JOURNAL OF VIROLOGY, Apr. 1995, p. 2565–2573 Vol. 69, No. 4
0022-538X/95/$04.00+0

Tetracycline Repressor-Regulated Gene Repression in Recombinant Human Cytomegalovirus

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Received 3 November 1994/Accepted 17 January 1995

The tetracycline repressor (TetR)-regulated gene expression system from *Escherichia coli* was used to control gene expression in recombinant human cytomegalovirus (HCMV). To adapt the TetR system in HCMV, derivatives of the viral US11 (early) gene promoter, which controls the β-glucuronidase reporter gene, were constructed by systematic insertion of the tetracycline operator (*tetO*) sequences. Gene expression from constructs containing two or three appropriately placed *tetO* sequences adjacent to the TATA box were efficiently repressed by a TetR-VP16 fusion protein (tTA) in a transient expression system. Efficient repression (50- to 120-fold) also occurred in tTA-expressing stably transfected human cells which were infected with recombinant HCMV containing a US11 promoter surrounded by three *tetO* sequences. The tTA-mediated gene repression was relieved in the presence of 1 μg of tetracycline per ml. The results of this study are significant in three respects. (i) This is the first demonstration that a TetR-derived protein can be used to efficiently repress gene expression in a mammalian system. (ii) Efficient repression was dependent on the presence of the transcriptional activation domain from the herpes simplex virus type 1 VP16 protein. (iii) The ability to regulate gene expression in a controlled fashion in order to elucidate viral gene function is an important development in the HCMV field. The tTA-mediated gene repression system may be extremely useful for creating host-range mutants in essential genes in order to study their role in the HCMV replicative cycle, a system that is otherwise exceedingly difficult to genetically dissect.

Human cytomegalovirus (HCMV), a species-specific betaherpesvirus, is an important ubiquitous pathogen that causes clinical disease in congenitally infected newborns and immunosuppressed and immunocompromised adults (1). Sequence analysis of the HCMV genome indicates that the 230-kb genome of HCMV contains more than 200 potential open reading frames (6). The biological functions of most of these genes are unknown. Although many genes are likely to be essential for HCMV replication in tissue culture, the identification and function of these genes have been difficult to study because of the lack of a complementing cell culture system (HCMV grows well only in primary human diploid fibroblasts in culture) in which to isolate and propagate HCMV null mutants. Therefore, alternative strategies for identifying essential genes of HCMV and their biological roles in the viral replicative cycle are needed. In light of this, we sought to develop a system in which expression of HCMV genes could be regulated by a prokaryotic repressor protein.

Positive and negative regulation of gene expression in mammalian cells by prokaryotic repressor proteins or derivatives thereof has been reported previously by several laboratories (reviewed in reference 17). Expression of *lac* operator-containing promoters can be repressed by the Lac repressor (LacI) in several mammalian cell systems (4, 7, 10, 22) and recombinant vaccinia virus (40, 41, 53). Also, a LacI-based fusion protein (LacI-VP16) was shown to activate gene expression in stably transformed human, murine, and rat cells (2, 36). However, the tetracycline repressor protein (TetR) system was chosen for development here because of the specificity of TetR to its operator (34) and the relatively high affinity between tetracycline and TetR (47).

In the *Escherichia coli* tetracycline resistance operon, expression of the tetracycline repressor gene and expression of the tetracycline resistance gene are negatively regulated by TetR homodimers consisting of 23-kDa monomers (20, 29). Binding of TetR homodimers to the two tetracycline operator (*tetO*) sequences overlapping the two divergent promoters of the tetracycline repressor and resistance genes inhibits transcription of both genes (3, 21). When tetracycline is bound to TetR, the operator-binding property of *tetR* is abolished (21), thereby allowing expression from the tetracycline resistance operon.

Control of gene expression in a eukaryotic system by TetR-mediated repression was first reported in a plant system (12, 14). When *tetO* sequences were appropriately positioned in the cauliflower mosaic virus (CaMV) 35S promoter, expression from the linked reporter gene was regulated by TetR. In contrast to repression, it was recently reported that gene expression in mammalian cells could be controlled by TetR-mediated transcriptional activation (18, 51). In this case, TetR was fused with the C-terminal acidic domain of herpes simplex virus type 1 (HSV-1) VP16 protein, a transcriptional transactivator. This fusion protein (tTA) binds to multiple *tetO* sequences upstream of the TATA box in a minimal promoter and thereby induces expression of the linked gene. However, in the presence of tetracycline, tTA no longer binds to the *tetO* sequences on the minimal promoter and only low-level basal expression occurs. Although TetR-mediated repression and activation systems were successfully adapted to plant and mammalian cells, respectively, the application of a TetR-mediated gene repression system in any mammalian or other animal virus system has not been reported to date.

In this article, we report the adaptation of a TetR-mediated gene repression system to control expression from HCMV.

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The prokaryotic β-glucuronidase reporter gene was placed under the control of the HCMV US11 (early) promoter containing *tetO* sequences. In transient assays, reporter gene expression was repressed by tTA but not by TetR. Recombinant HCMV mutants which contain the *tetO*-modified US11 promoter in place of the normal US11 promoter were isolated. Also, stably transformed cell lines expressing tTA have been established for use with these mutants. In recombinant HCMV mutant-infected cells, expression directed by the modified US11 promoter was repressed 50- to 120-fold in the absence, compared with the presence, of tetracycline. This tetracycline-sensitive TetR-mediated gene repression system may be useful for creating host-range or conditional lethal mutants in essential genes in order to study their role in the HCMV replicative cycle.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were isolated in this laboratory and used below passage 20. U373-MG astrocytoma cells were obtained from the American Type Culture Collection. Both cell types were grown in Dulbecco's modified Eagle medium (Mediatech) containing 10% fetal bovine serum (GIBCO) and 25 mM HEPES (*N*-2-hydoxyethylpiperazine-*N*′-2-ethanesulfonic acid). HCMV AD169 was obtained from the American Type Culture Collection; wild-type and recombinant HCMVs were propagated according to standard protocols.

DNA sequence. The numbering system of Chee et al. (6) for the HCMV AD169 DNA sequence (GenBank accession number X17403) is used in this report.

Reporter gene plasmids. pUS11pBgpA was used as the parental plasmid and consists sequentially of the US11 promoter containing a 465-base *Hin*dIII-*Xba*I fragment (bases 200856 to 200391), the β-glucuronidase gene, and the HSV-1 thymidine kinase polyadenylation signal (25). To insert synthetic *tetO* sequences adjacent to the TATA box of US11 promoter, an *Nhe*I restriction site was generated just upstream from the TATA box. The sequences between the *Aat*II and *Xba*I sites of pUS11pBgpA (bases 200471 to 200391) were replaced by a synthetic oligonucleotide containing the *Nhe*I restriction site at position −32 in relation to the US11 transcription initiation site at base 200395 (Fig. 1A) (25). The resulting plasmid, pUS11pBgpA-N, contains the new sequence 5′GAC**C GCTAGC**TATAAGA3′ instead of 5′GACTACATGCTATAAGA3′, with four base substitutions (boldface) upstream of the US11 TATA box (underlined). pUS11pBgpA-N was cleaved with *Nhe*I-*Bam*HI and then ligated with oligonucleotides containing one, two, or three TetR operator sequences within the US11 promoter and *Nhe*I-*Bam*HI cohesive termini (Fig. 1B to F and H). To construct p1opL (Fig. 1B), the oligonucleotide 5′CTAGC**ACTCTATCATTGATAGAGT**C TATAAGAACAGCCTTACAGCTTTTGAGTGTcTAGAG3′ and its complement were annealed and ligated {*tetO* sequences are in boldface type, TATA box sequences are underlined, and the putative transcription initiation site (on the basis of data from the US11 wild-type promoter [25]) is indicated in lowercase}. To construct p1opR (Fig. 1C), the oligonucleotide 5′CTAGCTATAAGA**CTC TATCATTGATAGAGT**AcAGCCTTACAGCTTTTGAGTCTAGAG3′ and its complement were annealed and ligated. To construct p2opLR (Fig. 1D), the oligonucleotide 5′CTAGC**ACTCTATCATTGATAGAGTC**TATAAGA**CTCTAT CATTGATAGAGT**AcAGCCTTACAGCTTTTGAGTCTAGAG3′ and its complement were annealed and ligated. To construct pR2opTA (Fig. 1E), the oligonucleotide 5′CTAGCTATAAGA**CTCTATCATTGATAGAGT**GA**cTCTATCA TTGATAGAGT**TCTAGAG3′ and its complement were annealed and ligated. To construct pL1R2opTA (Fig. 1F), the oligonucleotide 5′CTAGC**ACTCTATCAT GGATAGAGTC**TATAAGA**CTCTATCATTGATAGAGT**GA**cTCTATCATTGAT AGAGT**TCTAGAG3′ and its complement were annealed and ligated. To construct p3optx (Fig. 1G), the TATA box region of pUS11BgpA-N was excised with *Nhe*I-*Bam*HI and replaced with the 121-base *Eco*RV-*Bam*HI fragment from pUCA7-tx (39). This construct has a three-*tetO*-sequence arrangement similar to that of pL1R2opTA; however, ~60 bases of TATA box-proximal promoter sequences was derived from the CaMV 35S promoter. Essentially, the TATA box is TATATAA (instead of TATAAGA in US11), and there are a CAAT box motif and two AP-1-like sites upstream of the most upstream *tetO* sequence (Fig. 1G). To construct p1opTIS (Fig. 1H), the oligonucleotide 5′CTAGCTATAA GACAGCCTTACAGCTT**ACTCTAtCATTGATAGAGT**TTGAGTCTAGAG3′ and its complement were annealed and ligated. To construct pUS11Aat-7op (Fig. 2), pUS11pBgpA was partially digested with *Aat*II (−72 relative to the US11 transcription initiation site) and a 300-base *Xho*I-*Kpn*I fragment from pUHC13-3 (18) containing seven tandem *tetO* sequences was inserted. In this plasmid, the seven *tetO* sequences were positioned 45 bases upstream of the US11 TATA box. All plasmid DNA manipulations were done according to standard protocols (44).

TetR expression plasmids. The *tetR* gene was cloned into vector pIE (containing sequentially the HCMV major immediate-early enhancer promoter, a polylinker, and the HSV-1 thymidine kinase polyadenylation signal [28]) to yield pTetR. The TetR-coding region in pTetR is a 695-kb *Hin*cII fragment from pWH305 (38) to which optimal translation initiation sequences were introduced around the ATG start codon (35). The TetR protein encoded from pTetR has an additional alanine amino acid after the methionine start codon. To construct pTetR-NL, an oligonucleotide encoding the simian virus 40 T antigen nuclear localization signal (Pro-Lys-Lys-Lys-Arg-Lys-Val [30]) was ligated into pTetR such that it would be incorporated after the second TetR amino acid. The tTA fusion protein is encoded from pUHD15-1 as described previously (18). tTA is the 128-amino-acid acidic domain of the HSV-1 VP16 protein fused to the C terminus of TetR. To construct pUHD15-1-puro, the 1.25-kb *Pst*I-*Pst*I puromycin resistance gene-containing expression cassette from pUC4P (I. Kovesdi, American Cyanamid Co.) was inserted at the *Hin*dIII site of pUHD15-1 by blunt-end ligation.

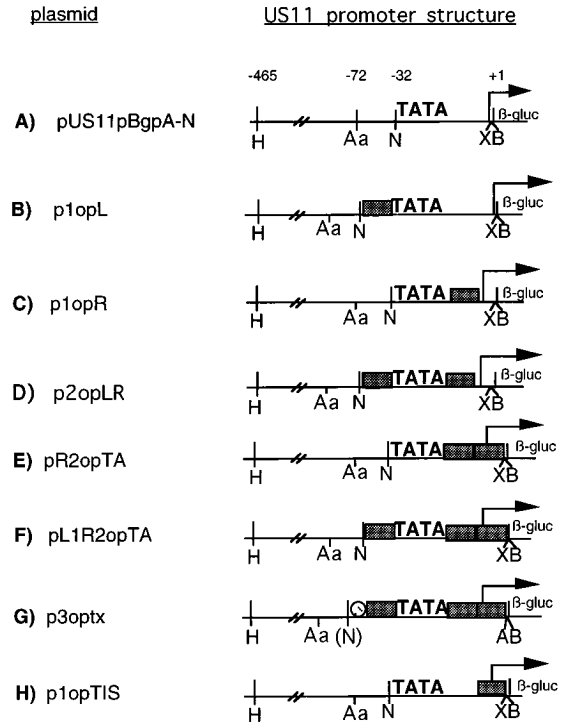
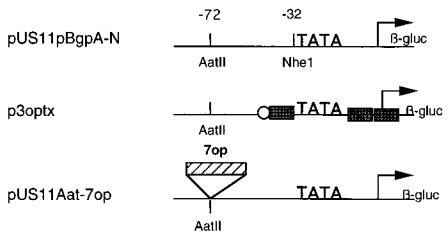


FIG. 1. Organization of US11 promoter plasmids. The HCMV US11 promoter sequence, −465 to +1 (relative to the transcription initiation site), controls expression of the β-glucuronidase (β-gluc) reporter gene in all plasmids. The locations of the TATA box (TATA), *tetO* (shaded rectangles), and CAAT box–AP-1 binding site motifs (circle) are indicated. H, *Hin*dIII; Aa, *Aat*II; A, *Asp* 718; N, *Nhe*I; X, *Xba*I; B, *Bam*HI. The restriction site shown in parentheses was lost as a result of the blunt-end ligation used to construct the plasmid.

Recombinant virus plasmids. pBgdUS11 (26) was modified to generate the plasmids for recombination into the HCMV genome. This plasmid consists of the 1.8-kb *Pst*I-*Xba*I fragment (bases 202207 to 200391, containing the US13, US12, and US11 promoter sequences), β-glucuronidase, and the 1.5-kb *Sal*I-*Sst*II fragment (bases 200171 to 198709, containing C-terminal US11 sequences and US10 sequences). To insert *tetO* sequences into the US11 promoter, pRV3optx and pRVL1R20pTA were constructed. pBgdUS11 was partially digested with *Hin*dIII and completely digested with *Bam*HI to remove the wild-type US11 promoter sequences. The corresponding *Hin*dIII-*Bam*HI *tetO*-modified US11 promoter fragments from p3optx or pL1R2opTA were ligated in place of the excised wild-type promoter fragment. The resulting plasmids were designated pRV3optx and pRVL1R2opTA, respectively.

Transfection for recombinant HCMV. Plasmid and wild-type HCMV AD169 DNAs were transfected into HFF cells by a modified calcium phosphate coprecipitation technique as described previously (26). After 14 days, the medium was removed and the cells were overlaid with 0.5% agarose in modified Eagle medium (GIBCO) containing 150 μg of X-Glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Biosynth) per ml. Blue (recombinant virus-containing) plaques were picked several days after the overlay was added. Recombinant viruses were plaque purified three times.

DNA blot analysis. Viral DNA was electrophoresed through 0.7% agarose gels. The DNA was blotted to Nytran membranes (Schleicher & Schuell), UV cross-linked, prehybridized, and then hybridized with DNA probes as described previously (26).



	+ pUC119	+ pUHD15-1	+ pTetR	+ pTetRNL
mock	2 ±1	1 ±1	1 ±1	1 ±1
pUS11pBgpA-N	496 ±16	379 ±95 (1.3)	445 ±47(1.1)	414 ±40(1.2)
p3optx	2733±118	67 ±19 (41)	750 ±56(3.6)	509 ±8 (5.4)
pUS11Aat-7op	334 ±46	1041±61(0.32)	ND	ND

FIG. 2. Repression assay for TetR and TetR derivatives. The schematic shows the US11 promoter region of the three plasmids used in this experiment. The symbols were described in the legend to Fig. 1, except that the hatched rectangle indicates seven tandem *tetO* sequences. β-Glucuronidase activity (picomoles of product per 0.1 μg of protein per min) was determined after cotransfection of the indicated plasmids in the HCMV-infected transient expression system. Cotransfection with pUC119 was done as a negative control. The results shown are the means ± standard deviations of three independent transfections. Fold repression (in parentheses) was calculated relative to the pUC119 data. ND, not determined.

Transient expression and β-glucuronidase assay. HFF cells and U373-MG cells were transfected in 60-mm-diameter plates when they were 70 to 80% confluent. Ten micrograms of supercoiled plasmid DNA was transfected per plate in 2 ml of Dulbecco's modified Eagle medium containing 200 μg of DEAE-dextran (M_w of 500,000; Pharmacia) per ml. After 4 h at 37°C, the cells were shocked with 20% dimethyl sulfoxide in 1× HEPES-buffered saline (pH 7.05) for 2 min. The cells were washed twice with phosphate-buffered saline (PBS), and growth medium was added. After incubation for 24 h, these plates were infected with the HCMV wild-type strain, AD169, at a multiplicity of infection of 4 PFU per cell. Cell extracts were made at 20 h postinfection, and the β-glucuronidase assay was performed as described previously (26). The amount of total protein in the cell extracts was determined by the Bradford method as described in a kit from Bio-Rad.

Production of polyclonal antisera. Purified TetR protein was obtained from G. Ellestad (American Cyanamid Co.). Polyclonal antisera against TetR were obtained after five intradermal injections into female New Zealand White rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, Pa.).

Western blot analysis. Proteins were extracted from cells and analyzed by Western blotting (immunoblotting) with an enhanced chemiluminescence kit (Amersham) as described previously (28). The primary anti-TetR rabbit polyclonal antibody was diluted 1:500 for use; the donkey anti-rabbit immunoglobulin G-horseradish peroxidase-conjugated secondary antibody (Amersham) was diluted 1:10,000 for use.

Stable transformation. U373-MG cells were transfected in 60-mm-diameter plates by the standard calcium phosphate method (44). The transfection mixture contained 10 μg of pUHD15-1-puro (previously linearized within the prokaryotic plasmid backbone sequences). Two days posttransfection, the cells were trypsinized and replated in 82-mm plates; after an additional 24 h, puromycin (Sigma) was added to a final concentration of 0.375 μg/ml. After 2 weeks, puromycin-resistant colonies were picked and expanded into cell lines. Each cell line was tested for tTA fusion protein expression by Western blot analysis.

Immunofluorescence. HFF cells were transfected by the DEAE-dextran method and infected at 24 h posttransfection as described above. After an additional 24 h, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 according to a standard protocol (19). To reduce the nonspecific fluorescence, the TetR antiserum was twice adsorbed to fixed-permeabilized HFF cells, each for 30 min at room temperature. The transfected cells were incubated at room temperature for 30 min with 30 μl of normal human serum diluted 1:10 in PBS containing 3% bovine serum albumin (BSA) (PBS-BSA) to block any Fc receptors which might be present as a result of HCMV infection. Subsequent antibody incubations and washes were done according to a standard protocol (19). The primary antibody was TetR antiserum diluted 1:100 in PBS-BSA. The secondary antibody was fluorescein-conjugated goat anti-rabbit immunoglobulin G diluted 1:250 in PBS-BSA. After incubation with the secondary antibody, the cells were incubated with 100 ng of 4′,6-diamidino-2-phenylindole for 5 min at room temperature to stain the nuclei. Immunofluorescence was visualized with a Zeiss Axioplan microscope.

One-step growth analysis. Confluent monolayers of parental U373-MG cells and stably transformed tTA-expressing cell lines in 35-mm-diameter plates were infected at a multiplicity of infection of 4 PFU per cell with the HCMV wild-type strain, AD169. After adsorption for 2 h at 37°C, the inoculum was removed and fresh medium was added to each plate. Virus was harvested daily, and the titer was determined as previously described (26).

RESULTS

General strategy. Initiation of transcription is an early step in gene expression and requires the formation of a functional transcription initiation complex in which RNA polymerase and various accessory transcription factors bind to or associate with the promoter sequences (42, 52). To regulate gene expression by TetR in HCMV, our strategy was to interfere with the formation of a functional transcription initiation complex. In this strategy, TetR proteins bind to operator sequences appropriately positioned within the promoter of the gene of interest, thereby interfering with either the binding of general transcription factors to promoter sequences or protein-protein interactions necessary for the formation of a functional transcription initiation complex, essentially blocking transcription (11–14). TetR-mediated gene regulation can be controlled by tetracycline: tetracycline binds to TetR, causing a reduction in the affinity of TetR for its operator sequences, resulting in derepression of gene expression. To test the utility of TetR in controlling gene expression in HCMV, the US11 gene promoter was chosen as a model. The following features are relevant to this choice. (i) The US11 gene is expressed with early kinetics in HCMV-infected cells and has been shown to be regulated by upstream sequences in transient expression assays (25). (ii) The US11 gene product, a 32-kDa nonstructural glycoprotein, is nonessential for viral growth in vitro, allowing for replacement of the US11 coding region by a reporter gene in the context of the viral genome (26). To facilitate insertion of TetR operator sequences within the US11 promoter, an *Nhe*I restriction site was created 5 bases upstream of the US11 TATA box in the US11 promoter–β-glucuronidase reporter gene plasmid (pUS11pBgpA) to yield pUS11pBgpA-N (Fig. 1A). Thus, replacement of the 38-base *Nhe*I-*Bam*HI fragment from pUS11pBgpA-N with *tetO*-containing sequences yields the modified US11 promoter constructs (Fig. 1). It was previously shown in a plant system that expression directed by a modified CaMV 35S promoter containing three *tetO* sequences adjacent to the TATA box (i.e., one upstream and two downstream) was efficiently repressed in the presence of TetR (12). To determine if this arrangement was also efficient in repression of expression directed by the HCMV US11 promoter, a 121-base region from the modified CaMV 35S promoter (essentially containing a CAAT box motif, two AP-1-like sites, and a TATA box with surrounding *tetO* sequences [39]) was inserted in place of the 38-base TATA box-containing *Nhe*I-*Bam*HI fragment of the US11 promoter to yield p3optx (Fig. 1G). Thus, a modified US11 promoter which contains the same number and positioning of *tetO* sequences as the TetR-repressible CaMV 35S promoter (12) was constructed. In a transient assay system in the absence of TetR, expression from p3optx, like that from pUS11pBgpA-N, was dependent on HCMV infection (data not shown), which is typical of a virus early promoter and consistent with previous results (25), but the level of expression was increased fivefold (Fig. 2 [pUC119 column]). This increase may be due to the presence of sequences resembling a CAAT box motif and AP-1 sites (39), which bind transcriptional activation factors and are not present in the wild-type US11 promoter. Most importantly, the

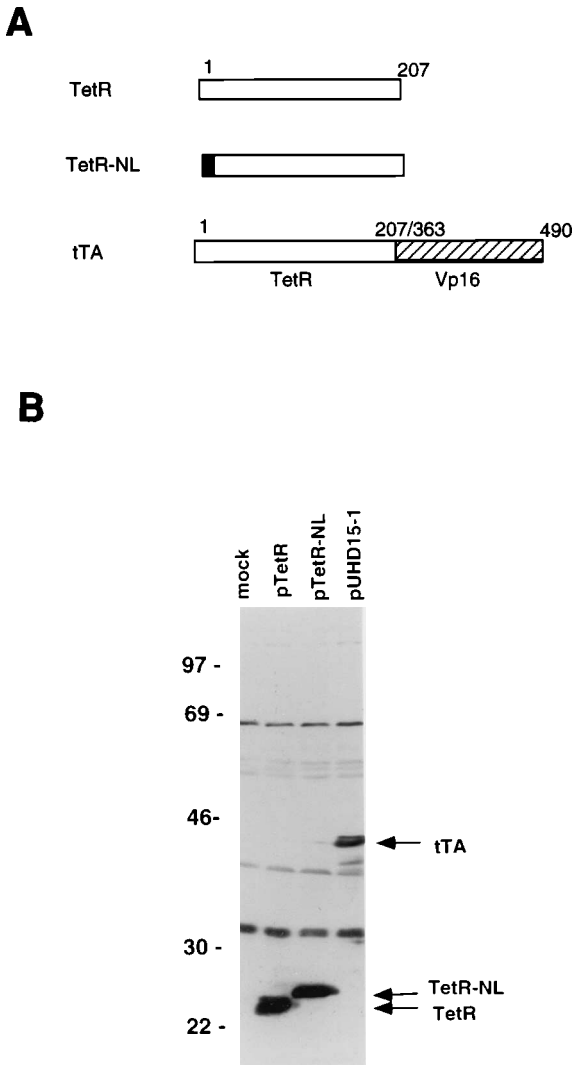


FIG. 3. Expression of TetR and derivatives. (A) Schematic representation of the structures of TetR and the derivatives used in this study. The shaded region of TetR-NL represents the insertion of the simian virus 40 T antigen nuclear localization signal between amino acids 1 and 2 of TetR. The hatched rectangle is the C-terminal transactivating domain of HSV-1 VP16. (B) Western blot analysis of TetR and derivatives. HFF cells were transfected with plasmids encoding TetR or a derivative. The cells were infected with HCMV at 24 h posttransfection, and total cellular proteins were harvested at 48 h posttransfection. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot analysis was performed with the TetR polyclonal antibody. The locations of TetR and the derivatives are indicated on the right; the positions of molecular weight markers (in kilodaltons) are indicated on the left.

data indicate that the presence of *tetO* sequences in the TATA box transcription initiation site area does not prevent HCMV-induced transcription.

Expression and intracellular localization of TetR. To control gene expression from operator-containing promoters, mammalian expression plasmids encoding various forms of TetR were constructed (Fig. 3A). In pTetR, the TetR gene was placed under the control of the HCMV major immediate-early promoter and contained optimal translation initiation sequences (35) around the ATG initiation codon. pTetR was modified to introduce the simian virus 40 T antigen nuclear localization signal after the second TetR codon, yielding pTetR-NL (Fig. 3A), to ensure efficient transport to the nucleus of this modified TetR protein (TetR-NL). pUHD15-1 encodes the tTA fusion protein, a modified TetR protein in which TetR is fused to the 128-amino-acid C-terminal acidic domain of the HSV-1 VP16 protein (18). This fusion protein was previously shown to be effective in activation of promoters containing multiple tandem TetR operators upstream of the TATA box in mammalian cell systems (18). TetR expression from these plasmids was monitored by Western blot analysis after transient expression in HFF cells (Fig. 3B). Large amounts of both TetR and TetR-NL were detected; the molecular weight of TetR-NL was slightly higher than that of TetR because of the nuclear localization signal. tTA was present at slightly lower steady-state levels than was TetR or TetR-NL.

Because viral gene transcription occurs in the nucleus of HCMV-infected cells, it was important to verify that TetR derivatives can get to the nucleus for transcriptional repression. The intracellular localization of TetR proteins was determined by immunofluorescence after transient expression (data not shown). The unmodified TetR protein was present throughout the cell, whereas TetR-NL protein was detected exclusively in the nucleus, indicating proper recognition of the nuclear localization signal. Like TetR, tTA appeared in both the nucleus and the cytoplasm. This result indicated that amino acids derived from the C-terminal region of VP16 do not cause detectable differences in the efficiency of nuclear localization of TetR proteins. Previous data from biochemical fractionation studies also indicated that tTA was present in both the nuclear and cytoplasmic cell fractions (18). Thus, although all TetR proteins appeared to be present in the nucleus, only TetR-NL was specifically localized there.

Effect of TetR derivatives on expression from the three-operator-modified US11 promoter. To determine the effect of the TetR proteins on transcription from the three-operator-modified US11 promoter (p3optx [Fig. 1G]) compared with the US11 promoter lacking *tetO* sequences (pUS11pBgpA-N), cotransfection assays were performed with HFF cells (Fig. 2). In the absence of TetR-derived proteins (i.e., the pUC119 cotransfection), HCMV-induced expression was detected from both promoters. In the presence of tTA (i.e., pUHD15-1), expression from the *tetO*-modified promoter was repressed ~40-fold, whereas essentially no repression was observed from the wild-type promoter. It is particularly noteworthy that TetR and TetR-NL caused only a three- to fivefold repression of expression from the *tetO*-modified promoter. The degree of repression caused by TetR-NL, which is specifically localized in the nucleus, was essentially the same as that caused by the unmodified TetR, which is found throughout the cell. Thus, the tTA protein was the only form of TetR which was able to efficiently repress expression, suggesting that the VP16 domain is required for the negative effect on gene expression.

Binding of tTA to operator sequences in a promoter is typically associated with transcriptional activation, not repression (18, 50, 51). Expression from pUS11Aat-7op, which contains a modified US11 promoter with seven tandem operators 45 bases upstream of the TATA box, was stimulated threefold in the presence of tTA (Fig. 2). This was dependent on the VP16 domain, because no stimulation was observed in the presence of a truncated form of tTA which lacked most of the VP16 domain (data not shown).

Operator positioning. To determine the optimal *tetO* sequence positioning for efficient repression by tTA, additional plasmid constructions were made and tested with a transient expression assay (Table 1). A plasmid that contains three *tetO* sequences surrounding the US11 TATA box, analogous to p3optx but lacking the CAAT box motif, is pL1R2opTA (Fig.

TABLE 1. tTA repression of expression from *tetO*-modified US11 promoter plasmids

Plasmid	β-Glucuronidase activity after cotransfection with[a]:		Fold repression[b]
	pUC119	pUHD15-1	
Mock	3 ± 2	2 ± 1	1.5
pUS11pBgpA-N	1,020 ± 50	700 ± 70	1.5
p1opL	1,190 ± 200	550 ± 130	2
p1opR	1,940 ± 140	200 ± 15	10
p2opLR	700 ± 40	80 ± 10	9
pR2opTA	1,060 ± 70	40 ± 3	27
pL1R2opTA	590 ± 10	20 ± 8	30
p3optx	5,700 ± 200	110 ± 10	52
p1opTIS	3,500 ± 400	430 ± 15	8

[a] β-Glucuronidase activity (picomoles of product per 0.1 μg of protein per min) was determined after cotransfection of the indicated plasmids in the HCMV-infected transient expression system. Cotransfection with pUC119 was done as a negative control. The results shown are the means ± standard deviations of three independent transfections.

[b] Fold repression was calculated relative to the pUC119 data.

1F). Other plasmids containing one or two *tetO* sequences in various positions relative to the US11 TATA box are shown in Fig. 1B to E and H). Insertion of *tetO* sequences within the US11 promoter caused an up to twofold reduction in HCMV-induced promoter activity in the absence of tTA (i.e., pUC119 column). Expression from plasmids containing three *tetO* sequences or two downstream *tetO* sequences (i.e., pL1R2opTA, p3optx, and pR2opTA) was repressed ~30- to 50-fold in the presence of tTA. An intermediate (8- to 10-fold) reduction in expression was observed when one *tetO* sequence was present just downstream of the TATA box (i.e., p1opR and p1opTIS), whether or not it overlapped the putative transcription initation site. Very little repression was observed with a single *tetO* sequence just upstream of the TATA box in the presence of tTA (i.e., p1opL). Accordingly, the upstream *tetO* failed to cause more than the intermediate level of repression when it was present in conjunction with a downstream *tetO* sequence (compare the results with p1opR and p2opLR). The data indicate that multiple operators downstream of the TATA box are more important for transcriptional interference than either a single operator or two operators, one on either side of the TATA box, in the US11 promoter system. The cumulative transient expression data (Fig. 2 and Table 1) indicated that tTA can either activate, repress, or have no effect on HCMV-induced gene expression, depending on the number or positioning of the *tetO* sequences in the US11 promoter. It is also noteworthy that, unlike tTA, neither the unmodified TetR protein nor TetR-NL was able to efficiently repress expression from either pR2opTA or pL1R2opTA (data not shown).

Recombinant virus with operator-modified US11 promoter. To determine whether tTA could efficiently repress gene expression from the context of the HCMV genome, the *tetO*-modified US11 promoter–β-glucuronidase expression cassettes from p3optx and pL1R2opTA were transferred to new plasmids (pRV3optx and pRVL1R2opTA, respectively), which were used to make recombinant virus. These plasmids were designed such that, upon recombination with viral genome, the modified US11 promoter and β-glucuronidase gene would replace the endogenous wild-type US11 promoter and coding sequences, respectively, by virtue of the viral flanking sequences in these recombination plasmids (Fig. 4). After linearization, the plasmids were cotransfected with wild-type HCMV strain AD169 virion DNA into HFF cells to generate recombinant HCMV mutants harboring a tetracycline-responsive

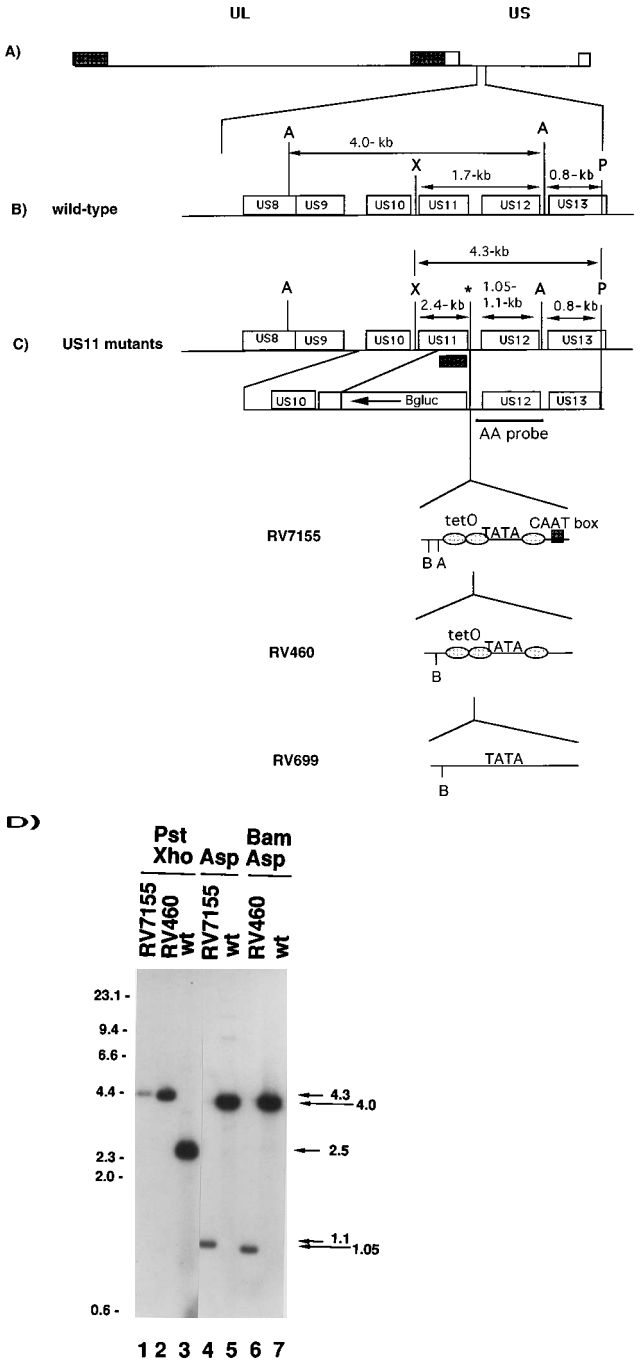


FIG. 4. Organization and DNA blot analyses of HCMV wild-type and recombinant virus DNAs. (A) Diagram of HCMV genomic DNA, including the unique long (UL) and unique short (US) components, which are flanked by repeated DNA (rectangles). (B) Expansion of the US8-US13 open reading frame region of wild-type DNA. (C) Genomic organization of mutants used in this study. The shaded ovals indicate *tetO*. The second line in panel C shows the general organization of the plasmids used for recombination with wild-type HCMV DNA to create the mutants. The *tetO*-modified US11 promoter region for each mutant is shown. RV699 was reported previously (26). A, X, P, and B indicate *Asp* 718, *Xho*I, *Pst*I, and *Bam*HI restriction sites, respectively. The asterisk indicates the position of the *Bam*HI and *Asp* 718 sites (when present), as shown in the *tetO*-modified promoters. (D) DNA blot analysis. Either wild-type HCMV (wt) or recombinant DNA (RV7155 and RV460) was digested with *Pst*I-*Xho*I, *Asp* 718, or *Asp* 718-*Bam*HI, electrophoresed, blotted, and hybridized with the 1.1-kb AA probe, as shown in panel C. The migration of a λ phage *Hin*dIII DNA molecular weight marker is given in kilobases to the left. The locations and sizes of hybridizing DNA fragments are indicated to the right.

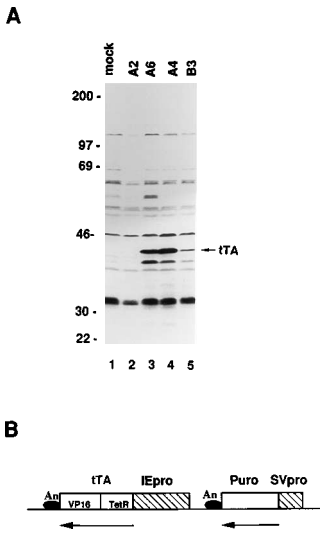


FIG. 5. (A) Western blot analysis of stably transfected tTA-expressing cell lines. U373-MG cells (permissive for HCMV) were transfected with pUHD15-1-puro (Puro) and selected for resistance to puromycin. Parental U373-MG cells (mock) and four puromycin-resistant cloned cell lines (A2, A6, A4, and B3) were examined for expression of the tTA fusion protein 24 h postinfection with the HCMV wild-type strain (multiplicity of infection of 2 PFU per cell). In this case, the cells were infected only to boost the level of tTA expression from the immediate-early promoter (IEpro). The position of the ~42-kDa tTA fusion protein is indicated. (B) Schematic representation of the relevant regions of pUHD15-1-puro. An, antibody; SVpro, SV40 promoter.

β-glucuronidase gene. Mutant viruses obtained from the pRV3optx and pRVL1R2opTA transfections were plaque purified and designated RV7155 and RV460, respectively (Fig. 4C). To verify recombination of the *tetO*-modified β-glucuronidase expression cassettes within the viral genome, DNA blot analysis was performed (Fig. 4D). The 1.1-kb AA probe (Fig. 4C) hybridized to an ~4.3-kb *Xho*I-*Pst*I DNA fragment in digests of RV7155 and RV460 genomic DNAs. The size of the hybridizing *Xho*I-*Pst*I DNA fragments reflects the ~1.8-kb net increase in genome size of the mutants compared with that for wild-type HCMV. The presence of the *tetO*-modified US11 promoter in the mutant viruses was confirmed as a result of hybridization of the AA probe to 1.1-kb *Asp* 718 and 1.05-kb *Asp* 718-*Bam*HI DNA fragments in digests of RV7155 and RV460 DNA, respectively (Fig. 4D). Some of these restriction sites are not present in the wild-type US11 promoter but appear only in the *tetO*-modified promoters, as shown in Fig. 4C. Thus, the DNA blot analyses confirmed that the *tetO*-modified β-glucuronidase expression cassettes were intact within the genomes of RV7155 and RV460.

TetR-regulated gene expression in recombinant HCMV. In order to test these recombinant viruses, stably transfected cell lines expressing tTA were established. U373-MG astrocytoma cells were transfected with pUHD15-1-puro, a plasmid containing both tTA and puromycin resistance gene expression cassettes. After selection in the presence of puromycin, colonies of stably transfected cells were picked and expanded into cell lines. tTA expression was assessed by Western blot analysis, and cell lines were selected for further use on the basis of their differential expression of tTA. Expression of tTA was not detected in cell line A2, low levels of tTA were present in cell line B3, and relatively high levels of tTA were detected in cell lines A4 and A6 (Fig. 5). Single-cycle growth analyses were performed to determine the growth kinetics of the HCMV wild-type strain, AD169, in cells expressing low or high levels of tTA (cell lines B3 or A4 and A6, respectively) compared with those for parental U373-MG cells. Wild-type HCMV replicated equally well in all three cell lines, as in parental U373 cells (data not shown), indicating that the presence of tTA has no significant effect on viral growth in tissue culture, consistent with the specificity of the TetR-derived DNA binding domain of tTA for *tetO* (34).

TABLE 2. Repression of β-glucuronidase activity in RV7155-infected stably transformed tTA-expressing cell lines

Cell line	β-Glucuronidase activity in infected cells[a]		Fold specific repression
	With tetracycline	Without tetracycline	
A4	940 ± 40	15 ± 1	60
A6	1,540 ± 130	13 ± 1	120
B3	4,930 ± 270	1,260 ± 120	4
A2	8,490 ± 1,840	8,990 ± 1,110	1
U373-MG	2,630 ± 260	2,650 ± 130	1

[a] Duplicate plates containing either parental U373-MG cells or stably transformed cells (A4, A6, A2, and B3) were infected with RV7155 at a multiplicity of infection of 0.5 PFU per cell, in the presence or absence of 1 μg of tetracycline per ml. At 20 h postinfection, cell lysates were made and β-glucuronidase activity was determined. β-Glucuronidase expression is given in picomoles of product per 0.1 μg of protein per min. Values are the means ± standard deviations of three independent experiments.

The ability of tTA to repress expression of *tetO*-containing genes in recombinant HCMV was initially tested by infecting each of the selected stably transfected cell lines, as well the untransfected parental cells, with RV7155 at a multiplicity of infection of 0.5 PFU per cell, either in the presence or in the absence of tetracycline. In general, the amount of specific repression correlated with the level of tTA in the cells (Table 2). No repression was observed in parental U373 cells or A2 cells, which did not express detectable amounts of tTA. Tetracycline-sensitive repression was observed in the cell lines expressing tTA. Expression in B3 cells was specifically repressed 4-fold, while expression in the A4 and A6 cells, which had the highest levels of tTA, was specifically repressed 60- to 120-fold. To determine if gene expression could be efficiently repressed with higher input amounts of virus, A4 cells were infected with RV7155 at different multiplicities of infection in the presence or absence of tetracycline (Table 3). Greater than 80-fold specific repression was observed at multiplicities of infection of 2.5 PFU or less per cell. However, even at a multiplicity of infection of 10 PFU per cell there was 33-fold specific repression.

The recombinant virus experiments of Tables 2 and 3 involved the use of RV7155, which has the US11 promoter modified with ~60 bases from the CaMV 35S promoter (CAAT box, AP-1 sites, and TATA box), as well as three *tetO* sequences (Fig. 4C). A more authentic US11 promoter is present in the recombinant virus designated RV460, which has three *tetO* sequences adjacent to the US11 TATA box (Fig. 4C). Expression from the *tetO*-modified US11 promoter in RV460 was compared with expression from the wild-type US11 promoter in RV699 (Table 4). In both viruses, the US11 promoter controls the expression of the β-glucuronidase reporter gene, which is in the place of the US11 coding region (Fig. 4C). Expression from the *tetO*-modified promoter in RV460 was specifically repressed 46-fold in A4 cells, but there was no repression of the wild-type US11 promoter (i.e., RV699). The specificity of the tTA protein to *tetO*-containing promoters was further attested in a control experiment. In parental U373-MG cells, which do not express any TetR-related proteins, tetracycline-sensitive repression of the US11 promoter in RV460 or

TABLE 3. β-Glucuronidase activity in A4 cell lines and parental U373-MG cells infected with serial dilutions of RV7155

Multiplicity of infection	β-Glucuronidase activity in[a]:					
	A4 cell lines		Fold specific repression	Parental U373 cells:		Fold specific repression
	Without tetracycline	With tetracycline		Without tetracycline	With tetracycline	
10	76 ± 19	2,540 ± 950	33	7,090 ± 1,740	7,155 ± 1,965	1
5	43 ± 5	2,570 ± 1,030	60	6,620 ± 1,440	7,248 ± 2,241	1
2.5	27 ± 1	2,190 ± 800	81	6,850 ± 1,060	7,215 ± 2,275	1
1.2	18 ± 1	1,740 ± 560	97	5,480 ± 2,430	4,828 ± 1,223	1
0.6	13 ± 1	1,200 ± 170	93	2,530 ± 930	2,520 ± 1,120	1
0.3	9 ± 2	950 ± 90	106	1,140 ± 120	1,090 ± 80	1

[a] Duplicate plates of either parental U373-MG cells or stably transformed tTA-expressing A4 cells were infected with RV7155 at the multiplicities of infection indicated in either the presence or the absence of 1 μg of tetracycline per ml. At 20 h postinfection, cell lysates were made, β-glucuronidase activity (picomoles of product per 0.1 μg of protein per min) was measured, and specific repression was determined as described for Table 1.

RV699 was not detected (Table 4). Expression from recombinant virus which contains the two-*tetO*-sequence-modified US11 promoter (identical to the arrangement depicted in pR2opTA [Fig. 1E]) was also specifically and efficiently repressed (25- to 35-fold) in A4 and A6 cells (32). The cumulative data are consistent with results from the transient expression assays in that TetR-mediated repression of gene expression from the HCMV genome is specific for *tetO*-containing promoters and is not due to a fortuitous interaction with elements present in the 60-base portion of the CaMV 35S promoter region present in the modified US11 promoter of RV7155.

DISCUSSION

Regulation of mammalian cell or animal virus gene expression by a TetR-based gene repression system was not reported previously. Our goal was to determine whether a tetracycline-sensitive repression system could be established in HCMV. A system was developed in which the tTA fusion protein was used to efficiently repress expression of a reporter gene under the control of a *tetO*-containing promoter in recombinant HCMV. Our model system involved the use of the moderately active HCMV US11 early promoter (25). Efficient tTA-mediated gene repression in HCMV (i.e., 50- to 120-fold) was dependent on (i) adequate amounts of tTA in infected cells (Table 2), (ii) the number and location of the *tetO* sequences in the viral promoter (Fig. 2 and Table 1), and (iii) the multiplicity of infection (Table 3). This repression was sensitive to low, nontoxic concentrations of tetracycline (1 μg/ml), conditions under which tTA does not bind to operator sequences (18, 21), resulting in induction (i.e., derepression) of expression.

TABLE 4. β-Glucuronidase activity in A4 cells and parental U373-MG cells infected with RV460 and RV699

Virus	Cell line	β-Glucuronidase activity in infected cells[a]		Fold specific repression
		Without tetracycline	With tetracycline	
RV460	A4	5 ± 1	230 ± 20	46
RV460	U373-MG	240 ± 10	220 ± 2	1
RV699	A4	350 ± 7	320 ± 20	1
RV699	U373-MG	430 ± 50	450 ± 20	1

[a] Duplicate plates containing either parental U373-MG cells or stably transformed tTA-expressing A4 cells were infected with RV460 or RV699 at a multiplicity of infection of 1 PFU per cell in either the presence or the absence of 1 μg of tetracycline per ml. At 20 h postinfection, cell lysates were made, β-glucuronidase activity (picomoles of product per 0.1 μg of protein per min) was measured, and specific repression was determined as described for Table 1.

TetR was shown previously to repress expression from a *tetO*-containing polymerase II promoter in tobacco plants (12–14) and polymerase III-driven suppressor tRNA genes in the eukaryotic slime mold *Dictyostelium discoideum* and the yeast *Saccharomyces cerevisiae* (8, 9). However, unlike the results from these systems, TetR was unable to efficiently repress gene expression in the HCMV system (Fig. 2). Also, TetR-NL, which is specifically localized in the nucleus, was unable to mediate efficient repression (Fig. 2). Thus, the mechanism by which specific repression is facilitated by tTA, although dependent on the operator-binding function of the TetR portion, requires the presence of the VP16 domain (Fig. 2). No differences in the intracellular distributions were detected by immunofluorescence as a result of the fusion of TetR with the VP16 domain compared with unmodified TetR (data not shown). The data suggest that the presence of the VP16 acidic activation domain on tTA seems to be mechanistically important for efficient repression, because TetR and TetR-NL were both ~10-fold less efficient than tTA in the repression assay (Fig. 2). Alternatively, the difference in mass between tTA (~42 kDa) and the other TetR proteins may be important. Studies are in progress to determine the requirements for efficient repression in this system. The presence of the VP16 domain in DNA binding protein fusions is typically associated with gene activation (18, 43, 51). In contrast, a previous study with a LacI-VP16 fusion protein (LAP348) indicated that either activation or repression could result, depending on the positioning of the *lac* operator in a promoter (36). A similar situation occurs in the tTA gene regulation system (Fig. 2 and Table 1). In the LacI-VP16 system, however, maximal repression was only 8-fold (36), about 5- to 10-fold lower than what we observed with tTA.

In addition to the VP16 domain, efficient repression in this HCMV system required at least two *tet* operators just downstream of the TATA box (Table 1). Although the most downstream *tetO* sequence overlapped the transcription initiation site in these promoters, a single *tetO* sequence which overlapped the transcription initiation site repressed at an intermediate level (eightfold) (Table 1). VP16 has been shown to directly interact with TBP and coactivator $TAF_{II}40$ (both of the TFIID complex), as well as basal transcription factor TFIIB, and, more recently, coactivator PC4 (15, 16, 23, 33, 37, 46). Therefore, the mechanism of repression by tTA may involve either steric hindrance in the formation of the transcription preinitiation complex by basal transcription factors and/or inappropriate positioning of the basal transcription factors or

coactivators as a result of their interactions with the VP16 domain. In RNA blot analyses with a β-glucuronidase probe representing the entire gene, transcripts with β-glucuronidase sequences were not detected in either RV460- or RV7155-infected A4 cells in the absence of tetracycline (32). This is consistent with anticipated mechanism of tTA-mediated repression at the transcriptional level. Furthermore, the apparent absence of mRNAs containing β-glucuronidase sequences indicates that tTA binding to sites just downstream of the TATA box did not result in the formation of a functional preinitiation complex downstream of the β-glucuronidase translation initiation codon. Hence, the utility of this system should not be limited to genes with short 5′ untranslated leader sequences, as tested in this study.

HCMV has a limited host range in tissue culture, growing well only in primary (nonimmortal) human diploid fibroblasts. Therefore, creation of stably transfected human fibroblast cell lines to complement lethal defects in mutant virus is not practical because of the finite life span of primary cells in culture. Our interest in developing a TetR-mediated gene regulation system in HCMV is based on the inability to create true null mutants in order to study the functions of viral essential genes. Replacement mutagenesis strategies involving the insertion of prokaryotic reporter genes, such as β-galactosidase and β-glucuronidase, have been used by our laboratory and others to create HCMV deletion mutants (5, 26, 31, 45, 48). In these cases, the HCMV mutants can be purified only if the deletion is a nonlethal event, thereby identifying genes which are dispensable for virus growth in tissue culture. To date, over 20 dispensable HCMV genes have been identified by replacement mutagenesis, including the elucidation of genes involved in major histocompatibility complex class I heavy-chain downregulation (24) and viral early cytopathic effect (27). Although the replacement mutagenesis strategies have utility, there is a need for additional systems with which to study HCMV essential gene function.

Recently, a heavy metal-dependent inducible gene expression system was used to regulate gene expression in HCMV (49). In this case, the *E. coli lacZ* reporter gene was placed under the control of the mouse metallothionein promoter and introduced into the *Hin*dIII-O region of HCMV by replacement mutagenesis. Expression of the *lacZ* gene in the recombinant virus was induced up to 24-fold by the presence of zinc. However, general application of this system to study gene function in HCMV may be restricted for the following reasons. (i) Proper temporal or quantitative regulation of the metallothionein promoter (i.e., similar to that of the endogenous promoter of the gene of interest) will likely be difficult to achieve (a problem common to all activation schemes). (ii) There are potential pleiotropic effects of heavy metals on other cellular or viral promoters or processes.

The tetracycline-sensitive tTA repression system is expected to be a useful genetic tool for studying the function of HCMV essential genes. In this system, the gene of interest is controlled by its own promoter, which is modified only with several *tet* operators. In the case of our US11 model promoter, HCMV-induced gene expression was only minimally affected as a result of the insertion of two or three *tetO* sequences in the absence of tTA (Tables 1 and 4) (32). Although the 50- to 120-fold repression of gene expression observed in the presence of tTA is substantial (Tables 2 to 4), higher levels of repression may be attainable by incorporation of a nuclear localization signal into tTA in order to increase the intranuclear concentration of this repressor. For reasons yet to be determined, the amount of repression observed (i.e., in the absence of exogenous tetracycline) varies, depending on the type of serum used in the media. The results reported here were obtained with fetal calf serum in the media. Similar results were obtained by omitting the serum from the media. However, fivefold lower levels of repression were observed when calf serum was used. Hence, it is important to test various sources or types of serum in order to obtain the optimal repression levels.

Although the main advantage of the system described herein is efficient repression of gene expression, an additional attribute of a TetR-based system is that gene repression can be reversed (i.e., derepressed) relatively quickly by the addition of tetracycline, which has a high affinity for the repressor. For example, the effect of depression of the gene of interest at various times in the replicative cycle can be determined by the addition of tetracycline to the media. Others have demonstrated that transcriptional reversal due to tetracycline binding to TetR or derivatives occurs within several hours after tetracycline addition (9, 18). Although the effective induction time may vary from gene to gene, an induction period of several hours is reasonable in the HCMV system, because the replication cycle is 3 to 4 days. For viral genes which are expressed at both early and late times, it should be possible to determine the effect of expression only at early or late times postinfection by the presence or absence of tetracycline. Thus, we anticipate that a TetR-regulated gene expression system may be an appropriate system for studying the biological roles of HCMV genes. Recombinant virus containing the US11 gene under the control of the *tetO*-modified US11 promoter was recently isolated; expression of the US11 gene was shown to be repressed by tTA in A4 cells in a tetracycline-sensitive fashion, as predicted (32). Application of the tTA repression system for studying the functions of several HCMV genes is currently in progress.

ACKNOWLEDGMENTS

We thank Lei Sun for excellent technical assistance and Ian Mohr for critical reading and suggestions concerning the manuscript.

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