A Novel *cis* Element Essential for Stimulated Transcription of the p41 Promoter of Human Herpesvirus 6

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The p41 DNA-binding protein of human herpesvirus 6 is an apparent processivity factor important for viral DNA replication. The p41 promoter was characterized to understand how this processivity factor is regulated. A single transcription start site and a functional TATA box are located 48 and 74 bp, respectively, upstream of the start codon. A reporter construct containing 1,027 bp of the sequence upstream of the p41 start codon was inactive in uninfected T cells but functioned as a strong promoter in human herpesvirus 6-infected cells. Mutational analysis identified a 21-bp element (the EA site) which is located at –73 to –52 bp relative to the transcription start site and is essential for promoter activity. The ability of the EA site to stimulate transcription optimally appears to be strictly dependent upon its distance from the p41 basal promoter. The EA site contains three overlapping sequences, a CAAT-enhancer-binding protein (C/EBP) transcription factor recognition site and two repeat elements. Mobility shift assays using the EA site identified four binding activities (C1 to C4). C1 and C2 are present in both uninfected and infected cells and do not contain C/EBP factors. In infected cells, point mutation of the EA site abrogates C1 and C2 binding activities and destroys transcriptional activity of the p41 promoter. C3 and C4 are present in uninfected cells only and were found to contain C/EBP factors. These findings indicate that in infected cells, transcriptional stimulation of the p41 promoter by the EA site requires C1 and C2 binding activities. These results further suggest that transcriptional activity may also depend upon the elimination of C3 and C4 binding activities.

Human herpesvirus 6 (HHV-6) is a T-cell-tropic virus isolated in 1986 from patients with lymphoproliferative disorders and AIDS (32). Seroepidemiological studies have revealed that HHV-6 is widespread in the healthy adult population and that seroconversion occurs early in life (20). It has been established that HHV-6 is the causative agent of roseola infantum (exanthem subitum), a disease of early childhood which is benign in most cases. HHV-6 may also play a role in certain lymphoproliferative disorders (1, 36) and, of current medical relevance, it is implicated as a cofactor in human immunodeficiency virus (HIV) infections. Coinfection of HIV-infected cells with HHV-6 results in transcriptional stimulation of the HIV long terminal repeat and increased replication of HIV (9, 12, 23). In addition, HHV-6 infection can upregulate expression of CD4, the HIV cell surface receptor, in both CD4⁺ CD8⁺ T cells and natural killer cells (22, 24). In this way, HHV-6 infection is able to make nonpermissive cells susceptible to HIV infection.

HHV-6 has a double-stranded (ds) DNA genome of approximately 170 kb consisting of a unique long region flanked by 10-kb direct repeats (26). Sequence and hybridization analyses indicate that HHV-6 is most closely related to human cytomegalovirus (HCMV) (18, 28). A number of HHV-6 genes have been identified by their homologies to HCMV genes, as well as to genes conserved among all human herpesviruses, e.g., DNA polymerase, glycoprotein H, and major capsid protein (14, 21, 34). Products encoded by other HHV-6 genes have been identified by using monoclonal antibodies (2, 5, 13).

We previously reported the cloning of an HHV-6 gene

encoding a 41-kDa nuclear protein (p41) which is expressed at early times of infection (2). p41 is highly homologous to infected cell protein 36 (ICP36) of HCMV, and its location in the HHV-6 genome is analogous to the location of ICP36 in the HCMV genome. Also, p41 binds DNA with the same affinity as does ICP36. Because ICP36 is a processivity factor which stimulates viral replication through its association with HCMV DNA polymerase, a similar function for p41 is likely. The processivity factors of human herpesviruses (11, 15, 27) are believed to be essential for viral propagation, since a herpes simplex virus type 1 mutant carrying an alteration in the UL42 processivity factor gene was defective for viral replication (25).

Since it is likely to be essential for viral replication, analysis of p41 expression is important in understanding the growth and propagation of HHV-6. In this study, we examined transcriptional regulation of p41. The p41 promoter was identified and shown to be highly active in HHV-6-infected cells but inactive in uninfected cells. In addition to a single transcriptional start site and a functional TATA element, the p41 promoter was shown to contain a novel 21-bp element that is critical for transcriptional activity in HHV-6-infected cells. This 21-bp element (the EA site) has a strict spatial requirement for its activity and contains three overlapping elements, a CAAT-enhancer-binding protein (C/EBP) consensus site and two repeat elements. The EA site binds four major complexes, C1 to C4. Two of the complexes, C1 and C2, recognize the EA site in both infected and uninfected cells, and their binding to the site is not mediated by C/EBP factors. However, two other complexes, C3 and C4, which recognize the EA site in uninfected cells only, were shown to contain C/EBP factors. The findings indicate that p41 promoter activity in infected cells requires C1 and C2 complex binding and further suggest that p41 promoter activity is dependent upon elimination of the C3 and C4 complexes.

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MATERIALS AND METHODS

Virus and cell lines. The HSB-2 human T-cell line was grown in RPMI 1640 (Gibco) and 10% fetal calf serum. HSB-2 cells were infected with the GS strain of HHV-6. Infection was maintained by addition of uninfected HSB-2 cells to infected cells displaying a >80% cytopathic effect.

HHV-6 genomic library construction and screening. Purified HHV-6 virus, kindly provided by Dharam Ablashi, was prepared as previously described (1). Viral DNA was prepared by resuspending virions in an equal volume (500 μ l) of 100 mM NaCl–10 mM Tris-HCl (pH 8.0)–25 mM EDTA (pH 8.0)–0.5% sodium dodecyl sulfate–0.5 mg of proteinase K (Boehringer Mannheim) per ml, incubated for 5 h at 37°C, and extracted with phenol-chloroform, and the DNA was ethanol precipitated. HHV-6 viral DNA (1 μ g) was digested with *Eco*RI and ligated into the dephosphorylated *Eco*RI site of lambda ZAP II phage arms (10 μ g) (Stratagene). Ligated phage were packaged by using Gigapack II Gold Packaging Extract (Stratagene). Phage were propagated in *Escherichia coli* XL-1 blue to yield a library with greater than 99% recombinant phage. The library was screened by using the Genius DNA Detection Kit (Boehringer Mannheim) and the open reading frame (ORF) of p41 as a probe (2). pBluescript plasmid vectors containing the cloned inserts were obtained from positive phage by *in vivo* excision (Stratagene). Sequencing of isolated clones verified that they encoded the p41 ORF and upstream sequences.

DNA sequence analysis. Sequencing of ds plasmid DNA was performed by the dideoxynucleotide chain termination method using synthetic oligonucleotide primers (33). Both strands of the upstream region of the HHV-6 genomic clone encoding the p41 ORF were sequenced. Sequence data were analyzed by using the University of Wisconsin Genetics Computer Group Sequence Analysis Package and the PIR and Tfsites data bases on a Microvax 3300 (8).

Primer extension analysis. Total cytoplasmic RNA was isolated from uninfected HSB-2 T cells and HSB-2 T cells nonsynchronously infected with HHV-6 as previously described (29). A synthetic oligonucleotide (5'-GATCACGGT GATCACGATGATGATCTCGACTACCG-3') was ³²P end labeled and annealed to 20 μ g of RNA in hybridization buffer (150 mM KCl, 10 mM Tris-Cl [pH 8.3], 10 mM EDTA) at 65°C for 90 min. Reactions were carried out in 30 μ l of reverse transcriptase buffer (20 mM Tris-HCl [pH 8.3], 10 mM MgCl₂, 5 mM dithiothreitol [DTT], 0.2 μ g of actinomycin D per ml, 1 mM deoxynucleoside triphosphates) with 1 U of reverse transcriptase (SuperScript II; Gibco) at 42°C for 60 min. A 105- μ l volume of an RNase reaction mixture (100 μ g of salmon sperm DNA per μ l, 20 μ g of RNase A per ml) was added to each sample, and the combination was incubated at 37°C for 15 min. Samples were phenol-chloroform extracted and ethanol precipitated. The products were fractionated on an 8% polyacrylamide–7 M urea gel and visualized by autoradiography at –80°C with Kodak XAR film and intensifying screens (DuPont).

S1 nuclease protection. A 425-bp DNA probe was prepared by PCR of the HHV-6 genome with an oligonucleotide primer complementary to an internal region of the p41 ORF (5'-AGTTTCCAGCTTTT-3') and a *Kpn*I-containing primer complementary to a sequence upstream from the ORF (5'-AAAAAAGGTACCGAATGATTGATTGA-3'). The PCR product was 5' end labeled with [γ -³²P]ATP (Amersham) and digested with *Kpn*I to generate a probe labeled at one end. S1 nuclease analysis was performed as previously described, with a heat-denatured probe (4).

Plasmid constructs. The parental vector used for all constructs was a modified pUC119 plasmid in which a region from the *Sac*I site to the *Bgl*II site was deleted. The chloramphenicol acetyltransferase (CAT) gene was subcloned into the *Bam*HI and *Hind*III sites of pBluescript (Stratagene) to generate pBICAT. The full-length p41 promoter was generated by PCR-assisted cloning with the HHV-6 genome as the template and a primer containing a synthetic *Kpn*I site that hybridizes at the 5' end of the p41 genomic clone in conjunction with a 3' primer containing a synthetic *Hind*III site that hybridizes to the sequences immediately upstream of the start codon of the p41 ORF. This PCR product was subcloned into the *Kpn*I and *Hind*III sites of the pBICAT construct. The entire p41 promoter-CAT cassette was subcloned into the *Kpn*I and *Hind*III sites of the modified pUC119 vector to generate pEA-979CAT. The promoter deletion series was generated by PCR of promoter fragments as described above, and fragments were subcloned into the *Kpn*I and *Hind*III sites of pEA-979CAT after removal of the full-length promoter. The p0CAT construct was generated from pEA-979CAT by removal of the p41 promoter, blunt ending, and religation. Oligonucleotide-directed mutagenesis was used to generate pEA-979delCAT, pEA-979TATAmut CAT, and pEA-979 mutCAT (Amersham Mutagenesis System). The pEA-73insCAT insertion constructs were prepared as follows. A p41 basal promoter product (bp –33 to +48) was generated by PCR with a 5' primer containing synthetic *Cl*aI and *Kpn*I sites and a 3' primer containing a synthetic *Hind*III site as described above. We prepared a second basal promoter which differed only by inclusion of five cytosine residues 3' of the *Cl*aI site. These two PCR products were subcloned into the *Kpn*I and *Hind*III sites of pEA-979CAT after removal of the full-length promoter. An EA site (bp –73 to –33) PCR product was generated by using a 5' primer containing a *Kpn*I site and a 3' primer containing a *Cl*aI site and subcloned into the *Kpn*I and *Cl*aI sites of the two basal promoter constructs to generate the 6- and 11-bp pEA-73insCAT constructs. The 8- and 13-bp insertion constructs were generated from the 6- and 11-bp insertion constructs, respectively, by *Cl*aI digestion, Klenow filling in, and religation. A 606-bp insertion construct was generated by subcloning a 600-bp PCR product from pBluescript SK– (Stratagene) into the *Cl*aI site of pEA-73ins6CAT. The constructs were confirmed by sequencing.

Transfections-infections and CAT assays. HSB-2 cells were transfected with CAT plasmids by the DEAE-dextran transfection method (4). Briefly, 2×10^7 cells were washed and resuspended in a solution containing 200 μ g of DEAE-dextran (Sigma) per ml and 10 μ g of plasmid DNA. The suspension was incubated for 45 min at 37°C, and cells were shocked by addition of dimethyl sulfoxide (final concentration, 10%) for 2 min. Cells were washed and resuspended in RPMI containing fetal calf serum. For infection, one-half of the transfected cells were added to an equal number of HHV-6-infected HSB-2 cells displaying a greater than 80% cytopathic effect. The other half of the transfected cells were added to an equal number of uninfected HSB-2 cells. CAT assays were performed as previously described (10), and reactions were incubated for either 30 or 60 min.

Nuclear extract preparation. We collected 10^9 uninfected HSB-2 cells or HHV-6-infected cells displaying >80% cytopathic effect, washed them in phosphate-buffered saline, and resuspended them in 2 packed cell volumes of cell lysis buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.0], 3 mM MgCl₂, 40 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 5% glycerol, 8 μ g of aprotinin per ml, 2 μ g of leupeptin per ml, 0.2% Nonidet P-40). Samples were incubated for 5 min at 4°C, and nuclei

were pelleted and resuspended in 2 packed nuclear volumes of extraction buffer (20 mM HEPES [pH 7.9], 0.42 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol). Samples were incubated for 45 min at 4°C with gentle shaking and centrifuged, and supernatants were dialyzed for 5 h at 4°C against 50 volumes of dialysis buffer (20 mM HEPES [pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol). Dialyzed samples were aliquoted and stored at -70°C.

Mobility shift assays. Probes used for mobility shift assays were prepared by annealing complementary synthetic oligonucleotides with overhanging ends (see Table 1). Probes were radiolabeled by Klenow filling of ends with [³²P]dCTP. For the binding reactions, 15 µg of nuclear cell extract was incubated at 30°C for 30 min in binding buffer (50 mM HEPES [pH 7.9], 300 mM KCl, 5 mM MgCl₂, 2.5 mM EDTA, 5 mM DTT, 50% glycerol) with 50,000 cpm of the radiolabeled probe and 2.5 µg of poly(dI-dC). Samples were run on a 4% nondenaturing polyacrylamide gel which was dried and visualized by autoradiography.

Nucleotide sequence accession number. The GenBank-EMBL accession number for the p41 promoter sequence is L29124.

RESULTS

The p41 gene of HHV-6 has a single transcription start site.

Two major RNA transcripts of 2.6 and 2.1 kb and a minor transcript of 1.7 kb were detected in HHV-6-infected cells by Northern (RNA) blot analysis using the p41 ORF as the probe (2). To map the 5' end(s) of these transcripts precisely, primer extension and S1 nuclease protection were performed on cytoplasmic RNA from uninfected and HHV-6-infected HSB-2 T cells. For primer extension analysis, an oligonucleotide complementary to a 5' internal region of the p41 ORF was used (Fig. 1A). An extended product of 91 nucleotides was generated with RNA from HHV-6-infected cells (Fig. 1B), whereas no extension products were obtained with RNA from uninfected cells. The position of this single transcription (48 bp 5' to the start codon) was confirmed with two other oligonucleotide primers complementary to different regions within the p41 ORF (data not shown).

To examine the transcriptional 5' end of p41 by S1 nuclease analyses, a genomic probe was required. A 3.7-kb *Eco*RI genomic fragment that included the entire p41 ORF was isolated from an HHV-6 genomic phage library that had been prepared from purified viral DNA. By using this genomic clone, the sequence of the 1,027-bp region which is 5' to the ATG start codon of the p41 ORF was determined. S1 nuclease analysis was performed with a 425-bp PCR-derived probe that extended from an internal region of the p41 ORF to 318 nucleotides 5' of the p41 start codon (Fig. 1A). In HHV-6-infected cells, a single protected fragment of 155 nucleotides was detected, while in uninfected cells, no protected fragments were observed (Fig. 1C). This is consistent with the results obtained by primer extension analysis and confirms that there is a single transcription start site for the p41 gene which is located 48 bp 5' of the translation start codon. These results indicate that in the HHV-6 viral genome, the 5' untranslated regions of the p41 transcripts are contiguous with the p41 ORF and thus are not derived from upstream splicing events.

Sequence of the p41 promoter region. The sequence of the genomic region immediately upstream from the start codon of p41 is shown in Fig. 2A. A TATA box (TATATAA) is present 26 bp 5' of the p41 transcription start site (arrow). Analysis of

FIG. 1. The p41 gene contains a single transcription start site. (A) Schematic representation of primer extension and S1 nuclease protection experiments. The ORF of p41 (open rectangle) and the transcription start site (bent arrow) are indicated. For primer extension, an oligonucleotide (shaded rectangle) recognizing a 5' region of the ORF of p41 mRNA was used to generate a 91-nucleotide product. For S1 nuclease analysis, a 425-bp probe (dashed line) spanning the p41 ORF and the immediate 5' region generated a protected product of 155 bp (solid line). (B) Primer extension. RNAs (20 µg) from uninfected and HHV-6-infected HSB-2 T cells were analyzed. A plasmid (pBluescript) sequencing ladder was used to size the products. (C) S1 nuclease analysis. RNAs (25 µg) from uninfected and HHV-6-infected HSB-2 T cells were analyzed. A control plasmid (pBluescript) sequencing ladder was used to determine the product sizes.

the protein-coding content of this upstream region identified an ORF whose termination codon is 68 bp 5' to the p41 transcription start site. A data base search revealed that the protein encoded by this HHV-6 ORF is homologous to the C-terminal portion of the large subunit of ribonucleotide reductase protein UL45 of HCMV, with 17.3% identity and 43.6% conservation (Fig. 2B). In the HCMV genome, the UL45 ORF is located immediately upstream of the UL44 ORF, which encodes the processivity factor ICP36, the homolog of HHV-6 p41 (Fig. 2B) (7). Therefore, there is conservation of gene organization in these regions of HHV-6 and HCMV. This further confirms the relatedness between these two viruses.

Identification of a cis element required for p41 promoter activity. To examine the ability of the region immediately upstream of the p41 ORF to function as a promoter, the entire 1,027-bp segment 5' of p41 was inserted upstream of the reporter CAT gene to obtain pEA-979CAT (Fig. 3A). HSB-2 T cells were transfected with pEA-979CAT and either cocultured with an equal number of HHV-6-infected cells displaying a greater than 80% cytopathic effect or cocultured with an equal number of uninfected HSB-2 cells. There was a large stimulation of CAT activity in the transfected-infected cells compared with the transfected-uninfected cells (Fig. 3B). The p0CAT construct, which lacks the p41 upstream region, failed

FIG. 2. Sequence and organization of the HHV-6 viral genome in the region upstream of the p41 ORF. (A) Sequence of the p41 promoter region. Denoted are the ATG of p41 (bracketed), the transcriptional start at +1 (bent arrow), and the TATA element (underlined). Indicated are nucleotide positions relative to the transcription start site (left side) and amino acid numbers (right side). (B) Comparison of genomic organization between HHV-6 and HCMV. ORFs of the virally encoded proteins are indicated. Sequence analysis comparisons were done by using the Wordsearch and Bestfit programs in the University of Wisconsin Genetics Computer Group DNA sequence analysis package.

to produce CAT activity in uninfected or infected cells. These results indicate that the 1,027-bp region 5' of the p41 ORF functions as a strong promoter in HHV-6-infected cells.

To delineate the elements within the 1,027-bp sequence that are essential for p41 promoter activity in HHV-6-infected cells, a mutational analysis was performed (Fig. 4). First, the importance of the TATA box located 26 bp 5' of the p41 transcription start site was examined by changing the TATATAA sequence to TAGCGCA (Fig. 4, bottom construct). This alteration produced a 17-fold decrease in promoter activity, confirming that the p41 TATA box functions as a basal transcription element (Fig. 4).

Next, a 5' deletion series of the 1,027-bp promoter was generated. As seen in Fig. 4, deletions of sequences up to -73 bp relative to the transcription start site had no effect on promoter activity. A further 21-bp deletion (-52 construct) resulted in an eightfold decrease in promoter activity in infected cells. Eliminating all sequences up to the TATA box (-33 construct) produced a basal promoter construct which had only half of the activity of the -52 construct. All constructs were inactive in uninfected cells, demonstrating that this promoter is stimulated specifically during HHV-6 infection. The deletion series indicates that sequences between bp -73 and -52, relative to the p41 transcription start site, are critical for promoter activity. To substantiate the importance of this 21-bp region, an internal deletion of sequences between -73 to -52 was made in the context of the full-length promoter. This internal deletion decreased promoter activity to the same

FIG. 3. The region upstream of the p41 ORF acts as a strong promoter in HHV-6-infected cells. (A) Schematic representation of pEA-979CAT, which contains the p41 promoter region linked to the CAT gene. The bent arrow indicates the transcription start site. (B) The p41 promoter is activated in HHV-6-infected T cells. CAT activities of pEA-979CAT and the promoterless construct p0CAT in infected and uninfected HSB-2 T cells were compared. CAT reactions were carried out for 30 min. The percent conversion of chloramphenicol to its acetylated products is indicated by the histogram, which represents the average results of three experiments.

extent as that observed when all sequences up to -52 bp were eliminated.

The sequence of the -73 to -52 region, where p41 inducible promoter activity maps, is shown in Fig. 5A. The 3' flanking sequence is also indicated, since the -52 deletion, which essentially eliminated all promoter activity, could have disrupted a portion of a *cis*-acting element. Data base inspection revealed a C/EBP consensus recognition site (3, 31) within the -73 to -52 region. The C/EBP transcription factors are a

FIG. 4. Identification of critical elements of the p41 promoter by mutational analysis. Indicated for each construct is the transcription start site of the p41 gene (bent arrow), the reporter CAT gene (rectangle), and the sequence of the wt (TATATAA) or mutant (TAGCGCA) TATA box. The numbers to the left refer to nucleotide positions relative to the transcription start site. Constructs were analyzed for CAT activity in both infected and uninfected HSB-2 cells. Quantitations represent the average results of three experiments.

FIG. 5. Analysis of the p41 promoter response element, the EA site. (A) The 21-bp EA site contains a C/EBP recognition element and overlapping repeat elements. The sequence of the EA site within the promoter of the p41 gene is designated by capital letters, and the flanking 3' promoter sequence is indicated by lowercase letters. The numbers refer to nucleotide positions relative to the transcription start site (arrow). The sequences of the C/EBP and repeat elements are coaligned with the sequence of the EA site. In the C/EBP consensus, K is G or T, N is any base, and Y is pyrimidine. The asterisks above the EA site sequence designate nucleotides chosen for mutation in panel B. (B) Point mutations that alter the C/EBP consensus and overlapping repeat sequences destroy p41 promoter activity. Indicated is the p41 promoter driving the CAT gene and the sequences of the TATA box and the EA sites of the wt (pEA-979CAT) and double point mutant (pEA-979mutCAT) constructs. CAT activities of the p41 promoter constructs in infected and uninfected cells are indicated. Quantitations represent the average results of three experiments.

family of bZIP proteins which are expressed in many tissues (3, 6, 16, 30, 35). It is of interest that two of these family members, Ig-EBP-1 and NF-IL-6, are expressed in T cells (3, 30). In addition, the -73 to -52 region also contains an imperfect repeat of the sequence TGTA(A/G)T with a 2-bp spacer and an inverted repeat TGTAG with a 1-bp spacer (Fig. 5A).

A double point mutation was introduced in the -73 to -52 region of the full-length promoter (Fig. 5B). This double point mutation, which altered the C/EBP consensus site, as well as the overlapping direct and indirect repeat sequences, reduced the activity of the p41 promoter in HHV-6-infected cells to the same extent as that observed when the entire -73 to -52 region was deleted, further underscoring the functional importance of this DNA element in transcription. The -73 to -52 region is hereafter referred to as the EA site.

Spacing of the EA site in the p41 promoter is critical for optimal activity. When a *cis* element of the p41 promoter (-73 to -34 bp) that included the EA site was cloned directly upstream of the TATA-like elements of both the simian virus 40 and thymidine kinase basal promoters and examined for HHV-6 inducibility, only twofold increases in CAT activity were observed (data not shown). With the heterologous constructs, no increases of activities were produced when the EA site was multimerized, reversed in orientation, or systematically repositioned to change the helical register relative to the basal promoters (data not shown). One possibility to account for the low activities of the heterologous constructs was lack of precise spacing between the basal promoters and the EA site.

We wondered whether altering the position of the EA site within its natural p41 promoter might have a deleterious effect on the stimulatory ability of the element. We generated constructs that introduced 6, 8, 11, 13, or 606 bp between the EA site and the p41 basal promoter (Fig. 6). Whereas the activity of the wild-type (wt) construct (p-73EACAT) was

FIG. 6. Activity of the EA site is spacing dependent. (A) Constructs: pEA-33CAT, natural p41 basal promoter, which includes the TATA box (-33) and sequences up to the start codon of p41 (+48); pEA-73CAT, includes the natural p41 basal promoter and the EA site (-73 to -33). Insertions of 6, 8, 11, 13, and 606 bp were made in the natural promoter between the EA site and the TATA box. (B) Fold CAT activity in HHV-6-infected cells of the natural p41 promoter construct, pEA-73CAT, and the insertion (Ins) constructs relative to the basal promoter construct, pEA-33CAT. The values represent the average results of three experiments.

increased 14-fold over that of the p41 basal promoter construct (pEA-33CAT) in HHV-6-infected cells, the activity of each insertion construct was increased only three- to fourfold (Fig. 6B). The low activities observed with the insertion constructs are thought to be due to alteration of spacing rather than helical register, since in the Ins11 construct, the helical register of the EA site relative to the basal promoter is restored. Not surprisingly, similar low activity levels were observed with insertion constructs containing the EA site in the reverse orientation. Activity was not obtained for any of these constructs in uninfected cells. These findings indicate that precise spacing of the EA site relative to the natural basal promoter is critical for optimal stimulation of the p41 promoter in HHV-6-infected cells.

Differential EA site binding activity in uninfected and HHV-6-infected cells. To determine whether the EA site of the p41 promoter functions in factor recognition, electrophoretic mobility shift assays were performed. When a ds oligonucleotide containing the wt EA site (Table 1) was labeled and incubated with nuclear extract from HHV-6-infected HSB-2 cells, two binding complexes, C1 and C2, were observed (Fig. 7, compare lanes 1 and 2). These C1 and C2 complexes were specifically eliminated by addition of an unlabeled wt EA site competitor (lanes 3 to 5). Competition was not demonstrated with an oligonucleotide containing the same double point

TABLE 1. Oligonucleotides used in this study

Designation	Sequence
EA wt site ^a	gacTGTAGTGATGTAATAAAATGTTGGCGG ACATCACTACATTATTTTACAACCGCCctag
EA mutant site ^a	gacTGTAGCGATTTAATAAAATGTTGGCGG ACATCGCTAAATTATTTTACAACCGCCctag
C/EBP consensus recognition site	ttaaCAATTGGGCAATCAGG GTTAACCGTTAGTCCttaa

^a Uppercase letters represent nucleotides between positions -73 and -47 relative to the transcription start site of the p41 promoter.

mutation in the EA site that had abrogated the functional activity of the p41 promoter (Fig. 7, lanes 6 to 8) (Table 1). In the converse experiment, complexes C1 and C2 were not observed when the mutant EA site (Table 1) was used as the probe in an electrophoretic mobility shift assay (data not shown). These results point to a direct relationship between a sequence-specific requirement for C1 and C2 binding activity and transcriptional activity of the wt EA site in HHV-6-infected cells.

To determine whether the EA site-binding activity is different in uninfected cells, an electrophoretic mobility shift assay was performed with nuclear extract from uninfected HSB-2 T cells. Besides the C1 and C2 complexes observed with the infected cell extract (Fig. 8, lane 2), two additional complexes, C3 and C4, were detected with the uninfected cell extract (lane 6). C3 and C4 consistently migrated as diffuse complexes under

FIG. 7. Specific binding of nuclear factors from HHV-6-infected cells to the EA site. A ds oligonucleotide containing the wt EA site (Table 1) was used as the probe. The labeled wt EA site oligonucleotide was incubated with HHV-6 nuclear extract either alone (lane 2) or with increasing molar amounts of unlabeled competitor wt EA (lanes 3 through 5) or mutant (mut) EA (lanes 6 through 8) ds oligonucleotides. Lane 1 contained the probe alone. C1 and C2 refer to complexes specifically eliminated by competition.

FIG. 8. Loss of C/EBP binding activity to the EA site in HHV-6-infected cells. Nuclear extracts from infected cells (lanes 2 to 5) or uninfected cells (lanes 6 to 9) were incubated with a ds oligonucleotide containing the labeled wt EA site as a probe, either alone (lanes 2 and 6) or with a 500-fold molar excess of a competitor ds oligonucleotide containing the wt EA site (lanes 3 and 7), the mutant (mut) EA site (lanes 4 and 8), or a C/EBP consensus site (lanes 5 and 9). Lane 1 contained the probe alone. C1, C2, C3, and C4 refer to complexes specifically eliminated by competition.

a variety of gel conditions which employed different ionic strengths, temperatures, and buffering conditions. Competition with a wt, but not a mutant, EA oligonucleotide eliminated C1 and C2 in infected cells (lanes 3 and 4) and C1 to C4 complex binding in uninfected cells (lanes 7 and 8). In addition complexes C1 to C4 were not observed when the mutant EA site (Table 1) was used as the labeled probe in an electrophoretic mobility shift assay.

Since the EA site contains a C/EBP consensus recognition element, it was important to determine whether any of the C1 to C4 binding activities involve actual C/EBP family members. When a C/EBP CAAT box consensus site (3, 17, 31) (Table 1) recognized by C/EBP family members was used as the unlabeled competitor, only complexes C3 and C4 from uninfected cells were eliminated (Fig. 8, lane 9). In contrast, there was no reduction in the binding activity of C1 or C2 from either infected or uninfected cells (Fig. 8, lanes 5 and 9). It is important to note that the C2 binding activity in the uninfected cell extract was clearly resolved when the C3 binding activity was specifically eliminated by competition (compare lanes 6 and 9). These results indicate that C/EBP family members are present in complexes C3 and C4 and that HHV-6 apparently downregulates the C3 and C4 binding activities, since they are absent in infected cells.

DISCUSSION

The p41 DNA-binding protein of HHV-6 is a putative processivity factor, on the basis of its homology to ICP36 of HCMV, and is proposed to be essential for viral replication

(2). As we have shown in this study, an ORF immediately upstream of the p41 promoter encodes a homolog of the HCMV large subunit of ribonucleotide reductase, further underscoring the relationship between HHV-6 and HCMV. Although the genomic organization and functionality of p41 and ICP36 appear to be similar, the proteins differ markedly in transcriptional regulation. The gene for ICP36 utilizes three distinct transcriptional start sites, each separated by approximately 50 nucleotides and each having a corresponding upstream TATA box element (19). Transcription from start sites 1 and 3 occurs at early times postinfection and persists until late times, while transcription from start site 2 occurs only at late times postinfection. Significantly, nearly the same level of promoter activity is observed with the full-length ICP36 promoter and a mutant construct in which all sequences upstream of the most proximal TATA element has been deleted. In contrast, the p41 promoter has a single transcription start site and a single TATA element and loses almost all activity when sequences upstream of the TATA element are deleted. Full activity of the p41 promoter is dependent upon the presence of a 21-bp element (EA site) located 19 bp upstream of the TATA box. This differential regulation of highly conserved processivity factor genes may reflect differences in the cellular tropisms of these herpesviruses.

The EA site contains a C/EBP recognition site, TGATGTAAT. Also contained within the EA site and overlapping the C/EBP site is a 6-bp direct repeat separated by a 2-bp spacer, TGTAGT(ga)TGTAAT, as well as a 5-bp inverted repeat separated by a 1-bp spacer, TGTAG(t)GATGT (Fig. 5A). HHV-6-infected cell nuclear extracts contain two binding activities, complexes C1 and C2, that specifically recognize the EA site. C1 and C2 appear to be critical for transcription, since a mutation in the EA site that disrupts their binding activity also diminishes p41 promoter activity. The fact that C1 and C2 are present in uninfected cells indicates that the factors that compose these complexes are not virally encoded or induced. Because C/EBP family members were shown not to be present in C1 or C2, since neither complex could be eliminated by competition with a C/EBP consensus element, it is possible that the other symmetrical repeat elements within the EA site are important for factor recognition. Indeed, the double point mutation that disrupted both binding activity and promoter activity also perturbed the half sites of the direct and indirect repeat elements of the EA site.

Interestingly, two other complexes, C3 and C4, are present in nuclear extracts of uninfected cells but not in those of infected cells. Factors present in C3 and C4 are C/EBP family members, since these complexes can be eliminated by competition with the C/EBP consensus element. The absence of these complexes in infected cells indicates that HHV-6 infection inhibits C3 and C4 binding activities. It is possible that in uninfected cells, C3 and C4 repress the p41 promoter and that in infected cells, C3 and C4 become downregulated to relieve this repression. Two C/EBP family members expressed in T cells, which may be present in the C3 and/or C4 binding complexes, are NF-IL-6 and IgEBP-1. NF-IL-6 is a transcription factor that upregulates expression of interleukin 6 via a C/EBP consensus site in the interleukin 6 promoter (3). Ig-EBP-1 is a ubiquitously expressed protein that recognizes the E sites in both the immunoglobulin H enhancer and the V_H1 promoter (30). The extent to which NF-IL-6 and/or Ig-EBP-1 are involved in recognition of the EA site and regulation of the p41 promoter has yet to be determined.

The EA site is unusual in that there is a spatial constraint on its ability to mediate transcriptional activation. Promoter activity is destroyed by small incremental insertions (e.g., 6 or 8

bp) and cannot be restored by producing a complete turn of the DNA helix (i.e., an 11-bp insertion). The importance of spacing may be relevant to how factors of the C1 and C2 complexes recognize the EA site and mediate transcription. Identification of factors that are present in the C1 and C2 complexes that recognize the EA site of the p41 promoter may reveal a novel form of transcriptional regulation that is important to T-cell biology.

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