bind to the target sequence TAATGARAT (R = purine) (9, 24, 37, 42, 43). HCMV is a betaherpesvirus, and HSV is an alphaherpesvirus. Our results suggest that these viruses, with different replication properties and cell specificities, achieve an acceleration of IE gene transcription through unique virion-associated transactivators interacting with different cellular transcription factors.

Promoters with upstream ATF or AP-1 sites responded to transactivation by UL82. The MIEP of HCMV contains four ATF sites and one AP-1 site. Transcription of three cellular proto-oncogenes, c-*fos*, c-*jun*, and c-*myc*, has been reported to be stimulated by HCMV infection in the presence of an inhibitor of de novo protein synthesis, suggesting the involvement of a virion-associated transactivator (3). The proto-oncogenes induced by HCMV are thought to be involved in cellular signal transduction and regulation of cell proliferation (3, 28, 29, 48). All three proto-oncogenes are also stimulated by the reagents which trigger cellular protein kinase A or C signaling pathways (14, 25, 28, 29, 38). Both ATF and AP-1 binding sites have been found upstream of the c-*fos* gene (44). The c-*jun* promoter contains one AP-1 binding site (28, 44). Although the c-*myc* promoter is inducible by reagents which stimulate the protein kinase A or C signaling pathway, the responsive *cis*-acting site has not been identified (19, 33), but c-*myc* does have an upstream E2F-responsive element (40). The SV40 enhancer contains two copies of AP-1 binding sites (31) and is stimulated by UL82. The HIV LTR has two AP-1-like motifs, GGGATCA and CTGACCT, that are not inducible by TPA (32). Expression of this viral promoter was not stimulated by UL82. Therefore, this is consistent with the interpretation that the specificity of the virion-associated transactivator is for viral or cellular promoters containing consensus ATF or AP-1 binding sites that are inducible by cAMP or TPA.

The core sequence of the 19-bp repeat element, TGACGTCA, contains a binding site for ATF. A purified ATF-bacterium fusion protein binds to the 19-bp repeat element and retards the migration of the DNA in an in vitro gel mobility shift assay (34a). The MIEPs from all CMVs analyzed to date contain the 19-bp repeat element. The HCMV, simian CMV, and murine CMV MIEPs are stimulated by UL82, and the amount of transactivation was proportional to their number of 19-bp repeats. Mutation at the ATF site within the 19-bp repeat element abolished responsiveness to UL82. Therefore, the pp71 tegument protein of HCMV can stimulate transcription from viral and cellular promoters containing upstream ATF sites.

Previous studies have shown that ATF and AP-1 are a family of related transcriptional factors (18, 19). The AP-1 binding site, TGAGTCA, differs from the ATF site, TGACGTCA, by the absence of a C · G base pair. There are at least 10 structurally distinct but related ATF genes and multiple forms of ATF and AP-1 proteins in the eucaryotic cell (17, 18). ATF can form either a homodimer or a heterodimer which binds to the TGACGTCA motif. AP-1 exists as either a *jun-fos* heterodimer or a *jun-jun* homodimer which binds to the TGAGTCA motif. However, the *jun* homodimers or *jun*-ATF heterodimers can bind to both ATF and AP-1 binding sites (18, 36). Nevertheless, each protein binds more strongly to its cognate site than to heterologous sites. Therefore, ATF or AP-1 could be binding to the TGACGTCA motif in the 19-bp repeat element.

Since transactivation of the MIEP of HCMV or the cellular proto-oncogene promoters by virions is independent of de novo protein synthesis, it is possible that pp71 activates host transcriptional factors by posttranslational modi-

FIG. 4. Effect of UL82 on the level of transcription from both wild-type and various mutant enhancer-containing MIEPs or the isolated repeat elements upstream of the enhancer-minus MIEP. Transfection, cytoplasmic RNA isolation, and RNase protection assay and an internal TK RNA control were as described for Fig. 3. (A) Diagram of the various plasmids used in the transient-transfection assays. The promoter structures and relative locations of various individual repeat elements from both the wild type and mutants are shown. The consensus sequence for the various *cis*-acting elements are designated below. (B) Autoradiogram of the steady-state level of CAT RNA and TK internal control RNA. Lanes: 1, cells transfected with RSV TK and probed with both the TK and CAT riboprobes for control; 2, pCAT760 plus pLink760; 3, pCAT760 plus pCMV71; 4, p*dl*36CAT plus pLink760; 5, p*dl*36CAT plus pCMV71; 6, p*dl*NdeCAT plus pLink760; 7, p*dl*NdeCAT plus pCMV71; 8, p*dl*14CAT plus pLink760; 9, p*dl*14CAT plus pCMV71; 10, p*dl*760CAT plus pLink760; 11, p*dl*760CAT pus pCMV71; 12, pIE193CAT plus pLink760; 13, pIE193CAT plus pCMV71; 14, pIE183CAT plus pLink760; 15, pIE183CAT plus pCMV71; 16, pIE213CAT plus pLink760; 17, pIE213CAT plus pCMV71; 18, pIE163CAT plus pLink760; 19, pIE163CAT plus pCMV71; 20, ^{32}P-labeled TK and CAT riboprobes not treated with RNase; 21, standard size DNA markers (Std). The sizes of the TK or CAT riboprobes and the protected RNAs are indicated by arrows. (C) Effect of UL82 on the CAT activity from both wild-type and various mutant enhancer-containing MIEPs or the isolated repeat elements upstream of the enhancer-minus MIEP promoter. Transfection and quantitation of CAT activity were done as described for Fig. 3. The relative promoter activity is reflected by the percent conversion of [^{14}C]chloramphenicol to the 3′-acetylated derivative and is plotted for each reporter plasmid in the presence and absence of pp71. The numbers of the various known *cis*-acting elements located upstream of each promoter and the fold stimulation of CAT activity are designated at the bottom.

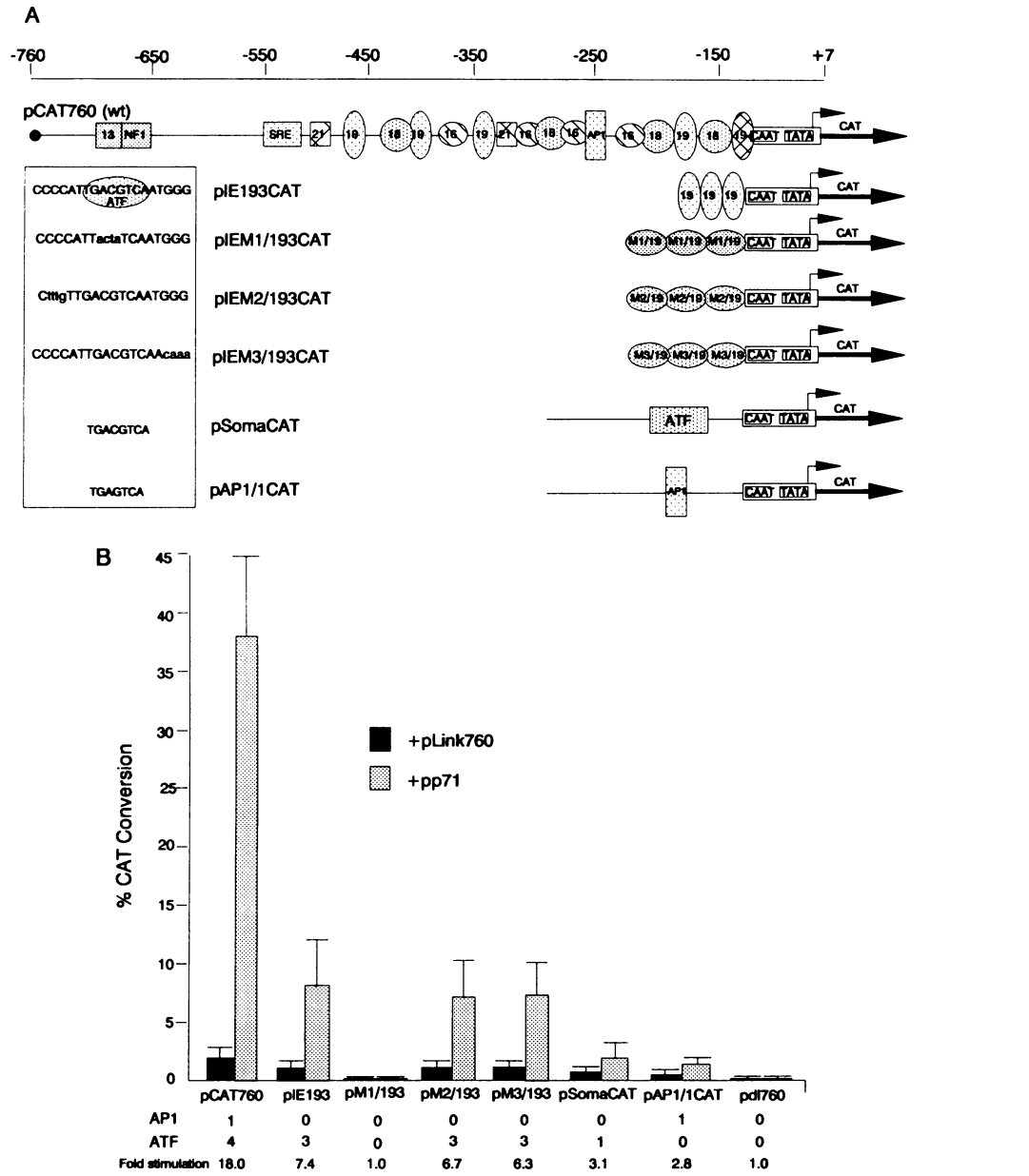


FIG. 5. Effects of point mutations within the ATF motif on the responsiveness of the promoter to transactivation by UL82. (A) Wild-type and mutant 19-bp repeat constructs. Mutant oligonucleotides of the 19-bp repeat sequence were constructed to contain point mutations in the ATF binding site (M1/19; CCCCATTactaTCAATGGG), in the 5′-flanking region of the ATF binding site (M2/19; CtttgTTGACGTCAATGGG), and in the 3′-flanking region of the ATF binding site (M3/19, CCCCATTGACGTCAAcaaa). The consensus ATF binding site is in boldface type and underlined. Three copies of each mutant 19-bp repeat were cloned into the *Xba*I site upstream of p*dl*760CAT. The additional plasmids are pSomaCAT and pAP1/1CAT. The known or mutant *cis*-acting sites upstream of the promoter are designated at the left. (B) Quantitation of CAT activity. Each reporter plasmid was cotransfected with either pLink760 or pCMV71 into HFF cells. After 48 h, cells were lysed and assayed for CAT activity. The relative promoter activity is reflected by the percent [^{14}C]chloramphenicol converted to a 3′-acetylated derivative and is plotted for each reporter plasmid in the presence and absence of pp71. Numbers of ATF and AP-1 binding sites present upstream of each promoter tested and the fold stimulation of CAT activity are designated at the bottom.

fication of preexisting proteins. Whether pp71 interacts directly or indirectly with these eucaryotic transcription factors is not known. It seems unlikely that pp71 is a DNA-binding protein. Basic amino acid domains, which are involved in DNA binding, are not evident. In vitro-translated pp71 did not bind to the DNA sequence containing the 19-bp repeat element (34a). pp71 could achieve target gene specificity via protein-protein interaction with transcriptional regulatory elements. It is also possible that pp71 removes an inhibitor binding to the ATF or AP-1 proteins and consequently activates the transcriptional factor. Lastly, pp71 may act by triggering a cascade of catalytic events of the cellular protein kinase A or C signaling pathways or activating the Ca^{2+}-calmodulin-dependent protein kinase and caus-

ing phosphorylation and subsequent activation of preexisting transcription factors (11, 50, 62). ATF has been shown to function as a substrate for depolarization-activated Ca^{2+}-calmodulin-dependent protein kinase (50).

Although the specific mechanism of how the UL82 gene products activate transcription of the IE1 and IE2 genes of HCMV and perhaps cellular genes is not understood, it should be possible to further characterize these interactions by identifying the viral protein domains involved and the cellular sites of action. Further studies are necessary to determine the functional role of these viral tegument proteins in influencing viral and cellular gene transcription.

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