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The Human Cytomegalovirus 2.7-Kilobase RNA Promoter Contains a Functional Binding Site for the Adenovirus Major Late Transcription Factor

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We have examined the factors which influence the expression of a major 2.7-kilobase (kb) early transcript encoded by the long repeat of the human cytomegalovirus (HCMV) strain AD169 genome. Previously, by deletion analysis, we determined that the promoter for this early RNA consisted of multiple *cis*-acting elements (Klucher et al., J. Virol. 63:5334–5343, 1989). Using extracts prepared from HeLa cells as well as from infected and uninfected foreskin fibroblasts, we also obtained evidence for the interaction of a cellular factor with one of these elements. In this study, we have further defined the specificity and functional importance of this binding. On the basis of DNase I footprinting and methylation interference assays, we localized the site of interaction to a region (nucleotides −113 to −106 relative to the mRNA start site) which contains homology to the binding site for the adenovirus major late transcription factor (MLTF), also referred to as the upstream stimulatory factor (USF). The contact points of binding between the cellular factor and the guanine residues within this segment were consistent with the pattern of binding for USF/MLTF. Additionally, by using oligonucleotides containing the binding sites for USF/MLTF from the adenovirus major late promoter and the HCMV 2.7-kb RNA promoter as competitors in gel retardation assays, we were able to show that USF/MLTF bound to the two promoters with similar affinity. Correlation of the binding activity with in vivo functional importance was provided by mutagenesis and transient-expression assays. A point mutation within the HCMV USF/MLTF site lowered the affinity of binding 5- to 10-fold and decreased the inducible activity of the HCMV 2.7-kb RNA promoter by approximately 50%. Furthermore, the addition of the HCMV USF/MLTF site to a minimal 2.7-kb RNA promoter containing only the TATA sequence resulted in an increase in HCMV inducible transcriptional activity of 6- to 20-fold. However, the HCMV USF/MLTF site could not functionally substitute for the TATA sequence. These studies further support the idea that for maximal response to the HCMV infection, the 2.7-kb RNA promoter requires multiple *cis*-acting sequences, two of which include the binding sites for USF/MLTF and TFIID.

Human cytomegalovirus (HCMV) is an important human pathogen causing a diverse array of disease states (38). A member of the herpesvirus family, HCMV contains a DNA genome which is temporally expressed following infection of permissive cells (11, 32, 58, 59). Immediate-early (IE) genes are the first genes expressed and do not require de novo protein synthesis for their transcription (11, 23 24, 32, 53, 58–60). There are at least three transcription units, IE1, IE2, and IE3, encoded within the major immediate-early region of HCMV (11, 21, 23, 50, 51, 53, 58–60). Expression of the IE1 and at least some of the IE2 gene products is mediated via a strong enhancer element which is responsive to both virus-specific and cellular *trans*-acting factors (8, 13–15, 20, 22, 25, 40, 41, 45, 52).

Early genes are the next class of genes to be expressed and require one or more HCMV IE gene products for their transcription (6, 48). Although numerous HCMV RNAs are expressed at early times after infection, many appear to be subject to different regulatory controls. In this regard, at least three differential patterns of early-gene expression can be identified as the infection progresses. A 2.7-kilobase (kb) RNA expressed from the long repeat of the genome (*Eco*RI fragments O and W in strain AD169; Fig. 1) is representative of one group. It is detected at early times postinfection and continues to increase in abundance until the midpoint of the infection (after viral DNA replication begins) (33). In contrast, a second early RNA, 1.2 kb in size, shows a different temporal control. This transcript, which is also encoded within the long repeat, demonstrates a major increase in abundance from the midpoint until late in the infection (33). Finally, a third group, represented by a family of transcripts encoded by HCMV AD169 *Eco*RI fragments R and d, is detected early after infection, with its steady-state levels remaining approximately the same throughout the infection (49).

We have been studying the sequence requirements and *trans*-acting factors necessary for the regulated expression of the HCMV early class of transcripts. In a previous study, we showed that multiple *cis*-acting sites important for promoter activity were present within the regulatory region of the HCMV early gene encoding the 2.7-kb transcript described above (26). In particular, one of these regions (corresponding to nucleotides −114 to −95 relative to the start site of the RNA) interacted with a cellular protein. This region (Fig. 1c) contained a sequence with homology to the previously identified binding site for the cellular factor called the upstream stimulatory factor (USF; also referred to as the major late transcription factor [MLTF] and upstream element factor) (4, 34, 43). USF/MLTF was initially described as a HeLa cell nuclear protein with a molecular mass of 43 to 55 kilodaltons which bound to and stimulated in vitro transcription from the adenovirus major late promoter (10, 36,

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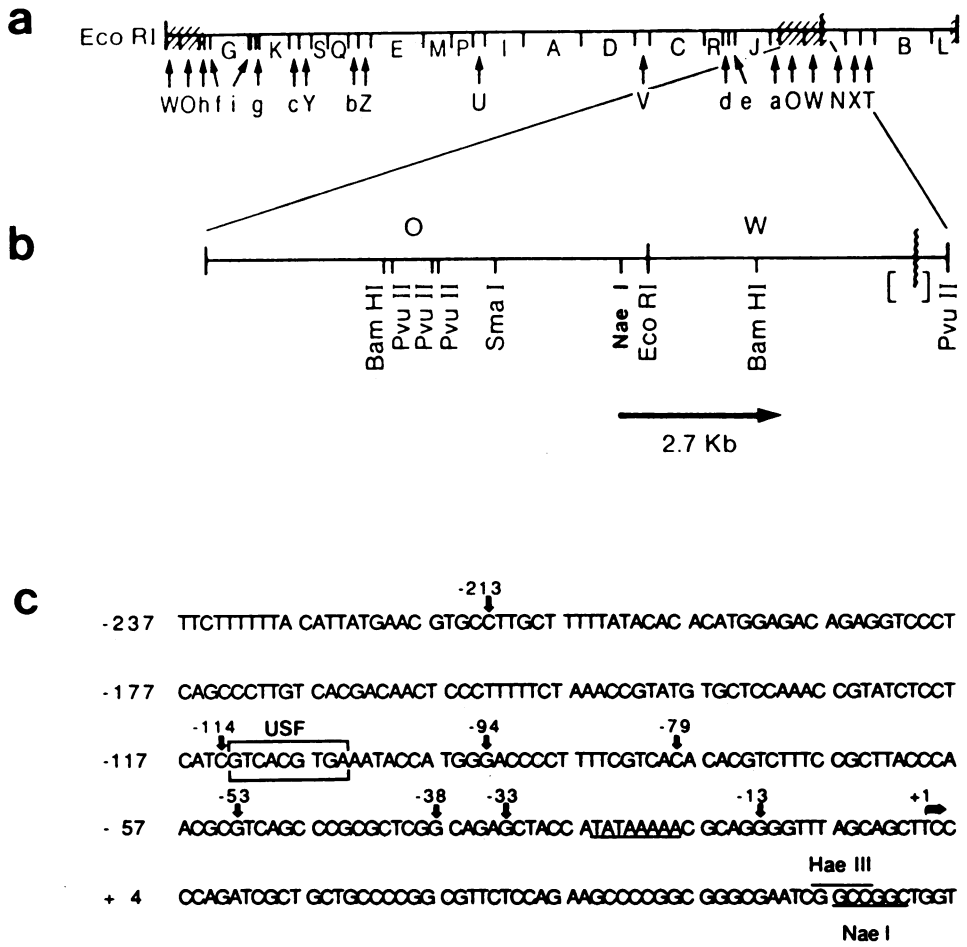

FIG. 1. (a) Diagram of the HCMV strain AD169 genome structure and *Eco*RI restriction endonuclease sites and fragments (arrows). Hatched areas represent repeated regions within the genome. (b) Map of the 2.7-kb early RNA encoded by *Eco*RI fragments O and W. A single copy of the RNA is shown. The *Nae*I site at +56 (relative to the RNA start site) is indicated. The wavy line in brackets denotes the position of the heterogeneous L-S junction. (c) Nucleotide sequence of the HCMV 2.7-kb RNA promoter region (sequence data from Greenaway and Wilkinson [17]). The nucleotide numbering relative to the RNA start site is shown at the left. The 5′ endpoints of deletion mutations described by Klucher et al. (26) are shown above the sequence by vertical arrows. The USF/MLTF-binding site (−113 to −105) is boxed.

43, 44). Subsequently, this factor was found to play a role in transcription of various cellular genes, including the rat γ-fibrinogen (9), the mouse metallothionein I (5), and the *Xenopus laevis* oocyte factor IIIA (18) genes. Recent reports also indicate that USF/MLTF may be altered in response to poliovirus infection (27) as well as play a role in the E1A induction of an adeno-associated virus gene (7).

In this work, we have studied the binding of USF/MLTF to the promoter of the HCMV gene encoding the 2.7-kb early RNA. Protein-DNA interactions and in vivo promoter activity were studied to show the specific binding of USF/MLTF to this promoter as well as its role in regulating transcription of this gene.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts obtained from Stephen Spector (University of California, San Diego) were maintained in Dulbecco modified Eagle medium (Irvine Scientific) containing 10% fetal bovine serum. HCMV strain AD169 was obtained from the American Type Culture Collection. Methods for cell culture and viral infection have been described before (55). HeLa cell suspension cultures were a gift from M. Karin (University of California, San Diego) and were maintained in Joklik modified minimum essential medium containing 5% fetal bovine serum.

Molecular cloning. Restriction enzymes used were obtained from Bethesda Research Laboratories, Inc., and used as recommended by the manufacturer. Competent *Escherichia coli* DH5 cells (Bethesda Research Laboratories, Inc.) were used as recommended by the suppliers for transformation of recombinant plasmids.

p89WOCATUSF and p89WOCAT2.7 were constructed by first cleaving p89WOCAT (containing promoter sequence from −33 to +56) (26) with *Hin*dIII, a site within the polylinker 5′ to the promoter, and *Bal*I, a site within the chloramphenicol acetyltransferase (CAT) coding sequence. The resulting 5.4-kilobase-pair (kbp) fragment which contained vector sequences and the 3′ end of the *cat* gene was then isolated. p89WOCAT was also cleaved with *Bam*HI, a site within the polylinker 5′ to the promoter, and *Bal*I, and the resulting 600-bp band was isolated. This 600-bp fragment

contained the HCMV 2.7-kb RNA promoter fused to the 5′ end of the *cat* gene. p89WOCATUSF was generated by ligating the 5.4-kbp and 600-bp fragments with a double-stranded oligonucleotide containing DNA sequence from −116 to −99 relative to the 2.7-kb RNA start site. This oligonucleotide contained a *Hin*dIII site at its 5′ end and a *Bam*HI site at its 3′ end. p89WOCAT2.7 was generated by ligating the 5.4-kbp and 600-bp fragments with a DNA fragment containing sequences from −213 to −34. This fragment also contained a *Hin*dIII site at its 5′ end and a *Bam*HI site at its 3′ end.

p69WOCATUSF was constructed in the same manner as p89WOCATUSF except that p69WOCAT (containing promoter sequence from −13 to +56) (26) was used as the starting vector.

p269WOCATpmUSF, containing a point mutation in the HCMV USF/MLTF site, was constructed by digesting p269WOCAT (containing promoter sequence from −213 to +56) (26) with *Hin*dIII and *Sst*I, a site within the polylinker 3′ of the HCMV promoter, and ligating the resulting 300-bp band with a *Hin*dIII-*Sst*I-cleaved M13mp18. A site-specific mutation changing a guanine to an adenine at nucleotide-106 relative to the 2.7-kb RNA start site was made by using the Muta-gene M13 in vitro mutagenesis kit (Bio-Rad Laboratories) and an oligonucleotide primer (5′-CGTCACGTAAA ATACCATGG-3′) containing the desired mutation, as described by the manufacturer. The mutated DNA was cloned directly upstream of the *cat* gene in pSV01 (48) which had been digested with *Hin*dIII and *Sst*I.

DNA sequence analysis. The site-specific mutation in p269WOCATpmUSF was characterized by the dideoxy chain termination method of DNA sequencing (42). The GemSeq K/RT Sequencing System (Promega Biotec, Inc.) was used in conjunction with the M13 universal primer. [α-^{35}S]dATP (1000 Ci/mmol) was purchased from Amersham.

Cell transfection. DEAE-dextran monolayer transfection was used for transient-expression assays. Human foreskin fibroblast monolayers were transfected with plasmid DNAs by the DEAE-dextran technique previously described by Staprans et al. (48). Cells were infected with HCMV at a multiplicity of infection of 5 at approximately 24 h posttransfection. In all experiments, duplicate flasks of transfected cells were plated for each condition under study.

Transient-expression assays. Approximately 24 h postinfection cells were harvested. When more than one plasmid was being analyzed, the contents of each flask were split into two parts: one for the analysis of CAT activity, the other for the preparation of nuclear DNA and assessment of transfection efficiency. Soluble extracts were prepared and assayed for CAT activity essentially as described by Gorman et al. (16) except that the acetyl coenzyme A concentration was increased to 4 mM for longer reaction times (10 h). Transfection efficiency was determined by isolation of transfected cell nuclear DNA and determination of relative amounts of plasmid DNA sequences present as described by Staprans et al. (48).

Cell extracts. Human foreskin fibroblasts were infected with HCMV in suspension at a multiplicity of infection of 3. Cells were harvested at 20 h postinfection, and nuclear extracts were prepared essentially as described by Dignam et al. (12) with the following modifications. Cells were lysed with a Dounce homogenizer with an A-type pestle. Nuclei were suspended in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA [ethylene glycol-bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2 mM dithiothreitol, 25% glycerol, and 0.1 volume of 4 M ammonium sulfate. After 30 min of rotation at 4°C on a Lab Quake, the nuclear suspension was centrifuged at 100,000 × *g* for 1 h. Protein in the supernatant was precipitated with 0.33 g of solid ammonium sulfate per ml and dialyzed as described by Dignam et al. (12). Nuclear extracts from mock-infected HeLa cells were prepared as described above. Protein concentrations were determined as described by Bradford (3).

Gel retardation assay. Probes for gel retardation assays were prepared by gel-purifying DNA fragments containing sequences from −213 to +56 relative to the 2.7-kb RNA start site and from −261 to +30 relative to the adenovirus major late promoter RNA start site and 3′-end labeling with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. The probe (0.5 ng) was mixed with 20 μg of poly(dA-dT) (Sigma Chemical Co.) and 50 ng of a 200-bp pGEM1 *Hpa*II fragment in a buffer described by Andrisani et al. (1). Appropriate specific DNA competitors were added in various molar excesses over probe. Crude HeLa nuclear extract (15 μg) was added to the probe mix and incubated at room temperature for 30 min. After binding, 3 μl of a solution containing 50% glycerol, 0.1% (wt/vol) bromophenol blue, and 0.1% (wt/vol) xylene cyanol was added, and the mix was electrophoresed on a 4% acrylamide gel as described by Speck and Baltimore (46). The gel was dried and subjected to autoradiography.

DNase I footprinting assays. Probes containing DNA sequences from −213 to +56 relative to the 2.7-kb RNA start site were 3′- or 5′-end labeled and incubated in the presence or absence of nuclear extracts as described above (75 μg of HeLa extract or 15 μg of infected-fibroblast extract). After 30 min, various amounts of DNase I were added to the reaction mixtures and allowed to digest at room temperature for 1 to 5 min before EDTA was added to 50 mM to stop the digestion. The mixture was run through a nitrocellulose filter (0.45-μm pore size) to separate the free from the bound fraction. The bound fraction was eluted off the nitrocellulose with 0.5 M ammonium sulfate–0.1% sodium dodecyl sulfate–1 mM EDTA, followed by phenol-chloroform-isoamyl alcohol (50:48:2) extraction and ethanol precipitation. The samples were suspended in formamide loading buffer and run on a 5% acrylamide–8 M urea sequencing gel.

Methylation interference assays. Probes containing DNA sequences from −213 to +56 relative to the 2.7-kb RNA start site were 3′-end labeled on the upper or lower strand, followed by methylation of G residues with dimethyl sulfate (31). Gel retardation assay mixes were scaled up fivefold and run as described above. DNA from bound and free bands was isolated, followed by chemical cleavage at G residues with piperidine. Equal counts of bound and free fractions were run on 5% acrylamide–8 M urea sequencing gels.

RESULTS

DNase I footprinting. In a previous study (26), we obtained evidence from gel retardation assays that a specific protein-DNA interaction was occurring between a cellular protein and a segment of HCMV DNA spanning nucleotides −114 to −95 relative to the start site of the major 2.7-kb RNA (Fig. 1c). To define the exact boundaries of this binding site and to determine whether HCMV infection modifies these boundaries, we used DNase I footprinting assays. For this experiment, a DNA fragment spanning nucleotides −213 to +56, in which the upper strand was 5′-end labeled, was incubated

with nuclear extracts prepared from human fibroblasts infected with HCMV for 20 h or from uninfected HeLa cells. After binding of proteins to the promoter, DNase I was used to digest the unprotected phosphodiester backbone of DNA, followed by separation of the bound fraction from the free fraction and denaturing gel electrophoresis. Both HeLa and infected-fibroblast extracts gave identical profiles, showing protection of sequences from −116 to −98 relative to the RNA start site (Fig. 2a). Similar experiments with a DNA fragment in which the lower strand was 3′-end labeled showed that both HeLa and infected-fibroblast extracts, as well as uninfected human fibroblast extracts (data not shown), gave complete protection from DNase I digestion of sequences from nucleotides −116 to −101 relative to the RNA start site. In addition, there appeared to be partial protection of sequences from nucleotides −100 to −84 in the presence of the HeLa extract in this experiment. This partial protection was not seen consistently and was probably due in part to the amount of extract used in the assay. The appearance of a DNase I-hypersensitive site at nucleotide −90 in the presence of the infected fibroblast extract was also noted. This hypersensitive site was also seen in some DNase I footprinting experiments with uninfected HeLa extracts (data not shown). The bracketed sequence in Fig. 2b shows the major region protected from DNase I digestion with extracts from infected fibroblast or uninfected HeLa cells.

Competition studies. Examination of the HCMV DNA sequence protected in the DNase I digestion experiment revealed the presence of a region of homology to the binding site of the cellular factor USF/MLTF (4, 43). This binding site was first identified as an important *cis*-acting regulatory element within the adenovirus major late promoter (19, 35, 61). To determine whether USF/MLTF was also binding at this site within the HCMV promoter, competition studies with defined oligonucleotides were conducted. The HCMV 2.7-kb RNA promoter (−213 to +56) and the adenovirus type 2 major late promoter (−261 to +30) were 3′-end labeled and incubated with HeLa cell nuclear extracts along with various amounts of oligonucleotide competitors containing either the HCMV sequences (−116 to −99) or the adenovirus major late promoter USF/MLTF-binding site (−66 to −47). The addition of HeLa cell extract to the HCMV promoter resulted in the formation of a single retarded complex (Fig. 3). The adenovirus promoter showed three major retarded complexes, one of which had roughly the same migration on the gel as the complex seen with the HCMV promoter. A 50-fold molar excess of either of the oligonucleotide competitors was sufficient to severely inhibit the formation of the complex with either the HCMV or adenovirus promoter. As a negative control, we showed that a 100-fold molar excess of an oligonucleotide containing the AP-1-binding site from the human collagenase gene had no effect on the formation of either complex. These results suggest that USF/MLTF binds to the HCMV promoter with roughly the same affinity as it does to the adenovirus promoter.

Methylation interference studies. To identify specific sites of contact between this cellular factor and the HCMV promoter, we used methylation interference assays. The HCMV promoter from −213 to +56 was 3′-end labeled on its upper or lower strand and methylated at guanine residues with dimethyl sulfate. The methylated probes were then incubated with HeLa cell nuclear extracts, and the bound and unbound bands were separated on a nondenaturing acrylamide gel. A promoter fragment that is methylated at a

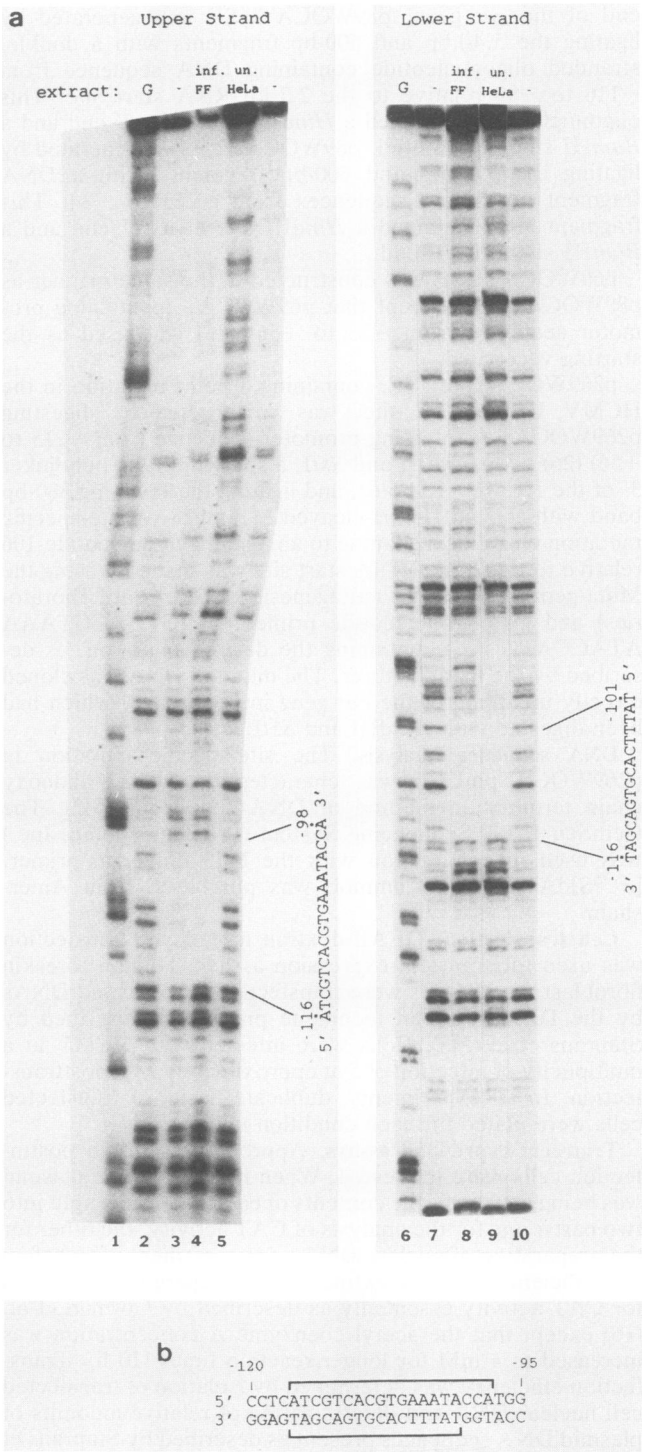

FIG. 2. DNase I footprint analysis of the HCMV 2.7-kb RNA control region. (a) The upper strand (left) was 5′-end labeled, and the lower strand (right) was 3′-end labeled and incubated with no protein (—, lanes 2, 5, 7, and 10), 15 μg of 20-h-postinfection HCMV-infected fibroblast extract (inf. FF, lanes 3 and 8), or 75 μg of uninfected HeLa extract (un. HeLa, lanes 4 and 9), followed by DNase I digestion. G chemical sequence reactions (lanes 1 and 6) were run as standards. (b) The nucleotides protected from DNase I digestion are indicated by the bracketed region.

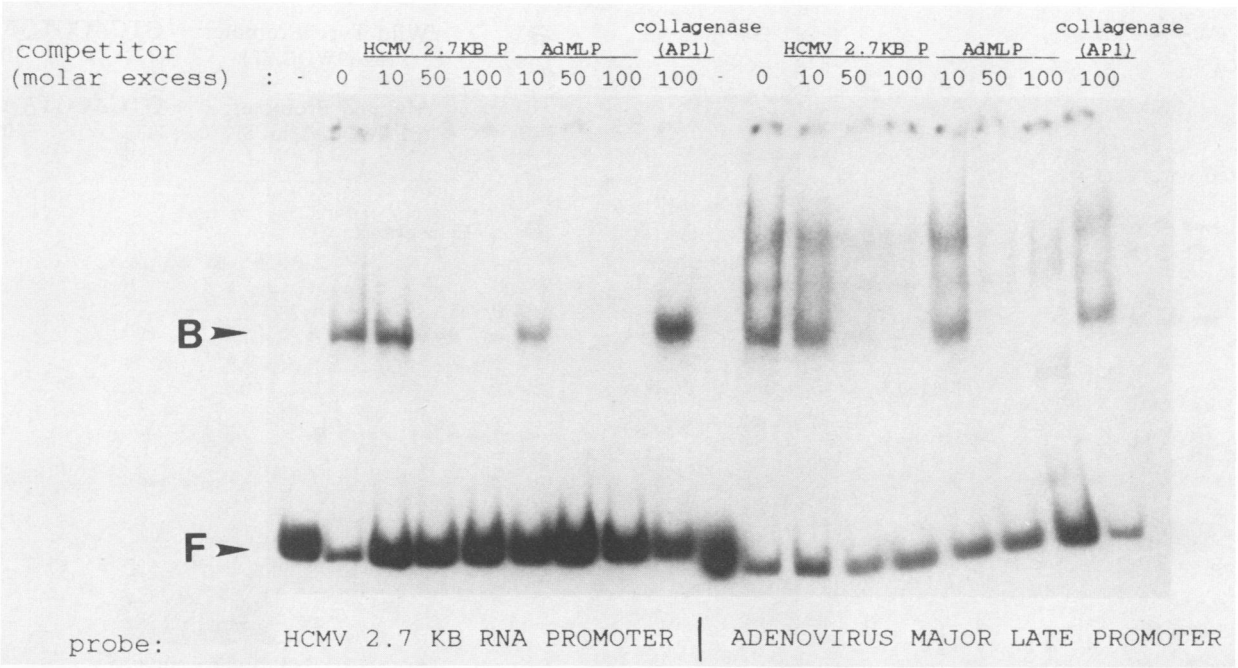

FIG. 3. Competition analysis with the HCMV 2.7-kb and adenovirus major late promoter USF/MLTF-binding sites. The HCMV 2.7-kb RNA promoter (HCMV 2.7KB P) (left) (−213 to +56) and the adenovirus major late promoter (AdMLP) (right) (−261 to +30) were 3′-end labeled and incubated with uninfected HeLa extract and various amounts of oligonucleotide competitors, followed by nondenaturing gel electrophoresis. The competitors and their molar excesses used are indicated at the top: HCMV 2.7-kb promoter (−116 to −99), adenovirus major late promoter (−66 to −47), and human collagenase promoter containing an AP-1-binding site (−79 to −60) (2). The probe used is indicated at the bottom. Arrows: B, protein-DNA complex; F, unbound probe.

guanine residue which interferes with binding will not be present in the bound fraction in this assay. The isolated bands were then cleaved with piperidine at the methylated guanine residues, followed by denaturing gel electrophoresis. Nucleotides −113, −108, and −106 on the upper strand (Fig. 4a) and nucleotides −111 and −109 on the lower strand were absent in the bound fraction while present in the free fraction, indicating their importance in the binding of this cellular factor. All of the G residues detected were contained within the DNase I-protected sequence. It should be noted that similar methylation interference results were seen with the USF/MLTF-binding site in the adenovirus major late, rat γ-fibrinogen (9), and mouse metallothionein I (5) promoters. However, based on these methylation interference results, the HCMV USF/MLTF-binding site (Fig. 4b) is in the opposite orientation relative to the binding sites in the other promoters.

Mutagenesis and promoter activity. The above studies indicated that USF/MLTF was binding to the HCMV 2.7-kb RNA promoter but did not address the question of function. To determine the potential role of USF/MLTF binding at this site in transcriptional control, we first examined the effect of a site-specific mutation on factor binding and promoter strength. For this experiment, the guanine nucleotide at position −106 in p269WOCAT was changed to an adenine to generate a plasmid designated p269WOCATpmUSF (Fig. 5a). When this mutant promoter was used as the probe in gel retardation experiments, no distinct retarded complexes were seen, and the smear observed suggested a destabilization of the DNA-protein complex (Fig. 5b). Additional gel retardation experiments with the wild-type promoter as the probe and both wild-type (p269WOCAT) and mutated (p269WOCATpmUSF) promoters as competitors revealed that the mutated USF/MLTF binding site could compete for the formation of the retarded complex, but had an approximately 5- to 10-fold-lower affinity for binding USF/MLTF than the wild-type binding site (Fig. 6). A region of the somatostatin promoter containing a cyclic AMP response element (CRE) had no effect on complex formation even at a 400-fold molar excess. When the *cat* construct containing the mutated promoter was used in transient-expression assays, we detected an approximately 50% decrease in inducible promoter activity relative to the wild-type promoter (data not shown).

To further address the question of whether the HCMV USF/MLTF-binding site could function as a transcriptional regulatory element, we generated an HCMV promoter construct, p89WOCATUSF, containing a single HCMV USF/MLTF-binding site positioned directly upstream of the TATA box (at nucleotides −56 to −48 relative to the RNA start site). With the use of this construct we could determine directly whether the HCMV USF/MLTF site could function independently of the other *cis*-acting sites we had previously mapped upstream of the TATA sequence (26). The construct p89WOCATUSF, as well as constructs containing the TATA box alone (p89WOCAT) and a wild-type promoter (p89WOCAT2.7) as controls, were then assayed for promoter activity in transient-expression assays following HCMV infection (Fig. 7). The construct containing the TATA box alone gave a low but measurable amount of CAT activity, approximately 0.4 to 1.9% of the activity from the wild-type construct. The addition of the HCMV USF/MLTF site (in p89WOCATUSF) resulted in CAT levels approximately 6- to 20-fold greater than that seen with the construct containing the TATA box alone, indicating that sequences containing the HCMV USF/MLTF-binding site can contrib-

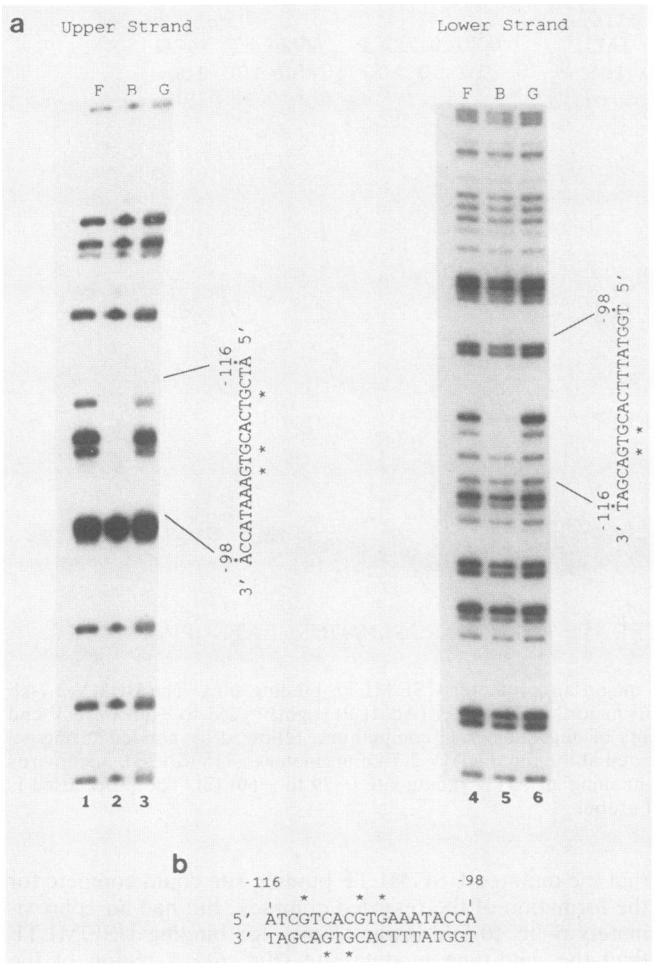

FIG. 4. Methylation interference analysis of USF/MLTF binding to the HCMV 2.7-kb RNA promoter. (a) Upper (left) and lower (right) strands were 3′-end labeled, methylated, and incubated with 75 μg of uninfected HeLa extract. Protein-DNA complexes were separated on nondenaturing polyacrylamide gels. Unbound (F, lanes 1 and 4) and bound (B, lanes 2 and 5) DNA was isolated, cleaved with piperidine, and subjected to denaturing polyacrylamide gel electrophoresis. G chemical sequence reactions (lanes 3 and 6) were run as standards. (b) Close contact points between USF/MLTF and the 2.7-kb RNA promoter are shown. Contacted guanine residues are indicated by stars.

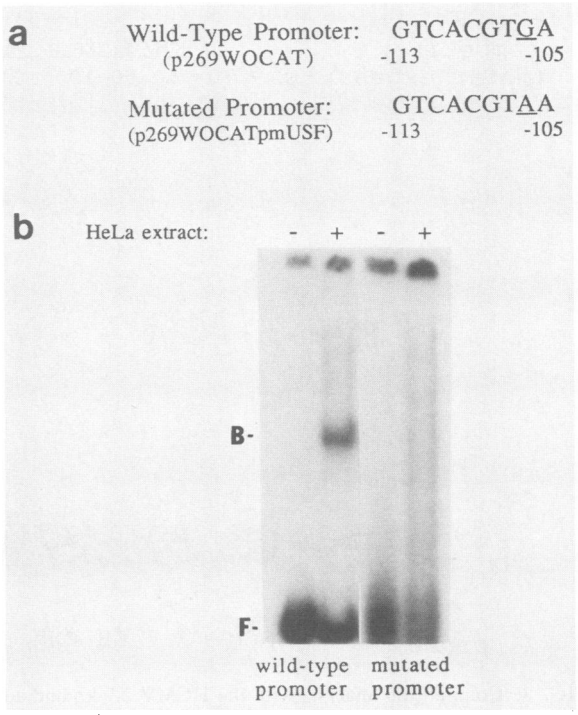

FIG. 5. Effect of a point mutation in the USF/MLTF site on factor binding. (a) Site-specific mutagenesis was used to make a point mutation in the HCMV USF/MLTF-binding site. Sequences of wild-type (p269WOCAT) and mutated (p269WOCATpmUSF) binding sites are shown. The mutated nucleotide is underlined. (b) Wild-type (p269WOCAT) and mutated (p269WOCATpmUSF) promoters (−213 to +56) were 3′-end labeled and incubated with (+) or without (−) 15 μg of uninfected HeLa extract, followed by nondenaturing gel electrophoresis. B, Protein-DNA complex; F, unbound probe. The promoter used is shown at the bottom.

ute to the activity of this promoter independent of the other *cis*-acting sequences present upstream of the 2.7-kb RNA TATA box. However, the further 6- to 24-fold increase seen when all of the previously identified elements were present (in p89WOCAT2.7) highlighted the importance of these other sites to maximal promoter activity.

Recently, it has been reported that some transcription factor-binding sites can functionally replace a TATA box in mediating promoter response to adenovirus E1A activation (39). To investigate whether the HCMV USF/MLTF site could substitute for the TATA box within the 2.7-kb RNA promoter, we generated a construct, p69WOCATUSF, which contained a USF/MLTF-binding site at nucleotides −36 to −28 relative to the RNA start site but did not contain a TATA box. This construct was transfected into human fibroblasts, followed by HCMV infection. In addition, a plasmid which did not contain a TATA box (p69WOCAT) and a plasmid which did contain a TATA box (p89WOCAT) were also assayed. As described above, the construct containing only a TATA box gave a low but measurable amount of inducible promoter activity. In contrast, neither p69WOCAT nor p69WOCATUSF had any inducible promoter activity (Fig. 7), indicating that the HCMV USF/MLTF site by itself could not functionally substitute for the TATA box.

DISCUSSION

Viral genes expressed early after infection require the coordinated expression or activation of both viral and cellular factors for maximal expression. We have studied the promoter of the HCMV 2.7-kb early RNA to better understand the regulated expression of HCMV early genes. Previously, we determined that HCMV IE genes were capable of activating this promoter and that the TATA box alone was sufficient for induction by the HCMV infection (26). However, the expression from a 5′ deletion mutant containing only the TATA box was 50- to 100-fold lower than that from the wild-type promoter, suggesting that other upstream sequences are necessary for maximal expression.

One potential candidate for an upstream regulatory site within the 2.7-kb RNA promoter was previously localized to nucleotides −114 to −95 (26). Deletion of this site caused a two- to three-fold reduction in inducible promoter activity, and a cellular protein was found to bind to this site. We

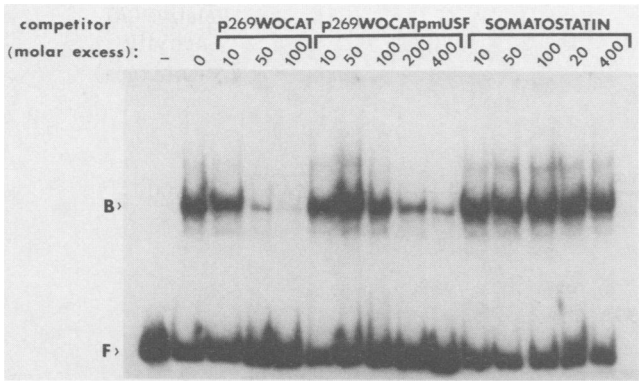

FIG. 6. Competition analysis with the wild-type and mutated HCMV USF/MLTF-binding sites. The wild-type HCMV 2.7-kb promoter (−213 to +56) was 3′-end labeled and incubated with uninfected HeLa extract and various amounts of competitors, followed by nondenaturing gel electrophoresis. The competitors and their molar excesses used are indicated at the top. p269WOCAT is the wild-type HCMV 2.7-kb RNA promoter (−213 to +56); p269WOCATpmUSF is the HCMV 2.7-kb RNA promoter (−213 to +56) containing a point mutation in the USF/MLTF-binding site at nucleotide −106 (see Fig. 5a); somatostatin is the somatostatin promoter (−71 to +53) containing a CRE (37). B, Protein-DNA complex; F, unbound probe.

noted that the consensus sequence for the binding site of the cellular factor USF/MLTF (4, 43) was contained within this region. To further establish the specificity of the binding activity, oligonucleotides containing the HCMV 2.7-kb binding site (−116 to −99) or the adenovirus major late promoter USF/MLTF-binding site (−66 to −47) were synthesized and used as competitors in gel retardation assays (Fig. 3). When the HCMV promoter (−213 to +56) was used as the probe, a 50-fold molar excess of either competitor severely reduced complex formation, with the adenovirus USF/MLTF site appearing to be slightly more effective as a competitor. The reciprocal experiment with the adenovirus promoter (−261 to +30) as the probe yielded identical results, with the adenovirus USF/MLTF site again slightly more effective as a competitor. However, it is difficult to determine whether this small (less than threefold) apparent difference in affinity between the adenovirus USF/MLTF site and the HCMV USF/MLTF site is real or the result of the differences in length or flanking sequences present in the two oligonucleotides.

Additional proof that USF/MLTF was binding to the 2.7-kb RNA promoter was provided by the results of methylation interference assays. We were able to show that the contact points of binding between the cellular factor and the guanine residues within the 2.7-kb promoter were consistent with the pattern of binding for USF/MLTF. Furthermore, mutation of one of these guanine contact points to an adenine substantially reduced the binding of USF/MLTF to the 2.7-kb RNA promoter, as assayed by gel electrophoresis. Interestingly, the site within the 2.7-kb RNA promoter is in the opposite orientation relative to the USF/MLTF binding sites within the adenovirus major late, the rat γ-fibrinogen, and mouse metallothionein I promoters. What effect this has on the transcriptional activity of the promoter is unclear. Lennard and Egly (29) reported no differences in the in vivo or in vitro transcriptional activity of the adenovirus major late promoter with the USF/MLTF site in the normal or inverted orientation, while Chang et al. (7) found an approximately twofold difference in the adenovirus E1a induction of an adeno-associated virus promoter containing an inverted USF/MLTF site compared with the normal orientation.

Two key questions relating to the importance of the HCMV USF/MLTF-binding site were considered in this study. First, can it function as a transcriptional regulatory element in vivo, and second, what role does it play in regulating the activity of the 2.7-kb RNA promoter during HCMV infection? In response to the first question, we were able to show that when the HCMV USF/MLTF-binding site was added to a minimal HCMV promoter (the 2.7-kb RNA promoter containing no upstream sequences other than a TATA box) at a position similar to its location in the adenovirus major late promoter, the activity from the HCMV promoter was increased 6- to 20-fold relative to the minimal promoter activity.

Further support for the functional importance of the interaction of this cellular factor with the 2.7-kb RNA promoter was provided by the mutagenesis studies. A single base change within the HCMV USF/MLTF-binding site lowered the binding affinity 5- to 10-fold and resulted in an approximately 50% loss of inducible promoter activity. This reduction in transcriptional activity was similar to that noted previously by 5′ deletion analysis (26). It is interesting that there was a larger relative change in transcriptional activity following addition of the HCMV USF/MLTF-binding site to the minimal HCMV 2.7-kb RNA promoter than following deletion or mutagenesis of the site. One possible explanation for these differences is that other regulatory sites within the wild-type promoter might in some way partially compensate for the loss of the USF/MLTF-binding site. Alternatively, the position of the USF/MLTF site relative to the initiation site of transcription may have an effect on the relative strength of the two promoters. This latter possibility seems less likely, since the USF/MLTF site does not appear to have a rigid dependence on position for its function. For example, the USF/MLTF-binding sites within the adenovirus major late, rat γ-fibrinogen, mouse metallothionein I, and *Xenopus* factor IIIA promoters are approximately 60, 80, 100, and 270 bp, respectively, from the start site of transcription, and yet all show an approximately three- to fivefold decrease in promoter activity upon their deletion or mutation (5, 9, 18, 34, 54).

In a previous study, it was shown that, like the HCMV 2.7-kb RNA promoter, another HCMV early promoter (that specifying the family of transcripts encoded by *Eco*RI fragments R and d) contains multiple regulatory elements (48). These promoters share little sequence homology other than a TATA box, and from deletion analyses of both promoters it would appear that the TATA element is sufficient for the viral induction of transcription, albeit at a greatly diminished level. A more difficult question to address has been the role, if any, of the other *cis*-acting sites in the temporal regulation of these promoters during the HCMV infection. Are they merely binding sites for constitutive cellular factors that fine tune the activity of the promoter once it is induced by the HCMV infection? Alternatively, do the factors binding at these sites play a direct role in the initial induction? Finally, are any of the factors themselves induced or modified during the course of the HCMV infection?

There are several studies which indicate that USF/MLTF may be affected by infection with other viruses. In one case, as measured by gel shift assays and UV-dependent protein cross-linking procedures, it was found that a modification of USF/MLTF was temporally linked to the general decrease in transcription occurring during infection of the cells with

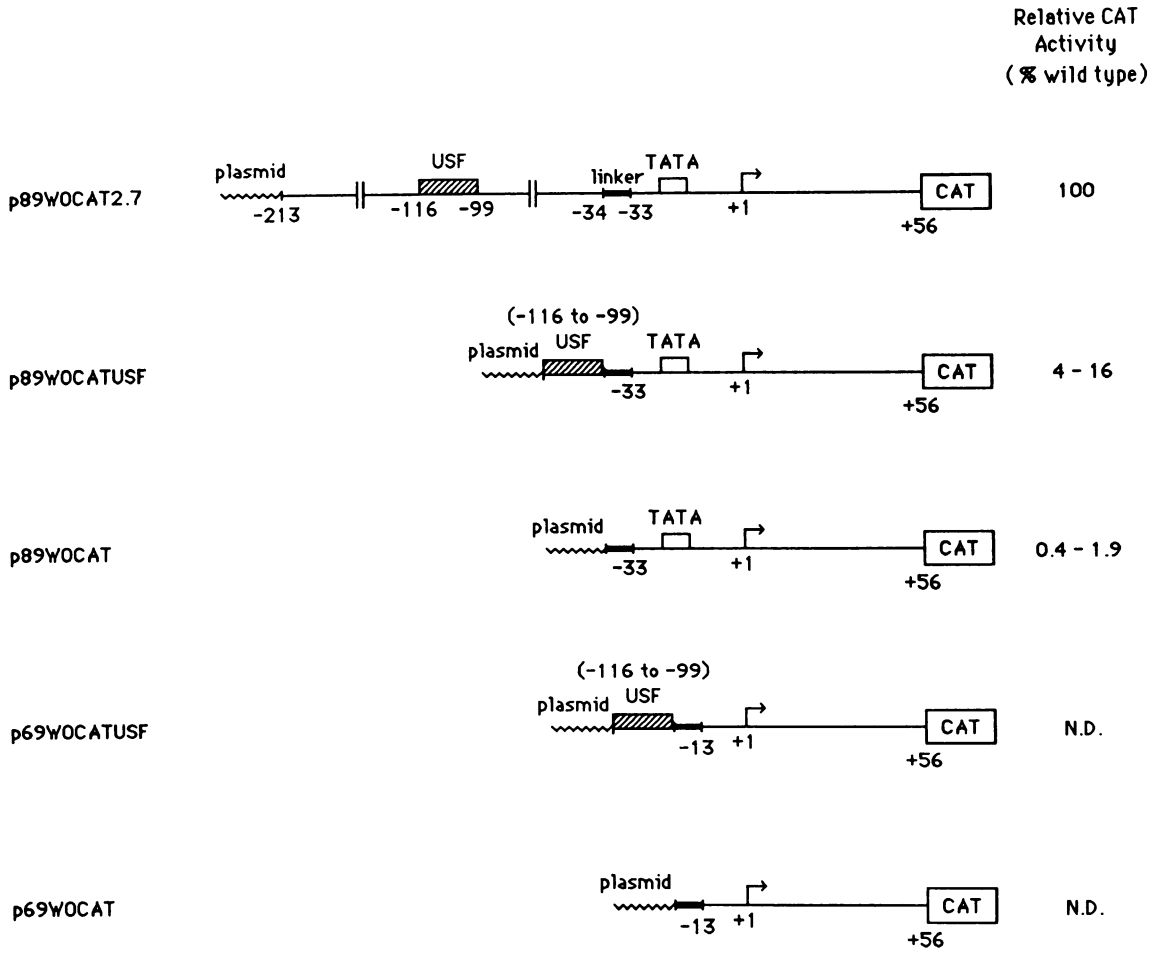

FIG. 7. Transient expression assays of plasmids containing USF/MLTF-binding sites. Plasmids were transfected in duplicate into human fibroblasts by DEAE-dextran, followed by HCMV infection. Cells were harvested and assayed for CAT activity as described in Materials and Methods. The plasmid transfected is shown at the left, and the CAT activity relative to that with the wild type (p89WOCAT2.7) is shown at the right. The data shown represent the range from four separate experiments. p89WOCAT2.7, p89WOCATUSF, and p69WOCATUSF are described in Materials and Methods. p89WOCAT and p69WOCAT were described previously (26). The hatched box represents the sequence containing the HCMV USF/MLTF-binding site (−116 to −99), the open box indicates the TATA sequence, the solid bar represents an 8-bp linker sequence, and the wavy line represents plasmid sequence. The RNA start site is at +1. N.D., No detectable induction of promoter activity by HCMV infection.

poliovirus (27). In contrast, Leong et al. reported no differences in the sequence-specific DNA-binding activity of USF/MLTF with extracts from adenovirus-infected or mock-infected HeLa cell extracts (30). We also have found no evidence for such modifications of USF/MLTF during the HCMV infection. USF/MLTF-DNA complexes formed in the presence of uninfected and HCMV-infected nuclear extracts have identical mobilities on nondenaturing gels (26), and the patterns of DNase I protection are not significantly different with either extract.

Another example involves the adenovirus E1A gene product, which activates transcription from the other early viral promoters as well as from the major late promoter. This transcriptional induction appears to be mediated through multiple promoter elements, including the TATA box, a CRE/ATF-binding site, two E2F-binding sites, and the USF/MLTF-binding site (7, 39). Because HCMV can also activate adenovirus promoters and can complement an E1A-negative adenovirus mutant (47, 56, 57), we attempted to demonstrate that the HCMV USF/MLTF site was directly responsive to the HCMV infection. In the case of adenovirus, one method of demonstrating the role of the various promoter elements in the E1A-mediated transcriptional activation has been to link the elements to heterologous minimal promoters which are not themselves induced by the E1A protein (7, 28). Chang et al. used this approach to show that the addition of a USF/MLTF site from an adeno-associated virus promoter to a minimal simian virus 40 promoter results in the conversion of this formerly uninducible promoter to one which can be activated, although at a low level, by the adenovirus E1A protein.

We have been unable to use this approach because we have yet to identify a minimal promoter which is not induced by the HCMV infection. Rather, we attempted to use the strategy employed by Pei and Berk (39), who showed that the CRE/ATF-binding site or two E2F-binding sites could replace the TATA box and independently mediate the response to E1A. In our experiments, when we used a promoter in which the HCMV USF/MLTF site was directly substituted for the TATA box, we were unable to detect any response to the HCMV infection. Although these results suggest that the TATA box is crucial for transactivation of

this promoter by HCMV infection and that the HCMV USF/MLTF site cannot function by itself to mediate promoter response, we have recently obtained preliminary data which indicate that the TATA box may not be absolutely necessary for induction of this promoter. We found that an internal deletion (nucleotides −33 to −13 relative to the start site of the RNA) which removed the TATA box but which left the remainder of the upstream elements intact resulted in a promoter which could be induced by the HCMV infection, albeit at a 50-fold-lower level than the wild-type promoter. Whether this TATA box-independent activation is mediated by a single, as yet unidentified, upstream element or by multiple elements (including the USF/MLTF-binding site) acting synergistically remains to be established.

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