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Regulation of a Human Cytomegalovirus Immediate-Early Gene (US3) by a Silencer-Enhancer Combination

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The US3 open reading frame of human cytomegalovirus (HCMV) is transcribed at immediate-early (IE) times after infection. Upstream of the US3 promoter, between −84 and −259 bp relative to the transcription start site, there are five copies of an 18-bp repeat, referred to as $5R_2$. Between −340 and −560 bp there are seven copies of a 10-bp dyad repeat, referred to as $7R_1$. We investigated the roles of these repeats in transcription from the US3 promoter in human foreskin fibroblast or HeLa cells. In transient transfection assays, the region containing $5R_2$ up-regulated transcription and was responsive to the p65 subunit of NF-κB. The DNA region containing $7R_1$ down-regulated transcription from either the US3 promoter or a heterologous promoter in a position- and orientation-independent manner. Mutational analysis and transient transfections indicated that DNA containing the 10-bp dyad or one-half of the dyad was sufficient to cause repression of downstream gene expression. DNA probes containing one or more copies of the pentanucleotide sequence TGTCG specifically bound cellular proteins, as demonstrated by electrophoretic mobility shift assays and cold-competition electrophoretic mobility shift assays. Two different DNA-protein complexes were detected with DNA probes containing one or two copies of the pentanucleotide. In HCMV-infected cell nuclear extracts, one of the DNA-protein complexes was present in amounts inversely proportional to the amount of US3 transcription. Its formation was affected by dephosphorylation of the DNA-binding protein(s). Transient dephosphorylation of the cellular repressor protein may occur during HCMV infection. Repression of US3 transcription may relate to the number of pentanucleotides and the cellular proteins that bind to it. Twenty-one copies of a TRTCG motif (R = purine) were found clustered upstream of the US3 gene and also in the modulator upstream of the HCMV IE1 and IE2 genes.

Human cytomegalovirus (HCMV) infection is usually asymptomatic, but it is a significant source of morbidity and mortality to those persons with immature or compromised immune systems. Serious manifestations of this infection include congenital central nervous system malformations, fatal pneumonitis in transplant patients, and retinitis in the AIDS population (1, 30).

The viral genome contains 230 kbp of double-stranded DNA and is expressed in a temporally regulated cascade (15, 16, 33, 50, 51). The viral immediate-early (IE) gene products, which are transcribed prior to viral protein synthesis, transactivate viral and cellular gene transcription (12, 13, 23, 25, 30, 37, 40, 43, 48). IE transcription is restricted to four regions of the genome. The major site is in the large unique (UL) component of the genome. This region is referred to as the major IE transcription unit and encodes the viral proteins IE1 and IE2 (UL122-123). Other regions of IE transcription are UL36-38, US3, and TRS1 (reviewed in references 11 and 45). Although the US3 gene is nonessential for virus replication in cell culture (27), transactivation of early viral promoters by the IE1 and IE2 viral proteins is more efficient in the presence of US3 protein (13). The IE US3 gene is transcribed between 2 and 5 h after infection, and then transcription of the viral gene is repressed (47).

Weston and Barrell (53) reported five copies of an 18-bp repeat and six copies of a 10-bp repeat in the 570-bp sequence immediately upstream of the US3 promoter. The 18-bp repeat, referred to as R_2, is partially homologous to the 18-bp repeat upstream of the major IE promoter (MIEP) and functions as an enhancer. The R_2 sequence contains the consensus binding site for members of the NF-κB/Rel family of eukaryotic transcription factors. The 10-bp R_1 repeat sequence is a dyad symmetry of the pentanucleotide TRTCG. R_1 was not found to share homology with any known consensus binding sites for transcription factors. A seventh copy of this repeat was found at position −425 bp relative to the transcription start site.

We investigated the regulatory roles of the five-R_2-copy ($5R_2$) and seven-R_1-copy ($7R_1$) regions in transcription from the US3 promoter. We demonstrate that the $5R_2$ enhancer region responds to the p65 subunit of NF-κB and that the $7R_1$ region contains a silencer. Cellular proteins bound specifically to the R_1-containing DNA. One of the DNA-protein complexes requires just the pentanucleotide-containing DNA, and the other DNA-protein complex requires two pentanucleotides within the DNA. The DNA-protein complex requiring two pentanucleotides is sensitive to dephosphorylation. A transient loss of this DNA-protein complex during HCMV infection occurs at the time of maximal transcription of the US3 gene. Clusters of the TRTCG pentanucleotide-containing sequence are found upstream of the IE US3 gene and the major IE UL122-123 genes. The role of these sequences in the negative regulation of IE transcription is discussed.

MATERIALS AND METHODS

Virus and cell culture. Primary human foreskin fibroblast (HFF) cells were maintained at 37°C in 5% CO_2 and Eagle's minimal essential medium supplemented with 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Maintenance and propagation of HCMV Towne strain have been described previously (44). Infections were done at a multiplicity of infection of

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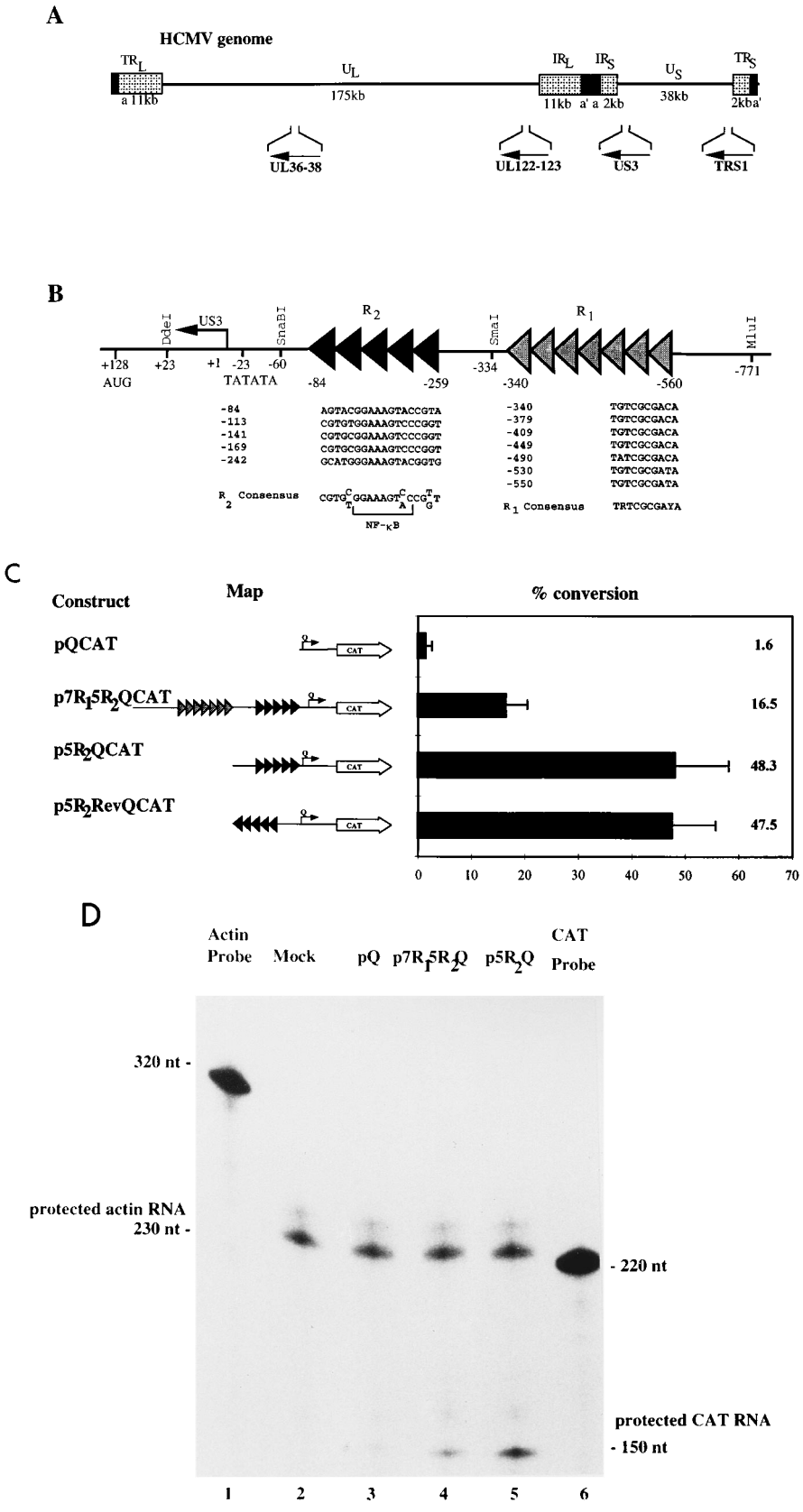

FIG. 1. Regulation of CAT gene expression by repeat elements upstream of the HCMV US3 gene. (A) Map of the entire HCMV genome in its prototypical arrangement, with the IE transcription units designated. TR and IR indicate the terminal and internal repeats, respectively. The unique sequences are represented by U. For both the repeats and the unique sequences, L and S represent the long and short components, respectively. The a sequences are denoted by a and a′. The

10. HeLa cells were maintained in the above medium or at a density of 5×10^5 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 μg of gentamicin per ml.

Enzymes. Restriction endonucleases were obtained from either Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England Biolabs, Inc., Beverly, Mass. T4 DNA ligase, the Klenow fragment of *Escherichia coli* DNA polymerase I, and calf intestinal phosphatase were acquired from Boehringer Mannheim Biochemicals, Indianapolis, Ind. *Taq* polymerase was obtained from Promega, Madison, Wis., and Perkin-Elmer, Norwalk, Conn. Potato alkaline phosphatase was purchased from Sigma Chemical Company, St. Louis, Mo. All enzymes were used according to the manufacturer's specifications.

Plasmid constructions. Plasmid pSVOCAT, which lacks a promoter upstream of the chloramphenicol acetyltransferase (CAT) gene but contains the simian virus 40 (SV40) polyadenylation signal downstream of the CAT gene, was used to construct the following plasmids (14). Plasmid p7$R_1$5R_2QCAT contains all of the R_1 and R_2 repeats, as well as the US3 promoter (denoted Q because of a previous name of this open reading frame), upstream of the CAT gene. The 1,081-bp *Hin*dIII-to-*Dde*I fragment containing the US3 promoter and repeats was isolated from the genomic clone pMSDT XbaI D (49). The R_1 repeats were removed by deleting the 437-bp fragment from the *Mlu*I site to the *Sma*I site, blunting the ends with the Klenow fragment of *E. coli* polymerase I, and religating to produce p5R_2QCAT. This plasmid contains the viral DNA from positions −334 to −60 relative to the transcription start site. Plasmid p7R_1QCAT was made by deletion of a 274-bp fragment from the *Sma*I site to the *Sna*BI site followed by religation. This plasmid contains the viral DNA from positions −771 to −334 relative to the transcription start site. pQCAT was similarly constructed by deleting the 711-bp fragment between the *Mlu*I and *Sna*BI sites. In pQCAT, only 83 bp of the US3 promoter between the *Dde*I and *Sna*BI sites (including the TATA box) remain. This plasmid contains −60 to +23 relative to the transcription start site.

The orientation of the repeats relative to the promoter was reversed by digesting p7$R_1$5R_2QCAT with restriction enzymes *Mlu*I and *Sna*BI and blunting with Klenow fragment. *Bgl*II linkers were ligated to both insert and vector DNA. After *Bgl*II restriction endonuclease digestion and ligation, the orientation of the insert in the plasmid was determined. Plasmid p7R_1Rev5R_2RevQCAT was digested with restriction endonuclease *Sma*I, and then *Bgl*II linkers were ligated to the blunt ends. After digestion with *Bgl*II, fragments of 437 and 274 bp were inserted to produce p5R_2RevQCAT and p7R_1RevQCAT, respectively.

For construction of p19CAT, the promoter was derived from the 86-bp *Hin*cII-*Xba*I DNA fragment of pCAT760dlRE (29), which lacks the IE2-mediated *cis* repression signal, and was inserted into the blunt end of the *Xba*I site of pCAT-basic (Pharmacia, Piscataway, N.J.). Plasmid p19CAT contains an ATF binding site (underlined), 5′-CCCATTGACGTCAATGGGG-3′, at position −223 bp relative to the transcription start site. After digestion at the *Hin*dIII site (−234), fill-in reaction with the Klenow fragment of *E. coli* polymerase I, and *Bgl*II linker ligation, plasmids p7R_1-19CAT and p7R_1Rev-19CAT were made by inserting the *Bgl*II-flanked 7R_1 fragment from p7R_1RevQCAT. *Nru*I digestion of p7R_1-19CAT followed by religation yielded p1R_1-19CAT. In this construct, 218 bp of genomic DNA remains including one copy of R_1 and two additional copies of its half-site, either TATCG or TGTCG. Plasmid p19CAT-7R_1 was made by inserting the 7R_1-containing DNA fragment into the *Bam*HI site at position +1600 of p19CAT. This location is downstream of the SV40 polyadenylation signal.

Plasmid pSK-1R_1Rev was created by inserting the *Bgl*II-flanked 7R_1-containing DNA fragment of p7R_1RevQCAT into the *Bam*HI site of pBluescript II-SK$^+$ (Stratagene Cloning Systems, La Jolla, Calif.) and then deleting six of the R_1 elements between the vector and genomic *Sal*I sites. Only 20 bp of viral DNA remain in this construct. Utilizing this plasmid as a template, we used the following primers to generate PCR products containing a new 5′ *Xho*I site and the mutations shown in lowercase type: m_1, 5′-CTAGTGCTcgagCCGGGcacg GCGACATG; and m_2, 5′-CTAGTGCTcgagCCGGGacgtactgacTG. The 28-bp *Xho*I fragments of these PCR products were then inserted into pBluescriptII-KS$^+$ at the *Xho*I site to create plasmid pKS-2$m_1$rev, pKS-1$m_2$rev, and pKS-1$m_1$for, with the "rev" and "for" notations designating reverse and forward orientations relative to the direction of transcription, respectively. The mutations were confirmed by sequencing with a Sequenase kit (United States Biochemical, Cleveland, Ohio) and [^{35}S]dATP (Amersham, Arlington Heights, Ill.). These plasmids were then digested with *Sau*96I, and the ends were made blunt with Klenow polymerase and deoxynucleoside triphosphates (dNTPs). *Hin*dIII linkers were ligated to the blunt ends and cleaved with restriction endonuclease *Hin*dIII. These mutant fragments were inserted into the *Hin*dIII site at −234 bp relative to the transcription start site of plasmid p19CAT. There are two copies of m_1 in p4Psyn-19CAT. Each of the plasmids p1Psynfor-19CAT and p1Psynrev-19CAT contains a single copy of m_2. Plasmid p0P-19CAT was made by mutating all sequences containing TRTCG and inserting the 38-bp *Hin*dIII fragment of the double-stranded m_3 oligonucleotide 5′-CCCAAGCTTCATGAGTCAAGACG TACTGACTTTTGAGCTTGGG-3′ into the same *Hin*dIII site of p19CAT.

Plasmid plink760 was used as a control and contains the enhancer-containing HCMV MIEP but no reporter gene sequences (24). The plasmid with the HCMV MIEP driving expression of the p65 subunit of NF-κB (pCMV-p65) was kindly provided by P. Baeuerle (42). Plasmid pSK-ΔSH, which served as the template to prepare a control probe for gel shift experiments, was created by deleting the *Sma*I-to-*Hin*cII region from pBluescript SKII$^+$. Plasmid pCH110 (Pharmacia) contains the β-galactosidase open reading frame driven by the SV40 promoter. All plasmids were purified in CsCl gradients twice (2).

Transfections. Equimolar DNA concentrations were estimated by restriction endonuclease digestion, gel electrophoresis, and comparison of ethidium bromide-stained bands. HFF cells were transfected with calcium phosphate precipitates on duplicate 100-mm-diameter plates as previously described (32). Precipitates contained 0.1 to 5 μg of reporter plasmid and 10 μg of carrier salmon sperm DNA per plate of cells. Dose response analyses were used to determine these concentrations as optimal. Transfections were done three to six times with different plasmid preparations and different cell isolates. In addition, plasmid pCH110 (Pharmacia), which has the SV40 promoter driving expression of the β-galactosidase gene, was used as a control for transfection efficiency. Cells were harvested 48 h after transfection. Protein concentrations of the cell lysates were determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.). β-Galactosidase activity was determined by the method of Herbomel et al. (21). CAT activities were determined in substrate excess as described by Gorman et al. (19). Acetylated derivatives were separated from nonacetylated [^{14}C]chloramphenicol by thin-layer chromatography, using a chloroform-methanol (95:5) solvent. The chromatographic plates were exposed to Kodak X-Omat AR film (Eastman Kodak Company, Rochester, N.Y.). For quantitative comparisons, the percent conversion was determined by AMBIS (San Diego, Calif.) image acquisition analysis, and standard deviations were calculated. These values were normalized to β-galactosidase activity and protein concentration to control for differences in transfection efficiency. Reported values are for assays done on 200 μg of protein.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously (18, 25) except that the nuclei were briefly homogenized a second time after their resuspension in buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM *N*-α-*P*-tosyl-L-lysine chloromethyl ketone, 2 μg of pepstatin per ml, 5 μg of leupeptin per ml).

DNA fragments used in EMSA were generated by using the T3 and T7 primers for PCR amplification of pSK-1R_1Rev for R_1 (P3-NX) and of pSK-ΔSH for control (B-SX). The mutant competitor fragments were generated by PCR amplification of their respective p19CAT plasmids, using the CAT and vector primers with sequences as follows: CAT, 5′-TTGGGATATATCAACGGTGG-3′; and vector, 5′-GGCTCGTATGTTGTGTGGAA-3′. Probes and competitor fragments were electroeluted from 12% polyacrylamide gels following digestion of the PCR products with *Xho*I and *Nci*I for P3-NX, P2-NX, and P1-NX, *Xho*I and *Spe*I for the control (B-SX), or *Hin*dIII for P0-H3. The concentrations of the DNA fragments were estimated by the ethidium dot method (39). Probes were generated by 3′-end-labeling DNA fragments with ^{32}P-dNTPs (Amersham) and the Klenow fragment of *E. coli* polymerase I to approximately 10^8 cpm/μg. Unincorporated dNTPs were removed by gel filtration chromatography with a Sephadex G50-150 spin column.

For EMSA, 6 to 10 μg of nuclear extract was incubated with various concentrations of poly(dI)-poly(dC) (Promega) at room temperature for 15 to 30 min in 20 μl of 10 mM HEPES (pH 7.9) containing 3.5 mM $MgCl_2$, 25 mM KCl, 0.5 mM EDTA, 5% glycerol, 1% polyvinyl alcohol, and 1 mM dithiothreitol. When

transcription units are named for the component of the genome in which they are located, and they are numbered as open reading frames from left to right according to Chee et al. (11). The arrow indicates the direction of transcription. (B) Detailed map of the US3 upstream regulatory region shows the orientation, number, and arrangement of each repeat as well as the restriction sites used in plasmid construction. Positions of the transcription and translation start sites are shown. The positions relative to the US3 transcription start site and the sequences of each of the R_1 and R_2 repeats are shown. The consensus sequences are below the individual sequences in the lists. Within the sequences, R indicates purine (A or G) and Y represents pyrimidine (T or C). The NF-κB sequence within R_2 is designated. (C) Percent conversion of [^{14}C]chloramphenicol to the 3′-acetylated derivatives in HFF cells. The mean percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives was normalized to the protein concentration of the extract and to the transfection efficiency of β-galactosidase-expressing internal control. The error bars represent standard deviations determined from at least three transfection experiments carried out in duplicate. Mean percents conversion are listed to the right. (D) Autoradiogram of the steady-state levels of CAT RNA and actin internal control RNA in HeLa cells. Cytoplasmic RNA was isolated, and the steady-state levels of RNA were determined by the RNase protection assay as described in Materials and Methods. Lanes: 1, ^{32}P-labeled actin riboprobe not treated with RNase; 2, mock-transfected; 3, pQCAT; 4, p7$R_1$5R_2QCAT; 5, p5R_2QCAT; 6, ^{32}P-labeled CAT riboprobe not treated with RNase. The sizes of the actin and CAT riboprobes and the protected RNAs are indicated and given in nucleotides (nt).

applicable, unlabeled competitor DNA was used in place of poly(dI)-poly(dC) during preincubation prior to probe addition. P3-NX, P2-NX, P1-NX, and P0-H3 were used at 60- and 120-fold molar excesses relative to moles of P3-NX probe used. For negative control, poly(dI)-poly(dC) was used at 0.0025 and 0.005 μg/μl to approximately equal the mass of the unlabeled competitor DNA fragments. The radioactive probe (~0.25 to 0.5 ng) was added after the preincubation, and incubation was continued at room temperature for an additional 15 to 30 min. DNA-protein complexes were separated from free probe by electrophoresis through 5% polyacrylamide gels containing 25 mM Tris (pH 8.5), 200 mM glycine, and 1 mM EDTA. Gels were dried and exposed to Kodak X-Omat AR film (Eastman Kodak) or Hyperfilm-MP (Amersham).

RNase protection. Construction of the plasmid for the CAT antisense riboprobe has been described previously (29). For the actin antisense riboprobe, a 220-bp fragment of the actin gene was cloned downstream of the T3 promoter in plasmid pBluescriptKSII (Stratagene). After linearization of the DNA templates, [^{32}P]RNA probe synthesis was performed as described by Krieg and Melton (28). Hybridization and RNase digestion conditions were as described previously (29). Cytoplasmic RNA was harvested from two 100-mm-diameter plates of transfected HeLa cells at 48 h after transfection as described previously (10, 24). RNA (40 μg) was hybridized with either riboprobe. The protected RNAs were subjected to electrophoresis in denaturing 6% polyacrylamide–urea gels.

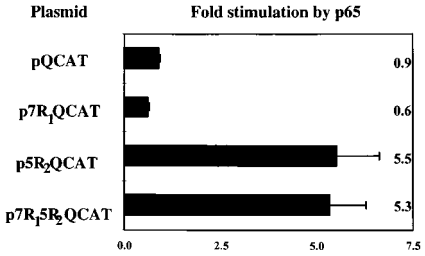

FIG. 2. Effect of the NF-κB p65 subunit on activity of the US3 promoter in constructs with or without the R_2 region. Mean fold stimulation of CAT activity relative to that of the control plasmid plink70 was determined. The CAT reporter plasmids (0.1 μg; see Fig. 1 for diagrams) were cotransfected with effector plasmid pCMVp65 (0.2 μg) or control plasmid plink760 (0.2 μg) into HFF cells as described in Materials and Methods. The error bars represent standard deviations determined from at least three transfection experiments. Fold stimulation values are listed to the right.

RESULTS

Effects of $7R_1$ and $5R_2$ on transcription from the US3 promoter. Figure 1A demonstrates the location of the US3 transcription unit relative to the other HCMV IE genes. The $7R_1$ and $5R_2$ repeat sequences and consensus sequences are designated in Fig. 1B. Weston (52) previously established that R_2 functions as an enhancer when placed either 5′ or 3′ of a heterologous promoter. Figure 1C diagrams the p$7R_1 5R_2$QCAT plasmid containing R_1 and R_2 repeats as they are in the context of the viral genome. This plasmid and modified plasmids pQCAT, p$5R_2$QCAT, and p$5R_2$RevQCAT were transfected into HFF or HeLa cells. Cell lysates were analyzed for either CAT activity or CAT RNA. Data from CAT assays were normalized to β-galactosidase activity from a cotransfected control plasmid, pCH110. RNase protection assay was as described in Materials and Methods. Deletion of the R_1R_2 region from p$7R_1 5R_2$QCAT to yield pQCAT reduced the mean percent CAT conversion from 16.5 (±4.8) to 1.6 (±0.3) (Fig. 1C). In contrast, deletion of the $7R_1$ region from p$7R_1 5R_2$QCAT to yield p$5R_2$QCAT increased the mean percent CAT conversion from 16.5 (±4.8) to 48.3 (±9.5) while mean percent conversion for the reverse orientation (p$5R_2$RevQCAT) was 47.5 (±7.1). Deletion of the $7R_1$ region caused approximately a threefold increase in steady-state CAT mRNA in transfected HeLa cells (Fig. 1D; compare lanes 4 and 5). Deletion of the $5R_2$ region to yield pQCAT reduced the level of steady-state CAT mRNA approximately eightfold (compare lanes 3 and 5).

The $5R_2$ sequence contains the consensus binding site for the eukaryotic transcription factor NF-κB/Rel (52). The prototypical NF-κB complex is composed of two subunits, p50 and p65 (42). A plasmid expressing p65 (0.2 μg per plate) was cotransfected into HFF cells with the previously mentioned plasmids (0.1 μg per plate). plink760, which does not contain the CAT gene, was cotransfected in parallel experiments as an enhancer/promoter control. For plasmids, p$7R_1 5R_2$QCAT and p$5R_2$CAT, activity was stimulated 5.3 (±0.9)- and 5.5 (±1.1)-fold, respectively. When plasmids pQCAT and p$7R_1$QCAT were cotransfected with the plasmid expressing p65, there were only 0.9 (±0.03)- and a 0.6 (±0.04)-fold increases in activity, respectively (Fig. 2). In transiently transfected cells, the p65 subunit of NF-κB stimulated expression from plasmids containing $5R_2$ but not from those lacking it. As its sequence predicts, $5R_2$ is an NF-κB-responsive enhancer.

To determine the effect of R_1 on the homologous basal US3 (Q) promoter, a fragment containing all seven of the R_1 elements ($7R_1$) was inserted into the *Sna*BI site (Fig. 1B) in either orientation (Fig. 3). The CAT activity of these constructs in HFF cells was then compared with that of constructs with or without the basal US3 promoter, pQCAT and pSVOCAT, respectively. If $7R_1$-containing DNA does negatively effect transcription, the highest activity would be from pQCAT, which gave only 1.6% CAT conversion after 1 h. Therefore, these assays were performed for 18 h. In transient transfection experiments, the mean percent CAT conversion from p$7R_1$QCAT was 3.7 (±0.5), while that of the reverse orientation (p$7R_1$RevQCAT) was 3.5% (±0.6). In contrast, the mean percent CAT conversion from pQCAT was 55.4 (±10.4). With no US3 promoter present, pSVOCAT yielded a mean percent CAT conversion of 5.3 (±0.3). The $7R_1$-containing DNA fragment repressed CAT expression averages of 15-fold when placed in the native orientation and 16-fold when placed in the reverse orientation. Therefore, with no NF-κB-responsive enhancer present, the $7R_1$ region was capable of repressing expression from the homologous basal promoter when placed upstream in either orientation.

The $7R_1$ region contains a silencer. Silencers negatively influence a variety of promoters, but similar to enhancers, they function independently of their orientation or position (7). To determine whether $7R_1$ contains a silencer, we inserted this region into a reporter plasmid with a heterologous enhancer-containing promoter driving CAT expression (Fig. 4A). In this plasmid, p19CAT, expression from the minimal HCMV MIEP is enhanced by the ATF consensus binding site. In HFF cells, the mean percent CAT conversion for the parent plasmid, p19CAT, was 50.0 (±1.0) (Fig. 4B). Insertion of the $7R_1$ region at position −234 bp relative to the transcription start site decreased the mean percent CAT conversion to 1.6 for both the native (p$7R_1$-19CAT) (±0.3) and reverse (p$7R_1$Rev-19CAT) (±0.4) orientations. When $7R_1$ was inserted downstream of the CAT gene at +1600 (p19CAT-$7R_1$), there was 13.7% (±0.4%) mean CAT conversion. The $7R_1$ region repressed transcription when placed upstream of the ATF binding site and the TATA box in either orientation or when placed downstream of the CAT gene. We conclude that the $7R_1$ DNA fragment is capable of repressing expression from either the homologous US3 promoter or the heterologous, modified MIEP regardless of position or orientation. We propose that the 437-bp $7R_1$ contains a silencer.

Effect of the R_1 and pentanucleotide-containing DNA. To delineate the *cis*-acting element in the $7R_1$ region that is responsible for transcriptional repression, we evaluated the functional role of the 10-bp R_1 element. We also evaluated the pentanucleotide TRTCG which forms but is not limited to the R_1 element. We placed either a genomic DNA fragment con-

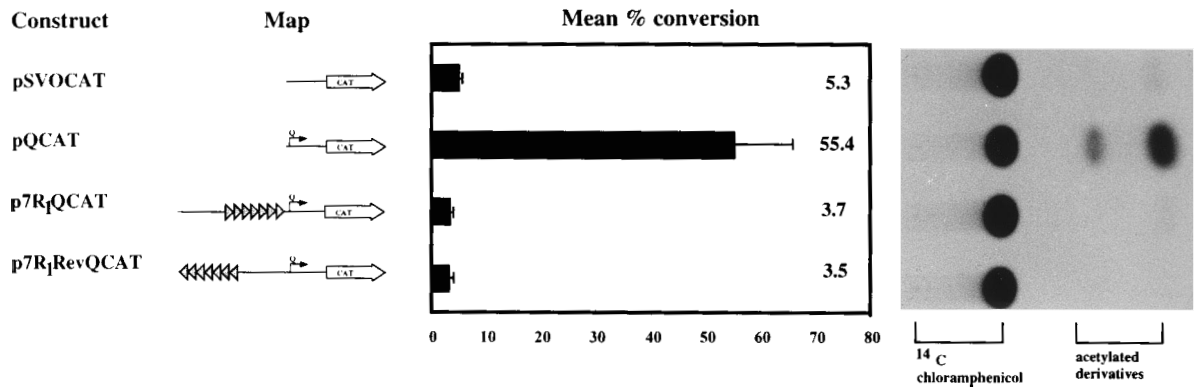

FIG. 3. Regulation of CAT gene expression from the US3 promoter with or without the $7R_1$ region. The Q promoter and the $7R_1$-containing DNA were inserted upstream of the CAT open reading frame in the promoterless vector pSVOCAT. Into this plasmid, pQCAT, the $7R_1$ repeats were inserted in either orientation. These constructs are depicted with Q representing the US3 promoter and shaded triangles representing R_1. Transfections were done in duplicate with 5 μg of reporter plasmid per plate of primary HFF cells as described in Materials and Methods and the legend to Fig. 1. The percent conversion of [^{14}C]chloramphenicol to the 3′-acetylated derivatives in HFF cells was determined as described in the legend to Fig. 1. The error bars represent standard deviations determined from at least three transfection experiments. Mean percents conversion are listed to the right. An autoradiogram of a representative experiment is shown. Positions of the [^{14}C]chloramphenicol and its acetylated derivatives are indicated below the autoradiogram.

taining four pentanucleotides (three TGTCG and one TATCG) or synthetic DNA sequences containing four, one, or no TGTCG pentanucleotides with flanking regions at −234 bp upstream of the modified MIEP of p19CAT (Fig. 5A). These constructs were transfected into permissive HFF cells, and equivalent amounts of protein extract were assayed for CAT activity and normalized to the value for the β-galactosidase internal control as described in Materials and Methods. The mean percent CAT conversion of the parent plasmid, p19CAT, was 50.6 (±2.2). Plasmids p4P-19CAT and p4Psyn-19CAT, which each contain four copies of the pentanucleotide sequence, yielded 1.6% (±0.3%) and 1.5% (±0.4%) conversion, respectively. Plasmids p1Psynfor-19CAT and p1Psynrev-19CAT, each with a single copy, gave 3.1% (±0.8%) and 2.7% (±0.5%) conversion, respectively. Conversely, plasmid p0P-19CAT, in which all of the sequences resembling TRTCG were mutated, restored activity to a mean 37.7% (±6.4%) conversion (Fig. 5B). These data suggest that DNA containing the pentanucleotide TGTCG plus flanking sequence represses downstream transcription when placed upstream in either orientation.

Binding of specific proteins. Silencers are bound by sequence-specific proteins (3, 4, 6, 34, 36, 38, 41, 46). To investigate the possibility that the pentanucleotide-containing DNA has a role in binding specific proteins, we synthesized a series of DNAs that contained various combinations of the TGTCG pentanucleotide or a mutation thereof (Fig. 6A). ^{32}P-labeled DNA fragments were used as probes in EMSA of either HFF or HeLa cell nuclear extracts. Four to five DNA-protein com-

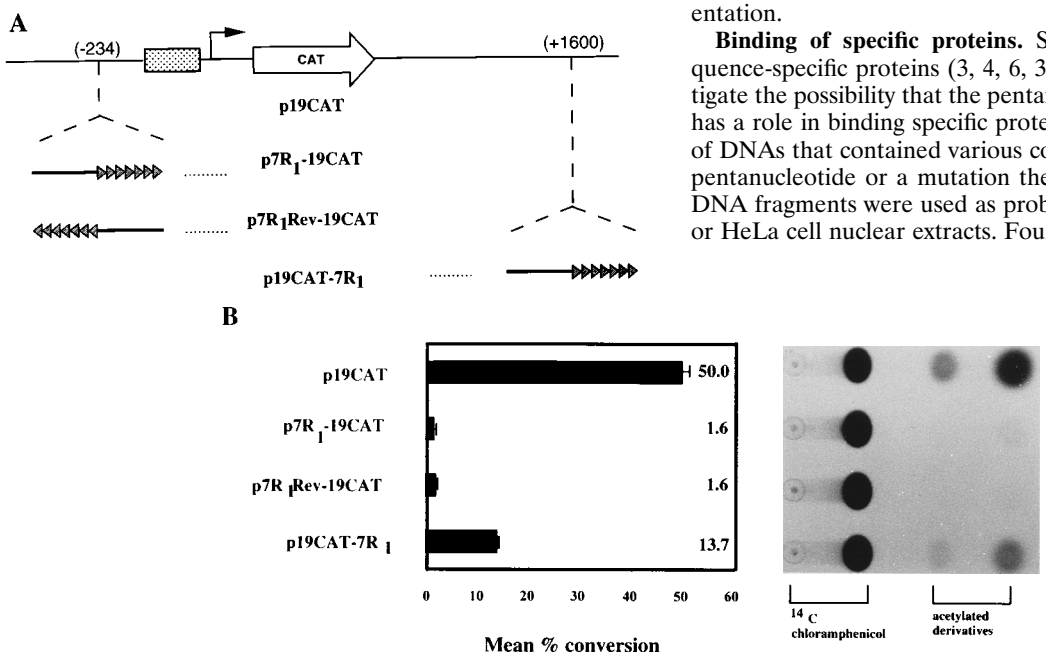

FIG. 4. Transfer of the HCMV US3 R_1-containing DNA to a heterologous promoter. (A) Diagram of the p19CAT construct and the various constructs derived from it by insertion of the $7R_1$-containing DNA. The stippled box represents the 19-bp element containing the ATF binding site upstream of the promoter. The small arrow represents the transcriptional start site. The CAT open reading frame is represented by the large arrow. The shaded triangles represent individual copies of R_1 and their orientations relative to the transcription start site. The heavy dark line represents flanking viral DNA. The dashed lines indicate the location of the insertion within the construct. The resulting plasmid names are listed. (B) Mean percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives. The ability of R_1 to repress transcription from the heterologous promoter was tested by transient transfections in HFF cells as described in Materials and Methods and the legend to Fig. 2. Mean percents conversion are listed. An autoradiogram of a representative experiment is shown. Positions of the [^{14}C]chloramphenicol and its acetylated derivatives are indicated below the autoradiogram.

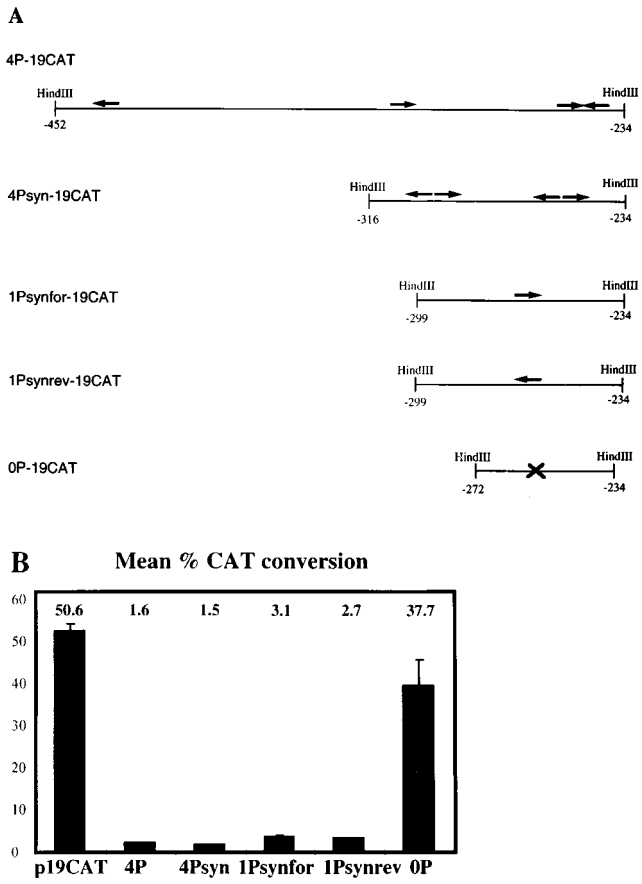

FIG. 5. Effect of TRTCG pentanucleotides on CAT gene expression. (A) Sequences of the wild-type and mutant DNA fragments inserted into the *Hin*dIII site at position −234 bp of plasmid p19CAT. Four copies of the pentanucleotide (designated by the arrow) are present in plasmid 4P-19CAT, with two of them arranged as a 3′-to-3′ inverted repeat. Four copies of the pentanucleotide arranged as two 5′-to-5′ inverted repeats (CGACATGTCG) are present in plasmid p4Psyn-19CAT. A single TGTCG pentanucleotide is present in the forward and reverse orientations in the plasmids p1Psynfor-19CAT and p1Psynrev-19CAT, respectively. Plasmid p0P-19CAT has all TRTCG motifs mutated. (B) Mean percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives. HFF cells were transfected in duplicate with 5 μg of the reporter plasmids and assayed for CAT activity as described in Materials and Methods and the legend to Fig. 1. Mean percents conversion are listed at the top.

plexes were detected, depending on the concentration of poly(dI)-poly(dC) (Fig. 6B). With HFF cell nuclear extracts, DNA-protein complex 1 was not always detectable. The probe lacking any TGTCG pentanucleotides (P0-H3) generated mainly DNA-protein complexes 2 and 4. DNA-protein complex 2 was very heterogeneous in electrophoretic mobility. Probe P1-NX, containing a single pentanucleotide (TGTCG), generated complex 3 and an additional, faster-migrating complex. The additional, faster-migrating complex was not detected at higher concentrations of poly(dI)-poly(dC), while complex 3 was still detected. Probe P2-NX, containing two pentanucleotides juxtaposed 5′ to 5′ (CGACATGTCG), or probe P3-NX, containing three pentanucleotides juxtaposed either 5′ to 5′ or 3′ to 3′, generated complexes 3 and 5. With the single-site probe P1-NX, a low amount of complex 5 was detectable after a longer exposure of the autoradiogram. The sequence flanking the pentanucleotide required to generate DNA-protein complex 3 or 5 is not known. These findings suggest that DNA-protein complexes 3 and 5 may require at least the sequence TGTCG but that complex 5 forms best when there is a duplication of the pentanucleotide juxtaposed 5′ to 5′. Pentanucleotides juxtaposed 3′ to 3′ (TGTCGCG ACA), which produce the R_1 motif, did not generate DNA-protein complex 5 (data not shown). Probes containing the pentanucleotide TATCG were not tested.

Cold competition-EMSA with HeLa cell nuclear extracts was used to confirm the sequence specificity for the DNA complexes. Five DNA-protein complexes were detected, and their relative amounts were influenced by the poly(dI)-poly(dC) concentration. With HeLa cell nuclear extracts, there was more complex 1 and less complex 3. With either HeLa or HFF nuclear extracts, the detection of the specific complex 5 and the nonspecific complexes 2 and 4 were similar. DNAs containing two pentanucleotides apposed 5′ to 5′ (P2-NX and P3-NX) competed efficiently for complex 5 (Fig. 6C). DNA containing a single pentanucleotide (P1-NX) competed poorly for complex 5 at 60-fold molar excess and slightly at a 120-fold molar excess (Fig. 6C). DNA lacking the pentanucleotide (P0-H3) did not compete for complex 5. Complex 3 was also not affected by cold competition with P0-H3. The pentanucleotides juxtaposed 3′ to 3′ did not compete for DNA-protein complex 5 (data not shown). While complex 3 can readily form on a single pentanucleotide motif, the efficient formation of complex 5 appears to require two pentanucleotide motifs that are juxtaposed 5′ to 5′.

Effect of HCMV infection. If repression of transcription of the US3 gene is due to the formation of DNA-protein complex 3 and/or 5 on the pentanucleotide sequences in the $7R_1$-containing DNA region, then those complexes should be detectable at all times except during maximal transcription from the US3 gene. To test this hypothesis, nuclear extracts were prepared from either mock- or HCMV-infected HFF cells at 6, 12, and 48 h after infection. EMSA with probe P3-NX (Fig. 7A) detected five DNA-protein complexes (Fig. 7B). The minor and faster-migrating complexes were not always detectable. The amounts of nonspecific DNA-protein complex 4 were approximately similar in all nuclear extracts tested and served as an internal control for protein concentration. DNA-protein complex 1 disappeared after infection, and DNA-protein complex 3 decreased in relative amount with time after infection. Only DNA-protein complex 5 had a temporal pattern similar to that of US3 transcription, i.e., greatly diminished early in infection, when the level of transcription is high, and abundant later, when the level of transcription is low (47). Since similar DNA-protein complexes were detected with mock nuclear extract, the DNA-binding proteins are likely of cellular origin. The significance of DNA-protein complex 3 and its relationship to DNA-protein complex 5 is not understood.

Involvement of phosphorylation in DNA-protein complex 5. Within 2 (data not shown) to 6 h after infection of HFF cells with HCMV, the DNA binding abilities of the proteins that generate DNA-protein complex 5 are altered (Fig. 7B, lane 3). However, DNA-protein complex 5 is reestablished within 12 h after infection. Since phosphorylation is frequently involved in cyclic regulation of proteins (26), we tested whether phosphatase treatment could alter complex formation. We performed EMSA with the P3-NX probe and nuclear extracts in the presence of increasing amounts of potato alkaline phosphatase. Upon phosphatase treatment, DNA-protein complex 3 was still detectable and increased slightly relative to DNA-protein complex 5 (Fig. 8). DNA-protein complex 5 decreased with increasing phosphatase (Fig. 8). Higher concentrations of potato alkaline phosphatase eliminated DNA-protein complex 5 (data not shown). When the concentration of phosphatase inhibitor was increased in the reaction (Fig. 8, lanes 5 to 8), DNA-protein complex 5 also increased. These data suggest

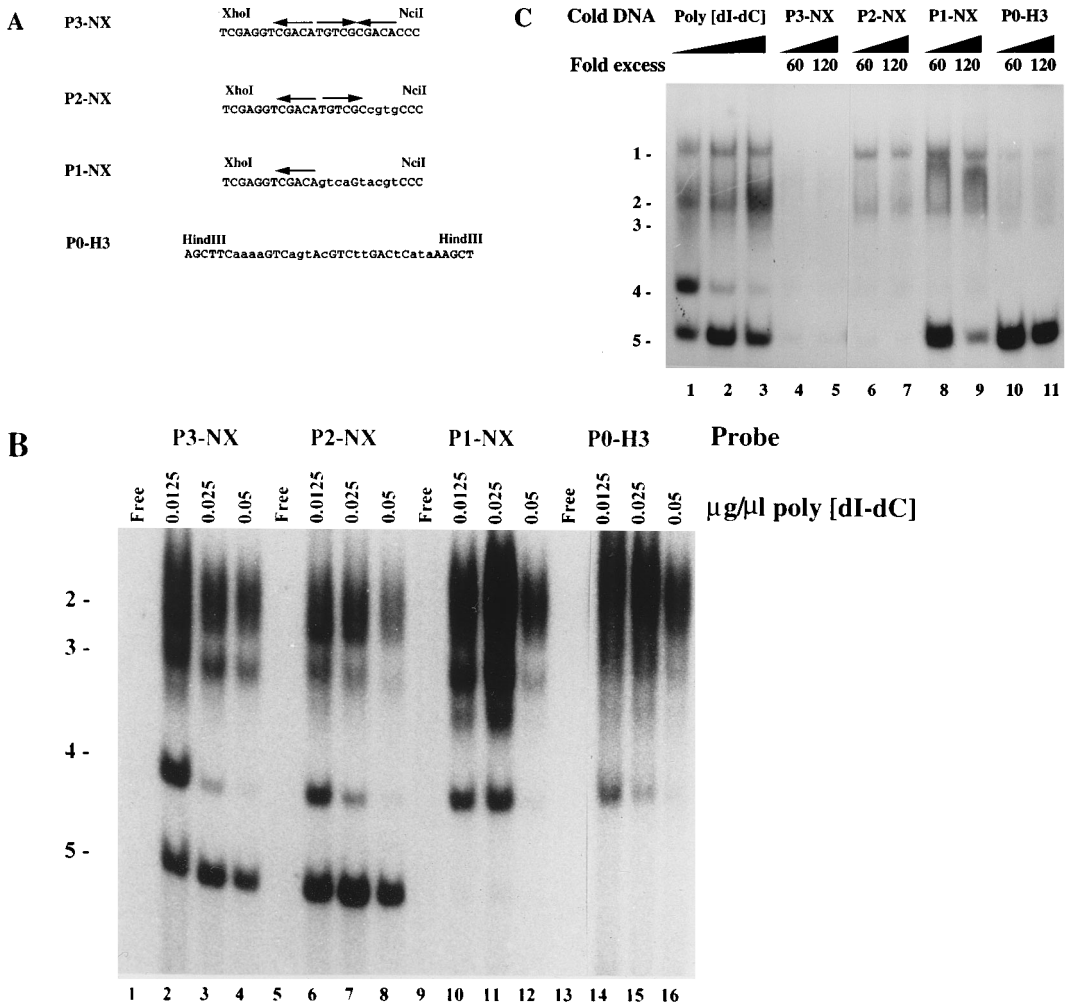

FIG. 6. Binding of cellular proteins to the pentanucleotide TGTCG-containing DNA. (A) Sequences of the probes. Arrows indicate pentanucleotides. P3-NX, P2-NX, and P1-NX have exactly the same flanking sequences except for the mutations designated in lowercase letters. P0-H3 has all TRTCG combinations mutated. (B) Autoradiogram of EMSA using HFF nuclear extracts and various probes in the presence of increasing amounts of poly(dI)-poly(dC). Probes are as follows: lanes 1 to 4, P3-NX; 5 to 8, P2-NX; 9 to 12, P1-NX; 13 to 16, P0-NX. Poly(dI)-poly(dC) concentrations ranged from 0.0125 to 0.05 μg/μl. (C) Autoradiogram of cold competition EMSA using HeLa cell nuclear extracts. Lanes: 1, probe P3-NX plus nuclear extract; 2 and 3, probe P3-NX plus nuclear extract in the presence of increasing amounts of poly(dI)-poly(dC); 4 to 11, probe P3-NX plus nuclear extract in the presence of 60- or 120-fold molar excess of nonradioactively labeled DNA as follows: lanes 4 and 5, P3-NX; lanes 6 and 7, P2-NX; lanes 8 and 9, P1-NX; lanes 10 and 11, P0-H3. The DNA-protein complexes are labeled 1 to 5.

that formation of DNA-protein complex 5 is affected by phosphorylation of one or more of its component proteins.

DISCUSSION

In this study, we have identified a silencer upstream of the HCMV US3 transcription start site (Fig. 1B). In the viral genome, 21 copies of a pentanucleotide, TRTCG, lie just upstream of 5 copies of an 18-bp enhancer, which is, in turn, adjacent to the promoter. The enhancer region is located from approximately −259 to −84 bp relative to the transcription start site (Fig. 1B) and is able to enhance activity of the US3 promoter. The consensus sequence contains the binding site for the eukaryotic transcription factor NF-κB/Rel. We were able to demonstrate that R_2 is an NF-κB-responsive enhancer by stimulating transcription of R_2-containing plasmids with the addition of a plasmid expressing p65.

The silencer region is located from approximately −560 to −340 bp relative to the transcription start site (Fig. 1B). In either HFF or HeLa cells, the $7R_1$ region represses transcription from the native promoter or from a heterologous promoter in a position- and orientation-independent manner. The half-site of R_1, the pentanucleotide TGTCG, can independently confer repression of a heterologous promoter in transiently transfected cells. The pentanucleotide-containing DNA fragment also functioned in an orientation-independent manner. When the HCMV US3 silencer is in its genomic context, it represses downstream gene expression similarly to other reported silencers including those of c-*mos*, human ε-globin, SCG10, human triglyceride lipase, mouse renin, and collagen II (8, 9, 20, 34, 41, 54). When placed upstream of five copies of an NF-κB binding site, it decreases activity of a reporter gene in transient transfection assays only threefold. In contrast, it is able to decrease expression from constructs containing a single ATF binding site upstream of the modified MIEP approximately 30-fold. In the absence of an enhancer element, it is capable of repressing all expression from the US3 promoter.

The HCMV R_1 region investigated contains 21 copies of a 5-bp motif between −334 and −771 bp that is also found to be clustered in the modulator between −760 and −1145 bp up-

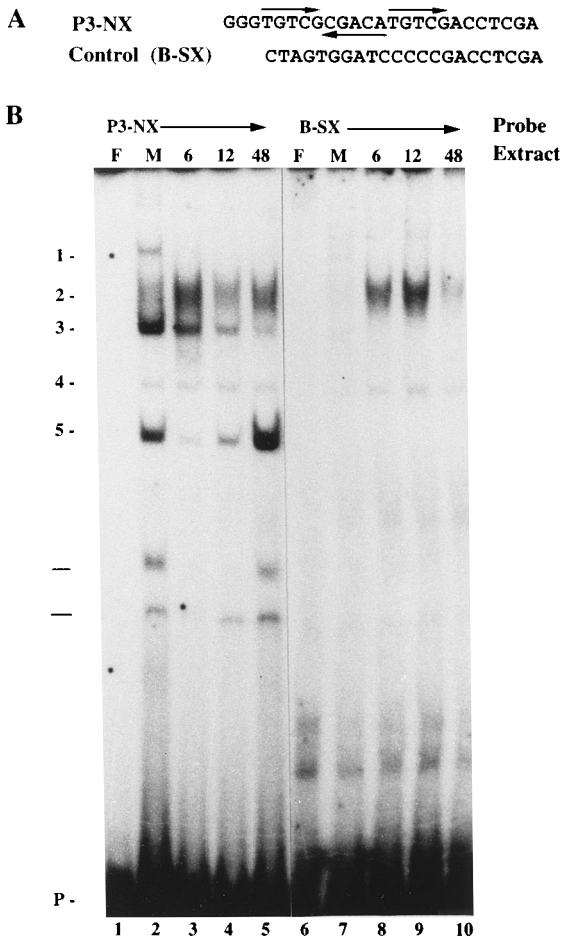

FIG. 7. EMSA of nuclear factors binding to the P3-NX probe at various times after infection of HFF cells with HCMV. (A) Sequences of the probes used. Probe P3-NX contains three copies of the pentanucleotide TGTCG (indicated by arrows) and some vector flanking sequence. Control probe B-SX contains the same vector sequence but has the pentanucleotides deleted. (B) EMSA using the P3-NX probe (lanes 1 to 5) and the control probe (lanes 6 to 10) with various nuclear extracts. Lanes: 1 and 6, no extract; 2 and 7, mock-infected HFF cells; 3 and 8, extract 6 h postinfection; 4 and 9, extract 12 h postinfection; 5 and 10, extract 48 h postinfection. The locations of the DNA-protein complexes and the free probe (P) are labeled on the left. DNA-protein complex 4 serves as an internal control for the same amount of nuclear extract in each lane. Complexes denoted by a line were not always detectable.

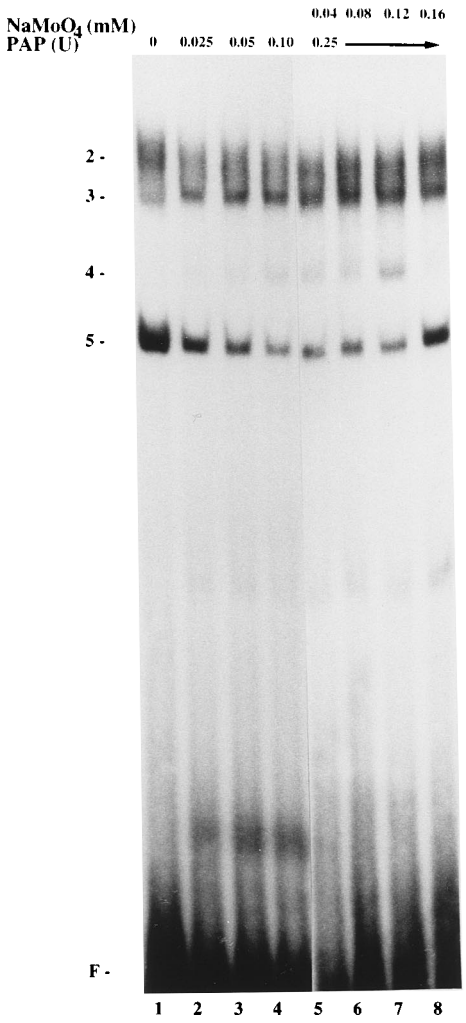

FIG. 8. Effect of dephosphorylation in the binding of HFF cellular protein to form DNA-protein complex 5. EMSA were performed with cellular nuclear extracts and ^{32}P-labeled P3-NX probe. In addition, increasing concentrations of potato alkaline phosphatase (PAP) with or without its inhibitor ($NaMoO_4$) were added. Positions of each of the DNA-protein complexes and of free probe (F) are indicated on the left.

stream of the MIEP (Fig. 9). Assuming a Poisson distribution, such clusters would be expected to occur at random only once every 10^{16} times in similar-size regions. Thus, the clustering of the pentanucleotide in these two regions of the viral genome may have a role in regulating IE transcription. The pentanucleotide consensus sequence is TRTCG, where R represents either purine. The sequences containing the TGTCG pentanucleotide were capable of repressing expression of a reporter gene in transient transfection experiments. Whether the TATCG pentanucleotide functions in the same manner as the TGTCG was not determined. The cluster of TRTCG motifs in the more complicated modulator may also function to repress transcription, but this possibility remains to be tested.

Results of EMSAs and cold competition-EMSAs indicate that the DNAs containing the pentanucleotide TGTCG bind specific cellular proteins in HFF or HeLa cell nuclear extracts to generate what we refer to as DNA-protein complexes 3 and 5. While the $7R_1$-containing DNA fragment contains both 5′ to 5′ (three) and 3′ to 3′ (eight) arrangements of either the TGTCG or TATCG pentanucleotide (Fig. 9A), only the 5′-to-5′ arrangement efficiently generated DNA-protein complex 5. The effect of the flanking sequences and the optimal number, distance, and orientation of these sites are unknown. Although only a single pentanucleotide is required for repression of gene expression in a transient transfection assay, the reason for the R_1 arrangement of the pentanucleotides remains to be determined by investigating wild-type and mutant elements in recombinant viruses.

Using a DNA probe that contained both of the viral genomic arrangements of TGTCG pentanucleotides, we found that these complexes change through the course of the HCMV replication cycle in HFF cells. DNA-protein complex 3 decreased through time, whereas DNA-protein complex 5 was significantly decreased in the very early stage of infection but returned by 12 h after infection. The US3 gene is transcribed primarily in the IE phase of infection, with its maximal expression between 2 and 5 h (47). The US3 promoter, like the MIEP in the UL component of the viral genome, contains a *cis* re-

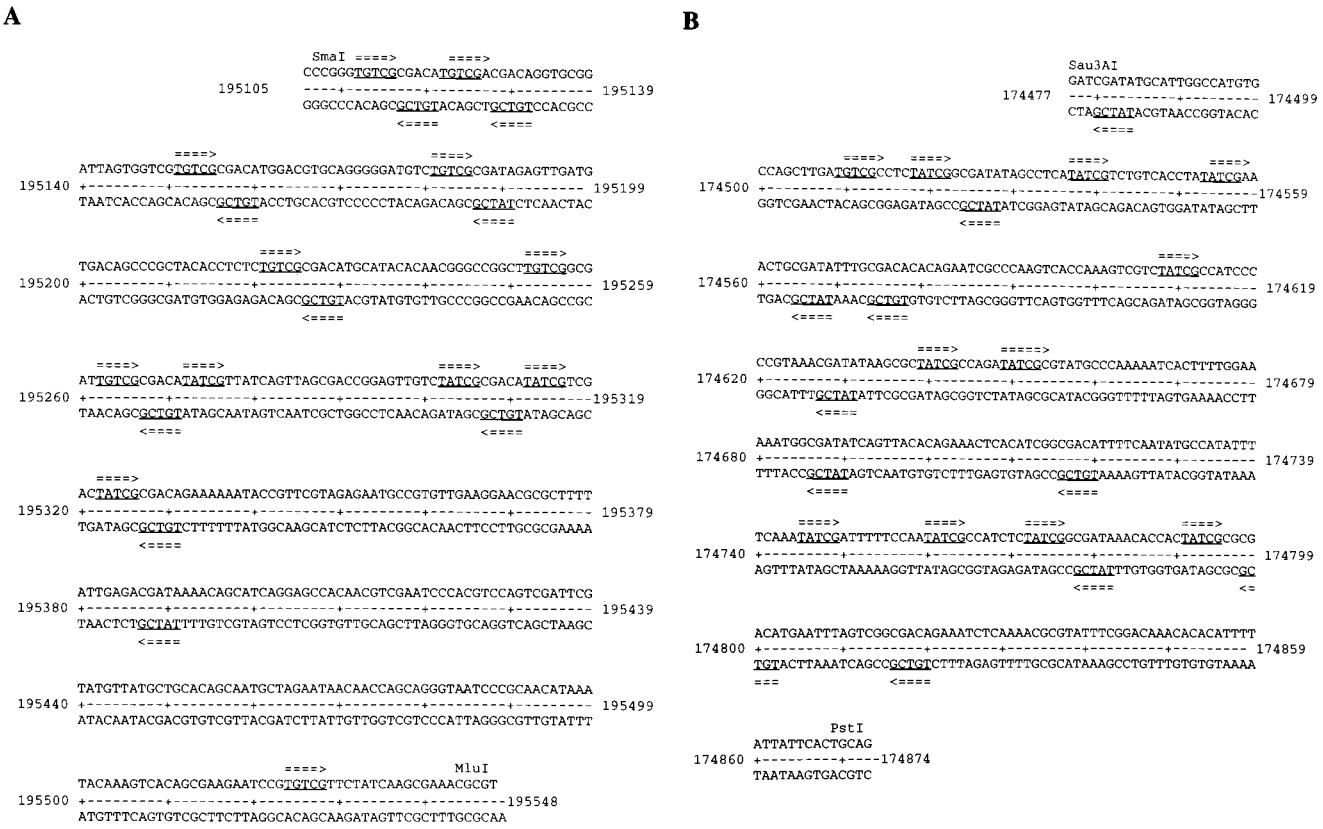

FIG. 9. Location of pentanucleotides within the upstream regulatory region of the IE US3 and UL122-123 genes. (A) Sequence of the US3 upstream region between the *Sma*I site at −334 (195,105 bp) and the *Mlu*I site at −771 (195,548 bp). The TRTCG pentanucleotides are underlined, and their orientations are indicated by arrows. (B) Sequence of the UL122-123 modulator between the *Sau*3AI site at −760 (174,477 bp) and the *Pst*I site at −1145 (174,874 bp). The TRTCG pentanucleotides are underlined, and their orientations are indicated by arrows. Base pairs are numbered according to Chee et al. (11).

pression signal between positions −1 and −13 relative to the transcription start site (5). The viral IE2 protein can bind to the *cis* repression signal and repress downstream transcription (31). Repression of US3 transcription at approximately 6 h after infection may be caused by IE2 protein binding. Repression at late times after infection (12 to 48 h) and after viral DNA replication may also require cellular repressor protein binding to the upstream silencer element associated with the R_1 sequences. While DNA-protein complex 3 or 5 may repress transcription from the US3 promoter in transiently transfected cells, DNA-protein complex 5 alone may repress US3 transcription late in HCMV infection. The difference in the cellular proteins that make up DNA-protein complexes 3 and 5 requires further investigation. Our experiments indicate that DNA-protein complex 5 is affected by dephosphorylation which may occur during the viral replication cycle.

We propose that the activation of transcription of the HCMV US3 gene at early times after infection is due in part to a release of the repression from the $7R_1$ silencer region. Transcriptional activation by the release of silencing has been proposed for other eukaryotic genes such as the interleukin-2 gene and the adenovirus E1A promoters (22, 35). Release of silencing may be due to a reversible modification or inactivation of a DNA-binding protein. These cellular proteins may influence the level of US3 or IE1/IE2 transcription in various cell types.

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