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Negative and Positive Regulation by a Short Segment in the 5'-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene†

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To analyze the significance of inducible DNase I-hypersensitive sites occurring in the 5'-flanking sequence of the major immediate-early gene of human cytomegalovirus (HCMV), various deleted portions of the HCMV immediate-early promoter regulatory region were attached to the chloramphenicol acetyltransferase (CAT) gene and assayed for activity in transiently transfected undifferentiated and differentiated human teratocarcinoma cells, Tera-2. Assays of progressive deletions in the promoter regulatory region indicated that removal of a 395-base-pair portion of this element (nucleotides −750 to −1145) containing two inducible DNase I sites which correlate with gene expression resulted in a 7.5-fold increase in CAT activity in undifferentiated cells. However, in permissive differentiated Tera-2, human foreskin fibroblast, and HeLa cells, removal of this regulatory region resulted in decreased activity. In addition, attachment of this HCMV upstream element to a homologous or heterologous promoter increased activity three- to fivefold in permissive cells. Therefore, a *cis* regulatory element exists 5' to the enhancer of the major immediate-early gene of HCMV. This element negatively modulates expression in nonpermissive cells but positively influences expression in permissive cells.

Since tissue specificity of some viruses is controlled at the transcriptional level by enhancer elements (6, 16, 18), we have examined characteristics of the regulatory region of the human cytomegalovirus (HCMV) major immediate-early (IE) gene in permissive and nonpermissive cells. Similar to simian virus 40 (28, 29), polyomavirus (29), and murine sarcoma virus (23), HCMV (9, 17, 21) is unable to grow in undifferentiated teratocarcinoma cells. However, when the cells are induced to differentiate, HCMV can replicate. For murine sarcoma virus (23) and polyomavirus (19), the defect is at the level of transcription in the enhancer element. We (21) and others (17) have shown that the block in HCMV replication is at the transcriptional level in the major IE gene. Further analysis by transfection demonstrates that the inability of this gene to be expressed in undifferentiated cells resides in the 5'-flanking sequence (21). A comparison of the structural features of this regulatory region with the active and inactive IE gene demonstrated the presence of constitutive and inducible DNase I-hypersensitive sites. The inducible DNase I sites which correlate with transcription were located in a region 5' to the enhancer. These results suggest that factors binding outside the enhancer region are necessary to induce the activity of the gene. Recently, footprinting analysis has demonstrated that two of the distal inducible DNase I sites occur in an area bound by nuclear factor 1 (NF-1) (14), a cellular DNA-binding protein originally isolated from HeLa cell extracts (20). The protein may have a multifunctional role in the cell, including replication (20) and gene regulation (15, 25). The presence of these NF-1-binding domains in the distal region of the regulatory sequence suggests that this protein plays a role in expression of the gene.

The HCMV regulatory region, defined by DNase I hypersensitivity, encompasses a region from the transcriptional start at nucleotides +1 to −1145. This sequence contains several different domains, including elements necessary for promoter function (30), a polymerase II transcriptional enhancer (4), and a cluster of NF-1-binding sites adjacent to the enhancer (14). Previous experiments have shown that the block in HCMV replication in human teratocarcinoma cells resides in the 5'-flanking sequence of the major IE gene (21). All of the components of this region described above may contribute to the activation or inactivation of the IE promoter in undifferentiated teratocarcinoma cells. To analyze each of the domains, we made a series of consecutive 5' deletions, using convenient restriction endonuclease sites, and attached these sequences to the chloramphenicol acetyltransferase (CAT) gene (Fig. 1). HCMV sequences which were used to direct expression of the CAT gene were cloned into the polylinker of pUC19 between the *Pst*I and *Bam*HI sites. All of the deleted HCMV regulatory sequences have a common 3' end at the *Sau*3A site at nucleotide +3. The HCMV regulatory elements (REs) were attached to the CAT gene by using a cartridge system designed by Gaffney et al. (8). For transient transfection experiments, the following parts of the HCMV regulatory region were used as promoters for *cat*: *Pst*I-*Sau*3A$_1$ (nucleotides −1145 to +3), *Sau*3A$_2$-*Sau*3A$_1$ (nucleotides −750 to +3), *Hin*cII-*Sau*3A$_1$ (nucleotides −601 to +3), *Hin*fI-*Sau*3A$_1$ (nucleotides −176 to +3), and a construct, *Pst*I-*Hin*cII-*Hin*fI-*Sau*3A$_1$ (nucleotides −1145 to −601 and −176 to +3), which deleted the enhancer. By in vitro transcription in cell-free assays, both the *Pst*I-*Sau*3A$_1$– and *Sau*3A$_2$-*Sau*3A$_1$–*cat* constructs initiate transcription at the proper start site (8a).

Undifferentiated Tera-2 cells which are nonpermissive for expression of the major IE gene were transfected by the calcium phosphate technique (12) with the HCMV-*cat* constructs described above. Promoter activity was measured as a function of CAT enzyme activity by the conversion of [^{14}C]chloramphenicol to its acetylated derivatives (10). Results of the undifferentiated Tera-2 cell transfection experiments are seen in Table 1. The numbers are averaged from

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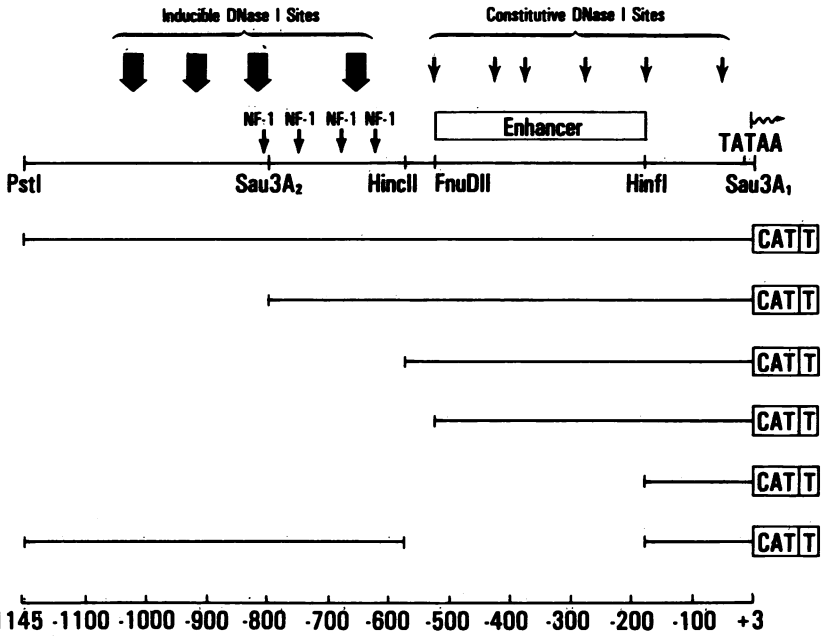

FIG. 1. HCMV IE promoter regulatory region-*cat* constructions: a map demonstrating the relative portions of the constitutive and inducible DNase I-hypersensitive sites in relation to known components of the major IE promoter regulatory region, i.e., the promoter (27), enhancer (4), and NF-1-binding region (15). Solid lines indicate portions of the IE promoter region which were attached to the *cat* gene by using a *Sau*3A$_1$ restriction site located 3 bases 3′ to the start of the mRNA. The promoterless *cat* gene, located on a 773-bp *Taq*I fragment, contains a 100-bp termination fragment (T) isolated from the 3′ end of the IE mRNA-5 region of HSV-1 (8). Numbers across the bottom indicate nucleotides.

three separate transfection assays and represent the percent conversion of chloramphenicol to acetylated forms per minute. The relative activity of the complete regulatory region (*Pst*I-*Sau*3A$_1$) with the promoter, enhancer, NF-1-binding region, and upstream element containing the two inducible DNase I-hypersensitive sites was increased twofold over that of promoterless *cat*. However, deletion of a 395-base-pair (bp) portion of the 5′-flanking sequence (nucleotides −750 to −1145) resulted in an 7.5-fold increase in CAT activity (*Sau*3A$_2$-*Sau*3A$_1$) relative to that with the complete HCMV regulatory region. This activity was retained with deletion of the NF-1-binding region (*Hinc*II-*Sau*3A$_1$), but removal of the enhancer region substantially decreased CAT activity (*Hin*fI-*Sau*3A$_1$ and *Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$).

In addition to the CAT assay, cytoplasmic RNA was isolated from Tera-2 cells transfected with the HCMV-*cat* constructs and the plasmid SV*tk neo* which contains the neomycin gene driven by the herpes simplex virus type 1 (HSV-1) *tk* promoter. Hybridization of RNA dot blots with a *cat* probe confirmed the results obtained with the enzyme assays (Fig. 2). The signal increased 10-fold with deletion of the region distal to the NF-1-binding sites (*Sau*3A$_2$-*Sau*3A$_1$ and *Hinc*II-*Sau*3A$_1$), whereas removal of the enhancer diminished the signal sevenfold (*Hin*fI-*Sau*3A$_1$ and *Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$). Hybridization of an equivalent quantity of RNA with a *neo* probe demonstrated the presence of similar amounts of message in each preparation. These results indicate that the various signals observed with the HCMV regulatory region deletions were not due to transfection artifacts. Therefore, these experiments indicate that a *cis* element 5′ to the NF-1-binding region negatively

TABLE 1. CAT assays for undifferentiated Tera-2 cells

Clone	% Conversion/min (SI)[a]	
	A	B[b]
*Pst*I-*Sau*3A$_1$	0.02 (2)	0.01 (1)
*Sau*3A$_2$-*Sau*3A$_1$	0.17 (17)	0.12 (12)
*Hinc*II-*Sau*3A$_1$	0.17 (17)	0.09 (9)
*Hin*fI-*Sau*3A$_1$	0.02 (2)	0.02 (2)
*Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$	0.03 (3)	0.03 (3)
cat	0.01 (1)	0.01 (1)

[a] Each number represents the conversion of chloramphenicol to its acetylated products per minute and consists of the average of three CAT assays. The range between experiments was less than 1% of the value shown. SI, Stimulation index, representing the increase in expression of HCMV-*cat* constructs relative to that of promoterless *cat*.

[b] Cells were superinfected with HCMV strain AD169 at a multiplicity of infection of 3 for 5 h before harvest at 48 h posttransfection.

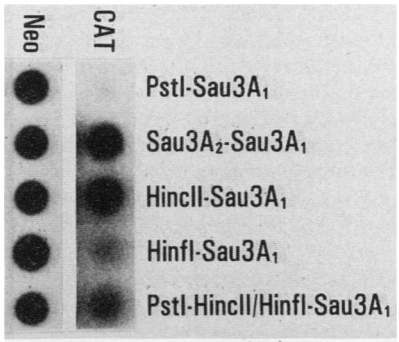

FIG. 2. *cat* RNA levels in transfected undifferentiated Tera-2 cells. RNA dot blot (31) of cytoplasmic RNA extracted from 10^7 undifferentiated Tera-2 cells transfected with 15 μg of the HCMV-*cat* constructs depicted in Fig. 1. Plasmid SV*tk neo* (15 μg) was cotransfected into cells as a control. Each well represents 20 μg of RNA. *cat* and *neo* gene probes were used.

modulates the function of the major IE regulatory region in the nonpermissive undifferentiated Tera-2 cells. In addition, the presence of the enhancer, but not the NF-1-binding region, is necessary for complete promoter function in these cells.

The HCMV IE promoter was shown to be *trans* activated by viral infection (26, 27) and is mediated by a virion component through a sequence 3′ to nucleotide −750 in the IE regulatory region. Although the HCMV major IE regulatory region in permissive cells has significant activity without the presence of viral superinfection, *trans* activation by a virion component is necessary for induction of the HSV-1 IE genes (2, 5). To determine whether a virion protein is necessary for activation of the IE promoter in undifferentiated Tera-2 cells, cells transfected with various HCMV-*cat* constructs were superinfected with virus (Table 1). For these CAT assays, cells were transiently transfected for 48 h and superinfected 5 h before the lysates were harvested. The experiments demonstrate that CAT activity from cells transfected with any of the *cat* plasmids did not change appreciably with or without superinfection. Similar results were obtained when cells were superinfected 16 h before harvest.

Since differentiation of Tera-2 cells results in permissive expression of the major IE gene, we examined the activity of the HCMV-*cat* constructs in these cells. Generally, experiments were done with Tera-2 cells exposed to 10^{-5} M retinoic acid for 7 to 10 days. Although minimal activity was detected in transfected undifferentiated Tera-2 cells with the complete HCMV regulatory region (*Pst*I-*Sau*3A$_1$), a high level of activity was observed in the differentiated cells (Table 2). Consecutive 5′ deletions in this regulatory region resulted in a concomitant decrease in CAT enzyme activity (*Sau*3A$_2$-*Sau*3A$_1$, *Hinc*II-*Sau*3A$_1$, and *Hin*fI-*Sau*3A$_1$). The HCMV-cat construct containing the enhancer and promoter (*Hinc*II-*Sau*3A$_1$) demonstrated a 2.5-fold increase in activity over that with the promoter alone (*Hin*fI-*Sau*3A$_1$). However, addition of the NF-1-binding region and upstream RE to the enhancer promoter increased CAT enzyme activity fivefold (*Pst*I-*Sau*3A$_1$). Therefore, although the region defined as the HCMV enhancer increases transcriptional activity, full transcriptional potential is attained only with the addition of the upstream sequences from nucleotides −601 to −1145. Interestingly, the region 5′ to the enhancer also demonstrated enhancerlike qualities by the ability of the 544-bp fragment to increase CAT activity threefold when it was attached to the HCMV promoter (*Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$). These data suggest that the HCMV IE regulatory region is composed of a modular motif of several distinctive regions which separately can enhance promoter function but together are additive in increasing transcription in permissive cells. However, in nonpermissive cells, the same *cis* sequence 5′ to the enhancer negatively modulates transcription.

HCMV superinfection of differentiated Tera-2 cells transfected with these HCMV-*cat* constructs resulted in a two- to threefold increase in CAT activity with all promoter deletions (Table 2). Superinfection elicited no significant difference in the response of any of the deleted HCMV promoters, suggesting a nonspecific increase in transcription due to viral infection.

To determine whether differentiated Tera-2 cells reflect permissive regulation of the major IE promoter, HeLa and human foreskin fibroblast cells were transfected with the HCMV-*cat* constructs. Similar to the situation in differentiated Tera-2 cells, deletion of the 395-bp upstream regulatory element (*Sau*3A$_2$-*Sau*3A$_1$) decreased activity two- to three-

TABLE 2. CAT assays for differentiated Tera-2 cells

Clone	% Conversion/min (SI)[a]	
	A	B[b]
*Pst*I-*Sau*3A$_1$	1.00 (50)	2.69 (44.8)
*Sau*3A$_2$-*Sau*3A$_1$	0.80 (40)	2.39 (39.8)
*Hinc*II-*Sau*3A$_1$	0.50 (25)	1.18 (19.7)
*Hin*fI-*Sau*3A$_1$	0.20 (10)	0.79 (13.1)
*Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$	0.60 (30)	0.96 (16.0)
cat	0.02 (1)	0.06 (1.0)

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.

fold in both human foreskin fibroblast and HeLa cells relative to that for the complete HCMV regulatory region (*Pst*I-*Sau*3A$_1$) (data not shown). In a finding also consistent with the differentiated Tera-2 cell results, when the region 5′ to the enhancer was attached to the promoter (*Pst*I-*Hinc*II–*Hin*fI-*Sau*3A$_1$), activity was increased fivefold relative to that with promoter alone (*Hin*fI-*Sau*3A$_1$).

The enhancement of transcription by the *cis* regulatory sequence prompted us to examine its ability to stimulate heterologous promoter function in cells permissive for IE expression. For these experiments, we fused the 395-bp HCMV RE (nucleotides −750 to −1145) to the 5′ end of the HSV-1 *tk* promoter. Transfection of differentiated Tera-2 cells with this construct resulted in a three- to fourfold increase in CAT activity relative to that with the *tk* promoter alone (Fig. 3). These experiments, consistent with the deletion studies, demonstrate a transcriptional enhancement correlating with the presence of the HCMV RE sequence and show that this activity is not promoter specific.

Negative regulation of viral enhancers in embryonal carcinoma (EC) cells has been reported for murine leukemia virus (11) and polyomavirus (13). The inability of these regulatory regions to function correlates with the inability of the viruses to replicate in these cells. In support of this idea, point mutations in the enhancer of polyomavirus allows this virus to replicate in undifferentiated EC cells (7, 24). Recent evidence suggests that undifferentiated EC cells contain a protein which functions like the adenovirus E1a protein (13). The E1a protein represses the wild-type polyomavirus enhancer (3) but does not repress enhancers with point mutations that allow growth in EC cells. These results indicate that the E1a protein and the repressor activity in EC cells act in a similar fashion by interacting with the same target sequence. We suggest that one possible mode of repression of the HCMV enhancer in human EC cells may follow a similar scenario. The potential target of this repressor protein would be a sequence between nucleotides −775 and −1145.

Although the presence of a repressor protein may account for the lack of HCMV expression, alternatively, the absence of an activator protein may also contribute to the inactivity of the promoter. One potential candidate would be the NF-1 protein which binds upstream of the enhancer region (14). Simian cytomegalovirus, which constitutively expresses a homologous IE gene in undifferentiated Tera-2 cells, contains a region in the regulatory sequence 5′ to the enhancer which is composed of 23 NF-1-binding sites (17), compared to four in HCMV. Simian cytomegalovirus and HCMV have high sequence homology in the proximal (nucleotides −1 to −500) 5′-flanking region of this gene (17). However, the presence of 19 more NF-1 sites in simian cytomegalovirus compared to HCMV suggests that the protein may have a

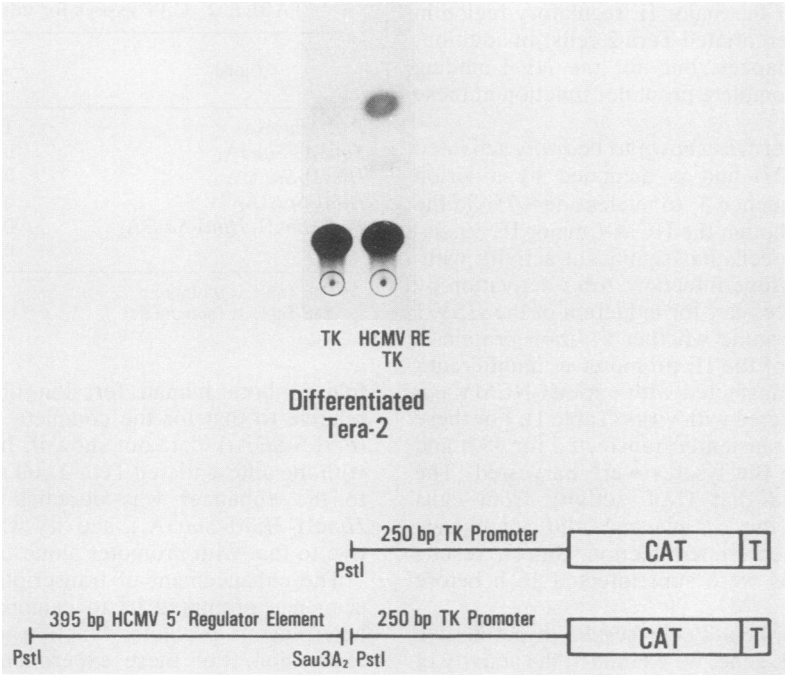

FIG. 3. Heterologous promoter enhancement by the HCMV RE fragment. The 395-bp HCMV RE (nucleotides −1145 to −750) was attached to the 5′ end of the HSV-1 *tk* promoter which controls *cat* expression. Both the HSV-1 *tk cat* and HCMV RE *tk cat* plasmids were transfected into differentiated Tera-2 cells and assayed for CAT activity.

role in activation. Interestingly, analysis of Tera-2 cells for the presence of the NF-1 protein reveals greater than a 10-fold decrease in the amount of this factor relative to that in the differentiated cells (L. Hennighausen, personal communication). Promoter activity was slightly diminished in differentiated Tera-2 cells by deletion of the NF-1 sites. However, the greatest effect of the binding of NF-1 may occur by changing the conformation of the regulatory region to allow binding of positive factors in transcription. Alternatively, binding of NF-1 may displace negative factors which prevent promoter activity. The constitutive activity of the simian cytomegalovirus IE regulatory region may represent a greater availability of NF-1 sites or the lack of the upstream regulatory sequence.

The identification of a dual function *cis* regulatory region 5′ to the enhancer indicates that sequences in addition to those previously described (4, 14, 27) are important in IE expression. In permissive cells, the decrease in activity due to progressive 5′ deletion in the IE promoter regulatory region indicates that several components rather than any one particular segment are involved in transcriptional enhancement. In fact, the *cis* RE by itself was capable of increasing homologous as well as heterologous promoter activity. Combining our current knowledge of this region, we conclude that the 5′-flanking sequence of the major IE gene appears to be composed of several distinct elements. These elements appear to be acting coordinately to contribute to DNA conformations important for accessibility to transcription factors and gene expression.

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