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Activation of the Major Immediate Early Gene of Human Cytomegalovirus by *cis*-Acting Elements in the Promoter-Regulatory Sequence and by Virus-Specific *trans*-Acting Components

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Upstream of the major immediate early gene of human cytomegalovirus (Towne) is a strong promoter-regulatory region that promotes the synthesis of 1.95-kilobase mRNA (D. R. Thomsen, R. M. Stenberg, W. F. Goins, and M. F. Stinski, Proc. Natl. Acad. Sci. U.S.A. 81:659–663, 1984; M. F. Stinski, D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein, J. Virol. 46:1–14, 1983). The wild-type promoter-regulatory region as well as deletions within this region were ligated upstream of the thymidine kinase, chloramphenicol acetyltransferase, or ovalbumin genes. These gene chimeras were constructed to investigate the role of the regulatory sequences in enhancing downstream expression. The regulatory region extends to approximately 465 nucleotides upstream of the cap site for the initiation of transcription. The extent and type of regulatory sequences upstream of the promoter influences the level of in vitro transcription as well as the amount of in vivo expression of the downstream gene. The regulatory elements for *cis*-activation appear to be repeated several times within the regulatory region. A direct correlation was established between the distribution of the 19 (5′ CCCC$^{A}_{G}$TTGACGTCAATGGG 3′)- and 18 (5′ $^{C}_{A}$CTAACGGGACTTTCCAA 3′)-nucleotide repeats and the level of downstream expression. In contrast, the 16 (5′ CTTGGCAGTACATCAA 3′)-nucleotide repeat is not necessary for the enhancement of downstream expression. In a domain associated with the 19- or 18-nucleotide repeats are elements that can be activated in *trans* by a human cytomegalovirus-specified component but not a herpes simplex virus-specified component. Therefore, the regulatory sequences of the major immediate early gene of human cytomegalovirus have an important role in interacting with cellular and virus-specific factors of the transcription complex to enhance downstream expression of this critical viral gene.

Diseases induced by human cytomegalovirus (HCMV), a herpesvirus, can range from congenital malformation in the newborn and pneumonitis in the immunocompromised adult to subclinical infection in the normal healthy individual. After infection, a complicated viral genome of 240 kilobases (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.

The relationship between cell types and HCMV gene expression is not understood. Although the virus infects many different cell types of the human host, it only replicates efficiently in cultured human fibroblast cells. The first viral genes expressed in these cells have been referred to as immediate early genes (7, 25, 49, 50). These are genes that do not require prior viral protein synthesis for efficient transcription. A cluster of three immediate early (IE) transcription units of HCMV were located in the larger unique component of the viral genome between approximately 0.709 and 0.751 map units (42). These regions have been designated IE1, IE2, and IE3. Transcription of these viral genes is unique relative to other herpesviruses because one region is highly transcribed compared to the others. We have referred to the highly transcribed region as IE1 or the major IE gene (42). This gene codes for a spliced mRNA molecule of approximately 1.95 kb (38). The sequence of the viral mRNA predicted a viral protein of 64 kilodaltons (kDa) (38). However, this protein migrates anomalously in denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels, and, consequently, it has been referred to as the 68- to 72-kDa major IE protein (3, 10, 40, 41, 42). This protein appears in the infected cell within 1 h after infection. It is a phosphorylated protein (10) that accumulates in the nucleus (27, 43).

The adjacent IE transcription unit, IE2, is transcribed in the same direction (R. M. Stenberg, P. R. Witte, and M. F. Stinski, submitted for publication) and codes for a variety of mRNAs ranging from 1.10 to 2.25 kb (42). These mRNAs are present at low concentrations relative to IE1 mRNA, and they code for IE proteins that are present in the infected cell at relatively low levels (40, 42). Recent evidence from our laboratory indicates that the mRNAs from IE2 contain the IE1 mRNA leader sequences due to differential splicing of the viral RNA molecules (Stenberg et al., submitted). However, an independent promoter in IE2 may also function at different times after infection (Stenberg et al., submitted).

The regulatory sequences upstream of IE1 may have an influence on transcription of IE2 as well as IE1 genes since both regions share leader sequences. The IE1 regulatory region has a series of repeat sequences each reiterated at least four times. These repeats are referred to as the 19-, 18-, and 16-nucleotide (n) repeats (44). There is also a 21-n sequence that is repeated twice. In vitro transcription analysis indicated that the promoter-regulatory sequence of IE1 competed efficiently for RNA polymerase II and other factors of the transcription complex when using a HeLa cell lysate (44). Sequences upstream of viral or eucaryotic genes can act to enhance downstream transcription by an unknown mechanism. It has been proposed that *cis*-acting DNA elements known as enhancers may provide an entry site for RNA polymerase II or some other component(s) of the transcriptional machinery (28, 47). The IE or alpha genes of herpes simplex virus (HSV) are unique relative to other animal DNA viruses because their regulatory elements re-

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spond to a viral component that induces elevated levels of gene expression. The *trans*-activation of HSV IE promoter-regulatory regions is due to a factor associated with the infecting virus and does not depend on the expression of IE genes (16, 19, 20, 21, 32–34).

In this report, we describe the regulatory elements of another herpesvirus, HCMV. The *cis*-acting domains are within a large region of approximately 465 base pairs (bp) and consist of elements that have an additive effect on downstream transcription. *trans*-Activation of the regulatory domains in stably transformed cells are virus specific, i.e., the regulatory domain responds to an HCMV-specified component but not an HSV-specified component. To determine the functional importance of these regulatory domains, we constructed deletions in the regulatory sequence. The wild-type or deleted sequences were ligated upstream of three different genes: thymidine kinase (TK), chloramphenicol acetyltransferase (CAT), or ovalbumin (OV). The effect of wild-type or mutated regulatory sequences on the level of RNA, protein, or enzymatic activity was measured.

MATERIALS AND METHODS

Virus and tissue culture. Human fibroblast cells and the growth of HCMV (Towne strain) were as previously described (40). The amount of infectious HCMV was determined by assays for plaques (53) or tissue culture infective doses (8). The HF strain of HSV-1 was obtained from G. Cohen (University of Pennsylvania, Philadelphia, Pa.) and assayed for infectious virus as previously described (6). Human 143 TK^- cells (5) were obtained from C. Croce (Wistar Institute, Philadelphia, Pa.).

Plasmid constructions. For the construction of recombinant plasmids, restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.), calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), Klenow DNA polymerase (Boehringer Mannheim), and T4 DNA ligase (New England BioLabs) were used according to the specifications of the manufacturers. The IE1 promoter-regulatory region of HCMV was located within a subclone of pXEP22 (44). A 760-bp fragment was isolated after *Sau*3A-I digestion and cloned into the *Bam*HI site of bacterial vector pAT153. In addition to regulatory sequences, the 760-bp *Sau*3A I fragment contains the CAAT and TATA boxes along with the cap site for the initiation of transcription (38, 44). The 3′ end of the *Sau*3A I fragment is located within the 5′ noncoding region of the IE1 gene at nucleotide +7. Three genes, TK, CAT, and OV, were selected to evaluate *cis*- and *trans*-acting regulatory domains in the HCMV IE promoter-regulatory region. The plasmid constructs are diagrammed in Fig. 1 and were constructed as follows. The 760-bp *Sau*3A I fragment was inserted upstream of the HSV TK gene at the *Bgl*II site which is 56 n upstream from the ATG site for the initiation of translation (26, 46). A clone, pRB103, containing the HSV TK gene was obtained from B. Roizman (University of Chicago, Chicago, Ill.). The 760-bp *Sau*3A I fragment was also inserted at the *Bam*HI site of the OV gene upstream of the ATG translation-initiation codon (54). A clone, pOV12, containing the OV gene was obtained from B. O'Malley (Baylor University, Houston, Tex.). The 3.4-kb *Bam*HI Q fragment of HSV containing the TK gene was necessary as a selectable marker and was inserted at the single remaining *Bam*HI site located at the 3′ end of the OV gene (Fig. 1). Plasmids pRB352 and pRB353 were obtained from B. Roizman (University of Chicago). Plasmid pRB352 contains the chicken OV gene fused to the HSV alpha-4 promoter-regulatory sequence. This plasmid also contains a TK gene linked to its natural beta promoter-regulatory sequence. Plasmid pRB353 is essentially the same as pRB352 except that the alpha-4 promoter-regulatory sequence is placed upstream of the TK gene as well as the OV gene (33).

To insert the HCMV promoter-regulatory sequences upstream of the CAT gene, the 760-bp *Sau*3A I fragment was inserted into vector plasmid plink153 which represents the polylinker from plink322 (22) inserted into the *Eco*RI site of bacterial vector pAT153. The viral promoter-regulatory sequences were isolated from the above recombinant plasmid by *Hin*dIII digestion, gel purified, and then inserted into pSVOCAT at the *Hin*dIII site (11), which is located upstream of the ATG translation initiation codon. The HCMV promoter-regulatory sequences inserted near the 5′ end of the TK, OV, or CAT genes were cloned in both orientations.

Deletions within the HCMV promoter-regulatory region from the *Nco*I site at −222 n were induced by *Bal* 31 digestion according to Maniatis et al. (22). *Bam*HI linkers were ligated to the ends of the DNA molecule, or the DNA molecules were treated with Klenow polymerase to fill in the ends and then blunt-end ligated as previously described (22). The sites of all *Bal* 31 deletions were determined by DNA sequencing by the chemical cleavage method (24). Deletions were also induced in pCATwt760 by site-specific deletion of the DNA fragments between the *Aat*II or *Nde*I sites (Fig. 1). The plasmid was linearized by partial digestion with *Aat*II or *Nde*I by the method of Maniatis et al. (22). After gel purification, the DNAs were recircularized with T4 ligase and cloned as described below. The site and size of the *Aat*II or *Nde*I deletions in the resulting constructs were determined by *Hin*cII and *Nco*I restriction endonuclease digestion followed by determination of DNA fragment sizes by gel electrophoresis. The locations of the above deletions and their relationship to the 21-, 19-, 18-, and 16-n repeats and the CAAT and TATA boxes are diagrammed in Fig. 2B. For the *Bal* 31 deletions, each deleted fragment of DNA was isolated by *Sau*3A-I restriction endonuclease digestion and inserted upstream of the TK, OV, or CAT genes as described above.

The DNAs were used to transfect *Escherichia coli* C600 Rec BC^- by the $CaCl_2$ precipitation method onto heat-shocked cells. Colony selection, small- and large-scale preparation, and DNA purification have been previously described (45).

Transfection. All transfections were done on duplicate 100-mm plates. All DNA concentrations were checked by comparisons of ethidium bromide stain intensity of the plasmids following gel electrophoresis. Plasmid DNAs were introduced into cells by transfection as calcium phosphate precipitates by a modification of the method of Graham and Van der Eb (13). For the biochemical transformation of human 143 TK^- cells, 1.0 or 0.5 μg of the pTK plasmid DNAs with 20 μg of sonicated salmon sperm DNA as carrier was added to subconfluent cells in 100-mm plates. For the transformation of human 143 TK^- cells with the OV plasmid DNAs (Fig. 1), 5 μg of specific plasmid DNA along with 20 μg of sonicated salmon sperm DNA as carrier was used. Generally, 20 to 200 hypoxanthine-aminopterin-thymidine (HAT)-resistant colonies were pooled, cultured, and tested for OV expression.

For the transformation of HeLa cells with the pCAT plasmid DNAs (Fig. 1), 5 μg of a pCAT plasmid with 20 μg of sonicated DNA as carrier was used as described above,

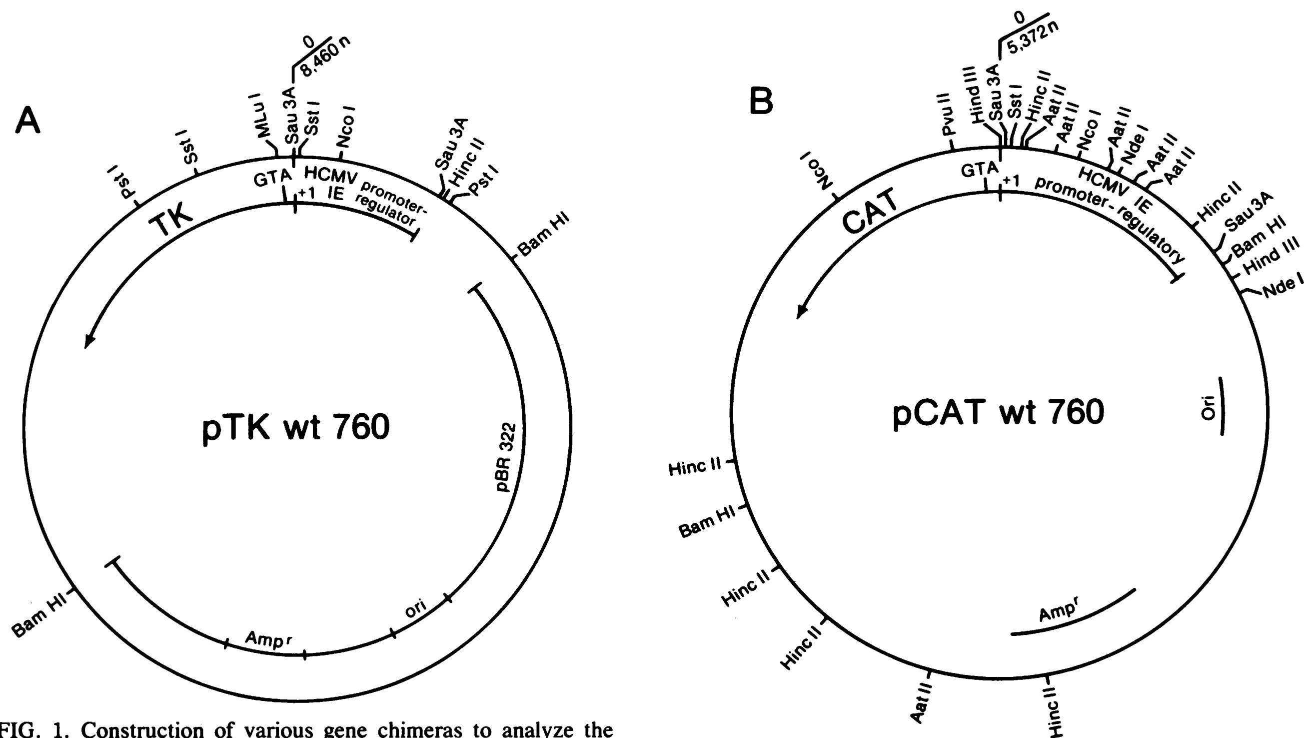
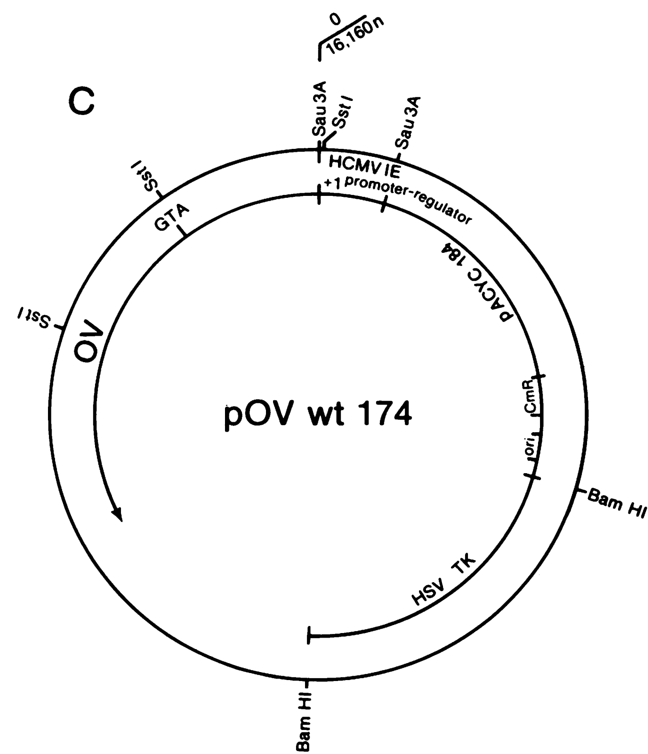


FIG. 1. Construction of various gene chimeras to analyze the effect of the HCMV major IE promoter-regulatory region on gene expression. Construction of the various chimeric plasmids is described in the text. The IE promoter-regulatory region was inserted in the correct orientation at (A) the *Bgl*II site upstream from the HSV TK gene (pTKwt760), (B) the *Hin*dIII site upstream from the procaryotic CAT gene (pCATwt760), or (C) the *Bam*HI site upstream from the chicken oviduct OV gene (pOVwt174). In the case of pOVwt174, the HSV TK gene was inserted at the *Bam*HI site located near the 3′ end of the OV gene.



except the calcium phosphate precipitate in the medium was on the cells for 16 to 18 h at 37°C. The cells were harvested at 48 h after transfection.

In vitro transcription. In vitro transcription was done by the method of Manley et al. (23) and as previously described (44). The pTK recombinant plasmids were cut with restriction endonuclease *Pst*I to generate linear templates. The in vitro transcribed RNA was fractionated by electrophoresis as previously described (42). After exposure of the dried gel to Kodak XAR film, the autoradiogram was scanned in a Beckman DU-8 scanner. The level of transcription was estimated relative to the wild type.

DNA sequence analysis. The methods used for labeling DNA in vitro and for sequence determination by the chemical modification and degradation procedure of Maxam and Gilbert (24) have been described (38, 44).

CAT assays. Cell harvesting and CAT assays were performed as described by Gorman et al. (11, 12). The cells were harvested at approximately 48 h after transfection. The acetylated derivatives were separated from nonacetylated chloramphenicol by ascending chromatography with a chloroform-methanol (95:5) solvent. The plates were exposed to Kodak XAR-2 film. For quantitative comparisons, the appropriate sections were cut from the thin-layer chromatography plate, and the amount of radioactivity was measured by liquid scintillation in a Beckman LS7500 counter. Preliminary experiments demonstrated that the assay was linear when at least 10^5 cpm appeared as the chloramphenicol 3-acetate product. The assay was linear for at least 120 min. All quantitative comparisons were made by measuring the chloramphenicol 3-acetate product, which renders valid estimates of CAT activity (36).

OV assay. HAT-resistant cells were infected with HCMV (5 PFU per cell) or HSV (1 PFU per cell), or they were mock infected. The cells were harvested at 16 h after infection. After the monolayer was washed twice with phosphate-

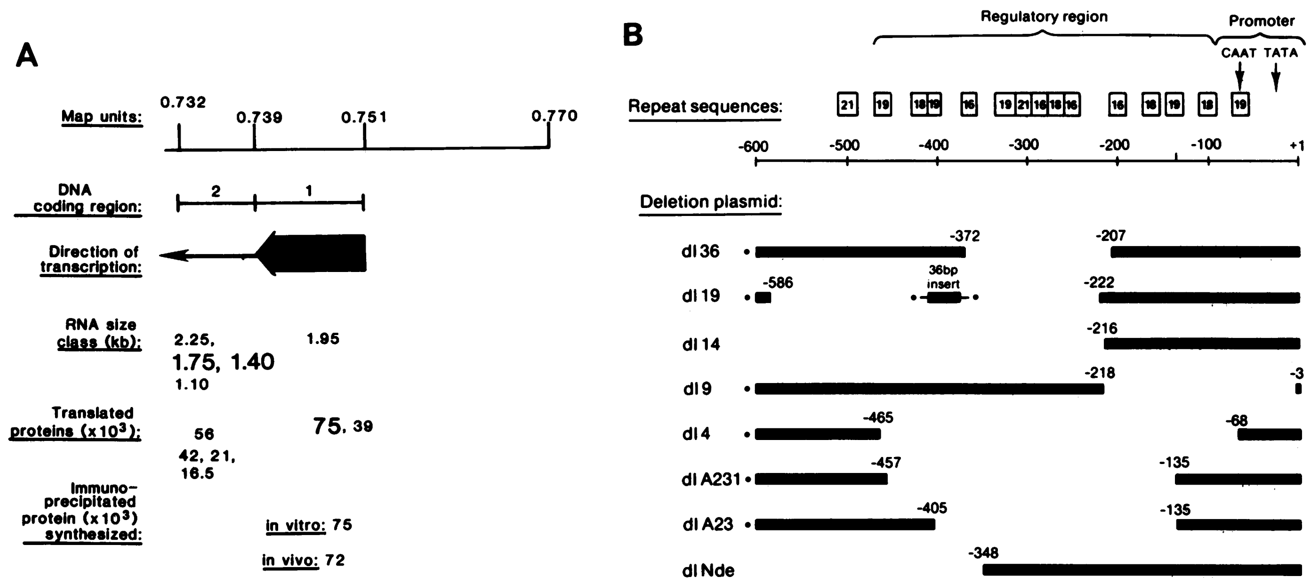


FIG. 2. HCMV transcription units IE1 and IE2 and the locations of the various repeat sequences and deletions in the HCMV major IE promoter-regulatory region. (A) Transcription units IE1 and IE2 (Stenberg et al., submitted) are located between 0.732 and 0.751 map units for the prototype orientation of the Towne strain. Arrows indicate the direction of transcription, and the thickness of the bar represents the relative abundance of the IE RNAs originating from the various coding regions. The RNA size classes and proteins encoded by the above DNA regions are listed. (B) The location of the promoter-regulatory sequences and the cap site are presented in the conventional orientation for a eucaryotic gene. The locations of the repeat sequences have been previously described (44) except for the 21-n repeats at −296 to −316 and −489 to −509. The consensus sequences of the 21-, 19-, 18-, and 16-n repeats are 5′ ACGGTAAATGGCCCGCCTGGC 3′, 5′ CCCC$^{A}_{G}$TTGACGTCAATGGG 3′, 5′ $^{C}_{A}$CTAAACGGGACTTTCCAA 3′, and 5′ CTTGGCAGTACATCAA 3′, respectively. The bars represent the nondeleted sequences. Deletions dl14 and dlNde are 5′ deletions, whereas the others are internal deletions.

buffered saline (PBS; pH 7.5), the cells were scraped into the buffer described above and pelleted by centrifugation. The cells were suspended in 0.5 ml of PBS containing 1% Nonidet P-40, 1% Tween 20, and phenylmethylsulfonyl fluoride (10 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and sonicated for 2 min in a Bransonic sonicator. Debris was removed by centrifugation, and the supernatants were stored at −20°C. The amount of OV present in the cell lysates was quantitated by a sandwich enzyme-linked immunosorbent assay (ELISA). Rabbit anti-OV antibody was purified by affinity chromatography as follows. After 50 mg of OV was dialyzed against PBS (pH 7.2), the protein was linked to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) according to the specifications of the manufacturer. The gel was washed first with 0.1 M acetate (pH 3.5) containing 10% dioxane and 0.5 M NaCl and then with 3.5 M $MgCl_2$. The gel was then washed with Tris hydrochloride-buffered saline (pH 7.2). Rabbit anti-OV serum was passed slowly through the gel twice. After being washed with Tris hydrochloride-buffered saline containing 0.5 M NaCl, the antibody was eluted with 0.1 M acetate (pH 3.5) containing 10% dioxane and 0.5 M NaCl. The antibodies were then dialyzed against PBS. The antibodies at a concentration of 1 mg/ml were diluted 1,000-fold in coupling buffer consisting of 0.1 M sodium carbonate (pH 9.6) containing 0.02% NaN_3 and then linked to Immulon flat-bottom plates (Dynatech Laboratories Inc., Alexandria, Va.) overnight. The wells were washed extensively with 0.9% NaCl and 0.05% Tween, and various dilutions of the antigen samples were added to the wells in duplicate. For an antigen standard, 1 to 100 ng of OV (Sigma) was added to the wells in duplicate. The antigen-antibody reaction was incubated for 2 h at room temperature, and then the wells were washed extensively as described above. Purified anti-OV antibody linked to bovine alkaline phosphatase (Sigma) by the method of O'Sullivan and Marks (31) was diluted 1,000-fold and added to the wells. After 2 h at room temperature, the wells were washed extensively as described above. Phosphatase substrate (Sigma) at a concentration of 1 mg/ml in 0.05 M sodium carbonate (pH 9.8) containing at 2 mM $MgCl_2$ was added to the wells. After 1 h at room temperature, the alkaline phosphatase reaction was stopped with 50 μl of 1 M sodium hydroxide. The amount of reaction product was determined by absorbance at 400 nm using an ELISA autoreader (Bio-Tek Instruments, Inc., Shelburne, Vt.). The amount of OV was determined from a standard curve with the linear range of the curve existing between 1 and 10 ng. The amount of OV was expressed as nanograms per milligram of protein. The amount of protein was determined with a protein assay dye reagent (Bio-Rad). The standard protein curve was generated with different concentrations of OV.

Immunoprecipitation of OV. HAT-resistant cells were infected with HCMV (5 PFU per cell), or they were mock infected. The cells were pulse labeled for 6 h with 50 μCi of [^{35}S]methionine per ml (1,225 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 3 h postinfection. After the cells were washed with PBS (pH 7.5), they were scraped into PBS containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and phenylmethylsulfonyl fluoride (10 μg/ml). After sonication for 2 min in a Bransonic sonicator, debris was removed by centrifugation. Immunoprecipitation was as previously described (42) except affinity-purified rabbit anti-OV antibody was used. Equal amounts of radioactive-cell lysate were used for the various immunoprecipitations. To block anti-OV antibody activity, 50 μg of cold OV was used. Immunoprecipitates were dissociated, and the polypeptides were fractionated by SDS-polyacrylamide gel electrophoresis as previously described (39, 40). Protein molecular

weight standards consisting of ^{14}C-labeled methylated proteins ranging in molecular weight from 97.4 kDa (phosphorylase B) to 14.3 kDa (lysozyme) were obtained from Bethesda Research Laboratories.

RESULTS

Construction of HCMV IE regulated gene chimeras. The 5′ end of the major IE gene of HCMV was mapped, and the sequence upstream of the cap site for the initiation of transcription was previously described by our laboratory (38, 44). The promoter-regulatory region is contained within a *Sau*3A I fragment of approximately 760 bp. Internal or 5′-end deletions were made from the *Nco*I site at −222 n as described above. Alternatively, site-specific deletions of DNA fragments were made at the *Aat*II or *Nde*I sites. The wild-type or deleted promoter-regulatory sequences were cloned in the correct orientation upstream of the ATG sites for the initiation of translation of the TK, CAT, or OV genes. Figure 1 illustrates the various gene chimeras. The promoter-regulatory region in the prototype orientation for HCMV (Towne) is upstream of two transcription units, IE1 and IE2, that are transcribed from right to left between approximately 0.732 and 0.751 map units (Fig. 2A). The location of the various deletions in the promoter-regulatory region in a conventional 5′-to-3′ orientation are diagrammed in Fig. 2B. The promoter-regulatory region, herein described, influences the transcription of transcription units IE1 and IE2.

Effect of deletion on in vitro transcription. The HCMV IE promoter-regulatory region efficiently initiates transcription of HCMV DNA in a HeLa cell lysate (44). To investigate the relationship between HCMV IE regulatory sequences and transcription, equal concentrations of the HSV TK gene linked to the wild-type or deleted promoter-regulatory sequences were transcribed by using a HeLa cell lysate as described above. Transcription was performed on templates of DNA cut with restriction endonuclease *Pst*I. With the wild-type (pTKwt760) DNA template, a predicted RNA of 812 n should be synthesized. The size of the RNA molecule as determined by electrophoresis agreed with the distance of the *Pst*I site from the HCMV cap site (Fig. 3, lane 2). The RNA would contain 7 n specified by HCMV DNA and 805 n specified by HSV DNA. The autoradiogram is overexposed for this RNA to facilitate detection of the RNAs described below. When the CAAT and TATA boxes were deleted (pTKdl9), no in vitro transcription was detected (Fig. 3, lane 10). When the CAAT and TATA boxes were present but the regulatory sequences were deleted back to −465 (pTKdl4), in vitro transcription was detectable but reduced 98.7% relative to that of wild-type sequences (Fig. 3, lane 6). When the upstream sequences were retained to −216 or to −222, transcription was reduced approximately 50% as seen with pTKdl19 or pTKdl14, respectively (Fig. 3, lanes 5, 7, and 9). The plasmid pTKdl36 renders a significantly higher level of in vitro transcribed RNA than did the wild-type sequences (Fig. 3, lane 4). The amount of RNA was estimated to be approximately 66% higher than in the wild type. The effect of the deletion in pTKdl36 was investigated further and is described below. These viral DNAs were transcribed by RNA polymerase II since the presence of alpha-amanitin inhibited transcription (Fig. 3, lanes 3 and 8). This experiment illustrated that the HCMV regulatory sequences upstream of the CAAT and TATA boxes of the IE1 gene play a direct role in the transcription of the downstream genes. The amount in vitro transcription of the HCMV-TK chimeras correlated with in vivo expression determined by the transformation of human 143 TK^- cells to a TK^+ phenotype.

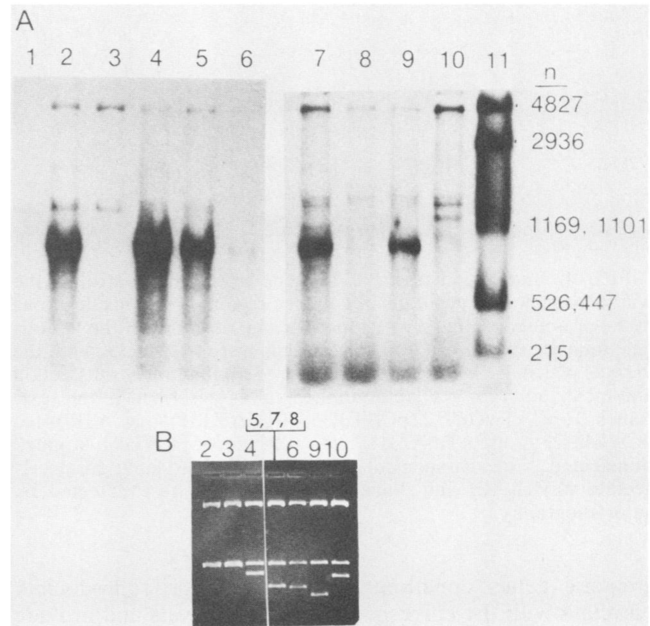


FIG. 3. In vitro transcription of the HSV TK gene using the HCMV major IE promoter-regulatory region. Wild type or various deletions in the sequence were inserted at the *Bgl*II site upstream of the TK gene as described in the legend to Fig. 1. The DNA templates were digested with *Pst*I, which cuts the DNA upstream of the promoter-regulatory insertion site and 812 n downstream from the HCMV cap site (Fig. 1). RNA was synthesized in standard reactions with 2.5 μg of DNA template and then extracted, denatured, and fractionated by electrophoresis in denaturing 1.5% agarose gels as described in the text. (A) Lanes: 1, no DNA; 2, pTKwt760 DNA; 3, pTKwt760 DNA plus alpha-amanitin; 4, pTKdl36 DNA; 5, pTKdl19 DNA; 6, pTKdl4 DNA; 7, pTKdl19 DNA; 8, pTKdl19 DNA plus alpha-amanitin; 9, pTKdl14 DNA; 10, pTKdl9 DNA; 11, molecular weight markers. (B) Ethidium bromide-stained DNA of the plasmids described above digested with restriction endonuclease *Pst*I. The lower DNA band represents the *Pst*I fragment containing the HCMV promoter-regulatory region or various deletions adjacent to part of the TK gene.

The transformation ratio was high with pTKwt760 and pTKdl36, but reduced by approximately 50% with pTKdl19 and pTKdl14 and by approximately 90% with pTKdl4 (data not shown).

***cis*-Acting elements are additive.** The in vitro transcription and biochemical TK transformation results suggested that the HCMV *cis*-acting regulatory elements were distributed over a distance of at least 465 n upstream from the cap site. Therefore, additional deletions were constructed by making site-specific deletions of DNA fragments at the *Aat*II and *Nde*I sites (Fig. 2B). The restriction endonuclease *Aat*II was selected because it cuts the regulatory sequences just upstream of the CAAT box in each of the 19-n repeat sequences, which are repeated five times (Fig. 2B). If the DNA was partially digested, ligation could result in the reconstitution of a 19-n repeat sequence. However, the ligation at the *Nde*I-digested DNA would result in a 5′-end deletion of the regulatory sequences (Fig. 2B). To better quantitate the level of downstream expression, the wild type and the *Bal* 31, *Aat*II, or *Nde*I deletions in the regulatory sequence were either inserted or constructed in proper orientation to the CAT gene so that the sensitive assay for acetylated [^{14}C]chloramphenicol could be used. Previous studies have shown that measurement of enzyme levels expressed from

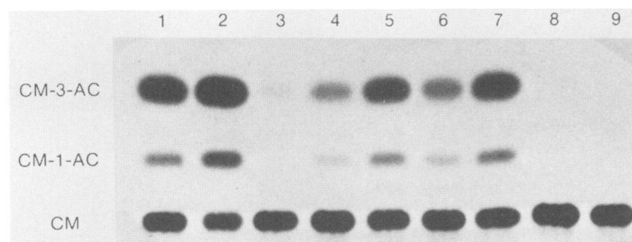


FIG. 4. Expression of the CAT gene under the control of the wild-type major IE promoter-regulatory region or various deletions in the promoter-regulatory region. The map positions of the various deletions are shown in Fig. 2. HeLa cells were transfected with the various plasmids. Extracts were prepared at 48 h after transfection and incubated with [^{14}C]chloramphenicol as described in the text. Lanes: 1, pCATwt760; 2, pCATdl36; 3, pCATdl19; 4, pCATdl14; 5, pCATdlA23; 6, pCATdlA231; 7, pCATdlNde; 8, pCATdl4; 9, mock transfected. Chloramphenicol (CM) and its acetylated forms, 1-acetate (CM-1-AC) and 3-acetate (CM-3-AC), were detected by autoradiography.

chimeric genes containing the CAT region reproducibly correlate with the corresponding mRNA levels and provide rapid and very sensitive estimates of promoter activity (11, 12, 17, 51). HeLa cells were transfected with the various recombinant plasmids and assayed for CAT enzyme activity as described above. Figure 4 demonstrates a correlation between the level of CAT enzyme activity and the extent of retained DNA sequence of the upstream regulatory elements. The activity of pCATdl36 was significantly higher than that of the wild type (Fig. 4 and Table 1). The amount of upstream expression appears to be correlated with the presence of the 19- or 18-n repeats and the distance of these repeats from the promoter. For example, dl36 would position four 19-n repeats or the combination of 19- and 18-n repeats adjacent to the promoter (Fig. 2B). Deletion of the 16-n repeats had no negative effect, and in this case there was a positive effect on the promoter-regulatory activity (Table 1). Recombinant plasmids with only two 19-n repeats such as pCATdl19, -14, and -A231 had only 4 to 31% of the activity of the wild type (Fig. 4A and Table 1). The lower activity of dl19 in an in vivo assay system is difficult to explain. It might be related either to the inadvertant insertion of a 36-bp fragment of DNA or to an inhibitory effect due to the remaining upstream sequences (Fig. 2). These inhibitory effects were not detected by the in vitro transcription assay (Fig. 3). When the upstream sequences are missing such as in pCATdlNde, the activity of the promoter is higher (Table 1). Recombinant plasmids with three 19-n repeats, i.e., pCATdlA23 and pCATdlNde (Fig. 2), had significantly higher activity, ranging from 96 to 99% relative to that of the wild type (Fig. 4 and Table 1). The activity of the promoter with no upstream regulatory sequence was represented by pCATdl4, which had only 1.4% of the activity of the wild type.

Reinsertion of wild-type regulatory sequences 328 n upstream of the promoter. Chimeric plasmid pCATdl4 had the lowest promoter activity detected (Table 1). This plasmid contained the natural promoter, i.e., CAAT and TATA boxes according to sequence analysis, but the regulatory sequences were deleted back to −465 (Fig. 2B). At 328 n upstream of this promoter in pCATdl4 was a convenient *Bam*HI site for the insertion of the 760-bp *Sau*3A I fragment (Fig. 1). A recombinant plasmid was constructed with the 760-bp *Sau*3A I fragment inserted in proper orientation 328 n upstream of the promoter in pCATdl 4; i.e., the wild-type promoter-regulatory sequence was inserted with the same number of repeats of all types plus one extra 21-n repeat from the pCATdl4 regulatory region. This plasmid was designated pCAT 4SB. Reinsertion of wild-type sequences upstream of the promoter enhances downstream expression of the CAT gene (Fig. 5 and Table 1). However, the amount of CAT expression was not restored to the level of the wild-type promoter-regulatory sequences demonstrated with pCATwt760 (Fig. 5). The lower activity of pCAT4SB might be due to the upstream 328 n between the promoter and the insert or to a positional effect on transcription. The insertion of the promoter-regulatory region 328 n upstream would result in two potential RNA cap sites. The data suggest that there is a positional effect on transcription with the HCMV IE promoter-regulatory region.

***trans*-Activation is virus specific.** An HSV virion component has been reported to enhance in *trans* the transcription from the HSV IE promoter-regulatory region upstream of various genes (1, 4, 30, 32, 33, 34). For example, the HSV-1 alpha-4 promoter-regulatory region upstream of the OV gene can be activated in *trans* by HSV infection when the gene is associated with a stably transformed cell (33). To determine whether the HCMV IE promoter-regulatory region was also

TABLE 1. Quantitative evaluation of *cis*-acting HCMV elements for the expression of recombinant CAT genes

Plasmid	No. of repeats				% (relative to wt)
	21	19	18	16	
pCATwt760	2	5	4	4	NA[a]
pCATdl36	1	4	3	1	237.8
pCATdl19	0	2	2	1	4.0
pCATdl14	0	2	2	1	21.7
pCATdlA23	1	3	2	0	95.8
pCATdlA231	1	2	1	0	31.4
pCATdlNde	1	3	3	3	99.2
pCATdl4	1	0	0	0	1.4
Control[b]	0	0	0	0	0.3
pCAT4SB[c]					18.7

[a] NA, Not applicable.
[b] Nontransfected cells.
[c] pCAT4SB contains the whole *Sau*3A I (760-bp) fragment 328 n upstream of the promoter in pCATdl4, and, consequently, all the wild-type repeat sequences are present plus one extra 21-bp repeat.

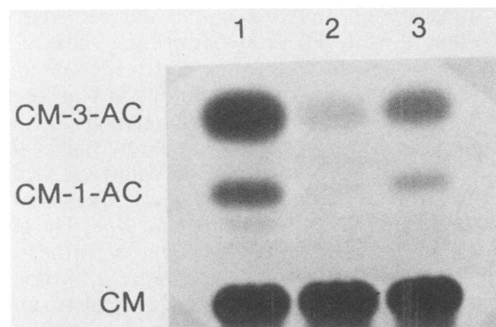


FIG. 5. Insertion of wild-type regulatory sequences 328 n upstream of the promoter. HeLa cells were transfected, and the extracts were prepared and assayed as described in the legend to Fig. 4. Lanes: 1, pCATwt760; 2, pCATdl4; 3, pCAT4SB. Chloramphenicol (CM) and its acetylated forms, 1-acetate (CM-1-AC) and 3-acetate (CM-3-AC), were detected by autoradiography.

activated in *trans*, the promoter-regulatory region or deletions of this region were inserted upstream of the OV gene. To select for stably transformed cells, the TK gene was inserted downstream of the 3′ end of the OV gene (Fig. 1C). Human 143 TK^- cells were transfected with plasmids containing the HCMV or the HSV IE promoter-regulatory regions upstream of the OV gene, and HAT-resistant cells were selected. These cells were infected with HCMV or HSV, or they were mock infected, and the amount of OV associated with cell lysates was determined by ELISA as described above. The data presented in Table 2 demonstrate that the *trans*-activation of herpesvirus promoter-regulatory regions is virus specific in a stably transformed cell. HCMV stimulates the HCMV IE promoter-regulatory region but not the HSV promoter-regulatory region. On the other hand, HSV stimulates the HSV alpha-4 promoter-regulatory region but not the HCMV promoter-regulatory region. The degree of *trans*-activation of the herpesvirus IE promoters varies somewhat according to the culture of HAT-resistant cells isolated. Similar levels of *trans*-activation were detected at early (2 to 3 h) as well as late times (14 to 16 h) after infection.

trans-Activation of OV expression by HCMV was also demonstrated in HAT-resistant cells pulse labeled with [^{35}S]methionine. Immunoprecipitates of OV were fractionated in denaturing SDS-polyacrylamide electrophoresis gels. The amount of labeled OV immunoprecipitated was significantly higher from infected cells compared to the mock-infected cells (Fig. 6A, lanes 3 and 4). Since OV is a secretory protein as predicted from its known function in the hen oviduct, the extracellular medium of the mock-infected cells was concentrated 10-fold and analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. A protein similar in molecular weight to the ^{14}C-labeled OV standard protein was detected (Fig. 6B, lane 3). When 50 μg of cold OV was mixed with the ^{35}S-labeled proteins, the immunoprecipitation of OV was inhibited (Fig. 6B, lane 4). However, secretion of OV was not higher in the infected cells (data not shown).

Sequences required for *trans*-activation. To determine whether the sequences upstream of the HCMV IE promoter are required for *trans*-activation of genes in stably transformed cells, human 143 TK^- cells were transfected with wild-type or deletion regulatory sequences upstream of OV. The HAT-resistant colonies were pooled, cultured, and tested for *trans*-activation by HCMV infection as described above. In general, the basal level of OV expression was lower in cells transfected with the deletion plasmids, but there were also a few exceptions (Table 3). Approximately the same level of *trans*-activation was demonstrated to occur with both pOVwt174 and pOVdl361 (Table 3). The different clone numbers represent wild-type or deletion regulatory sequences upstream of the OV gene as shown in Fig. 1 and 2B, but the plasmids also contain the TK gene. For example, dl361 contains the dl36 promoter-regulatory region (Fig. 2B) upstream of the OV gene, and the extra digit designates the presence of the TK gene inserted near the 3′ end of the OV gene. Deletion of the 16-n repeats, such as in pOVdl361 (Fig. 2B), had no negative effect on *trans*-activation. The basal level of expression was always similar to that of the wild type. However, in vitro transcription or transient expression using the dl36 promoter-regulatory region always demonstrated a higher level of activity than did the wild-type sequences (Fig. 3 and 4). Deletion of some of the 19- or 18-n repeats reduced the level of *trans*-activation by approximately 50% as demonstrated with pOVdl197 and pOVdl147 (Table 3). The level of stimulation with these deleted promoter-regulatory regions was reproducible but admittedly at a low level. Cells containing pOVdl46 had no increase in OV expression after infection (Table 3). Therefore, the extent of the regulatory sequences upstream of the promoter influenced the level of *trans*-activation of OV expression.

TABLE 2. Virus-specific enhancement of herpesvirus IE promoters and OV expression in stably transformed cells

Plasmid	Virus promoter-regulatory sequence	OV (ng/mg of protein)[a]		
		Uninfected	CMV infected (5 PFU/cell)	HSV infected (1 PFU/cell)
Expt 1				
pOVwt174	CMV	4.7	14.1	2.5
pOVwt311	CMV[b]	ND[c]	ND	ND
pRB352	HSV	5.2	5.3	18.0
pRB353	HSV	3.9	4.1	17.8
Expt 2				
pOVwt174	CMV	3.8	26.4	3.5
pOVwt311	CMV	ND	0.4	ND
pRB352	HSV	3.3	3.0	6.8
pRB353	HSV	3.4	3.1	9.3

[a] Cells were harvested at 16 h postinfection.
[b] Promoter-regulatory region was in the opposite orientation.
[c] ND, Not determined (<10 ng).

***trans*-Activation does not require de novo viral protein synthesis.** To determine whether *trans*-activation was due to a component of the virus inoculum or was the result of de novo viral protein synthesis, HAT-resistant cells containing the wild-type (pOVwt174) or dl36 (pOVdl361) promoter-regulatory sequences upstream of the OV gene were either mock infected or infected with 5 PFU of HCMV per cell in the presence of 100 μM anisomycin. At early times (3 to 4 h) or after overnight incubation (16 h), cytoplasmic RNA was isolated, fractionated by denaturing gel electrophoresis, and blotted onto nitrocellulose filters as described above. The Northern blot was probed with the ^{32}P-labeled *Bam*HI fragment of pOV12 as previously described (42). With wild-type or dl36 promoter-regulatory sequences upstream of the OV gene, the amount of mRNA detected in the cytoplasm was higher in infected cells compared to mock-infected cells (Fig. 7). These results suggest that a virion component is involved in *trans*-activation.

DISCUSSION

After primary infection, the region transcribed in highest abundance is IE1 or the major IE gene (42). The promoter-regulatory region upstream of this gene consists of a series of different repeat sequences distributed up to −509 n from the cap site for the initiation of transcription (44). This relatively small region of approximately 500 n competes efficiently for RNA polymerase II and the other factors of the transcription complex (44). A few other regions of this 240-kb viral genome are also transcribed under IE conditions but at levels 8- to 20-fold lower relative to IE1 (42, 49). The high level of IE1 transcription is presumably due to a set of inducing sequences contained in the promoter-regulatory region. Deletion studies within this region indicated that the CAAT and TATA boxes, also referred to as the promoter element within 68 bp of the mRNA 5′ terminus, are required for the minimal level of gene expression. Sequences up-

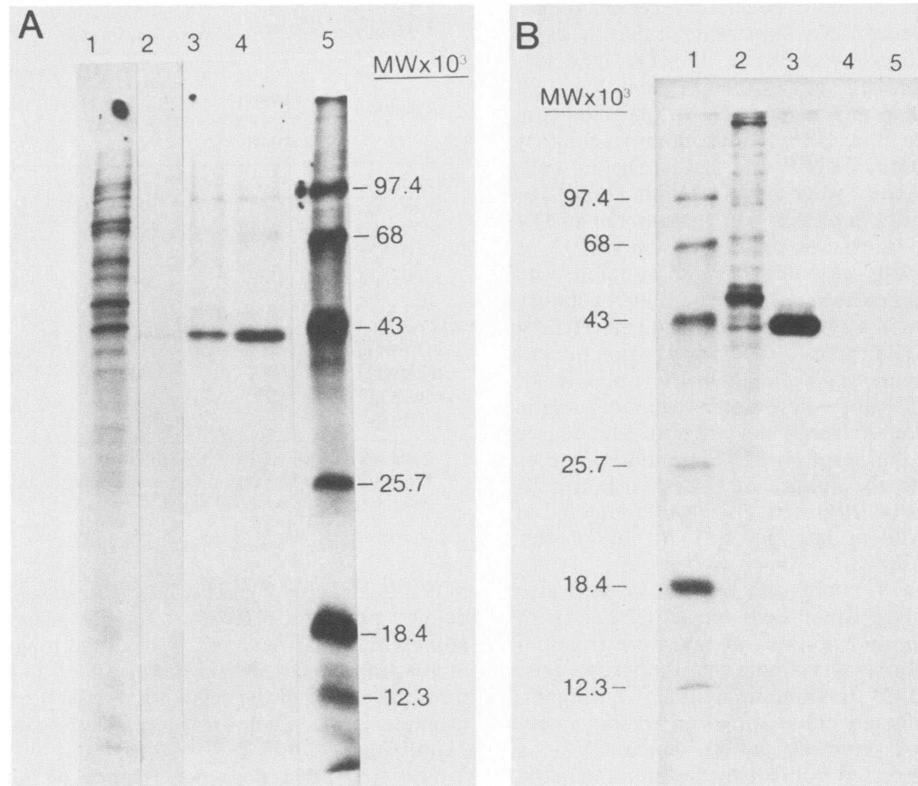


FIG. 6. Autoradiograms of OV antigen immunoprecipitated from cell lysates or extracellular medium with affinity-purified anti-OV antibody. Human 143 TK⁻ cells were transfected with pOVwt174 or pOVwt311 (contains the promoter-regulatory region in the incorrect orientation). HAT-resistant cells were selected as described in the text. Cells were either mock infected or HCMV infected and then pulse labeled for 5 h with [^{35}S]methionine at 3 h postinfection. Extracellular medium was concentrated 10-fold by lyophilization. Immunoprecipitation was performed with anti-OV antibody followed by the addition of *Staphylococcus aureus* Cowan I. The precipitated proteins were fractionated in denaturing polyacrylamide gels as described in the text. (A) Lanes: 1, 2-h exposure of the cell lysate; 2, immunoprecipitation of the pOVwt311 cell lysate; 3, pOVwt174 cell lysate; 4, pOVwt174 lysate of HCMV-infected cells; 5, molecular weight markers. (B) Lanes: 1, molecular weight markers; 2, extracellular medium; 3, immunoprecipitation of the pOVwt174 extracellular medium; 4, pOVwt174 extracellular medium plus 50 μg of cold OV; 5, pOVwt174 extracellular medium plus preimmune serum.

stream of the CAAT box function to enhance downstream expression of the HCMV major IE gene. The level of in vitro transcription of an adjacent gene was directly correlated with the type and amount of viral regulatory sequence upstream of the promoter. Deletion of the sequences between −207 and −372 (dl36) did not affect the level of downstream expression. Three of the four 16-n repeat sequences are located in this region. Therefore, it is proposed that the 16-n repeat or the surrounding sequences are not required for *cis*-activation. The higher level of expression with the dl36 gene chimeras may be due to the deletion of elements that suppress downstream expression. It is interesting that the 16-n repeat has a sequence very similar to the nuclear factor 1 consensus sequence, 5′ TGG$^{A}_{C}$NNNNNGC-CAA 3′ (15, 29, 35, 37). Our observations suggest that this sequence and possibly nuclear factor 1 binding are not involved in promoting downstream transcription. Alternatively, the dl36 TK or CAT gene chimeras may function well due to positioning of important *cis*-acting elements adjacent to the promoter. Although there is no critical distance requirement between an enhancer element and the promoter, the extent of stimulation decreases as the distance between the two elements increases. In the case of dl36, the 19- and 18-n repeats would be positioned closer to the promoter. The HCMV 18-n repeat sequence is not conserved in the related simian cytomegalovirus (CMV) (Colburn) which also has a strong IE promoter-regulatory region that is comparable in strength to that of HCMV. Therefore, the 18-n sequence may not independently provide

TABLE 3. Effect of deletion of the HCMV regulatory sequences on OV expression and *trans*-activation in stably transformed cells

Plasmid	OV (ng/mg of protein)[a]		Ratio of infected to uninfected
	Uninfected	Infected	
Expt 1			
pOVwt174	5.1	19.1	3.7
pOVdl361	3.2	10.4	3.3
pOVdl197	1.2	2.7	2.3
pOVdl147	2.1	2.8	1.3
pOVdl146	1.2	1.3	1.1
Expt 2			
pOVwt174	1.9	7.7	4.1
pOVdl361	1.8	7.0	3.9
pOVdl197	1.3	2.6	2.0
pOVdl147	0.5	1.3	2.6
pOVdl46	2.8	2.6	0.9

[a] Conditions were as described for Table 2.

an enhancer function or may be a regulatory element specific for human cells. In contrast, the 19-n repeat is highly conserved upstream of both HCMV and simian CMV (41). Therefore, it is proposed that the 19-n repeat or surrounding sequences such as the GC-rich motifs play an important role in the level of IE gene expression in both human and simian CMVs. However, the level of expression of the various gene chimeras in this investigation was correlated with the number of 19- and 18-n repeats and their surrounding sequences.

trans-Activation of HSV IE promoter-regulatory regions is due to a factor associated with the infecting virus (1, 4, 16, 33, 34). Experiments using the OV gene linked to the IE promoter-regulatory region of HSV or HCMV demonstrated that *trans*-activation is virus specific in stably transformed cells. Therefore, the induction sequence contained within the respective promoter-regulatory regions must be unique. There is little to no homology between the regulatory sequences of the HSV alpha-4 gene (19, 21) and the HCMV IE1 gene (44). These viruses have evolved to have unique cognitive and regulatory domains upstream of their IE genes and, presumably, the virus-specific component(s) involved in *trans*-activation is also a unique viral protein. With HSV, a viral tegument protein of approximately 65 kDa may represent the virion component involved in *trans*-activation (4). The HCMV component involved in *trans*-activation is currently being investigated.

Although other animal DNA viruses can code for proteins that act in *trans* to enhance the transcription of viral (9, 17) or cellular (14) genes, the herpesviruses are unique in that a virus-specific protein involved in *trans*-activation is a part of the HSV virion and presumably the HCMV virion. The domain involved in *trans*-activation of the HSV IE promoter-regulatory region is upstream of the promoter (34). The current observations with HCMV support the notion that the *cis*-acting domains are located at identical or closely proximal positions to the regions responsive to *trans*-activation by the virus-specified component. Like the *cis*-acting domains, the domains responsive to *trans*-activation are distributed over a long distance and are additive in enhancing transcription. These regions may represent binding sites for a virus-specified protein or for other factors which interact with it.

Our results obtained with a number of deletion mutants indicated that the 19- or 18-n repeats of the HCMV IE1 regulatory region are important elements for enhanced gene expression. The 19-n repeat sequence, 5′ CCCCA$^{A}_{G}$GTTGACGTCAATGGG 3′, has a characteristic CAAT box; one of which forms part of the promoter at −68 n (44). The CAAT box is a sequence thought to be involved in the binding of RNA polymerase II (2). In addition, there are GC-rich motifs surrounding the 19-n repeat that may be involved in binding transcription factors. The 19-n repeat sequences are nearly perfect palindromes. These sequences may interact to form cruciform structures as previously proposed (44). Secondary structures may facilitate an entry site for some component(s) of the transcriptional machinery.

Enhancers are suggested to be binding sites for tissue-specific transcription factors (28, 47, 48, 52). The HCMV regulatory region may serve as a binding site for a cellular transcription factor as well as a virus-specified transcription factor or other factors which interact with it. Hence the sequences have a *cis*-acting domain as well as an adjacent or identical domain that can be activated in *trans* in stably transformed cells by an HCMV-specified component but not an HSV-specified component.

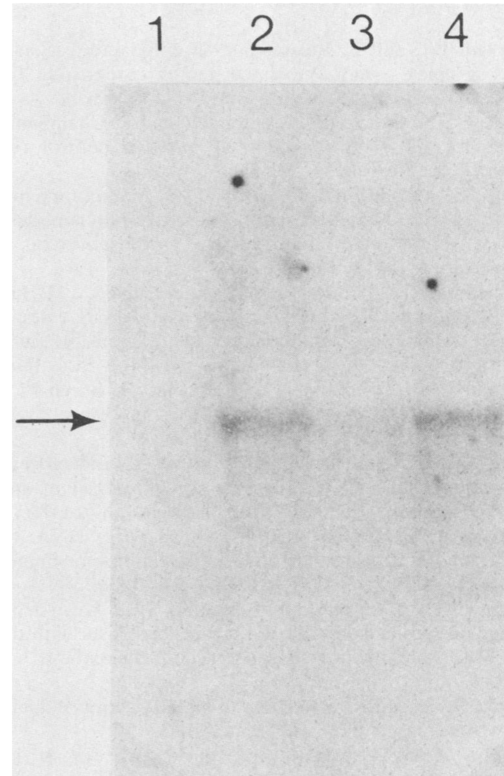


FIG. 7. Effect of HCMV infection on the steady-state level of OV-specific cytoplasmic RNA. Stably transformed cells containing pOVwt174 or pOVdl361 in the presence of anisomycin were either mock infected or infected with 5 PFU of HCMV per cell. Cytoplasmic RNA was isolated, fractionated, and blotted as described in the text. The blot was probed with ^{32}P-labeled *Bam*HI DNA of pOV, and the RNA band was detected by autoradiography. Lanes: 1, mock-infected pOVwt174; 2, HCMV-infected pOVwt174; 3, mock-infected pOVdl361; 4, HCMV-infected pOVdl361.

Since the IE1 gene is highly expressed relative to the other IE genes, the IE1 regulatory region exerts its major enhancing effect on the adjacent IE1 gene that codes for a 72-kDa protein (42). However, this regulatory region also exerts an effect on the adjacent region referred to as IE2. The viral RNAs from this region can be linked to the leader exons of IE1 by differential RNA processing (Stenberg et al., submitted). Enhancement of IE1 gene expression may be required during the first 3 to 4 h of infection. The major IE protein itself, a cellular protein, or another IE gene protein may act as a positive regulator of transcription of other HCMV genes, similar to the EIA protein of adenovirus (14).

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LITERATURE CITED

1. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
2. **Benoist, C., K. O'Hare, R. Breathnach, and P. Chambon.** 1980. The ovalbumin gene sequence of putative control regions. Nucleic Acids Res. **8**:127–142.
3. **Blanton, R. A., and M. J. Tevethia.** 1981. Immunoprecipitation of virus-specific immediate-early and early polypeptides from cells lytically infected with human cytomegalovirus strain AD169. Virology **112**:262–273.
4. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
5. **Campione-Piccardo, J., W. E. Rawls, and S. Bacchetti.** 1979. Selective assay for herpes simplex viruses expressing thymidine kinase. J. Virol. **31**:281–287.
6. **Cohen, G. H., R. K. Vaughan, and W. C. Lawrence.** 1971. Deoxyribonucleic acid synthesis in synchronized mammalian KB cells infected with herpes simplex virus. J. Virol. **7**:783–791.
7. **DeMarchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map locations for immediate-early, early and late RNAs. Virology **114**:23–38.
8. **Furukawa, T., A. Fioretti, and S. Plotkin.** 1973. Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication. J. Virol. **11**:991–997.
9. **Gaynor, R. B., and A. J. Berk.** 1983. Cis-acting induction of adenovirus transcription. Cell **33**:683–693.
10. **Gibson, W.** 1981. Immediate-early protein of human cytomegalovirus strains AD169, Davis, and Towne differ in electrophoretic mobility. Virology **112**:350–354.
11. **Gorman, C. M., G. T. Merlin, M. D. Willingham, I. Pastan, and B. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. U.S.A. **79**:6777–6781.
12. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
13. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
14. **Greene, M. R., R. Triesman, and T. Maniatis.** 1983. Transcriptional activation of cloned human β-globulin genes by viral immediate-early gene product. Cell **35**:137–148.
15. **Guggenheimer, R. A., B. W. Stillman, K. Nagata, F. Tamanoi, and J. Hurwitz.** 1984. DNA sequences required for the in vitro replication of adenovirus DNA. Proc. Natl. Acad. Sci. U.S.A. **81**:3069–3073.
16. **Herz, C., and B. Roizman.** 1983. The α promoter regulator-ovalbumin chimeric gene resident in human cells is regulated like the authentic α4 gene after infection with herpes simplex virus 1 mutants in α4 gene. Cell **33**:145–151.
17. **Imperiale, M. J., L. T. Feldman, and J. R. Nevins.** 1983. Activation of gene expression by adenovirus and herpes simplex regulatory genes acting in *trans* and by a *cis*-acting adenovirus enhancer element. Cell **35**:127–136.
18. **Lang, J. C., D. A. Spandidos, and N. M. Wilkie.** 1984. Transcriptional regulation of a herpes simplex virus immediate early gene is mediated through an enhancer-type sequence. EMBO J. **3**:389–395.
19. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
20. **Mackem, S., and B. Roizman.** 1982. Regulation of α genes of herpes simplex virus: the α 27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. J. Virol. **43**:1015–1023.
21. **Mackem, S., and B. Roizman.** 1982. Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. Proc. Natl. Acad. Sci. U.S.A. **79**:4917–4921.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. U.S.A. **77**:3855–3859.
24. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing with base specific chemical cleavage. Methods Enzymol. **65**:499–560.
25. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblast permissively infected by human cytomegalovirus strain AD169. Virology **125**:31–46.
26. **McKnight, S. L.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. **8**:5949–5964.
27. **Michelson-Fiske, S., F. Horodniceanu, and J. C. Guillon.** 1977. Immediate early antigens in human cytomegalovirus-infected cells. Nature (London) **270**:615–617.
28. **Moreau, P., H. R. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon.** 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. **9**:6047–6069.
29. **Nagata, K., R. A. Guggenheimer, and J. Hurwitz.** 1983. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. U.S.A. **80**:6177–6181.
30. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans* activation by herpesvirus infection. J. Virol. **52**:522–531.
31. **O'Sullivan, M. J., and V. Marks.** 1981. Methods for the preparation of enzyme-antibody conjugates for use in enzyme immunoassay. Methods Enzymol. **73**:147–166.
32. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24**:555–565.
33. **Post, L. E., B. Norrild, T. Simpson, and B. Roizman.** 1982. Chicken ovalbumin gene fused to a herpes simplex virus α promoter and linked to a thymidine kinase gene is regulated like a viral gene. Mol. Cell. Biol. **2**:233–240.
34. **Preston, C. M., M. G. Cordingley, and N. D. Stow.** 1984. Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. J. Virol. **50**:708–716.
35. **Rawlins, D. R., P. J. Rosenfeld, R. J. Wides, M. D. Challberg, and T. J. Kelly.** 1984. Structure and function of the adenovirus origin of replication. Cell **37**:309–319.
36. **Shaw, W.** 1975. Chloramphenicol acetyltransferase from resistant bacteria. Methods Enzymol. **43**:737–754.
37. **Siebenlist, U., L. Henninghausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-*myc* gene in Burkitt lymphoma. Cell **37**:381–391.
38. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. **49**:190–199.
39. **Stinski, M. F.** 1977. Synthesis of proteins and glycoproteins in cells infected with human cytomegalovirus. J. Virol. **23**:751–767.
40. **Stinski, M. F.** 1977. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26**:686–701.
41. **Stinski, M. F., R. M. Stenberg, and W. F. Goins.** 1984. Structure and function of the human cytomegalovirus immediate early genes, p. 399–421. *In* F. Rapp (ed.), Herpesvirus. UCLA Symposia on Molecular and Cellular Biology, vol. 21. Alan R. Liss, Inc., New York.

42. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. **46:**1–14.
43. **Tanaka, S., M. Otsuka, S. Ihara, F. Maeda, and Y. Watanabe.** 1979. Introduction of pre-early nuclear antigen(s) in HEL cells infected with human cytomegalovirus. Microbiol. Immunol. **23:**263–271.
44. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. U.S.A. **81:**659–663.
45. **Thomsen, D. R., and M. F. Stinski.** 1981. Cloning of the human cytomegalovirus genome as endonuclease *Xba*I fragments. Gene **16:**207–216.
46. **Wagner, M. J., J. A. Sharp, and W. C. Summers.** 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. **78:**1441–1445.
47. **Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon.** 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell **32:**503–514.
48. **Wasylyk, B., C. Wasylyk, and P. Chambon.** 1984. Short and long range activation of the SV40 enhancer. Nucleic Acids Res. **12:**5589–5607.
49. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41:**462–477.
50. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus: transcription at immediate early and early times after infection. J. Virol. **38:**446–459.
51. **Weeks, D. L., and N. C. Jones.** 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral genes. Mol. Cell. Biol. **3:**1222–1234.
52. **Weiher, H., M. Konig, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219:**626–631.
53. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. **135:**253–259.
54. **Woo, S. L. C., W. G. Beattie, J. F. Catterall, A. Dugaiczyk, R. Staden, G. C. Brownlee, and B. W. O'Malley.** 1981. Complete nucleotide sequence of the chicken chromosomal ovalbumin gene and its biological significance. Biochemistry **20:**6437–6446.