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The Human Cytomegalovirus Origin of DNA Replication (ori_{Lyt}) Is the Critical *cis*-Acting Sequence Regulating Replication-Dependent Late Induction of the Viral 1.2-Kilobase RNA Promoter

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Plasmid constructs containing the 1.2-kb RNA promoter from the long terminal repeat region of human cytomegalovirus (HCMV) display the early-phase regulation of this promoter but lack the characteristic late induction (E. J. Wade, K. M. Klucher, and D. H. Spector, J. Virol. 66:2407–2417, 1992). To determine if the HCMV origin of replication (ori_{Lyt}) was necessary and sufficient for the late induction of the 1.2-kb RNA promoter, we cloned a 9.6-kbp segment of the origin of replication onto the p456 OCAT plasmid containing the 1.2-kb RNA promoter. This plasmid was designated ori456 OCAT. A control construct, which contains all of the same sequences as the ori456 OCAT construct except that a 2.4-kbp segment derived from HCMV *Eco*RI segment U is inverted in orientation to disrupt the origin function, was designated inv456 OCAT. After electroporation into human fibroblast cells and infection with HCMV 24 h later, ori456 OCAT replicated and showed the same early and late transcription pattern as the authentic viral 1.2-kb RNA. Under similar conditions, the inv456 OCAT neither replicated nor showed late induction. Experiments using plasmids synthesized in bacteria lacking methylation activity demonstrated that the late induction was not dependent on the change in methylation state of the plasmids. Ganciclovir, an inhibitor of the HCMV DNA polymerase, was used to demonstrate the replication dependence of the expression of the virally encoded 1.2-kb RNA, while the nearby early 2.7-kb RNA was unaffected. Ganciclovir also inhibited the late induction of the chloramphenicol acetyltransferase gene from ori456 OCAT, while expression from inv456 OCAT increased. Site-specific mutations in two previously identified important regulatory elements of the 1.2-kb RNA promoter, the AP1-binding site and the CATA site, indicated that these sites continue to contribute to promoter activity at late times but that the replication-dependent late induction acts independently of these sites. Possible mechanisms underlying the late induction are discussed.

Human cytomegalovirus (HCMV) is a significant pathogen in humans (17). Like other members of the herpesvirus family, HCMV regulates its gene expression in a temporally ordered manner upon infection of a permissive host cell (3, 15, 22, 23). After the initial expression of the immediate-early genes, characterized by the ability to be transcribed in the absence of de novo protein synthesis, the early genes are expressed. These genes require prior protein synthesis for their transcription and thus appear to rely on the action of virally encoded or induced transactivators (3, 10, 15, 19, 22–24). Our laboratory has been interested in the nature of the *cis*-acting sequences and *trans*-acting factors (both viral and cellular) responsible for the regulation of this class of transcripts.

We and others have shown previously that the 1.2-kb RNA encoded in the long terminal repeat section of the genome is a member of the early gene class (8, 9, 16). Unlike other early promoters that we have analyzed, the 1.2-kb RNA promoter undergoes a late induction of approximately 10-fold after the onset of viral DNA replication, and this induction is sensitive to the presence of phosphonoacetic acid (PAA), a specific inhibitor of the viral DNA polymerase. A plasmid construct utilizing the 1.2-kb RNA promoter to drive expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene displays the same pattern of early expression but lacks the late induction characteristic of the virally encoded 1.2-kb RNA promoter. This late induction was also not observed for a plasmid that contained the entire promoter and gene for the 1.2-kb RNA, as well as 1.7 kbp upstream and 2.4 kbp downstream of the gene. Since these results suggested that the proximal *cis*-acting sequences were not responsible for the late induction, we hypothesized that the viral origin of replication (ori_{Lyt}) might function as a key distal *cis*-acting sequence.

Johnson and Everett found the herpes simplex virus type 1 (HSV-1) ori_s to be necessary for the full level of expression of the US11 true late gene (12). In their experiments, the increase in RNA transcription was proportional to the level of DNA amplification, suggesting that template amplification is the key determinant of RNA transcription rate. Snowden et al. also found that inclusion of the HSV-1 ori_s on β-galactosidase reporter gene constructs driven by promoters of the early, delayed early, and late classes gave a temporal expression pattern similar to that of their viral genomic counterparts (18). However, the level of induction for all three promoters was much less than the level of template amplification, suggesting a relative decrease in the efficiency of template usage. This was true even for the true late gC gene, which requires replication for its efficient expression. They suggested that competition for a multifunctional factor involved in both transcription and replication could explain this decrease in transcription efficiency.

We used a similar approach to determine if the HCMV ori_{Lyt} is necessary and sufficient to confer the late induction on the 1.2-kb RNA promoter. The HCMV origin of replication is

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in the long unique section of the genome, near the junction of *Eco*RI fragments I and U in strain AD169 (1, 5, 14). It appears to be a complex region of considerable size, with approximately 1.5 kbp constituting the minimal origin of replication and flanking sequences adding significantly to its efficiency (14). The HCMV origin of replication has not yet been extensively characterized, but other herpesvirus origins of replication are sometimes associated with enhancers (11).

In this study, we demonstrate that the HCMV origin of replication is necessary and sufficient for the late induction of the 1.2-kb RNA promoter when present on a plasmid. Despite a low level of amplification of the plasmid templates containing HCMV ori_{Lyt}, an increase in transcription rate equivalent to that of the viral 1.2-kb RNA gene is observed from these plasmid templates. A control plasmid containing all the same sequences, but with a nonfunctional origin of replication, does not show this increase in transcription rate. Addition of ganciclovir, a specific inhibitor of the HCMV DNA polymerase, inhibits the late induction of the 1.2-kb RNA but does not affect the nearby 2.7-kb early RNA. The 1.2-kb RNA promoter is also subject to inhibition by ganciclovir when present on replication-competent templates.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were a gift from Stephen Spector (University of California at San Diego). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum and Mito+ serum extender (Collaborative Research Incorporated). HCMV AD169 was obtained from the American Type Culture Collection. Methods for cell culture and viral infection have been described elsewhere (20).

Molecular cloning. To construct ori456 OCAT, an intermediate construct containing the intact HCMV origin of replication was needed. It was assembled from HCMV AD169 *Eco*RI fragment I digested with *Bal*I and *Eco*RI and with *Eco*RI fragment U digested with *Sst*I and *Eco*RI. The vector was prepared by digesting pGem-1 (Promega) with *Sst*I and *Hin*cII, followed by treatment with calf intestine alkaline phosphatase (CIAP) to inhibit self-ligation. These three DNA fragments were ligated together and used to transform *Escherichia coli* DH5α competent cells. The resulting colonies were screened with several restriction enzymes to confirm the structure of the recombinants. This intermediate construct was designated pGIU. It was cleaved with *Pvu*II and ligated with *Hin*dIII linkers (Stratagene). Excess linkers and the pGem-1 vector were digested away with *Hin*dIII, and the resulting 9.6-kbp fragment was gel purified. This fragment was ligated to p456 OCAT (21), which had been linearized with *Hin*dIII and treated with CIAP, and used to transform *E. coli* DH5α competent cells. The resulting colonies were screened to identify one with the orientation shown in Fig. 1A. This clone was designated ori456 OCAT.

For the construction of inv456 OCAT, three separate intermediate constructs were necessary. HCMV AD169 *Eco*RI fragment U was cleaved with *Eco*RI and ligated into an *Eco*RI-digested, CIAP-treated Bluescript II KS- vector (Stratagene). Orientation was selected such that the *Pvu*II site in fragment U was proximal to the *Sst*I site of the vector. This construct was designated bU. The second intermediate construct, pGI, was generated by cleaving pGIU, described above, with *Eco*RI, gel purifying the larger fragment, and religating it. pGI was used as the vector for the bU fragment following digestion with *Eco*RI and treatment with T4 DNA polymerase and CIAP. bU was digested with *Pvu*II, and the 2.4-kb fragment was gel purified and ligated with the pGI vector to generate pGI (U inv). Orientation was selected such that fragment U was joined to fragment I at the *Pvu*II end, rather than at the *Eco*RI end as it is found in the viral genome. Inv456 OCAT was created by ligation of p456 OCAT, digested with *Hin*dIII and treated with CIAP, to the 9.6-kb *Hin*dIII fragment of pGI (U inv). This generated a construct sharing all the sequences of ori456 OCAT but with the segment derived from fragment U in the opposite orientation (Fig. 1B).

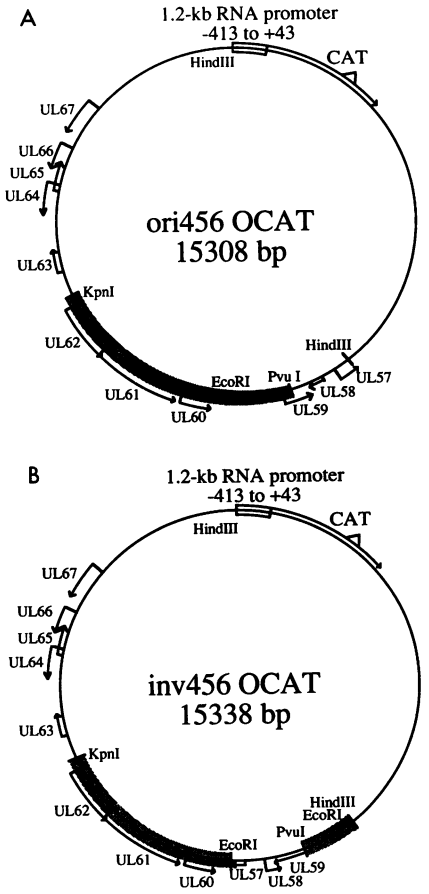

FIG. 1. (A) Map of ori456 OCAT. The open box at the top represents the 1.2-kb RNA promoter from nt −413 to +43 relative to the cap site. It drives expression of the bacterial CAT gene. Sequences extending clockwise from the CAT gene to the *Hin*dIII site on the lower right are identical to those in the parental p456 OCAT (21). Other sequences are derived from the HCMV origin of replication and flanking sequences. The shaded region between the *Kpn*I site and the *Pvu*I site constitutes the minimal origin of replication (14). All known large open reading frames (2) are depicted as arrows to show orientation and the absence of open reading frames stretching into the 1.2-kb RNA promoter region. (B) Map of inv456 OCAT. This construct is identical to ori456 OCAT except that the region between the *Hin*dIII site at the lower right and the *Eco*RI site at the extreme bottom is reversed in orientation, thus separating components of the minimal origin of replication (shaded regions). Also a 30-bp segment from Bluescript II KS- is present between the p456 OCAT sequences and the inverted origin sequences.

The plasmid DNAs for the experiments using unmethylated input DNA were generated by transforming SCS110 (Stratagene) bacteria cells with ori456 OCAT or inv456 OCAT. These bacteria lack both the *dam* and *dcm* methylation activ-

ities present in the *E. coli* DH5α cells that were used to generate the other plasmids. Lack of sensitivity to *Dpn*I was confirmed prior to electroporation into HFF cells.

Additional derivatives of ori456 OCAT and inv456 OCAT, containing various point mutations described previously (21), were made by joining the 9.6-kb ori or inv *Hin*dIII fragment from ori456 OCAT or inv456 OCAT, respectively, to the *Hin*dIII-linearized and CIAP-treated vector containing the proper mutation. In each case, orientation was selected to correspond to that of ori456 OCAT or inv456 OCAT, and the presence of the point mutations was confirmed by sequencing of the promoter region. Sequencing was performed with the Sequenase sequencing kit (United States Biochemical) as recommended by the manufacturer. The primer used has the sequence 5′-AGC GGG GCG TCC GAA G-3′ and anneals to the DNA between nucleotides (nt) +13 and +27.

For Southern analysis, the plasmid DNA was detected by using a construct designated pG0.5 EDCAT. This construct has a 533-bp *Eco*RI-to-*Dpn*I fragment of the CAT gene from pSV01 ligated into the *Eco*RI and *Sma*I sites of pGem-1, respectively. To generate a probe for hybridization, the pG0.5 EDCAT construct was digested with *Eco*RI and *Bam*HI, and the 537-bp fragment was isolated by gel purification and labeled with ^{32}P, using either the Boehringer Mannheim or United States Biochemical random priming kit as recommended by the manufacturer.

For detection of CAT reporter mRNA transcripts in electroporated cells, a new probe was made. p456 OCAT was digested with *Hin*dIII and *Eco*RI, and the 0.76-kbp fragment was isolated by gel purification and ligated into pGem-1 that had been similarly digested and treated with CIAP. The resulting construct was designated p456 GEMCAT. p456 GEMCAT was linearized with *Ava*I and used to generate ^{32}P-labeled riboprobes 282 nt in length, using T7 RNA polymerase and the Promega riboprobe kit. This riboprobe was found to generate far less background than previous constructs used to detect CAT mRNA, as a result of the absence of viral sequences in the riboprobe.

Electroporation experiments. HFF cells were trypsinized and replated at a density of 3.5×10^6 cells per 15-cm-diameter tissue culture dish the day prior to electroporation. On the day of the experiment, the cells were collected by trypsinization and pelleted by low-speed centrifugation. Cells were washed one time in phosphate-buffered saline or OptiMEM (Gibco-BRL) and resuspended at 10^7 cells per ml in OptiMEM. The DNAs to be used were aliquoted into the cuvettes, usually in a volume of 10 μl of reporter (ori456 OCAT or inv456 OCAT, or their derivatives, at 100 ng/μl) and 3.75 μl of pGem-1 at 400 ng/μl as a control for electroporation efficiency. HFF cells (0.5 ml) were added to each cuvette and pulsed once at 1.5 kV/cm and 300 μF, using a BTX Transfector 300 electroporation system and type 620 cuvettes with a 2-mm gap. The cells were removed at once from the cuvette and divided equally between two 15-cm-diameter tissue culture dishes.

Electroporated cells were infected at 24 h postelectroporation, at a multiplicity of infection of approximately 5. The plating protocol ensured that the cells were at a density that was optimal for efficient infection. In some experiments, cells were maintained in the presence of 40 or 400 μM ganciclovir (Syntex).

Times of harvest were as indicated in the relevant figure. Cells were harvested for DNA analysis and CAT analysis as described previously (21). CAT analysis was performed as previously described (4).

DNA analysis. The extracted DNA (10 μg) was digested first with *Hin*dIII, and then half (5 μg) was removed and digested with *Dpn*I. The digested DNAs were separated electrophoretically and transferred to nylon membranes (Nytran; Schleicher & Schuell). These filters were hybridized to the random-primed, [α-^{32}P]dCTP-labeled p0.5 EDCAT fragment described above and similarly labeled pGem-1. Hybridization and washing conditions were as previously described (21).

In addition, a small aliquot (400 ng) of linearized DNA was saved for slot blot analysis. The DNA was blotted on a Schleicher & Schuell Minifold apparatus, using the protocol recommended by the manufacturer.

RNA analysis. CAT results were confirmed by direct analysis of the CAT mRNAs in the electroporated cells. Total cell RNA was purified by using the Promega RNAgents Total RNA isolation system and the protocol recommended by the manufacturer. Treatment of the RNA with DNase I was found to be necessary to eliminate all DNA. This was done by resuspending the RNA in DNase buffer (10 mM Tris [pH 7.6], 1 mM EDTA, 1 mM magnesium acetate) and adding approximately 130 U of DNase I (Gibco-BRL). Incubation was for 30 min at 37°C, followed by purification on a G-50 spin column and precipitation with sodium acetate and ethanol. Prior to use, the RNA was quantitated by spectrophotometry and divided into 20- or 40-μg aliquots.

RNA slot blot analysis was performed as previously described (21). RNase mapping was also done as previously described (21), except that *Ava*I-linearized p456 GEMCAT was used to generate the riboprobe.

Northern (RNA) analysis. HFF cells were infected with HCMV in the absence or presence of 40 or 400 μM ganciclovir, and the cells were refed daily with medium containing freshly added drug. At various times postinfection (p.i.), RNA was purified from approximately 10^7 cells per condition, using the RNAgents Total RNA isolation system (Promega) and the protocol recommended by the manufacturer. The PolyATtract isolation system (Promega) was used to select poly(A) RNA. Two micrograms of poly(A)-selected RNA was electrophoretically separated on a gel of 1% agarose in 1× 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer (40 mM MOPS [pH 7.0], 50 mM sodium acetate, 1 mM EDTA) and 2.2 M formaldehyde and transferred to a 0.45-μm-pore-size nylon membrane. The membrane was prehybridized at 42°C for 4 h in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–150 mg of low-molecular-weight herring sperm DNA per ml. The membrane was probed with a 5.3-kbp, ^{32}P-labeled, random-primed fragment from HCMV *Eco*RI fragment O, bounded by *Pst*I and *Eco*RI sites. The *Eco*RI site is the one forming the W/O border. The hybridization solution was the same as the prehybridization solution except for the addition of 1/5 volume of 50% dextran sulfate and the labeled probe. Washes were two times in 6× SSPE–0.1% SDS at room temperature with shaking for 15 min each time, two times in 1× SSPE–0.1% SDS at 37°C for 15 min each time, and one time in 0.1× SSPE–0.1% SDS at 60° for 1 h. Prior to autoradiography, the membranes were rinsed quickly several times with 0.1× SSPE to eliminate any residual SDS. Results were quantified with a Molecular Dynamics PhosphorImager.

RESULTS

Induction of plasmid replication by HCMV infection. To determine if the HCMV origin of replication was an essential *cis*-acting element involved in the late induction of the 1.2-kb RNA promoter, a construct was made which included this region on the p456 OCAT reporter plasmid used previously to

characterize the regulation of the 1.2-kb RNA promoter (21). This construct, designated ori456 OCAT, is diagrammed in Fig. 1A. The 1.2-kb RNA promoter is shown as an open box at the top of the figure. The shaded region represents the sequence subsequently shown to be the minimal origin of replication (1, 5, 14). All known long open reading frames are marked with arrows (2).

A second construct was created to act as an important control in these experiments. The inv456 OCAT construct, diagrammed in Fig. 1B, has all of the same sequences as ori456 OCAT plus 30 nt derived from a Bluescript II KS- polylinker, but the region derived from HCMV AD169 *Eco*RI fragment U is reversed in orientation relative to ori456 OCAT and the viral genome. (Note the orientation of the UL59 open reading frame for comparison.) This separates as yet unidentified components of the minimal origin of replication, disabling its function as an origin.

A time course experiment was performed to compare replication of the viral genome with that of electroporated ori456 OCAT and inv456 OCAT constructs. HFF cells were electroporated with 1 μg of the ori456 OCAT or inv456 OCAT construct, along with 0.75 μg of pGem-1 to act as a control for electroporation efficiency and plasmid loss. At 24 h after electroporation, the cells were infected with HCMV at a multiplicity of infection of 5. RNA, DNA, and protein were harvested from the same experiment at various times p.i. to allow quantitation of replication and gene expression from the viral and plasmid genomes.

A slot blot analysis was performed on the ori456 OCAT time course to assess viral DNA replication. Total DNA was digested with *Hin*dIII, serially diluted, and transferred to a nylon membrane. Serial fivefold dilutions began at 250 ng of DNA, with the exception of those for the 96-h-p.i. DNA, which began at 10 ng. The membrane was probed with a 955-bp *Pvu*II-to-*Sma*I segment of the 1.2-kb RNA coding region. The results are shown in Fig. 2A. The 14- and 24-h time points reflect the input level of DNA from the infecting virus, prior to significant replication. A fivefold increase in the level of viral DNA takes place between 24 and 48 h p.i. A further 25-fold increase in viral DNA takes place between 48 and 96 h. The electroporated but uninfected control cells harvested at the same time as the 96-h-p.i. samples show no detectable signal, indicating that this probe is specific for the viral sequences and does not detect plasmid sequences. The amount of viral DNA detected was similar for the inv456 OCAT electroporation time course (data not shown).

The extent of plasmid DNA replication was assessed by a *Dpn*I sensitivity assay. *Dpn*I cleavage requires methylation of its recognition sequence, G^5m-ATC, and since the input plasmid DNA was synthesized in Dam$^+$ DH5α *E. coli* it is sensitive to digestion by *Dpn*I. Plasmid DNA which is replicated in the HFF cells is hemi- or unmethylated, leading to a loss of sensitivity to *Dpn*I. Replication of the ori456 OCAT construct, but not the inv456 OCAT construct, is demonstrated in Fig. 2B. Each lane contains 5 μg of total DNA digested with *Hin*dIII (lanes H) or *Hin*dIII and *Dpn*I (lanes D). The membrane was probed with a 537-bp fragment of the CAT gene isolated from plasmid pG0.5 EDCAT. The CAT gene is present on a single 5.7-kbp *Hin*dIII fragment of both the ori456 OCAT and inv456 OCAT plasmids. Complete digestion of these plasmids with *Hin*dIII and *Dpn*I generates a 1.1-kbp fragment containing CAT sequences complementary to the probe.

The inv456 OCAT plasmids electroporated into cells infected for 48 or 96 h remained susceptible to digestion with *Dpn*I, indicating that replication had not occurred. In contrast,

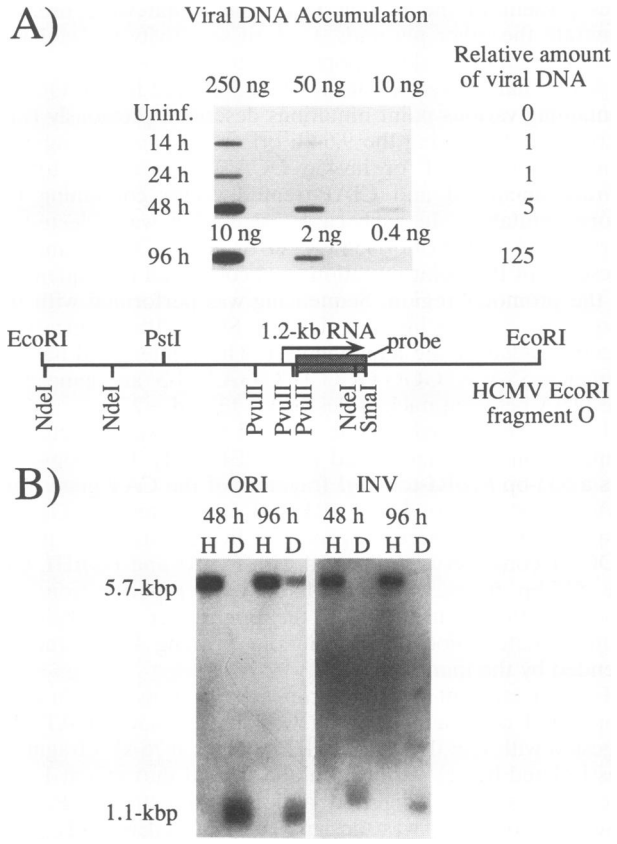

FIG. 2. Temporal analysis of viral and plasmid DNAs. HFF cells were electroporated with ori456 OCAT and mock infected (Uninf.) or infected with HCMV 24 h later. Cells were harvested at the indicated times p.i., and DNA was isolated. (A) DNAs digested with *Hin*dIII were serially diluted in fivefold increments, beginning with 250 ng for all except the 96-h sample, for which dilution began at 10 ng, and slot blotted onto a Nytran filter. The filter was probed with a 955-bp *Pvu*II-to-*Sma*I segment of HCMV *Eco*RI fragment O to detect the abundance of viral templates capable of coding for the 1.2-kb RNA. The probe used is diagrammed below blot. (B) The same DNAs from the 48- and 96-h time points were digested with *Hin*dIII (lanes H) or *Hin*dIII and *Dpn*I (lanes D), separated on 0.8% agarose gels, transferred to a nylon membrane, and probed with a 535-bp segment of the CAT gene to detect the replication status of the electroporated plasmid DNA. The same procedure was followed for cells electroporated with inv456 OCAT. The 5.7-kbp band corresponds to the *Hin*dIII fragment containing the CAT gene, while the 1.1-kbp band is the *Hin*dIII-*Dpn*I digestion product for unreplicated templates. The 5.7-kbp band at 96 h in lanes D reflects replicated plasmid DNA.

the ori456 OCAT plasmids were susceptible to digestion at 48 h, but *Dpn*I-resistant plasmids were observed at 96 h. However, approximately half of the ori456 OCAT plasmids remained sensitive to *Dpn*I, indicating that some but not all templates underwent replication. Ori456 OCAT is replicated during the same time interval in which the viral templates underwent their largest amplification. However, the fact that ori456 OCAT undergoes at most a 2-fold amplification, while during this same interval an approximately 25-fold increase in viral DNA was observed, indicates that ori456 OCAT replicates less efficiently than the viral genome or that only a small fraction of the input plasmid is efficiently replicated.

Temporal expression of the 1.2-kb RNA promoter on the viral genome and plasmid constructs. The temporal kinetics of

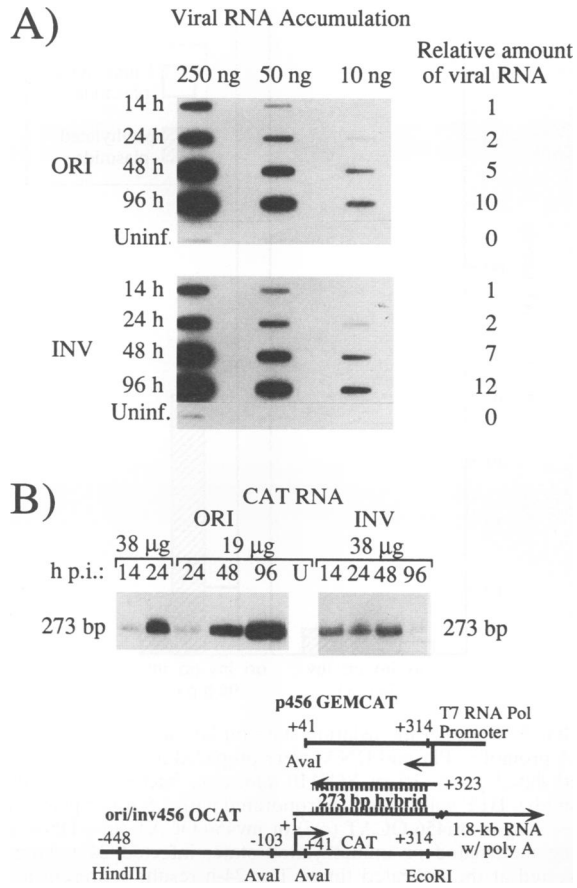

FIG. 3. Temporal analysis of viral and hybrid CAT RNAs. (A) HFF cells were electroporated with ori456 OCAT or inv456 OCAT, infected with HCMV or mock infected (Uninf.) 24 h later, and harvested for total RNA at the times indicated. The mock-infected samples were harvested at the same time as the 96-h-p.i. samples. RNAs were serially diluted in fivefold increments and slot blotted onto a Nytran filter. The filter was probed with the a 955-bp *Pvu*II-to-*Sma*I fragment of HCMV *Eco*RI fragment O (see Fig. 2A) to detect the virally encoded 1.2-kb RNA. The blot was scanned, and the relative amount of viral RNA detected is indicated at the right. (B) The same RNA samples were hybridized to 4×10^5 cpm of riboprobe derived from T7 RNA polymerase (Pol)-mediated transcription of p456 GEMCAT plasmid linearized with *Ava*I and gel purified. Hybridization reactions were digested with RNase T_1 and run on denaturing gels. The band represents the 273-bp protected fragment of the CAT mRNA, which is diagrammed at the bottom. This figure represents a composite of two gels run with RNA from the same electroporation. The 24- to 96-h samples from ori456 OCAT electroporated cells are from one gel, using 19 µg for each hybridization, while all other samples are from a second gel, using 38 µg for each hybridization. Autoradiography of the dried gels was for 5 (for 38-µg samples) or 10 (for 19-µg samples) days.

the 1.2-kb RNA transcribed from the superinfecting viral genomes are shown in Fig. 3A. This slot blot analysis used total RNA from the ori456 OCAT and inv456 OCAT time course experiments described above. Serial fivefold dilutions began at 250 ng for all time points. For this slot blot, the 955-bp *Pvu*II-to-*Sma*I probe from the 1.2-kb RNA transcribed region (Fig. 2A) was used to detect the 1.2-kb RNA coded for by the superinfecting viral genomes. It is evident that the kinetics of accumulation of viral RNA were similar for both the ori456 OCAT and inv456 OCAT time course experiments. The viral 1.2-kb RNA is readily detectable by 14 h p.i. and increases almost twofold by 24 h, two- to threefold more by 48 h, and another twofold by 96 h p.i. Only a low level of background hybridization was detected for the uninfected control samples. The results were consistent with our previous studies, which showed that the 1.2-kb RNA continues to increase its rate of synthesis throughout the period of viral DNA replication.

Total RNA harvested from the same experiment was used in an RNase protection assay in the amounts indicated in Fig. 3B, to quantify the level of CAT mRNA transcribed from the electroporated plasmids. The total RNA was hybridized with a 282-nt, ^{32}P-labeled riboprobe generated by T7 RNA polymerase-mediated transcription from an *Ava*I-linearized p456 GEMCAT plasmid. The 273-bp protected hybrid is diagrammed at the bottom of Fig. 3. The products of the hybridization reactions were digested with RNase T_1 and run on a 4% denaturing acrylamide gel. The figure is a composite of two gels run with RNA prepared from the same experiment. For comparison, the 24-h time point is shown twice for the ori456 OCAT electroporated cells, once from each gel, and the level of CAT mRNA was assumed to be the same in subsequent quantitation.

In HFF cells electroporated with ori456 OCAT, a low level of CAT mRNA is detected at 14 h p.i. CAT mRNA levels are approximately 4-fold higher by 24 h p.i. and then increase almost 10-fold by 48 h p.i. Between 48 and 96 h p.i., another threefold increase in CAT mRNA takes place. The major increase between 24 and 96 h p.i. correlates with the peak of viral replication in this experiment (Fig. 2A). Although ori456 OCAT plasmid replication is not detectable until 96 h p.i. (Fig. 2B), a low level most likely occurs before 48 h p.i. These results suggest that even a low level of replication is sufficient to produce a substantial increase in the transcription rate of the CAT mRNA.

The results presented above contrast with those from cells electroporated with inv456 OCAT. CAT mRNA is detected from the inv456 OCAT templates at 14 h p.i. but does not increase substantially thereafter. An almost fourfold decline in CAT mRNA levels is observed between 48 and 96 h p.i. These results correlate well with our previous results for the parental p456 OCAT plasmid, which also reached peak mRNA levels early and declined at late times (21). The decline at late times is most likely due to competition from the highly amplified viral templates.

PhosphorImager analysis of the data presented in Fig. 3B is shown in Fig. 4. Results are shown as a percentage of the CAT mRNA detected at the 96 h p.i. time point of the ori456 OCAT time course. Also shown are the results of the CAT enzyme activity assay performed with samples from the same time course experiments. Amounts of extract that produced results in the linear range of the assay were used.

CAT enzyme activity resulting from the ori456 OCAT construct increased approximately threefold between 14 and 24 h p.i., fivefold between 24 and 48 h p.i., and ninefold between 48 and 96 h p.i. The results show that the inv456 OCAT plasmid was slightly more active than the ori456 OCAT plasmid at 14 h p.i. However, CAT activity was stable between 14 and 24 h p.i. for the inv456 OCAT plasmid and increased only 1.5-fold by 48 h p.i. The drop in CAT mRNA levels measured between 48 and 96 h p.i. was not reflected in the level of CAT activity, which remained essentially stable during this interval.

The temporal induction pattern of ori456 OCAT-encoded CAT mRNA, measured by RNase protection and CAT enzyme activity, is similar to that of the viral mRNA, differing mainly in its greater magnitude of the late induction but not in

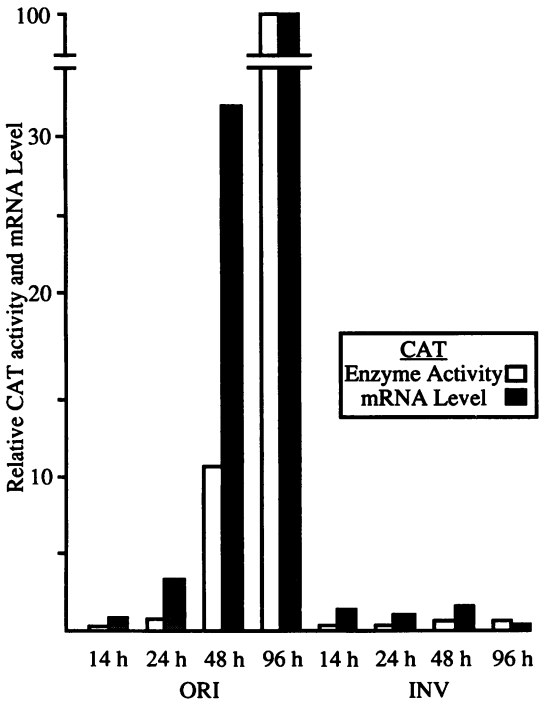

FIG. 4. Correlation between CAT enzyme activity and CAT mRNA from ori456 OCAT and inv456 OCAT time course experiments. CAT mRNA levels from Fig. 3B were quantified and expressed as a percentage of the amount of CAT mRNA measured from the 96-h-p.i. time point of the ori456 OCAT time course. Results are presented as the gray bars. CAT enzyme activity was measured for each of the samples, using an amount of protein extract that produced results in the linear range of the assay. Results were similarly expressed as percentages of the ori456 OCAT value at 96 h p.i. and are represented by open bars.

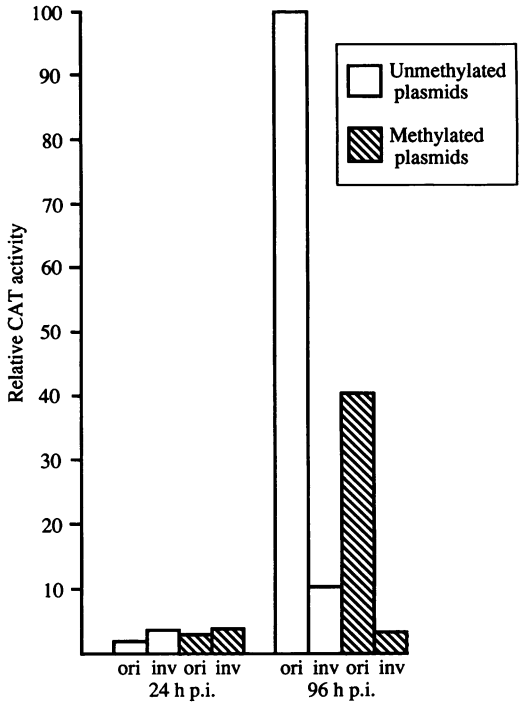

FIG. 5. Effect of methylation state on late induction of the 1.2-kb RNA promoter. Plasmid DNAs were prepared in either *E. coli* DH5α (methylated plasmids) or SCS110 *dam dcm* bacteria (unmethylated plasmids). HFF cells were electroporated with 1.5 μg of pGem-1 plus either 2 μg of ori456 OCAT (ori) or inv456 OCAT (inv) DNA, either in the methylated or unmethylated state, infected 24 h later, and harvested at the indicated times. The 24-h results represent a single sample, while the 96-h results represent averages of duplicate samples. CAT enzyme activity is represented as a percentage of the value measured in the unmethylated ori456 OCAT plasmid electroporated cells harvested at 96 h p.i. The amount of extract used in each assay was such that the values of CAT activity were in the linear range.

its timing. In contrast, the inv456 OCAT-encoded CAT mRNA shows no induction beyond levels reached by 14 h p.i., and even the CAT enzyme activity peaked by 48 h p.i. at levels only slightly higher than at 14 h p.i. If the induction of the 1.2-kb RNA promoter at late times in the infection was due to a late gene product capable of *trans* activating the promoter, it should have had an equal stimulatory effect on the inv456 OCAT plasmid. Since this is not the case, we conclude that the late induction likely occurs only on templates capable of replication.

Measurement of CAT activity indicates that its temporal induction pattern accurately reflects that of the CAT mRNA, albeit with a slight delay. Since CAT activity is easier to measure and reflects the pattern of transcription of the 1.2-kb RNA promoter, subsequent experiments were done using CAT activity as the measure of promoter transcription rate.

Effect of methylation on gene expression directed by the replicating and nonreplicating plasmids. In the experiments described above, the input constructs ori456 OCAT and inv456 OCAT were methylated on adenosine within the sequence GATC. However, in the eukaryotic cell, as the plasmid replicated, this site would no longer be methylated on the progeny DNA. To rule out the possibility that the differential gene expression from the replicating and nonreplicating plasmids was due to a change in the methylation state itself, ori456 OCAT and inv456 OCAT plasmid DNAs were isolated from SCS110 *dam dcm* bacteria. These DNAs were electroporated into HFF cells, while a parallel experiment made use of the methylated ori456 OCAT and inv456 OCAT plasmid DNAs. Cells were harvested at 24 and 96 h p.i. Results of the CAT enzyme activity assay are shown in Fig. 5, with all values expressed as a percentage of the activity of the unmethylated ori456 OCAT plasmids at 96 h p.i.

The level of CAT expression directed by the 1.2-kb RNA promoter was essentially the same for both ori and inv template configurations and both methylation states at 24 h p.i. Expression of the 1.2-kb RNA promoter from the unmethylated ori456 OCAT and inv456 OCAT templates was approximately 2.6-fold higher than that from the methylated templates at 96 h p.i. However, DNA analysis revealed that the increased expression was matched by an approximately twofold-higher level of unmethylated templates detected in the infected cells at 96 h p.i. (data not shown). This effect was not replication dependent, since the nonreplicating inv456 OCAT templates showed an equivalent increase in transcription and template number. The replication-dependent late induction is reflected by the ratio between the expression of ori456 OCAT construct and the inv456 OCAT construct. Since this ori/inv ratio was approximately 10.5 for both the methylated and unmethylated templates at 96 h p.i., it appears that no significant change in the replication-dependent late induction occurred due to the change in methylation state.

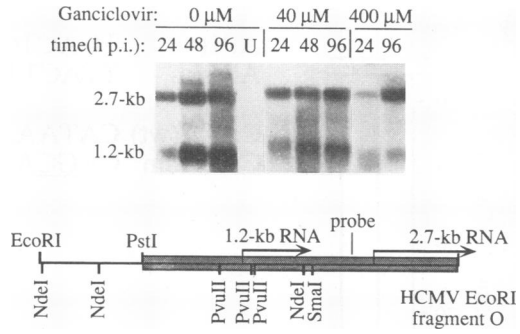

FIG. 6. Northern experiment showing differential effects of ganciclovir on the 1.2- and 2.7-kb early RNAs. HFF cells (10^7 per condition) were infected with HCMV at multiplicity of infection of 5 and harvested for total RNA at the times indicated. Lane U was from mock-infected cells, harvested at the same time as the 96-h-p.i. samples. Cells were maintained in the absence or presence of 40 or 400 μM ganciclovir, an inhibitor of the HCMV DNA polymerase, as indicated. Poly(A) RNA was purified from the total RNA harvests, and 2 μg of each was run on a 1% agarose–formaldehyde gel and transferred to a nylon membrane. The membrane was probed with a 5.3-kbp *Pst*I-to-*Eco*RI segment of HCMV *Eco*RI fragment O, diagrammed below, which detects both the 1.2- and 2.7-kb early RNAs.

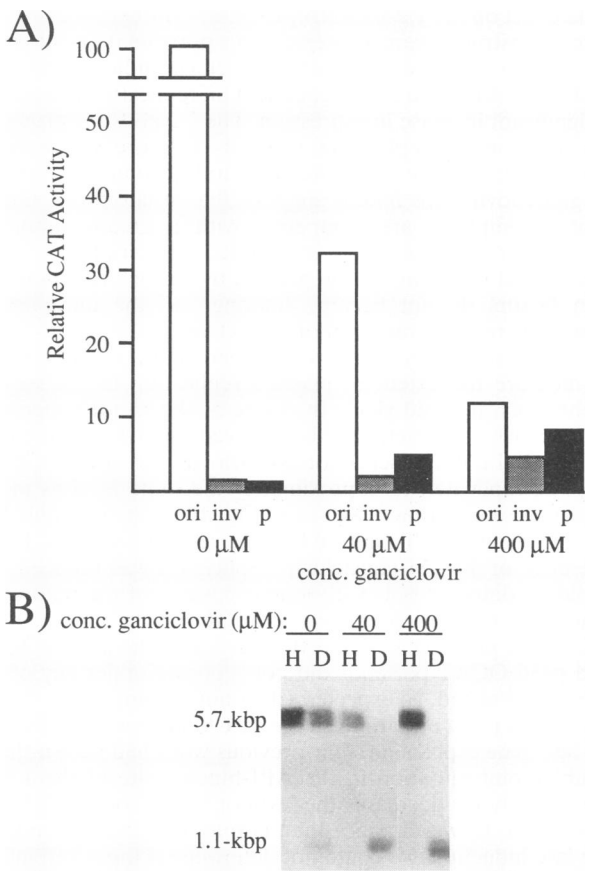

FIG. 7. Effects of ganciclovir on the late induction of the 1.2-kb RNA promoter on plasmid constructs. HFF cells were electroporated with 1.5 μg of pGem-1 plus 2 μg of plasmid ori456 OCAT (ori), 2 μg of inv456 OCAT (inv), or 0.74 μg of p456 OCAT (p), infected with HCMV 24 h later, and harvested at 96 h p.i. Cells were maintained in the absence or presence of 40 or 400 μM ganciclovir, and medium with fresh drug was added daily. (A) CAT enzyme activity results are shown as a percentage of that measured from ori456 OCAT electroporated cells at 96 h p.i. in the absence of ganciclovir and represent averages of duplicate samples. The amount of extract used in each assay was such that the values of CAT activity were in the linear range. (B) Total cell DNA was purified from the electroporated and infected cells, digested with *Hin*dIII (lanes H) or *Hin*dIII and *Dpn*I (lanes D), separated electrophoretically, transferred to a nylon membrane, and probed with a 535-bp segment of the CAT gene. Only results for the ori456 OCAT electroporated cells are shown.

The late induction of the 1.2-kb RNA promoter from the ori456 OCAT construct is dependent on DNA replication. In a previous report, we showed that PAA, an inhibitor of the viral DNA polymerase, could be used to block the late induction of the 1.2-kb RNA promoter encoded by the viral genome (21). In this study, we used ganciclovir, which is also an inhibitor of the viral DNA polymerase but is less toxic to the cells than PAA, to show that the late phase of induction of both the ori456 OCAT plasmid and the viral genomic 1.2-kb RNA was susceptible to inhibition.

We first performed a Northern (RNA) experiment to determine the effects of ganciclovir on the temporal expression patterns of the two major HCMV early RNAs of 2.7 and 1.2 kb coded for by the long terminal repeats of the viral genome. Previously we have shown that expression of the 1.2-kb RNA but not the 2.7-kb RNA was inhibited by PAA at late times p.i. HFF cells were infected with HCMV in the presence or absence of 40 or 400 μM ganciclovir. Poly(A)-selected RNA was prepared at 24, 48, and 96 h p.i. except for the 400 μM ganciclovir time course, which lacked the 48 h p.i. time point. Two micrograms of this poly(A)-selected RNA was used in a Northern analysis, and the results are shown in Fig. 6. A small amount of variation in the level of 2.7-kb RNA can be detected prior to 96 h, but at the later time point all three ganciclovir conditions show equivalent amounts of 2.7-kb RNA. In contrast, a clear effect of ganciclovir can be noted on the expression of the 1.2-kb RNA at late times. At 48 and 96 h p.i., 40 μM ganciclovir partially inhibited the late induction of the 1.2-kb RNA, while 400 μM ganciclovir was sufficient to completely inhibit the late induction of the 1.2-kb RNA. Thus, 400 μM ganciclovir blocks the replication-dependent late induction of the 1.2-kb RNA while having no significant effect on the expression of the nearby replication-independent 2.7-kb RNA at late times p.i.

If the ori456 OCAT plasmid was correctly reflecting the temporal expression of the 1.2-kb RNA, it should also be subject to inhibition by ganciclovir. We tested this hypothesis by electroporating HFF cells with ori456 OCAT, inv456 OCAT, or p456 OCAT (21), infecting them with HCMV 24 h later, and harvesting for DNA and protein at 96 h p.i. The cells were maintained in the absence or presence of 40 or 400 μM ganciclovir, as indicated. Results are shown in Fig. 7A. Values are shown as a percentage of the CAT enzyme activity for the ori456 OCAT construct in the absence of ganciclovir and represent averages of two assays.

In this experiment, the ori/inv ratio in the absence of ganciclovir was 75, somewhat higher than in previous experiments, reflecting a particularly good infection. The ori/inv ratio dropped to 15 in the presence of 40 μM ganciclovir and to 2.5 in the presence of 400 μM ganciclovir. This indicates a precipitous decrease in the replication-dependent late induction of the 1.2-kb RNA promoter mediated by ganciclovir.

CAT enzyme activities for inv456 OCAT and p456 OCAT are similar under all conditions tested, indicating that the 9.6 kbp of sequence present in the inv456 OCAT construct has

little effect on the 1.2-kb RNA promoter. Most significantly, all three constructs were expressed to approximately the same level in the presence of 400 μM ganciclovir, indicating that the ori456 OCAT construct is dependent on replication to undergo a significant increase in expression. The 1.2-kb RNA promoter encoded on nonreplicating plasmids is more active when amplification of the superinfecting viral genomes is inhibited by ganciclovir. This finding suggests that at late times, unreplicated templates are competing with a greatly amplified number of viral genomes for limited transcription factors. When viral replication is suppressed by ganciclovir, transcription factors do not become limiting and the unreplicated templates remain transcriptionally active.

A *Dpn*I sensitivity assay, as described above, was performed to measure the extent of plasmid replication in this experiment. Each plasmid yields the same 5.7-kb *Hin*dIII digestion product, which, if methylated, is cleaved to a 1.1-kbp fragment by *Dpn*I. In the absence of ganciclovir, the ori456 OCAT plasmid replicated to approximately the same level as in the time course experiment shown in Fig. 2B. Approximately half of the ori456 OCAT plasmid became resistant to *Dpn*I. In the presence of 40 μM ganciclovir, replication was reduced and could be detected only with long exposures of the autoradiograph. No replication of the ori456 OCAT plasmid is detectable in the presence of 400 μM ganciclovir. The inv456 OCAT and p456 OCAT plasmids did not replicate under any of the conditions tested, as expected (data not shown).

Effect of point mutations in the AP1-binding and CATA sites on late gene expression. Our previous work had shown that a double-point mutation in the AP1-binding site of the 1.2-kb RNA promoter decreases the level of expression by eightfold at 48 h p.i. However, since the plasmid used did not undergo the late induction, we could not determine if the AP1-binding site continues to be an important *cis*-acting site at late times in the temporal expression of the 1.2-kb RNA promoter. The AP1-binding site double-mutation construct was used as the starting point for construction of ori456 and inv456 constructs similar to the wild type but incorporating the point mutations. These were designated ori456 AP1dm and inv456 AP1dm. In addition, a double-point-mutation construct which mutated the CATAAA site to CAGCAA was cloned into the ori and inv constructs, to determine if the CATAAA site continues to function throughout the course of infection.

These constructs were electroporated into cells, infected 24 h later with HCMV, and harvested at 24 or 96 h p.i. The exact nature of the mutations and results of the CAT enzyme assay on the 96-h samples are shown in Fig. 8A. Results of the CAT assay are expressed as a percentage of the activity of the ori456 OCAT (wild-type) sample. Figure 8B shows the results of the *Dpn*I sensitivity assay of DNA isolated from the cells electroporated with the point mutation constructs. As expected, in each case, the ori constructs showed no significant replication at 24 h p.i. but did show significant replication by 96 h p.i. The inv constructs showed no evidence of *Dpn*I-insensitive plasmid at 24 or 96 h p.i., indicating that no replication had taken place (data not shown).

The point mutations are known to act negatively on the expression of the 1.2-kb RNA promoter, so a decrease in expression is expected. The key determinant of the effect of the mutation(s) on the late induction of the 1.2-kb RNA promoter is the ratio of the activity of the ori to inv plasmid construct. In this experiment, this ratios were 10.5 for the wild-type 1.2-kb promoter, 5.2 for the AP1dm plasmids, and 13.5 for the CATAdm plasmids. Thus, although the ori456 AP1dm plasmid expression is 15-fold less than the wild-type level, the decrease in the inv456 AP1dm expression is nearly as large. Therefore,

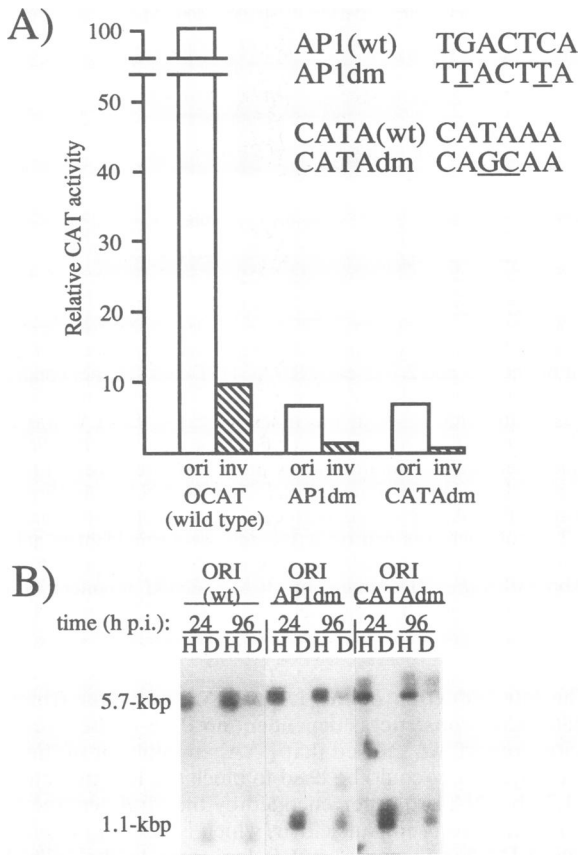

FIG. 8. Effects of point mutations on the late induction of the 1.2-kb RNA promoter. HFF cells were electroporated with 1.5 μg of pGem-1 and 2 μg of one of the following six plasmids: ori/inv456 OCAT (wild type [wt]), ori/inv456 AP1dm (AP1dm), or ori/inv456 CATAdm (CATAdm). Cells were infected with HCMV 24 h later and harvested for protein and DNA at 24 or 96 h p.i. (A) The precise mutations which define each of the point mutation constructs are illustrated at the upper right, with the mutated bases underlined. The CAT enzyme activity for each sample at 96 h p.i. is expressed as a percentage of that measured in ori456 OCAT electroporated cells. (B) *Dpn*I sensitivity assays were performed on the harvested DNAs as described in previous figure legends. Only results for the ori constructs are shown.

the AP1-binding site is still required for efficient transcription at late times but is not essential for the replication-mediated late induction.

Since the presumed function of the CATA site is binding of the TATA-binding protein to initiate RNA transcription, it follows that its function would be required throughout the entire course of infection. The 14-fold drop in measured promoter activity indicates that this site is indeed required for efficient transcription from the 1.2-kb RNA promoter. However, the ori/inv ratio of 13.5 indicates that this promoter is still responsive to replication of the plasmid, even when the CATA site is mutated.

DISCUSSION

Previously, we showed that the important *cis*-acting sequences of the 1.2-kb RNA promoter lie between nt −413 and +43 relative to the start site of RNA transcription (21). While

this construct, p456 OCAT, was inducible by infection with HCMV and featured the early induction of the 1.2-kb RNA, it lacked the characteristic replication-dependent late induction occurring after 24 h p.i. In this study we have shown that the replication-dependent late induction can be conferred on a plasmid construct by inclusion of the HCMV origin of replication.

The construct (ori456 OCAT) used to demonstrate the late induction of the 1.2-kb RNA promoter on a plasmid included 9.6 kbp of sequence derived from HCMV AD169 *Eco*RI fragments I and U. A control plasmid construct (inv456 OCAT) included of all the same sequences, with the addition of a 30-bp plasmid linker, but with the segment derived from *Eco*RI segment U inverted relative to its genomic orientation. Inv456 OCAT does not replicate under conditions that allow ori456 OCAT to replicate (Fig. 2B). The appearance of replicated plasmid, as assayed by *Dpn*I insensitivity, coincides with the largest increase in viral templates, between 48 and 96 h p.i. (Fig. 2A). However, the level of plasmid amplification is much lower than the level of viral genome amplification.

The virally encoded 1.2-kb RNA and the ori456 OCAT-encoded CAT mRNA follow similar temporal induction patterns throughout the entire time course, while inv456 OCAT-encoded CAT mRNA features the early but not the late induction (Fig. 3 and 4). One difference between the viral 1.2-kb RNA and the ori456 OCAT CAT mRNA was the magnitude of the late induction. The viral 1.2-kb RNA increased gradually over the time course, while the plasmid-encoded CAT mRNA showed a 30-fold increase between 24 and 96 h p.i. In addition, the DNA analysis (Fig. 2) indicated a much greater level of template amplification for the viral genomes than for the electroporated ori456 OCAT plasmids. The amount of replicating plasmid electroporated into the cells was low, in an effort not to oversaturate the replicative capacity of the infected cells. However, even at this low level of input DNA, a significant fraction remains unreplicated at 96 h p.i.

To exclude the possibility that the late induction was due to the change in the methylation state of the ori456 OCAT template, we compared the CAT enzyme activities of these methylated plasmids with the activity of plasmids synthesized in *dam dcm* bacteria. The key measure of the replication-dependent late induction is the ratio between the activity of the ori456 OCAT plasmid to that of the inv456 OCAT plasmid. This ratio remained the same for both the methylated and unmethylated plasmids at both 24 and 96 h p.i. (Fig. 5), though the unmethylated plasmids were both 2.6-fold more active at 96 h p.i. This indicated that the change in methylation state does not account for the replication-dependent late induction. The increased activity of the unmethylated plasmids seems to be a function of greater plasmid stability within the cells, though it could also reflect unmasking of a previously unrecognized promoter element operating at late times. However, the stable ori/inv ratio indicates this additional activity is neither synergistic nor cumulative with the replication-mediated effect.

In a previous study, we determined that the late induction of the virally encoded 1.2-kb RNA was subject to inhibition by PAA, a specific inhibitor of the viral DNA polymerase (21). We extended that observation here by using ganciclovir, another inhibitor of the viral DNA polymerase with less toxicity to the host cells, to inhibit the late induction of the 1.2-kb RNA without affecting expression of the nearby 2.7-kb early gene (Fig. 6). Ganciclovir at 400 μM completely blocked the replication-dependent late induction of the 1.2-kb RNA, while 40 μM ganciclovir inhibited but did not block the late expression of the 1.2-kb RNA. Thus, late gene expression was dependent on the level of viral DNA replication.

Having established that ganciclovir can prevent the late induction of the viral 1.2-kb RNA promoter, we showed that ganciclovir is also effective at blocking the late induction of the ori456 OCAT plasmid. Use of 400 μM ganciclovir was sufficient to limit CAT enzyme expression from the ori456 OCAT plasmid to approximately the same level as that from the inv456 OCAT and p456 OCAT plasmids (Fig. 7A). This result confirmed the replication dependence of the late induction of the ori456 OCAT-encoded 1.2-kb RNA promoter. Our finding that both inv456 OCAT and p456 OCAT expressed similar levels of CAT enzyme (Fig. 7A) ruled out a cryptic promoter in the HCMV *Eco*RI fragment I which transcribes through the 1.2-kb RNA promoter and into the CAT gene. These results also indicated that the sequences from the origin of replication do not appear to contain a significant replication-independent enhancer. The only exception to this would be if the enhancer straddled both sides of the *Eco*RI site defining the junction of *Eco*RI fragments U and I.

In the presence of ganciclovir, inv456 OCAT and p456 OCAT were more active than in its absence (Fig. 7A). Ganciclovir suppresses replication of the superinfecting virus, which could indirectly increase expression of the plasmids by lowering competition for limited transcription factors. This finding suggests that transcription factors interacting with the 1.2-kb RNA promoter are limiting at late times in the infection. Alternatively, the factors may have a higher affinity for replicating templates.

Although ganciclovir is effective at inhibiting the 1.2-kb RNA promoter, it is clear from the Northern experiment (Fig. 6) that the nearby 2.7-kb RNA promoter is unaffected. One possibility that would account for this difference is a *cis*-acting sequence present in the 1.2-kb RNA promoter, but lacking from the 2.7-kb RNA promoter, which participates with the replication complex in mediating the late induction. A construct with a mutation in such a sequence should demonstrate a significantly lower ori/inv ratio than the wild-type promoter. We previously identified two important *cis*-acting sequences in the 1.2-kb RNA promoter, a perfect match to the AP1-binding site consensus sequence at −75, and a CATA site at −30, which appears to act as the binding site for the TATA-binding protein. Point mutations in these sites were incorporated into ori456 and inv456 constructs and assayed for CAT enzyme expression (Fig. 8A). Mutation of the AP1-binding site lowered the 1.2-kb RNA promoter activity substantially, but the ori/inv ratio remained approximately 5. Mutation of the CATA sequence also did not alter the late inducibility, but ori456 CATAdm promoter strength was reduced 14-fold compared with the wild-type level. Therefore, it is clear that the AP1-binding site and the CATA site continue to be important determinants of promoter strength throughout the course of infection but do not appear to contribute to the replication-mediated late induction of the 1.2-kb RNA promoter. Thus, if a specific *cis*-acting sequence is responsible for the replication-dependent late induction of the 1.2-kb RNA promoter, it awaits a more extensive analysis.

Several mechanisms have been proposed to account for the effects of the HSV-1 ori$_s$ on promoter-reporter gene hybrid constructs. The results of Johnson and Everett (12) were consistent with a straightforward amplification model, whereby replication generates additional templates available for transcription, and both unreplicated and replicated templates are transcribed at an equivalent rate. However, they also suggested that the replicated templates may be more transcriptionally

active than the unreplicated templates as a result of enhanced access of transcription factors to the open replication complex.

Snowden et al. (18) analyzed the effect of concurrent and nonconcurrent replication on the transcription of three promoter-reporter gene fusion constructs from the early, delayed early, and late classes of HSV-1 genes. When template number was amplified approximately 50- to 100-fold prior to infection, all three promoters showed approximately proportional increased reporter gene activity. However, when replication and transcription were concurrent, the increased reporter gene activity was small compared with the increase in template number, although the promoters seemed to behave appropriately according to their temporal class. They proposed that competition for some dual-functional factor required for both transcription and replication limits promoter expression (18).

In our experiments with the HCMV ori_{Lyt}, we found that a very low level of template replication was sufficient to allow a high level of activation of the 1.2-kb RNA promoter. This makes template amplification unlikely to be the sole mechanism by which late 1.2-kb RNA levels are induced. However, our analysis provides no information on the proportion of templates that are transcriptionally active. The *Dpn*I sensitivity assays used to determine the extent of plasmid template replication indicated that at least 50% of the plasmids do not replicate by 96 h p.i. If only a small fraction of unreplicated templates are transcriptionally active and replication competent, and most or all of their replicated progeny are transcriptionally active, amplification could be the sole mechanism of the late induction. However, if similar fractions of both unreplicated and replicated templates are transcriptionally active, then amplification is insufficient to account for the results that we observed.

The results of Snowden et al. (18) suggest that transcription factors required by HSV at early times are not limiting, since amplification of templates by the simian virus 40 ori did not show evidence of competition for limited factors. Templates harboring the HSV-1 ori, however, showed competition for limiting factors late in the infection cycle. Therefore, it is possible that *trans*-acting factors which are abundant at early times become limiting at late times. A similar mechanism could account for the differential regulation of the 1.2- and 2.7-kb RNAs, if the 2.7-kb RNA reaches a critical limitation imposed by lack of key transcription factors, while the pool of transcription factors utilized by the 1.2-kb RNA promoter is relatively abundant. The increased activity of p456 OCAT in the presence of DNA polymerase inhibitors indicates that a limitation exists on the pool of transcription factors utilized by the 1.2-kb RNA promoter. However, similar experiments with the 2.7-kb RNA promoter suggest that the pool of transcription factors that it requires is even more limited (13). Thus, in the early phases of infection, the number of transcriptionally active templates could impose a limit on the total amount of a given gene product, while in later phases of the infection, there might be competition for scarce transcription factors.

If amplification is not solely responsible for the late induction of the 1.2-kb RNA promoter, then some other aspect of replication must account for the increased transcriptional activity. Passage of the replication complex could alter the balance of positive and negatively acting factors associated with the 1.2-kb RNA promoter. Alternatively, transcription factor(s) which are stably associated with the replication complex could have a dual role, one that is specific for the replication of the viral DNA and a second in which they enhance transcription of the 1.2-kb RNA promoter only when brought into its proximity. Herendeen et al. have established a precedent for such a mechanism. Working with bacteriophage T4, they found that three T4-encoded DNA polymerase accessory proteins stimulate transcription from T4 late promoters. A single-stranded break in the nontranscribed strand is recognized by these proteins, instead of a specific enhancer sequence (6). Experiments with catenanes established that a DNA-tracking mechanism is involved in conveying the transcription-activating signal from the break to the late promoter. In effect, the replisome behaves as a mobile enhancer on T4 late genes (7). We speculate that similar dual-function factors are associated with the eukaryotic replisomes and function to enhance transcription from promoters capable of responding to them. Further experiments should reveal whether the 1.2-kb RNA promoter behaves in this manner.

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REFERENCES

1. **Anders, D. G., M. A. Kacica, G. Pari, and S. M. Punturieri.** 1992. Boundaries and structure of human cytomegalovirus *ori*Lyt, a complex origin for lytic-phase DNA replication. J. Virol. **66:**3373–3384.
2. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
3. **DeMarchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map locations for immediate-early, early and late RNAs. Virology **114:**23–28.
4. **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.
5. **Hamzeh, F. M., P. S. Lietman, W. Gibson, and G. S. Hayward.** 1990. Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. J. Virol. **64:**6184–6195.
6. **Herendeen, D. R., G. A. Kassavetis, J. Barry, B. M. Alberts, and E. P. Geiduschek.** 1989. Enhancement of bacteriophage T4 late transcription by components of the T4 DNA replication apparatus. Science **245:**952–958.
7. **Herendeen, D. R., G. A. Kassavetis, and E. P. Geiduschek.** 1992. A transcriptional enhancer whose function imposes a requirement that proteins track along DNA. Science **256:**1298–1303.
8. **Hutchinson, N. I., R. T. Sondermeyer, and M. J. Tocci.** 1986. Organization and expression of the major genes from the long inverted repeat of the human cytomegalovirus genome. Virology **155:**160–171.
9. **Hutchinson, N. I., and M. J. Tocci.** 1986. Characterization of a major early gene from the human cytomegalovirus long inverted repeat; predicted amino acid sequence of a 30-kDa protein encoded by the 1.2 kb mRNA. Virology **155:**172–182.
10. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD169. J. Virol. **49:**363–370.
11. **Jankelevich, S., J. L. Kolman, J. W. Bodnar, and G. Miller.** 1992. A nuclear matrix attachment region organizes the Epstein-Barr viral plasmid in Raji cells into a single DNA domain. EMBO J. **11:**1165–1176.
12. **Johnson, P. A., and R. D. Everett.** 1986. DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucleic Acids Res. **14:**3609–3625.

13. **Klucher, K. M., D. K. Rabert, and D. H. Spector.** 1989. Sequences in the human cytomegalovirus 2.7-kilobase RNA promoter which mediate its regulation as an early gene. J. Virol. **63:**5334–5343.
14. **Masse, M. J. O., S. Karlin, G. A. Schachtel, and E. S. Mocarski.** 1992. Human cytomegalovirus origin of DNA replication (oriLyt) resides within a highly complex repetitive region. Proc. Natl. Acad. Sci. USA **89:**5246–5250.
15. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology **125:**31–46.
16. **McDonough, S. H., S. I. Staprans, and D. H. Spector.** 1985. Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. J. Virol. **53:**711–718.
17. **Nankervis, G. A., and M. L. Kumar.** 1978. Diseases produced by cytomegaloviruses. Med. Clin. North Am. **62:**1021–1035.
18. **Snowden, B. W., E. D. Blair, and E. K. Wagner.** 1988. Transcriptional activation with concurrent or nonconcurrent template replication has differential effects on transient expression from herpes simplex virus promoters. Virus Genes **2:**129–145.
19. **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.
20. **Tamashiro, J. C., L. J. Hock, and D. H. Spector.** 1982. Construction of a cloned library of the *Eco*RI fragments from the human cytomegalovirus genome (strain AD169). J. Virol. **42:**547–557.
21. **Wade, E. J., K. M. Klucher, and D. H. Spector.** 1992. An AP-1 binding site is the predominant *cis*-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. J. Virol. **66:**2407–2417.
22. **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.
23. **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.
24. **Wilkinson, G. W. G., A. Akrigg, and P. J. Greenaway.** 1984. Transcription of the immediate early genes of human cytomegalovirus strain AD169. Virus Res. **1:**101–106.