JOURNAL OF VIROLOGY, Feb. 1991, p. 897–903
0022-538X/91/020897-07$02.00/0

Vol. 65, No. 2

A *cis*-Acting Element in the Major Immediate-Early (IE) Promoter of Human Cytomegalovirus Is Required for Negative Regulation by IE2

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Received 27 September 1990/Accepted 19 November 1990

The major immediate-early promoter (MIEP) of human cytomegalovirus (CMV) contains a number of different enhancer elements in both repetitive and nonrepetitive sequences that influence the level of downstream transcription. This report describes a *cis*-acting element in the MIEP that responds to negative regulation by the IE2 gene product. Deletion analysis demonstrated that the *cis*-acting repressor element is located between the TATA box and the transcription initiation site from −13 to −1. The DNA sequence of the repressor element is 5′-CGTTTAGTGAACC-3′. The sequence is found in both the human and simian CMV MIEPs but not the murine CMV MIEP or in several other enhancer-containing promoters. The repressor element was isolated in a DNA fragment from −13 to +3 and was found to be functional in either orientation. It could be transferred to a heterologous enhancer-containing promoter and was functional when placed between the TATA box and the transcription initiation site. The element did not function when placed downstream of the transcription initiation site. Therefore, the *cis*-acting repressor element is position dependent. The role of the repressor element and the IE2 gene product in human CMV productive or latent infection is discussed.

Human cytomegalovirus (HCMV), a betaherpesvirus, replicates more slowly than the alphaherpesviruses. After high multiplicity of infection of cultured cells, the maximum rate of viral DNA replication occurs only after 3 days. Viral plaques are not visible until 10 to 14 days after infection (36, 37). In the human host, the virus is frequently associated with infections of the newborn that can persist for years. Although infection is normally asymptomatic in immunocompetent hosts, HCMV can cause serious or fatal disease in immunocompromised patients as well as in neonates (14). The rate of HCMV replication in cell culture or in the host is presumably influenced by a complicated series of regulatory events involving the viral and host genomes as well as their specific *trans*-acting factors.

The CMV genomes of both human and animal origin are large double-stranded DNAs of approximately 230 kb. There are several enhancers within the genome (2, 15, 40, 41). An unusually strong enhancer is located upstream of the major immediate-early promoter (MIEP) (2, 40). Within this regulatory region are elements that modulate expression in different cell types (19) and in undifferentiated and differentiated cells (29). There are a number of different repeat elements which contain consensus binding sites for known eucaryotic transcription factors (2, 37, 38, 40). DNA footprinting and in vitro transcription experiments indicate that both repetitive and nonrepetitive sequences in the enhancer may influence downstream transcription (7–9, 22, 40).

There are two transcription units downstream of the MIEP that are designated IE1 and IE2 (33, 34, 39). IE1 codes for an mRNA of 1.95 kb that consists of the first four consecutive exons (32). The first three exons of IE1 are alternatively spliced to a downstream fifth exon, which is designated the IE2 transcription unit. Three different size classes of mRNAs (2.25, 1.70, and 1.40 kb) originate either by the selection of different splice acceptor sites within exon 5 or by the alternate processing of a coding intervening sequence within exon 5 (36). Since the translation initiation codon is in exon 2, IE1 and IE2 have the same N-terminal 85-amino-acid sequence coded by exons 2 and 3.

The IE1 protein consists of 491 amino acid residues, is heavily phosphorylated, and is moderately acidic (33). The largest IE2 protein consists of 579 amino acid residues and is also phosphorylated (13, 30, 34). Both IE proteins are transported to the nucleus of virus-infected cells (25).

Transient expression assays have been used to investigate the possible functions of the HCMV IE1 and IE2 proteins. The IE1 protein positively regulates the MIEP to enhance expression of IE1 (5) and IE2 (20). The largest of the IE2 proteins independently transactivates a representative HCMV early promoter (4, 20). The IE1 and IE2 proteins act synergistically to increase expression from several early viral promoters (4, 6, 7, 13, 20, 26, 30). The IE2 protein also negatively autoregulates the MIEP in nonpermissive Vero cells (26) and in permissive human fibroblast cells (12, 31). We have investigated this repressive effect by IE2. We describe a *cis*-acting site positioned between the TATA box and the transcription initiation site of the MIEP that is required for negative regulation by IE2. The relevance of this negative effect on expression of the HCMV IE regulatory proteins is discussed.

MATERIALS AND METHODS

Virus and cell culture. Growth of human foreskin fibroblast (HFF) cells and propagation of HCMV (Towne) have been described previously (35). HeLa cells were prepared as described previously (28).

Plasmid constructions. Plasmid pCAT760, which contains the MIEP of HCMV upstream from the procaryotic chloramphenicol acetyltransferase (CAT) gene, has been described previously (38). Plasmid pTJ278 is a similar plasmid

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which contains the MIEP of simian CMV upstream of the CAT gene (kindly provided by G. Hayward). Plasmid pSV11CAT contains the MIEP of murine CMV upstream of the CAT gene (kindly provided by U. Koszinowski). Plasmids pRSVCAT, pSV2CAT, and pLTRCAT, containing the Rous sarcoma virus (RSV), simian virus 40 (SV40), and human immunodeficiency virus (HIV) enhancer-containing promoters upstream of the CAT gene, have been described previously (1, 10, 11). Plasmids pSV40IE1 and pLTRIE1 were constructed by replacing the respective viral promoters driving expression of the CAT gene by the HCMV IE1 promoter. For pSV40IE1, the SV40 promoter −55 to +60 was replaced by the HCMV MIEP −68 to +7. For pLTRIE1, the HIV long terminal repeat (LTR) promoter from −45 to +182 was replaced by the HCMV MIEP −68 to +7.

Various deletions and linker substitutions were made in pCAT760 (summarized in Fig. 3). For 5′ end deletions, pCAT760 was linearized by digestion with restriction endonuclease *Sst*I. The ends of the DNA molecule were made blunt by digestion with mung bean nuclease, and *Xho*I linkers were ligated to the blunt ends as described previously (21). After DNA sequencing, it was determined that the nuclease removed two additional base pairs. The resulting plasmid was designated pCAT760XhoI and was used for the construction of plasmids pIE1−20/−13, pIE1−20/−9, and pIE1−20/−1 (see Fig. 3). To construct plasmid pIE1−20/−13, pCAT760XhoI was linearized by *Xho*I restriction endonuclease digestion, the ends were made blunt, and the plasmid was recircularized by ligation. To construct plasmids pIE1−20/−9 and pIE1−20/−1, 5′ end deletions were generated by BAL 31 nuclease digestion after digestion by *Xho*I in pCAT760XhoI. The ends of the DNAs were made blunt, and *Xho*I linkers were ligated to the blunt ends as described above. After a secondary restriction endonuclease digestion, the modified 5′ ends containing the *Xho*I linker and the downstream CAT gene were gel purified and were substituted for the wild-type sequence downstream of the *Xho*I site in pCAT760XhoI. Therefore, MIEP sequences were unchanged upstream of −20. Downstream of −20, deleted sequences are substituted by *Xho*I linker sequence (diagramed in Fig. 3).

A slightly different approach was used to generate 3′ end deletions. The DNA fragment from bp −19 to +7 in the MIEP was isolated after *Sst*I and *Bgl*II restriction endonuclease digestion. This fragment was inserted into the polylinker of pGEM at the *Sst*I and *Bam*HI restriction endonuclease sites, and the resulting recombinant plasmid was designated pGEMRE. To construct plasmids pIE1+3/+8, pIE1−1/+8, pIE1−3/+8, pIE1−9/+8, and pIE1−12/+8, 3′ end deletions were made by BAL 31 digestion of the *Xba*I restriction endonuclease digested pGEMRE. After BAL 31 digestion and generation of blunt ends, the plasmids were recircularized by ligation. The region containing the deletions was isolated after *Sst*I and *Hin*dIII digestion and then substituted for the wild-type sequences in pCAT760 by ligation between the *Sst*I and *Hin*dIII sites.

In both the 5′ and 3′ deletions described above, linker DNA replaced all or part of the deleted DNA (diagramed in Fig. 3). Gaps represent base pairs not replaced by linker substitution. The DNA sequences of all plasmid DNAs were determined as described below.

Clones involving orientation, duplication, position, and transfer of the IE1-specific repressor element were constructed (diagramed in Table 3). Different orientations and duplications of the HCMV IE1 repressor element were obtained as follows. Plasmid pIE1−20/−9 was digested with *Xho*I (−14) and *Hin*dIII (+8) restriction endonucleases, and the ends of the DNA molecule were made blunt by treatment with T4 DNA polymerase as described by Maniatis et al. (21). This plasmid served as a vector plasmid for various insertions of the repressor element. The HCMV IE1 repressor element −13 to +3 was isolated from pGEMRE after *Sst*I and *Sph*I restriction endonuclease digestion. The ends of the DNA molecule were made blunt by treatment with mung bean nuclease, and then the DNA was ligated in either orientation and in duplication between −14 and +8. A variety of different recombinant plasmids was obtained (see Table 3).

The HCMV IE1 repressor element was positioned downstream of the transcription initiation site at +31 as follows. pCAT760 was digested with *Sst*I (−16) and *Hin*dIII (+8) restriction endonucleases, which removes the repressor element. The 59-bp polylinker of pGEM from the *Sst*I to the *Hin*dIII site was substituted for wild-type DNA in pCAT760 between the *Sst*I (−16) and *Hin*dIII (+8) sites. This plasmid was designated pCAT760dlRE. dlRE indicates deletion of the repressor element. After linearization of pCAT760dlRE by *Hin*dIII restriction endonuclease digestion and the generation of blunt ends by T4 polymerase-exonuclease digestion, the 17-bp repressor element containing blunt ends was inserted in either orientation. Therefore, wild-type DNA between −16 and +8 was replaced with linker DNA by the insertion of a 59-bp DNA fragment, and then the repressor element was inserted at the downstream *Hin*dIII site. Assuming that the transcription initiation site remains at the same position from the TATA box as determined for wild-type DNA (33), the 5′ end of the repressor element would now be located at +31. These plasmids were designated pIESRE1 and pIESRE1R. The latter plasmid has the repressor element in the opposite orientation (diagramed in Table 3).

The HCMV IE1 repressor element was positioned downstream of the SV40 TATA box as follows. After digestion of pSV2CAT DNA with *Nde*I (−571) and *Hae*III (−17) restriction endonucleases, the DNA fragment was isolated and substituted for HCMV DNA sequence in pIE1−20/−13 between the *Nde*I (−810) and *Sma*I (−16) sites. Therefore, the SV40 enhancer-promoter has the HCMV repressor element downstream of the SV40 TATA box from −16 to +7, which is located immediately upstream of the CAT gene. This plasmid was designated pSV40RE1 and is diagramed in Table 3. All plasmid DNAs containing the repressor element were analyzed by DNA sequencing as described below.

Plasmid pLink760 was used for enhancer control DNA as previously described (12). Plasmid pSVIE2, which can express the HCMV IE2 proteins, has also been described previously (20).

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

Transfection. Plasmid DNAs were banded twice in cesium chloride gradients and quantified spectrophotometrically. Equimolar DNA concentrations were checked by restriction endonuclease digestion of plasmid DNAs followed by gel electrophoresis and comparison of the intensities of ethidium bromide-stained bands. Permissive HFF cells in 100-mm culture dishes were transfected in duplicate with calcium phosphate precipitates as described previously (4, 13). Transfections were done multiple times with different plas-

mid preparations to control variations in transfection efficiencies. Measurements of uptake of various DNAs were evaluated as described previously (13) and were found to be similar.

CAT assays. Cell harvesting and CAT assays were performed as described by Gorman et al. (11). CAT activities were determined in the linear portion of the enzyme reaction. Acetylated derivatives were separated from nonacetylated [^{14}C]chloramphenicol by ascending chromatography with a chloroform-methanol (95:5) solvent. For quantitative comparisons, the radioactivity on the plate 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. For construction of the CAT RNA probe, pCAT760 was digested with *Sst*I (−16) and *Pvu*II (+200) restriction endonucleases, and the DNA was isolated and inserted at the *Sst*I and *Hin*dIII sites made blunt in the polylinker of pGEM. The resulting clone, pGEM-CAT, was linearized with *Eco*RI and used as a template for SP6 RNA polymerase. Synthesis by SP6 RNA polymerase on linear pGEM-CAT DNA produced an antisense RNA probe of 230 nucleotides (nt), in agreement with the predicted size. [^{32}P]RNA probe synthesis, hybridization, and RNase digestion conditions were as described by Krieg and Melton (16). Because CAT RNA is A+U rich, RNase digestions were at 25°C for 30 min. Cytoplasmic RNA was harvested from two 100-mm plates of transfected HeLa cells at 48 h after transfection as previously described (33, 34). Eighty micrograms of RNA from each transfection was hybridized to the antisense RNA probe at 40°C overnight. The protected fragments were subjected to electrophoresis on 6% polyacrylamide-urea gels. The bands were detected by autoradiography, and the level of CAT RNA was quantitated by scanning with a DU-8 spectrophotometer with the DU-8 gel-scanning Compuset module (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Effects of IE2 on various enhancer-containing promoters. Although the HCMV MIEP directs extremely strong downstream gene transcription of the viral IE genes, the IE2 proteins can negatively autoregulate the viral promoter in both nonpermissive Vero cells (26) and permissive HFF cells (12, 31). To determine whether negative regulation of the MIEP is HCMV specific, the MIEP of human, simian, or murine CMV upstream of the CAT gene was cotransfected with either control plasmid pLink760 or plasmid pSVIE2, which expresses the IE2 gene products of HCMV (20). Nonpermissive HeLa cells and permissive HFF cells were both transfected. After 48 h, cytoplasmic RNA was isolated from the HeLa cells and assayed for steady-state levels of CAT RNA to evaluate the level of transcription. Detection of CAT RNA in HeLa cells was preferred because these cells take up transfected DNA more efficiently than do HFF cells. It was more difficult to detect the CAT RNA in HFF cells. Cell lysates were prepared from the HFF cells and assayed for CAT enzyme activity to evaluate the level of expression of the CAT mRNA from the various promoters. Detection of CAT enzyme activity in the HFF cell was preferred because this is a permissive cell for HCMV replication.

Figure 1B illustrates the 230-nt antisense RNA probe used. The size of the CAT RNA protected from RNase digestion was predicted to be 200 nt. The 230-nt probe and

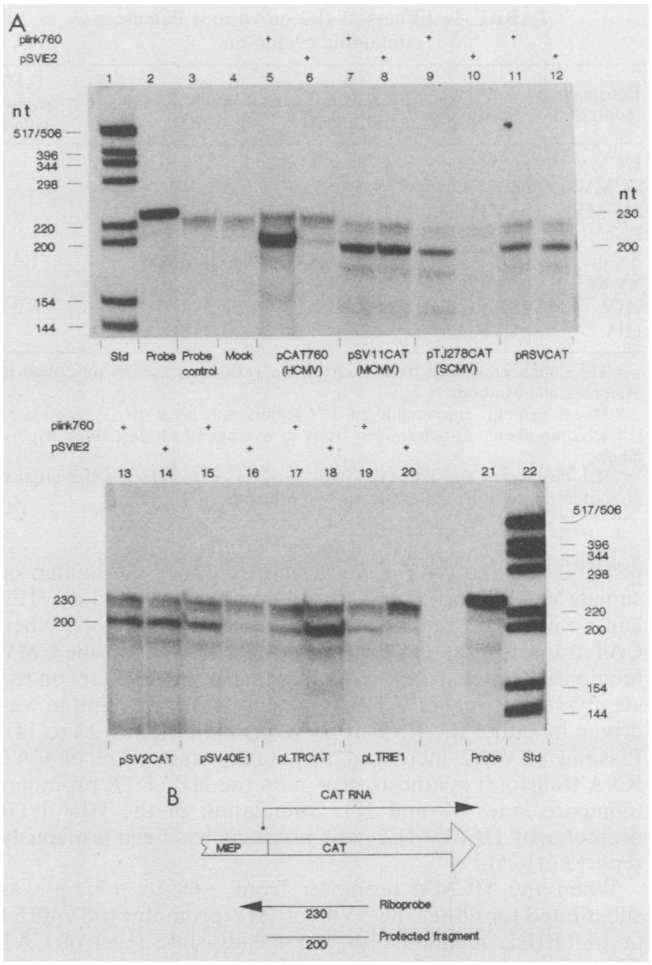

FIG. 1. (A) Effects of IE2 on the steady-state level of CAT RNA synthesis from various enhancer-containing promoters. HeLa cells were cotransfected with 25 μg of the indicator CAT gene driven by various enhancer-containing promoters plus either 30 μg of control DNA pLink760 or 30 μg of pSVIE2 as described in the text. Cytoplasmic RNA was isolated at 48 h after transfection and hybridized to a 230-nt ^{32}P-labeled antisense RNA probe. After RNase treatment, the protected RNA was fractionated in 6% urea-acrylamide gels and the bands were detected by autoradiography as described in Materials and Methods. (B) CAT RNA, size of the riboprobe, and size of the protected fragment. Lanes: 1 and 22, standard molecular size markers; 2 and 21, undigested ^{32}P-labeled RNA probe; 3, digested ^{32}P-labeled RNA probe; 4, mock-transfected cells; 5, pCAT760 plus pLink760; 6, pCAT760 plus pSVIE2; 7, pSV11CAT plus pLink760; 8, pSV11CAT plus pSVIE2; 9, pTJ278 plus pLink760; 10, pTJ278 plus pSVIE2; 11, pRSVCAT plus pLink760; 12 pRSVCAT plus pSVIE2; 13, pSV2CAT plus pLink760; 14, pSV2CAT plus pSVIE2; 15, pSV40IE1 plus pLink760; 16, pSV40IE1 plus pSVIE2; 17, pLTRCAT plus pLink760; 18, pLTRCAT plus pSVIE2; 19, pLTRIE1 plus pLink760; 20, pLTRIE1 plus pSVIE2.

the effect of RNase digestion on the probe alone are demonstrated in Fig. 1A (lanes 2 and 3). A protected fragment of 200 nt was not detected with RNA from mock-infected cells (lane 4). Cytoplasmic RNA from cells transfected with the CAT gene driven by either the human, murine, or simian CMV MIEP had protected a band of 200 nt (lanes 5, 7, and 9). The steady-state level of CAT RNA was significantly reduced (seven-fold) in cells cotransfected with plasmid

TABLE 1. Effects of IE2 on various enhancer-containing promoters

Enhancer source[a]	Reporter plasmid	Mean % conversion[b]		Fold repression	Fold stimulation
		pLink760	pSVIE2		
HCMV	pCAT760	47.1 ± 0.9	7.4 ± 2.1	6.4	
SCMV	pTJ278	49.5 ± 2.3	8.5 ± 4.9	5.8	
MCMV	pSV11CAT	47.0 ± 0.6	42.4 ± 3.0	1.1	
RSV	pRSVCAT	26.6 ± 4.3	23.8 ± 1.8	1.1	
SV40	pSV2CAT	20.0 ± 3.4	27.8 ± 3.8		1.4
SV40	pSV40IE1[c]	20.8 ± 0.8	2.4 ± 0.9	8.6	
HIV	pLTRCAT	7.5 ± 0.2	47.1 ± 4.4		6.3
HIV	pLTRIE1[c]	8.0 ± 0.2	2.0 ± 0.2	4.0	

[a] The enhancer source from each of the various viruses is described in Materials and Methods.

[b] Mean percent conversion of [^{14}C]chloramphenicol to 3′ acetylated [^{14}C]chloramphenicol, determined from an average of at least three experiments.

[c] IE1 designates the HCMV MIEP (−68 to +7) replacing the original promoters as described in Materials and Methods.

pSVIE2 and the CAT gene driven by either the human or simian MIEP (lanes 6 and 10). In contrast, plasmid pSVIE2 had no effect on the steady-state level of CAT RNA when CAT transcription was driven by the MIEP of murine CMV (compare lanes 7 and 8). In addition, IE2 had no effect on the steady-state level of CAT RNA when CAT transcription was driven by either the RSV or SV40 promoter (lanes 11 to 14). Plasmid pSVIE2 increased the steady-state level of CAT RNA (fourfold) synthesis driven by the HIV LTR promoter (compare lanes 17 and 18). Stimulation of the HIV LTR promoter by HCMV IE2 gene products has been previously reported (1, 5).

When the HCMV promoter from −68 to +7 bp was substituted for either the SV40 or HIV promoter (pSV40IE1 or pLTRIE1, respectively), the steady-state level of CAT RNA was reduced four- or fivefold, respectively, in cells cotransfected with plasmid pSVIE2 relative to control DNA pLink760 (compare lanes 15 and 16 and lanes 19 and 20).

pSVIE2 also repressed expression of CAT enzyme activity approximately sixfold when transcription of the CAT gene was driven by the human and simian CMV MIEPs in permissive HFF cells (Table 1). In contrast, pSVIE2 had no effect on CAT enzyme levels when we used the murine CMV MIEP or on CAT enzyme levels from pRSVCAT or pSV2CAT. In contrast, pSVIE2 stimulated pLTRCAT. When the HCMV IE1 promoter from −68 to +7 bp was substituted for the SV40 promoter in plasmid pSV2CAT or the HIV promoter in plasmid pLTRCAT, plasmid pSVIE2 significantly repressed CAT enzyme levels in transfected HFF cells (Table 1). The results obtained by RNase protection assay and CAT enzyme were in good agreement.

These experiments suggest that human and simian CMV have a *cis*-acting element that responds to repression by IE2 gene products and that a similar element is absent in the MIEP of murine CMV as well as the enhancer-containing promoters of RSV, SV40, and HIV. These experiments also suggest that the element is between −68 to +7 bp of the HCMV promoter.

Mapping the HCMV *cis*-acting element. Previous experiments indicated that substitution of the HCMV IE1 TATA box by the SV40 TATA box had no effect on negative regulation by IE2 (data not shown). Therefore, we made our initial deletion and substitution mutations downstream of the TATA box between −20 and +8 of the MIEP. All mutations were analyzed by DNA sequencing.

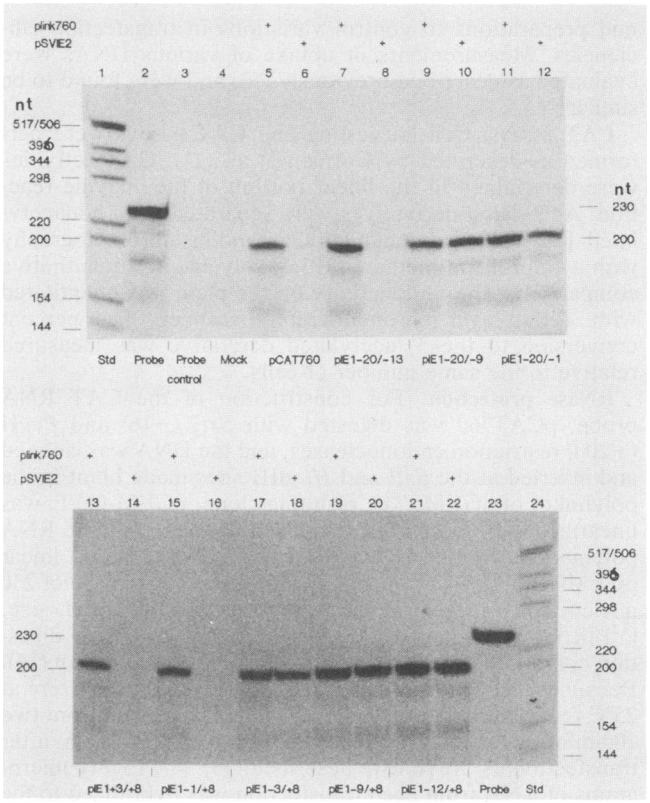

FIG. 2. Effects of IE2 on the steady-state level of CAT RNA synthesis from various deletion mutants in the HCMV MIEP. Transfection, cytoplasmic RNA isolation, and the RNase protection assay were as described in the legend to Fig. 1 and Materials and Methods. Lanes: 1 and 24, standard molecular size markers; 2 and 23, undigested ^{32}P-labeled RNA probe; 3, digested ^{32}P-labeled RNA probe; 4, mock-transfected cells; 5, pCAT760 plus pLink760; 6, pCAT 760 plus pSVIE2; 7, pIE1−20/−13 plus pLink760; 8, pIE1−20/−13 plus pSVIE2; 9, pIE1−20/−9 plus pLink760; 10, pIE1−20/−9 plus pSVIE2; 11, pIE1−20/−1 plus pLink760; 12, pIE1−20/−1 plus pSVIE2; 13, pIE1+3/+8 plus pLink760; 14, pIE1+3/+8 plus pSVIE2; 15, pIE1−1/+8 plus pLink760; 16, pIE1−1/+8 plus pSVIE2; 17, pIE1−3/+8 plus pLink760; 18, pIE1−3/+8 plus pSVIE2; 19, pIE1−9/+8 plus pLink760; 20, pIE1−9/+8 plus pSVIE2; 21, pIE1−12/+8 plus pLink760; 22, pIE1−12/+8 plus pSVIE2.

The steady-state level of CAT RNA was lower (sixfold) in cells cotransfected with plasmid pSVIE2 versus plasmid pLink760 when CAT transcription is driven by the HCMV MIEP, which agrees with the results given in Fig. 1 and Table 1 (Fig. 2; compare lanes 5 and 6). The steady-state levels of CAT RNAs with mutant plasmids pIE1−20/−13, pIE1+3/+8, and pIE1−1/+8 were also repressed by plasmid pSVIE2 (compare lanes 7 and 8, 13 and 14, and 15 and 16). In contrast, CAT RNA levels with mutant plasmids pIE1−20/−9, pIE1−20/−1, pIE1−3/+8, pIE1−9/+8, and pIE1−12/+8 CAT were not affected by plasmid pSVIE2 (compare lanes 9 and 10, 11 and 12, 17 and 18, 19 and 20, and 21 and 22).

In permissive HFF cells, CAT enzyme activities from mutant plasmids pIE1−20/−13, pIE1+3/+8, and pIE1−1/+8 were significantly repressed by plasmid pSVIE2, but mutant plasmids pIE1−20/−9, pIE1−20/−1, pIE1−3/+8, pIE1−9/+8, and pIE1−12/+8 were not (Table 2). The results obtained by RNase protection assay and CAT enzyme assay were in good agreement.

TABLE 2. Mapping of the *cis*-acting site involved in negative regulation of the MIEP by IE2

Reporter plasmid	Mean % conversion[a]		Fold repression
	pLink760	pSVIE2	
pCAT760	51.0 ± 4.0	8.3 ± 0.5	6.1
pIE1−20/−13[b]	48.3 ± 3.5	8.7 ± 5.3	5.6
pIE1−20/−9	48.7 ± 1.7	51.7 ± 3.2	
pIE1−20/−1	48.6 ± 2.6	51.2 ± 1.1	
pIE1+3/+8	50.7 ± 3.4	8.5 ± 0.7	6.0
pIE1−1/+8	51.0 ± 3.0	9.8 ± 3.7	5.2
pIE1−3/+8	49.1 ± 1.1	50.3 ± 0.8	
pIE1−9/+8	47.5 ± 4.6	52.6 ± 7.7	
pIE1−12/+8	46.7 ± 3.6	55.0 ± 10.2	

[a] Mean percent conversion of [^{14}C]chloramphenicol to 3′ acetylated [^{14}C]chloramphenicol, determined from an average of at least three experiments.

[b] Numbers designate the locations of the deletions upstream and downstream of the transcription initiation site (+1).

The mutational analysis is summarized in Fig. 3, which compares the DNA sequences between −29 and +8 bp of the MIEPs for human, simian, and murine CMV. Although human and simian CMV are similar in sequence, murine CMV is different. A mutation that altered the −2 bp position in the HCMV MIEP such as in pIE1−3/+8 abolished down-regulation of the MIEP by IE2 gene products. A mutation that maintained the −1 bp such as in pIE1−1/+8 was still repressed by IE2 gene products. Likewise, a mutation that altered the region downstream of −13 bp such as in pIE1−20/−9 was also no longer repressed by IE2 gene products. This finding indicates the *cis*-acting repressor element maps to a site between −13 and −1 bp of the MIEP. The sequence between human and simian CMV is identical

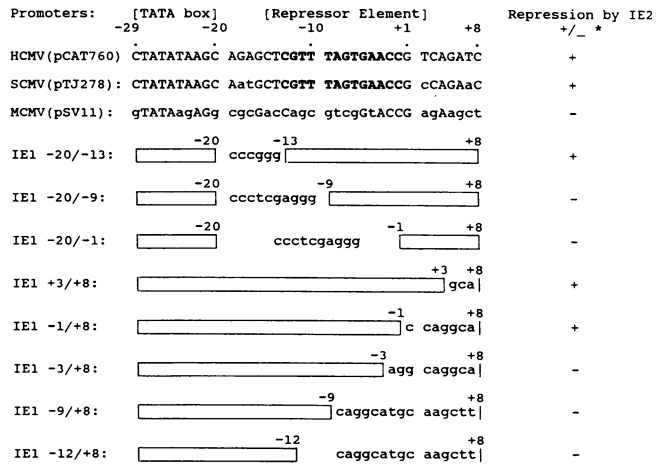

FIG. 3. DNA sequence comparison of the −29 to +8 regions of the MIEPs of human, simian, and murine CMV and deletion mutants in the HCMV MIEP. Capital letters designate homology, and small letters designate lack of homology. Bold letters indicate the *cis*-acting repressor element. The location of the TATA box is designated. The effects of the HCMV IE2 gene products on downstream expression from either the various CMV MIEPs or the various deletion mutants in the HCMV MIEP are summarized. Deleted wild-type DNA sequence was replaced by linker DNA. Small letters indicate the linker DNA. Gaps represent changes in the spatial arrangement of the DNA sequence relative to the wild-type HCMV MIEP sequence.

TABLE 3. Effects of orientation, duplication, position, or transfer of the repressor element on downstream CAT expression

Reporter plasmid	Orientation, duplication, or position of RE[a]	Mean % conversion[b]		Fold repression
		pLink760	pSVIE2	
pCAT760	−14 →	51.0 ± 3.6	84. ± 0.6	6.0
pIERE1R	−14 ←	48.0 ± 2.0	11.0 ± 0.4	4.4
pIERE2	−14 →→	49.0 ± 4.4	8.8 ± 1.9	5.6
pIERE2R	−14 ←←	49.2 ± 4.8	14.9 ± 0.2	3.3
pIERE3	−14 →→→	49.3 ± 5.1	8.3 ± 1.9	6.0
pIERE4D	−14 →→←←	49.3 ± 4.0	7.9 ± 1.4	6.2
pIESRE1	+31 →	45.3 ± 3.1	50.9 ± 3.8	
pIESRE1R	+31 ←	48.0 ± 1.0	52.3 ± 2.5	
pSV2CAT		20.0 ± 2.0	33.6 ± 2.9	
pSV40RE1[c]	−16 →	22.0 ± 2.0	4.5 ± 0.5	5.0

[a] The arrow indicates DNA sequence containing the repressor element between −13 and +3 of the HCMV MIEP. The 5′ insertion site of the repressor element into the vector plasmid is designated. The arrow also indicates the orientation of the repressor element. The number of arrows indicates the number of duplications of the repressor element.

[b] Mean percent conversion of [^{14}C]chloramphenicol to 3′ acetylated [^{14}C]chloramphenicol, determined from an average of at least three experiments.

[c] SV40 DNA sequence downstream of the SV40 TATA box from −16 to +7 was replaced by the HCMV repressor element as described in Materials and Methods.

for these bases, and both MIEPs exhibited IE2 mediated-negative regulation. In contrast, murine CMV does not have a similar sequence (Fig. 3), and it was not negatively regulated by IE2.

Effect of orientation, duplication, position, or transfer of the repressor element. A region of the HCMV MIEP containing the repressor element (−13 to +3) was inserted in both orientations and as tandem repeats into a mutant plasmid of pCAT760 lacking the region between −14 and +7. These various constructs (Table 3) were cotransfected into permissive HFF cells with either control plasmid pLink760 or plasmid pSVIE2. Regardless of the orientation or number of duplications of the repressor element (plasmid pIERE1R, pIERE2, pIERE2R, pIERE3, or pIERE4D), plasmid pSVIE2 repressed CAT enzyme levels as much as sixfold in the transfected HFF cells. The effect of repression was always slightly higher in the wild-type orientation (Table 3).

As described in Materials and Methods, we also positioned the repressor element at 31 bp downstream of the MIEP lacking the wild-type element. In these constructs, expression was no longer repressed by plasmid pSVIE2 regardless of the orientation of the element (Table 3). Therefore, negative regulation by the repressor element is position dependent.

We also substituted the HCMV MIEP repressor element between the TATA box and transcription initiation site of the SV40 promoter. Whereas plasmid pSVIE2 had no repressive effect on CAT enzyme levels from the parent plasmid pSV2CAT, when the HCMV repressor element was inserted at −16 as in plasmid pSV40RE1, CAT enzyme activity was reduced fivefold (Table 3).

DISCUSSION

The regulatory proteins IE1 and IE2 of HCMV influence the level of transcription from early viral promoters. Regulation of HCMV IE gene transcription presumably plays a central role in both productive or latent infection. These viral regulatory genes are located downstream of the enhanc-

er-containing MIEP. Transcription from the HCMV MIEP is extremely strong and is autoregulated by its encoded proteins, IE1 and IE2.

The level of transcription of the IE genes downstream of the MIEP is regulated differently at various times after infection (32, 34). Immediately after infection, the IE1 mRNA is approximately fivefold more abundant than the IE2 mRNAs. This may be due to preferential processing of the precursor viral RNA at the first polyadenylation signal. After approximately 5 h, the steady-state levels of the IE1 and IE2 mRNAs decrease significantly (32, 34). This transcriptional regulatory event may involve the IE2 protein and a *cis*-acting repressor element.

In this study, we have identified a *cis*-acting repressor element positioned immediately upstream of the transcription initiation site. When this control element was deleted or substituted by linker DNA, IE2 could no longer repress downstream transcription. The wild-type DNA sequence of the repressor element is 5′-CGTTTAGTGAACC-3′. The sequence is present in both human and simian CMV but not in murine CMV. It is not present in other heterologous enhancer-containing promoters from SV40, RSV, or HIV. However, the repressor element can be transferred to heterologous enhancer-containing promoters. The element functions in either orientation, but it must be positioned between the TATA element and the cap site. When positioned downstream of the cap site, the element does not function.

The viral IE2 protein may interact directly or indirectly with this element. Mutational analysis of the IE2 protein has suggested that the amino-terminal portion is not required for negative regulation. However, two regions positioned toward the carboxyl-terminal end of the protein are required (12, 31). One region between amino acid residues 428 and 452 contains a putative C2H2 zinc finger motif and the other, within the last 29 residues of the IE2 protein, contains a cluster of seven negatively charged residues. These observations suggest that a specific interaction involving the IE2 protein is required for negative regulation of the MIEP.

The location of the repressor element (−13 to −1) might permit IE2 to have a dominant effect over the transcription complex associated with the MIEP. To initiate RNA polymerase II transcription from a promoter containing a TATA element, TFIID binds to the TATA box and TFIIA stabilizes the complex. A bridge is believed to form between the RNA polymerase and the protein-TATA element complex via TFIIB (3). The position of the repressor element could allow the IE2 protein to destabilize the transcriptional complex at the cap site.

Two other herpesvirus regulatory proteins may also autoregulate transcription by interacting with *cis*-acting elements at the cap site. The herpes simplex virus ICP4 protein is a sequence-specific DNA-binding protein that can bind to sites in the promoter and cap site for the ICP4 gene as well as another regulatory viral gene designated ICP0. Mutations in the promoter of ICP4 can disrupt the DNA-binding activity of ICP4 and impair the negative regulatory activity (23, 24, 27, 28). Another herpesvirus regulatory protein, Zta of Epstein Barr virus, behaves somewhat differently. At low concentrations, Zta binds upstream of its own promoter. At high concentrations, it binds at the transcription start site (17, 18). Therefore, Zta may activate early in infection and then negatively autoregulate its own transcription after the Zta protein accumulates to a threshold level. The autoregulation of IE gene transcription may be a common property of herpesviruses, even though there is no common DNA sequence homology among the *cis*-acting repressor elements of herpes simplex virus, Epstein-Barr virus, HCMV, and murine CMV. Although the specific mechanism of how the IE2 protein of HCMV regulates transcription of the IE1 and IE2 genes is not understood, it should be possible to characterize these interactions by identifying the viral and cellular proteins involved and their cognate protein domains.

ACKNOWLEDGMENTS

We thank C. M. Stoltzfus and L. Turek for critical review of the manuscript. Thanks to the members of the laboratory for help and encouragement.

This work was supported by Public Health Service grant AI-13562 from the National Institute of Allergy and Infectious Diseases.

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