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Functional Analysis of the True Late Human Cytomegalovirus pp28 Upstream Promoter: *cis*-Acting Elements and Viral *trans*-Acting Proteins Necessary for Promoter Activation

ALISON S. DEPTO† AND RICHARD M. STENBERG*

Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501

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As a model for analyzing the regulation of human cytomegalovirus late genes, we investigated the 28-kDa phosphoprotein (pp28) gene region. Transcripts of 1.6 and 1.3 kb were expressed in wild-type human cytomegalovirus-infected cells but not in cells infected with a DNA-negative temperature-sensitive mutant (*ts*66), indicating that DNA replication is absolutely required for pp28 gene expression. Transient promoter activation studies revealed that the pp28 gene region upstream promoter (pp28US) functioned early when expressed independently of the viral genome. However, the promoter was not efficiently activated by immediate-early (IE) proteins but was activated equally well by both wild-type virus and *ts*66. Deletion analysis of the pp28US promoter indicated that sequences upstream of the CAP site between −107 and −32 were required for activation of the pp28 promoter. Within that region exist a 10-bp sequence at −90 (AGTGAT CGTG) and its inverted repeat at −32 which positively influence pp28 promoter function. Therefore, in the case of the pp28US promoter, viral proteins interact through discrete sequences to facilitate late gene expression.

Infection of permissive cells in vitro with human cytomegalovirus (HCMV) leads to an ordered sequential expression of viral genes (9, 28, 52, 53). On the basis of their times of transcription in infected cells, HCMV genes are divided into three broad classes (9, 52, 53). The first class of genes to be transcribed after viral infection are the immediate-early (IE) genes which require no de novo protein synthesis for their expression (8, 9, 52, 53). IE gene expression and protein function have been extensively characterized elsewhere (2, 7, 8, 11, 14, 18, 31, 35, 42–47, 49, 50, 52–54). IE proteins are required for subsequent HCMV early gene expression and regulation (2, 11, 40, 44) and may be required for late gene expression as well. Early genes are expressed after IE gene expression, and early transcription proceeds in the absence of viral DNA replication (9, 28, 52, 53). This is in contrast to late gene expression, which by definition occurs after the initiation of viral DNA synthesis. Viral RNA transcribed at early and late times originates from all regions of the genome (9, 28, 52, 53). Previous studies demonstrated that the abundance of early and late transcripts varies, depending on the region of the genome being transcribed (9, 28, 52, 53).

While many HCMV early and late genes have been previously identified, including genes coding for glycoproteins (3, 5, 6, 25, 33), structural proteins (3, 16, 27, 29, 32, 34, 37), regulatory and nonstructural proteins (3, 13, 19, 20, 23, 30), and ones of unknown function (2, 3, 7, 12, 17, 21, 22, 43), many of these studies involved physical mapping of the genes rather than addressing their expression and regulation. Therefore, we examined the kinetics of early and late RNA expression in order to identify true late RNAs which absolutely require DNA replication prior to expression.

In these studies, we used a temperature-sensitive, DNA-negative virus (*ts*66) to address the kinetics of expression of RNAs before and after viral DNA replication. This enabled us to identify RNAs expressed only under late conditions. We chose to examine RNA expression and promoter activation corresponding to the pp28 gene region. Our studies indicate that (i) the two pp28 gene region RNAs of 1.6 kb and 1.3 kb absolutely require DNA replication for their transcription, (ii) the pp28 gene region upstream promoter (pp28US) is activated through a discrete sequence containing a set of inverted repeats, and (iii) IE proteins are insufficient for activation of this late promoter.

Analysis of HCMV late gene expression. HCMV mutant *ts*66 is a DNA replication-negative virus at the nonpermissive temperature (11, 44). Therefore, a comparison of RNAs isolated from wild-type (WT) HCMV- and *ts*66-infected cells at late times after infection (72 h) will allow the identification of true late genes. Several putative true late genes were analyzed by isolating whole-cell RNAs from WT- or *ts*66-infected cells and subjecting the RNAs to Northern analysis. The results demonstrated that several RNAs were transcribed in WT- but not *ts*66-infected cells (Fig. 1). These transcripts were assigned to the true late class since they absolutely required viral DNA replication prior to expression. Three specific RNAs, a 1.5-kb RNA from IE2 which codes for a 40-kDa late protein (36, 43) and 1.6- and 1.3-kb RNAs from *Xba*I-C in the pp28 gene region (21, 27, 29), have been previously described. In addition to these RNAs, previously uncharacterized RNAs from *Xba*I-B (7.5, 6.0, and 4.4 to 2.4 kb), *Xba*I-L (1.2 kb) and *Xba*I-Q (1.4, 1.2, and 1.0 kb) were also detected. Numerous other true late RNAs were detectable but were not included in this study (data not shown).

Analysis of RNA expression from the pp28 gene region. Because we were interested in studying the expression and regulation of a true late gene, the pp28 gene region was chosen for further investigation (21, 27, 29) (Fig. 2C). Two RNAs, a 1.3-kb RNA which encodes the 28-kDa capsid phosphoprotein and a 1.6-kb RNA encoding a recently described 58-kDa virion phosphoprotein (21, 22), are tran-

* Corresponding author.
† Present address: Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

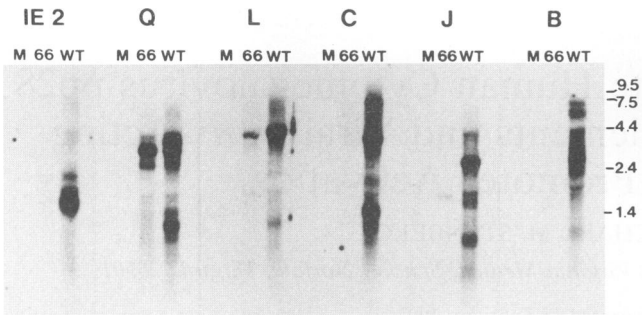

FIG. 1. Whole-cell RNA expression in *ts*66- and WT-infected cells. Whole-cell RNA was isolated from mock (M)-, *ts*66 (66)-, or WT HCMV (WT)-infected cells at 72 h after infection (11, 43). RNA from an equivalent number of cells was subjected to Northern blot analysis and hybridized to the *Xba*I fragments indicated at the top of each blot (51) or to a probe representing IE region 2 (43). The Towne strain of HCMV (20 to 25 PFU per cell) was used exclusively for these studies. Propagation of virus was performed as previously described (48). *ts*66, a temperature-sensitive DNA-negative mutant of HCMV Towne, has been previously described (11, 43) and was propagated at 33.5°C. The nonpermissive temperature for *ts*66 is 39.5°C. Molecular sizes are indicated in kilobases.

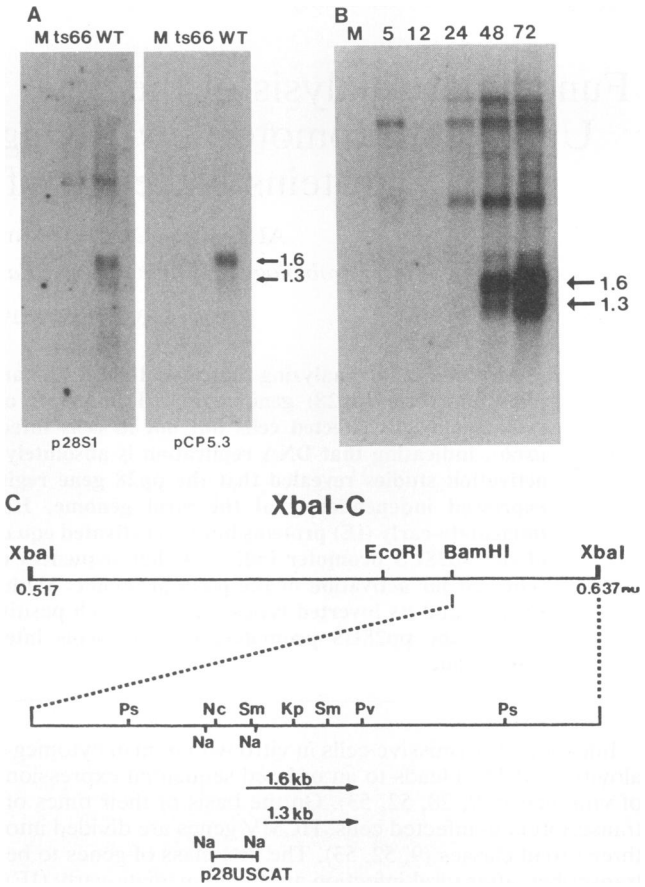

FIG. 2. Analysis of RNA expression from the pp28 gene. (A) Whole-cell RNA was isolated from mock (M)-, *ts*66 (ts66)-, or WT HCMV (WT)-infected cells at 72 h after infection. RNA from an equivalent number of cells was subjected to Northern blot analysis and hybridized with the 1.0-kb *Sma*I fragment and the 5.3-kb *Pst*I fragment of *Xba*I-C as indicated in panel C. (B) Time course analysis of pp28 RNA expression. Whole-cell RNA was isolated at the indicated hours after infection from cells infected with HCMV. Mock-infected cell RNA (M) (1 μg) and RNA from an equivalent number of infected cells were analyzed. The blot was hybridized with the 1.0-kb *Sma*I fragment of *Xba*I-C. The 1.3- and 1.6-kb RNAs are indicated to the right of the blots. (C) Restriction endonuclease map of the pp28 gene region. The 1.6- and 1.3-kb RNAs are shown below the expanded map. The pp28 promoter construct (p28USCAT) contains the 697-bp *Nae*I fragment representing the upstream promoter of the pp28 gene region (−600 to +97). This fragment was isolated and subjected to blunt-end ligation to add *Hin*dIII linkers to the DNA. After digestion with *Hin*dIII and purification of the fragment by acrylamide gel electrophoresis, the promoter fragment was cloned into pSV0CATd at the *Hin*dIII site. Enzyme reactions were carried out as described elsewhere (1, 26). Recombinants containing the fragment in both orientations were isolated (p28USCAT and p28USCATrev). Ps, *Pst*I; Na, *Nae*I; Nc, *Nco*I; Sm, *Sma*I; Kp, *Kpn*I; Pv, *Pvu*II.

scribed from this region. To further analyze the expression of these RNAs, Northern blot analysis was performed with probes specific for the pp28 gene region. Figure 2A demonstrates that the 1.6-kb and 1.3-kb RNAs were detected in WT- but not *ts*66-infected cells. In addition, neither the 1.3-kb nor the 1.6-kb RNA could be detected in *ts*66-infected cells upon longer exposure of the blot (data not shown).

We also assessed the expression of these RNAs during the course of HCMV infection. The results shown in Fig. 2B indicate that the 1.3- and 1.6-kb RNAs were not detected until 48 h after infection. At 72 h after infection, both RNAs were detected at much higher levels. Our results are consistent with those of previous studies which utilized the viral DNA polymerase inhibitor phosphonoacetic acid to study RNAs specific for the pp28 gene region (27). These studies taken together indicate that both RNAs are true late and absolutely require DNA replication for their expression.

Activation of the pp28 gene region upstream promoter. We used the promoter for the 1.6-kb RNA to study true late promoter activation. Because we were assessing the upstream or most 5′ promoter, we designated this construct p28USCAT (Fig. 2C). To test pp28US promoter activation, cells were transfected with p28USCAT and p28USCATrev and subsequently superinfected with HCMV. At 24 and 72 h after infection, cells were harvested and extracts were prepared and analyzed for chloramphenicol acetyltransferase (CAT) activity. The results demonstrate that although there was some activation of p28USCATrev, levels were six- to eightfold lower than those of p28USCAT (data not shown). Therefore, the pp28US promoter functions most efficiently in the appropriate orientation.

Analysis of early and late promoter activation. Previous analysis of the pp65 early-late promoter demonstrated that it was activated by IE proteins as well as by superinfecting virus (11). Therefore, activation of the true late pp28US promoter by IE proteins was assessed and compared with early-late promoter activation. Activation by IE1 and IE2 proteins was analyzed by transfecting cells with the pol (pPolCAT), pp65 (p65EHCAT), or the pp28 (p28USCAT) promoter-CAT plasmids in the presence of pSVH, a construct which expresses IE1 and IE2 (11, 44). The level of activation by IE proteins alone was compared with the level induced by viral superinfection during two experiments to determine whether IE proteins could sufficiently activate the pp28US promoter.

The data in Table 1 demonstrate that both pPolCAT and p65EHCAT are activated by IE gene products (pSVH) and that viral superinfection results in a 3.6- to 5.6-fold increase

TABLE 1. Quantitation of p28USCAT and p65EHCAT promoter activity[a]

Target	Effector	CAT activity	Fold activation[b]
Expt 1			
p28USCAT	pSV0d	0	
p28USCAT	pSVH	3.7	
p28USCAT	M	0	
p28USCAT	HCMV	51.6	14
p65EHCAT	pSV0d	0	
p65EHCAT	pSVH	34.8	
p65EHCAT	M	0	
p65EHCAT	HCMV	196.2	5.6
Expt 2			
p28USCAT	pSV0d	0	
p28USCAT	pSVH	1.9	
p28USCAT	M	0	
p28USCAT	HMCV	38.8	19.8
p65EHCAT	pSV0d	0	
p65EHCAT	pSVH	17.6	
p65EHCAT	M	0	
p65EHCAT	HCMV	63.6	3.6
pPolCAT	pSV0d	0	
pPolCAT	pSVH	6.8	
pPolCAT	M	0	
pPolCAT	HCMV	36.8	5.4

[a] Promoter activation was tested by transfection of CAT plasmids followed by superinfection with HCMV or by cotransfection with various IE gene plasmids exactly as previously described (11). M, mock.

[b] Fold activation is the increase in CAT activity of HMCV superinfection relative to IE1 and IE2 transfection.

TABLE 2. Activation of p28USCAT and p65EHCAT by *ts*66 and WT HCMV

Target	Effector (h postinfection)[a]	% Acetylation
p28USCAT	M	0
	*ts*66 (72)	36.2
	*ts*66 (96)	51.4
	WT (72)	24.2
	WT (96)	37.9
p65EHCAT	M	0
	*ts*66 (72)	74.3
	*ts*66 (96)	67.4
	WT (72)	88.1
	WT (96)	70.5

[a] Cells were harvested at the times indicated in parentheses. M, mock.

in CAT activity. In contrast, the pp28US promoter is activated poorly by IE1 and IE2 gene products but efficiently activated by viral superinfection. There is a 14- to 20-fold increase in activation of the pp28 promoter by viral superinfection compared with activation by the IE1 and IE2 proteins. In addition, whereas p28USCAT and pPolCAT are equivalently activated by superinfecting virus, only pPol CAT is efficiently activated by IE proteins. While an increase in activation by viral superinfection occurs for all promoters, the pp28US promoter is activated to much higher levels by superinfecting virus than by IE gene products. This indicates that the IE1 and IE2 proteins are insufficient for complete activation of the pp28US promoter and that other infected cell proteins are required. HCMV infection also caused a higher level of activation of the pol and pp65 promoters than did the IE proteins. While this could imply that additional proteins are necessary for the activation of these two early promoters, it is probably because of increased efficiency of infection versus transfection. Additional studies will be necessary to resolve this question.

Activation of pp28USCAT by *ts*66. It must be remembered that late gene expression is closely coupled to virus DNA replication. It is likely that the physical process of replicating DNA affords a level of regulation of late gene expression. However, transient promoter assays allow for an assessment of *trans*-acting proteins capable of influencing the expression of late genes. Our studies described above demonstrate that IE1 and IE2 proteins are not sufficient for complete activation of the pp28 promoter. This is in contrast to early pol (44) and pp65 (11) promoter activation as well as other early promoters studied which are efficiently activated by IE1 and IE2 gene products (2, 40). In addition, we have performed transient cotransfections using plasmid constructs capable of expressing IE proteins from the US3 (3, 54) and UL36-38 (3, 18, 50) gene regions in various combinations with IE1, IE2, and the pp28US promoter. The use of these constructs in analogous experiments resulted in no increase in pp28 promoter activity (data not shown). Therefore, IE proteins appear insufficient for late promoter function, indicating that true late promoters require additional viral proteins for expression.

Recently, the proteins required for activation of the ICP36 (23) late promoter have been characterized by Stasiak and Mocarski (41). Their studies demonstrated that a protein encoded by sequences in *Xba*I-I (TRS1) was required in combination with IE1 and IE2 proteins for complete activation of the ICP36 promoter. This protein, which is related to the US22 family (3), may also be required for pp28US promoter activation.

Because the pp28 promoter was activated at early times when outside the context of the viral genome, *ts*66 was employed to determine whether the proteins required for pp28US promoter activation were produced prior to viral DNA replication. Cells were transfected with p28USCAT or p65EHCAT and then superinfected 24 h later at the nonpermissive temperature with either *ts*66 or WT HCMV. Results in Table 2 indicate that both p65EHCAT and p28USCAT were activated by *ts*66. Activation of p28USCAT and p65EHCAT by *ts*66 or WT occurred at similar levels at both 72 and 96 h after infection. In addition, activation of the pp28US promoter by *ts*66 under transient conditions indicates that it does not require viral DNA replication for induced expression. Therefore, viral proteins necessary for pp28US promoter activation are produced prior to viral DNA replication and are likely to be IE and/or early proteins.

Analysis of repeated sequences in the pp28US promoter. The pp28US promoter sequence was analyzed to determine the *cis*-acting sequences responsible for its activation. An inspection of the pp28US promoter sequence revealed the presence of a number of direct (DR) and inverted (IR) repeats (Fig. 3). Other HCMV early and late promoters have been investigated elsewhere (2, 11, 17, 23, 40, 42) and shown to contain sequences upstream of the CAP site that are required for promoter activation. However, the sequences present in the pp28 promoter do not conform to consensus binding sites for known transcription factors (24) nor are

FIG. 3. Sequence of the AD169 pp28US promoter region (3). Differences from the Towne sequence are indicated above the AD169 sequence. *Nae*I sites used to clone the promoter are underlined. Inverted and direct repeats are indicated by bold lines. The TATA homology is indicated by a bold underline. +1 indicates the CAP site. Locations of the deletional mutants are indicated by vertical lines. Deletion mutants were generated by unidirectional exonuclease III digestion of p28dHCAT, a derivative of p28USCAT lacking the downstream *Hin*dIII site. This plasmid was digested with *Nde*I to linearize the plasmid in the vector 50 nucleotides upstream of the 5′ vector-promoter joining region (*Hin*dIII). The DNA was repaired with Klenow polymerase in the presence of α-phosphorothioate deoxynucleoside triphosphates and subsequently digested with *Hin*dIII. Treatment with exonuclease III was performed as specified by the supplier (Promega) by using 5 U of enzyme per μg of DNA, except that the reaction mixtures were incubated at 24°C to digest the DNA at approximately 100 bp/min. After digestion with S1 nuclease, repair with Klenow polymerase, and ligation for 16 h, the DNA was transformed into bacteria and the resulting clones were screened for desired mutations. To generate d24/26 and d25/26, two oligonucleotides complementary to the AD169 sequence, AD24 (CAAGCTTCACGATCACTATTAACGCGAGTCTCGGCCTAGCCGCGGC) and AD25 (CAAGCTTCTATTAACGCGAG TCTCGGCCTAGCCGCGGC), were annealed independently with AD26 (GCCGCGGCTAGGCCG) and extended with Klenow polymerase, resulting in a double-stranded DNA with a 5′ *Hin*dIII site and a 3′ *Sst*II site. These double-stranded oligonucleotides were digested with *Hin*dIII and *Sst*II and ligated into p28dHCAT at the *Hin*dIII and *Sst*II sites. Recombinants were isolated, sequenced, and tested for activation as described previously (11, 38).

they similar to sequences identified in other HCMV promoters (2, 11, 17, 40, 42). This may indicate that cellular regulatory DNA binding proteins play a lesser role in activation of this late promoter. To determine the minimal sequence required for pp28US promoter activation and to determine whether any of the direct or inverted repeat sequences play a role in activation, deletion analysis of pp28US promoter sequences was performed.

Promoter deletion mutants were generated and activation was assessed by superinfection of transfected cells. Figure 4 demonstrates that deletion to −107 (d43) resulted in little change in pp28US promoter activation. Deletion of a sequence containing one copy of IR4 (d105 at −72 and d24/26 at −40) resulted in a twofold decrease in activation, indicating that IR4 may have a role in promoter regulation. Consistent with this, deletion of sequence containing both copies of IR4 (d25/26 at −32) resulted in a 6.3-fold decrease in activation. As d24/26 and d25/26 differ only by the presence or absence of IR4, these data indicate that IR4 plays a role in promoter activation. These experiments revealed that the region upstream of the CAP site and TATA homology contain a number of potential regulatory elements, some of which are required for promoter activation.

As stated above, the proteins required for activation of the ICP36 late promoter have been characterized elsewhere (41). However, unlike pp28, the ICP36 late promoter does not require *cis*-acting sequences 5′ to the TATA for late promoter activation, suggesting that pp28 and ICP36 may utilize different mechanisms (23). Therefore, it is possible and even likely that additional viral *trans*-acting proteins influence other late genes such as pp28. Studies are under way to determine whether TRS1 or other regulatory proteins are required for pp28US activation.

While the pp28 promoter is efficiently activated at early times under transient conditions, pp28-specific RNAs are not expressed until late in infection and only after virus DNA replication. As is the case with other DNA viruses, these data imply that the pp28 promoter contains two regulatory components: one replication-dependent component that is regulated by the onset of viral DNA synthesis and another replication-independent component that functions as a consequence of viral *trans*-acting proteins. This is consistent with the regulation of herpes simplex virus late promoters which are also activated early when expressed independently of the viral genome (4, 10, 15, 39). It is likely that specific *cis*-acting sequences influence late gene expression independently of *trans*-acting factors which may exclusively control promoter function. In addition to IR4, the pp28US promoter contains repeated sequences that are apparently not important for promoter activation in a transient assay

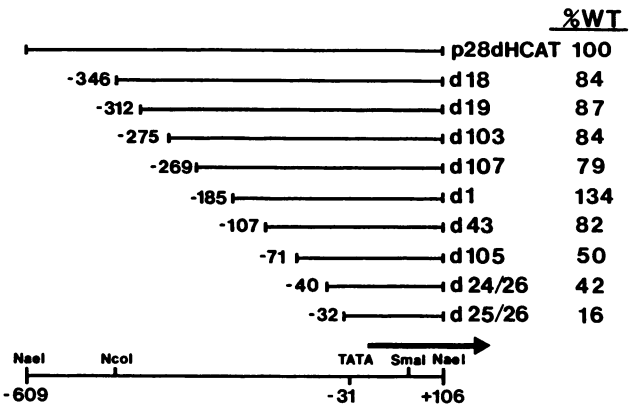

FIG. 4. Activation of the pp28US promoter deletion constructs. p28dHCAT and promoter deletion constructs were transfected into human fibroblast cells and then superinfected with HCMV. Seventy-two hours after infection, extracts were prepared and analyzed for CAT. Positions of the deletions are shown in reference to the distance from the CAP site (+1). Levels of promoter activation for the individual deletion constructs are shown on the right. %WT, percent acetylation relative to p28dHCAT. p28dHCAT contains the same promoter fragment as p28USCAT, except that the downstream *Hin*dIII site was destroyed.

system. However, it is possible and even likely that these sequences play a role in regulation of the pp28US promoter when it is in the context of the viral genome. These repeated sequences may bind viral and/or cellular proteins prior to genome encapsidation and subsequently repress late gene expression. The replication of viral DNA could result in a derepression of transcription because of the generation of progeny DNA which becomes available to the transcriptional machinery. As progeny DNA becomes associated with protein prior to encapsidation, late gene expression would again become repressed. Therefore, multiple mechanisms may control late gene expression in HCMV-infected cells, resulting in a class of true late genes which are expressed only after the virus is committed to complete the replication cycle.

Nucleotide sequence accession number. The GenBank accession number of the Towne sequence in Fig. 3 is M88497.

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