An ATF/CREB Site Is the Major Regulatory Element in the Human Herpesvirus 6 DNA Polymerase Promoter

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Human herpesvirus 6 (HHV-6) is a recently described T-cell pathogen whose medical relevance and molecular biology are just beginning to be addressed. As a first look at the regulation of viral genes, control of the HHV-6 DNA polymerase promoter was examined. Polymerase gene transcription in HHV-6-infected cells was found to initiate from a single site located 115 bases upstream of the translation start codon. A polymerase promoter-chloramphenicol acetyltransferase reporter gene construct failed to be expressed in uninfected T cells but was highly active in HHV-6-infected cells. Mutational data indicated that the polymerase promoter is TATA-less. Mutational analysis also revealed that the major upstream promoter regulatory element required for transcriptional activity in HHV-6-infected cells is a palindromic ATF/CREB transcription factor binding site. The significance of this site for promoter induction was further demonstrated by the fact that the polymerase ATF/CREB element, when appended to a heterologous basal promoter, is highly responsive to HHV-6 infection. Two protein complexes were found to bind in a specific manner to the ATF/CREB motif in both uninfected and HHV-6-infected T-cell nuclear extracts. Site-specific mutation of the ATF/CREB site resulted in loss of protein binding as well as loss of promoter activity in HHV-6-infected cells.

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood lymphocytes of patients with AIDS and with lymphoproliferative disorders (35). The virus was subsequently shown to be ubiquitous in the healthy adult population, with seropositivity rates in excess of 80% (2). Initial infection generally occurs between 1 and 3 years of age, and thereafter latent virus is thought to be carried for life (22). HHV-6 has been shown to cause exanthem subitum (roseola infantum), a benign rash and fever of early childhood, and is likely to be responsible for some cases of heterophile-negative infectious mononucleosis (37, 43).

The virus is predominantly tropic for CD4+ T lymphocytes but can infect several other cell types as well (2, 27). Since CD4 is the cellular receptor utilized by human immunodeficiency virus type 1 (HIV-1), a potential interaction between these two viruses has been investigated. Coinfection of T cells with HIV-1 and HHV-6 results in increased replication of HIV-1 in vitro (25). Also, HHV-6 infection of either CD8+ T cells or CD3+ natural killer cells causes upregulation of CD4, rendering these cells susceptible to HIV-1 infection (24, 26).

HHV-6 is shed intermittently in the saliva of healthy adults, without associated changes in antibody titer (22). This reactivation is a likely source of person-to-person transmission. HHV-6 reactivation, while generally asymptomatic in the healthy population, may have serious consequences in immuno suppressed individuals. In bone marrow transplant patients, HHV-6 is associated with suppression of marrow function, and in vivo studies indicate that HHV-6 can impair macrophage maturation (4).

HHV-6 has a double-stranded DNA genome of approximately 170 kb, consisting of a unique long region flanked by 10-kb direct repeats (28). The virus is predicted to encode more than 70 proteins. Partial sequence analysis has revealed that HHV-6 is most closely related to human cytomegalovirus (HCMV) and that the genomic organization of the unique long regions of HHV-6 and HCMV are largely collinear (19, 32).

The DNA polymerase gene of HHV-6 has been cloned and characterized (39); however, little is known about the regulation of this or any other HHV-6 gene. In this study, the HHV-6 DNA polymerase promoter region was analyzed. It was found that a promoter-CAT construct is not expressed in uninfected T cells but is highly upregulated following HHV-6 infection. The most important promoter upstream regulatory element has been identified as a palindromic binding site for the ATF/CREB (hereafter referred to as ATF) family of transcription factors. This element can strongly confer HHV-6 inducibility to a heterologous promoter.

MATERIALS AND METHODS

Cells and virus. The HSB-2 human T-cell line was grown in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum and antibiotics (2). HSB-2 cells were infected with the HHV-6 strain GS, and infected cell cultures were maintained by addition of uninfected HSB-2 cells to infected cells displaying cytopathic effect (15).

DNA sequence analysis. Sequencing of double-stranded plasmid DNA was performed by the dideoxy nucleotide chain termination method using synthetic oligonucleotide primers (36). Sequence data were analyzed by using the University of Wisconsin Genetics Computer Group sequence analysis package and the GenEMBL and STF sites data bases (7).

Primer extension. Total cytoplasmic RNA was isolated as previously described (33) from both uninfected and HHV-6-infected HSB-2 cells. A 20-base oligonucleotide (5’-CCTGTC TATCAAACAGTGT-3’) complementary to the polymerase 5’ untranslated region was 32P end labelled, and 100 cpm of the oligonucleotide was ethanol precipitated along with 20 μg of RNA. Primer extensions were carried out as described elsewhere (3). Samples were resuspended in hybridization buffer (133 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid] [pH 7.5], 1.0 M NaCl, 0.3 mM EDTA) and incubated overnight at 30°C. Following precipitation, samples...
were reverse transcribed in 40 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl2, 5 mM dithiothreitol (DTT), 0.6 mM each deoxynucleoside triphosphate, and 100 U of reverse transcriptase (Superscript; Bio-Rad Laboratories) for 90 min at 42°C. RNase A was added, and the mixture was incubated for 10 min at 37°C. Samples were phenol-chloroform extracted, ethanol precipitated, and separated on a 6% polyacrylamide–7 M urea gel. A sequencing reaction was run alongside to size the primer extension products. Autoradiography was performed on dried gels at −80°C with Kodak XAR film and intensifying screens (DuPont).

**S1 nuclease protection.** A 383-bp DNA fragment was PCR amplified from purified HHV-6 genomic DNA (16). The upstream primer (relative to the polymerase open reading frame) contained a KpnI restriction site. The downstream primer hybridized within the polymerase open reading frame. The PCR product was gel purified, 32P end labeled, and digested with KpnI to generate probe labeled at one end only. S1 protection assays were carried out as described elsewhere (3). The labelled probe was heat denatured, and 5 × 10⁴ cpm was added to 20 μg of cytoplasmic RNA prepared as described above. The samples were ethanol precipitated, resuspended in S1 hybridization buffer (80% formamide, 40 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM EDTA), heated to 65°C for 10 min, and incubated overnight at 30°C. After ethanol precipitation, S1 nuclease digestion was carried out in 0.28 M NaCl, 50 mM sodium acetate (pH 4.5), 4.5 mM ZnSO₄, 20 μg of single-stranded calf thymus DNA per ml, and 20 U of S1 nuclease (Boehringer Mannheim) for 60 min at 30°C. Samples were ethanol precipitated in the presence of 40 μg of tRNA per ml and then separated on a 6% polyacrylamide–7 M urea gel alongside a sequencing reaction mixture. Gels were autoradiographed as above.

**Plasmid constructs.** The polymerase promoter was amplified from purified HHV-6 (GS) genomic DNA (16) by using primers with added restriction sites on their ends (KpnI on the 5′ primer and HindIII on the 3′ primer). This 639-bp promoter fragment containing 524 bp upstream and 115 bp downstream of the transcription start site was subcloned into the pBlueScript KpnI and HindIII sites. The HindIII-to-BamHI fragment from pSV2CAT containing the chloramphenicol acetyltransferase (CAT) coding region, and simian virus 40 promoter signals was then subcloned into the HindIII and BamHI sites, yielding a pBluescript promoter-CAT construct. The entire KpnI-to-BamHI promoter–CAT cassette was then subcloned into the KpnI and BamHI sites of a modified pUC119 vector. The modification consisted of deleting the region from the SacI site in the pUC119 polylinker to the BglII site in the lacZ coding region. This pUC119 promoter–CAT construct was the parental plasmid used for all constructs reported in this study. The 5′ and 3′ deletion series were made by replacing the KpnI-to-HindIII promoter fragment with PCR products generated by using either a different 5′ or 3′ primer with the appropriate restriction site added on the end. The promoterless control plasmid was made by digesting the pUC119 promoter-CAT plasmid with KpnI and HindIII, blunt ending with Klenow fragment and T4 DNA polymerase, and rescaling with T4 DNA ligase.

**Site-specific mutageneis.** Mutagenesis of double-stranded plasmid DNA was accomplished by using the Transformer site-directed mutagenesis kit (Clontech). The target plasmid was always the pUC119 639-bp promoter–CAT construct. The selection primer introduced a single-base change, which inactivated the unique EcoRI site in the CAT gene without altering any CAT amino acid residues. Briefly, mutagenic and selection primers plus target plasmid DNA were denatured and rapidly cooled, the annealed primers were extended with T4 DNA polymerase, and the completed strands were ligated with T4 DNA ligase. Reactions were ethanol precipitated and electroporated into the *Escherichia coli* repair-deficient strain BMH 71-18 mutS. Cultures were grown overnight in Luria broth-ampicillin, and then miniprep DNA was isolated. The DNAs were digested with EcoRI, ethanol precipitated, and electroporated into *E. coli* JM109. Mutants were verified by sequencing. **Heterologous promoter constructs.** The polymerase promoter was removed from the pUC119 promoter CAT construct by digestion with KpnI and HindIII and replaced with the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) basal promoter (29). The TK fragment used was a PCR product containing sequences from −40 to +35 relative to the start site of transcription, amplified with primers having KpnI (5′ primer) and HindIII (3′ primer) sites on their ends. This construct was digested with KpnI and blunt ended with T4 DNA polymerase. Double-stranded blunt-end oligonucleotides containing the polymerase promoter ATF site, purine box, or both were then ligated into the blunt-ended TK-CAT vector. The top-strand oligonucleotides were as follows: ATF, 5′-GGGTATGGC GACCTGCACTGGGGAAG-3′; purine box, 5′-GCGGTTTTC GAAAAAGAGGAAGGTGCTG-3′. The double-stranded blunt-ended oligonucleotide containing both sites was produced by annealing the purine box top-strand oligonucleotide to the ATF bottom-strand oligonucleotide and filling in the ends with Klenow fragment. Constructs were verified by sequencing.

**Transfection-infection and CAT assay.** HSB-2 T cells were transfected by the DEAE-dextran method as described elsewhere (3). Briefly, 2 × 10⁵ cells per sample were washed with STBS (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, 0.5 mM MgCl₂), resuspended in 1 ml of STBS containing 10 μg of plasmid DNA and 200 μg of DEAE-dextran (Sigma) per ml, and incubated for 30 min at 37°C. Dimethyl sulfoxide was added to 10%, and after 2 min, cells were washed with STBS and then with RPMI 1640 and resuspended in 5 ml of RPMI 1640 supplemented with 10% fetal calf serum plus antibiotics. Sixty percent of the cells were split equally into two parts, to each of which was added either uninfected HSB-2 cells or an equal number of HHV-6-infected HSB-2 cells, approximately 50% of which displayed cytopathic effect. CAT assays were performed as described elsewhere (11). Quantitation was accomplished by radioanalytical scanning (AMBI Systems, Inc.) of thin-layer chromatography plates. Data shown are the averages of three or more experiments. The experimental variation between identical samples was 15% or less.

**Nuclear extract preparation.** Nuclear extracts were prepared essentially as described in reference 21. HSB-2 cells or HHV-6-infected HSB-2 cells, 50% of which displayed cytopathic effect, were washed twice with phosphate-buffered saline and lysed for 5 min at 0°C in 10 mM HEPEs (pH 7.0), 40 mM KCl, 3 mM MgCl₂, 5% glycerol, 8 μg of aprotinin per ml, 2 μg of leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% Nonidet P-40, and 1 mM DTT. Nuclei were collected and extracted for 30 min at 0°C in 2 packed cell volumes of 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 PMSF, and 25% glycerol. Microfuged extracts were dialyzed for 1 h at 4°C against 20 mM HEPEs (pH 7.9), 0.1 M KCl, 0.2 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, and 20% glycerol. Diazyed extracts were aliquoted and stored at −80°C. It was noted that nuclei isolated from infected-cell cultures contained large
amounts of debris due to cell lysis by HHV-6, which likely contributes to the protein content of the infected cell extracts. Thus, when identical protein quantities of infected and uninfected nuclear extracts are added to the mobility shift assay binding reaction mixtures, the actual amount of intact nuclear protein in the HHV-6-infected samples may be significantly less than with uninfected cell samples. It is therefore difficult to compare levels of binding activity between infected and uninfected cell extracts.

**Electrophoretic mobility shift assay.** Probes were generated by $^{32}$P end labelling of double-stranded blunt-ended oligonucleotides. The ATF oligonucleotide was the same as that used for the heterologous promoter construct: top strand, 5'-GGG TATGGCTGACGTCAGTGGGCAAG-3'. Competitions were performed with cold double-stranded blunt-ended oligonucleotides. The ATF mutant competitor was (top strand) 5'-GGGTATGGCTTAACTGGGCAAG-3'. This top-strand oligonucleotide was used to introduce the ATF mutation into the promoter-CAT plasmid. Binding reaction mixtures (20 μl) contained 5 μg of nuclear protein, 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg of poly(dI-dC), 0.2 ng of labelled probe, and competitor oligonucleotide if applicable. Reaction mixtures were incubated for 5 min at room temperature without probe to allow the competitor to bind, and then probe was added and the incubation was continued for 20 min at 30°C. Samples were separated on 4% nondenaturing polyacrylamide gels in 0.5 × Tris-borate EDTA buffer at 4°C. Autoradiography was performed as described above.

**RESULTS**

HHV-6 DNA polymerase gene transcription initiates at a single site. The nucleotide sequence of the DNA polymerase gene of HHV-6 (39) was used to design probes for S1 and primer extension analyses (Fig. 1). For S1 analysis, the probe consisted of single-stranded $^{32}$P-end-labelled DNA which spanned the region from 305 bp upstream to 78 bp downstream of the predicted translation start codon of the polymerase gene. The S1 probe protected a 194-nucleotide (nt)-length RNA from HHV-6-infected T cells but not from RNA from uninfected T cells. This indicates that polymerase gene transcription initiates 115 nt upstream of the translation start codon in HHV-6-infected cells. For primer extension analysis, the probe used was a 32P-end-labelled oligonucleotide with the 5' terminus located 45 nt upstream of the polymerase translation start codon. A single product of 71 nt, specific to HHV-6-infected cells, was observed, confirming the location of the polymerase gene transcription start site at 115 nt upstream of the translation start codon.

A polymerase promoter-CAT construct is highly upregulated in HHV-6-infected cells. An HHV-6 polymerase promoter DNA fragment containing sequences from −524 to +115 relative to the start site of transcription was cloned upstream of the CAT reporter gene coding region. The control plasmid was the same CAT vector without any HHV-6 sequences present. These constructs were tested by transfection into the HSB-2 T-cell line followed by the addition of uninfected T cells or HHV-6-infected T cells as a source of virus (Fig. 2). Following transfection, the control plasmid produced undetectable CAT activity levels (<1% conversion) when uninfected cells were added and was upregulated only slightly when HHV-6-infected cells were added. After transfection of the polymerase promoter-CAT construct, CAT activity was also undetectable when uninfected cells were added. However, when polymerase promoter-CAT-transfected cells were cocultivated with HHV-6-infected cells, a dramatic increase in activity to levels which are 50-fold greater than that of the control plasmid was observed. Thus, the HHV-6 DNA polymerase promoter is inactive in uninfected T cells but is highly induced following infection by HHV-6.

**FIG. 1.** Transcription start site mapping. (Left) S1 analysis of uninfected and HHV-6-infected cell RNA. The nucleotide length of the fragment protected by the polymerase mRNA is shown. The upper band is produced by reannealing of the labelled single-stranded probe with the unlabelled complementary strand generated in the PCR (see Materials and Methods). (Right) Primer extension assay with HHV-6-infected and uninfected cell RNA. The nucleotide length of the polymerase transcript is indicated. The oligonucleotide probe was run off the gel. Bands smaller than 71 nt seen with both infected and uninfected cell RNA are likely due to cross-hybridization of the probe with cellular sequences. For both S1 and primer extension, products were sized by using a sequencing ladder which was run alongside the assay products (not shown). A schematic of the S1 and primer extension assays is depicted below. Arrow, transcription start site. *,$^{32}$P-end-label.
The polymerase promoter is TATA-less. The polymerase promoter sequence is shown in Fig. 3. Inspection of the sequence revealed a CAAT box homology centered at nt -191. A stretch of 15 purine nucleotides is located at positions -97 to -83. This purine box has homology to Ets protein-binding sites (21, 40). A consensus palindromic ATB-binding site, TGACGTCA, was found adjacent to the purine box at position -76 to -68 (13, 14). No consensus TATA element is present at the typical -25 to -30 region; however, a TTTAAA motif is located at positions -34 to -29. This sequence is required for activity of the HSV-1 UL38 promoter and is located from nt -31 to -26 in the HSV-1 promoter (12). In addition, the HHV-6 polymerase promoter contains two other AT-rich motifs in the -30 region, AATATT at -48 and CATAA at -11. All three potential TATA sequences were altered by point mutation as shown in Fig. 4 (AT-rich mutants A, B, and C). The mutants were then tested by transfection into T cells followed by cocultivation with uninfected or HHV-6-infected cells. As with the wild-type promoter, these constructs showed undetectable CAT levels in uninfected cells (data not shown). None of these mutations caused a significant decrease in promoter activity in HHV-6-infected cells, since the variation in CAT activity between experiments, while usually less than 10%, occasionally reached 15%. This result strongly implies that the HHV-6 polymerase promoter is TATA-less.

The predominant upstream promoter element is the ATF site. In order to assess the contribution of the CAAT box, purine box, ATF site, or any other upstream sequences to polymerase promoter activity, a 5' deletion series was constructed in the polymerase-promoter-CAT plasmid. The deletion constructs were tested by transfection into T cells followed by cocultivation with uninfected or HHV-6-infected cells (Fig. 5). In uninfected cells, all constructs showed undetectable CAT levels (data not shown). This ruled out the existence of a negative upstream regulatory sequence which might have accounted for the lack of promoter expression in uninfected cells. In HHV-6-infected cells, deleting sequences from -524 to -97 relative to the transcription start site did not affect CAT activity. Thus, the CAAT motif at -191 is not involved in promoter activation in infected cells. Deleting 10 bases further, to -87, however, gave an approximately 30% (1.4-fold) drop in promoter activity. This deletion removes the 5' end of the purine box. Removing the remainder of the purine box, to -77, did not decrease activity any further. However, deleting the entire ATF site, to -67, resulted in a dramatic 13-fold drop in promoter activity. Deletion to +33 reduced activity twofold, to the background level obtained with the promoterless CAT plasmid. Thus, the ATF site appears to be the major upstream regulatory element, with the purine box playing a minor role.

The effect of the purine box and ATF site on promoter activity is shown in Fig. 4 (AT-rich mutants A, B, and C). The mutants were then tested by transfection into T cells followed by cocultivation with uninfected or HHV-6-infected cells. As with the wild-type promoter, these constructs showed undetectable CAT levels in uninfected cells (data not shown). None of these mutations caused a significant decrease in promoter activity in HHV-6-infected cells, since the variation in CAT activity between experiments, while usually less than 10%, occasionally reached 15%. This result strongly implies that the HHV-6 polymerase promoter is TATA-less.
activity was further assessed by point mutation (Fig. 4). For analysis of the purine box, two mutants were generated. Mutant A has 4 bases substituted in the 5' end of the purine motif, and mutant B has 7 bases changed in the 3' end. Relative to the full-length wild-type promoter, mutants A and B gave 7 and 14% decreases in promoter activity, respectively, in HHV-6 infected cells, which is within the observed experimental variation in CAT activity. Therefore, by point mutational analysis, the purine box does not appear to be important for promoter function. There are two possible explanations for the finding that deletion of the purine box (Fig. 5, −87 construct) produced a greater reduction in activity than did nucleotide substitutions. First, the bases critical to purine box function may not have been mutated in construct A or B (Fig. 4). Alternatively, the decrease in CAT levels seen with the 5' deletion to −87 may have resulted from a negative effect on the ATF site due to the juxtaposition of novel 5' sequences rather than from removal of the purine box. Regardless, the purine box does not appear to contribute in a major way to promoter activity. By contrast, the predominant role of the ATF site for promoter activation in HHV-6-infected cells was confirmed by point mutational analysis. Changing the four central bases in the ATF palindrome caused an 88% (8.3-fold) drop in promoter strength (Fig. 4).

**Sequences downstream of the transcription start site are not required for promoter activity.** To test for the presence of promoter regulatory sequences downstream of the transcription start site, a 3' deletion series was constructed in the polymerase promoter-CAT plasmid and tested by transfection-infection (Fig. 5, bottom). These constructs were also inactive in uninfected cells (data not shown). Deleting from +115 to +63 resulted in an approximately 30% increase in activity in HHV-6-infected cells. Since the length of the mRNA leader is altered by the 3' deletions, this relatively small change in CAT activity may be due to an effect on translation efficiency rather than on transcription. Alternatively, we cannot rule out the possibility that this deletion removed a weak negative control element. Further removal of sequences, to +13, had no

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**FIG. 5.** Polymerase promoter 5' and 3' deletion series. 5' deletions (top) and 3' deletions (bottom) are shown schematically (not to scale) with CAT activity in HHV-6-infected cells indicated to the right of each construct. The endpoint of each deletion relative to the transcription start site is shown to the left for 5' deletions and to the right for 3' deletions. The full-length promoter (−524 to +115) is normalized to 100% CAT activity. Locations of the purine box (GA) and the ATF site are indicated. The transcription start site is shown with an arrow. CAT assays were incubated for 1 h at 37°C. The endpoint of each deletion in the context of the nucleotide sequence is depicted in Fig. 3. The data shown are the averages of three experiments.
significant effect on promoter strength. We conclude that sequences downstream of +13 relative to the start site of transcription do not contain any important positive regulatory signals for promoter expression in HHV-6-infected cells. Deletion to –57, which should remove sequences involved in basal transcription, diminished activity almost to promoterless CAT background levels, as expected.

The ATF site can confer HHV-6 responsiveness to a heterologous promoter. Given the importance of the ATF element for promoter activation, we tested whether this site, in the absence of other polymerase promoter sequences, could confer inducibility to HHV-6 infection. The polymerase promoter ATF site, two copies of the purine box (GA box), or both elements in their natural orientations were placed upstream of the HSV-1 TK basal promoter (construct ATF-TK-CAT, GA×2-TK-CAT, or GA-ATF-TK-CAT, respectively) (Fig. 6). Two copies of the purine box were inserted because of the low level of activity associated with this element. The TK promoter sequences in these constructs included only the TATA box and transcription initiation regions, with no TK upstream activating elements present. The control TK-CAT construct contained no HHV-6 sequences upstream of the TK basal promoter. These plasmids were tested by transfection into T cells followed by cocultivation with uninfected or HHV-6-infected cells. The higher activity of the TK basal promoter compared with the natural polymerase promoter allowed the quantitation of CAT activities in uninfected cells for the TK constructs. In uninfected cells, addition of the ATF site, purine box, or both to the TK basal promoter had little effect on activity. Strikingly, however, in HHV-6-infected cells, addition of the ATF site upstream of the TK basal promoter (ATF-TK-CAT) resulted in 23-fold-increased activity over that of the control (TK-CAT) plasmid. The construct containing two copies of the purine box (GA×2-TK-CAT) showed only a twofold induction over control plasmid activity in infected cells. The construct GA-ATF-TK-CAT was used to examine the ability of the purine box to cooperate with the ATF site for activation of the TK basal promoter. This construct, which contained both the ATF site and the purine box linked together exactly as in the natural polymerase promoter, produced essentially the same 24-fold induction over control plasmid activity in infected cells as did the ATF-TK-CAT plasmid, which contained only the ATF site.

This indicates a lack of cooperativity between the two polymerase promoter elements in the context of the TK basal promoter and suggests that the purine box does not function under these conditions. Consistent with the mutagenesis results, this analysis suggests that the purine box plays a minor role in promoter activation. These results also confirm that the ATF site is a strong positive element in HHV-6-infected cells and demonstrate that this site is sufficient to confer HHV-6 inducibility to a heterologous basal promoter.

The ATF site specifically binds two protein complexes. To analyze protein binding to the ATF site, a double-stranded oligonucleotide containing the polymerase promoter ATF element was labeled for use in bandshift assays (Fig. 7). Two protein complexes, C1 and C2, were observed in both uninfected- and infected-cell nuclear extracts. Shorter exposures of the autoradiogram shown in Fig. 7 revealed that the ratios of the quantities of C1 to those of C2 are the same in both infected and uninfected cells. Although the overall levels of C1- and C2-binding activities appear to be higher in uninfected cells than in infected cells, the probable contribution of lytic cell material to the protein content of HHV-6-infected cell nuclear extracts (see Materials and Methods) makes the significance of this difference uncertain. Specificity of binding was demonstrated by the ability of excess unlabelled ATF site oligonucleotide, but not mutant ATF site oligonucleotide, to eliminate complexes C1 and C2. This result shows that the ATF site is specifically bound by two protein complexes.
DISCUSSION

We have cloned the DNA polymerase promoter of HHV-6 and shown that it is active in infected cells but not in uninfected cells. A 5′ deletion series and site-specific mutants indicate that a palindromic ATF site is the predominant upstream promoter element utilized in HHV-6-infected cells. ATF sites are involved in the regulation of a large number of cellular and viral promoters, e.g., the somatostatin and the adenovirus E4 promoters (13, 20, 31). ATF sites are capable of binding homo- or heterodimers of proteins belonging to the large family of cellular transcription factors referred to as bZIP, for basic-region leucine zipper proteins, which includes the CREB/ATF, Fos/Jun, and C/EBP subfamilies (30). The particular constituents of the protein dimers determine the DNA-binding specificity and whether binding will result in activation or repression. An additional level of regulation occurs through posttranslational modification, such as phosphorylation, which has been shown to affect either the DNA-binding or the activation abilities of several bZIP proteins (1, 10).

In this study, mobility shift assays have revealed that two protein complexes bind specifically to the HHV-6 polymerase ATF element. The presence of two complexes suggests the potential involvement of multiple bZIP proteins in regulatory interactions with the ATF site. In this regard, our preliminary results indicate that the complex C1, but not C2, contains the bZIP protein ATF-2 or an ATF-2-like factor, as an antiseraum directed against ATF-2 can block formation of C1 in mobility shift assays. The fact that the same two ATF-binding complexes are observed in both uninfected and HHV-6-infected cells but promoter activity is only observed in infected cells suggests several possible, non-mutually exclusive ways in which the ATF-binding proteins could be involved in promoter regulation. First, promoter induction may be mediated through interactions between the basal transcription machinery, ATF-binding proteins, and viral transactivators. Adenovirus E1A and HSV-1 VP16, among others, are thought to transcriptionally transactivate promoters by bridging upstream binding factors to the basal machinery (23, 38, 41). Interestingly, E1A has been shown to interact directly with ATF-2 and the basal factor TBP (6, 9). Second, transcriptional activation ability of the ATF-binding factor(s) may be induced by posttranslational modification, which may not affect migration of the complexes. Third, a change in bZIP dimerization partners is possible since many family members have similar molecular weights and thus may migrate indistinguishably in mobility shift assays. Finally, alterations in chromatin structure may occur during infection such that the polymerase ATF site becomes accessible for factor binding. Indeed, the HCMV immediate-early proteins appear to be capable of upregulating transcription in vitro by reversing repression resulting from assembly of DNA templates into chromatin (17). It is to be noted that in our mobility shift assays, the ATF probe is not likely to be packaged as chromatin and therefore would be available for protein binding in both infected and uninfected cell extracts.

The HHV-6 DNA polymerase promoter appears to be TATA-less, as mutation of any of the AT-rich motifs in the −30 region was without effect. Many TATA-less promoters have been described, most of which have initiator elements encompassing the transcription start site (34, 42). These elements determine start site placement, as does TFII D with TATA-containing promoters. Initiators have been grouped according to sequence conservation; however, none of these sequences could be identified in the polymerase promoter initiation region. Nevertheless, our 3′ deletion results suggest that the basal promoter resides between −57 and +13 relative to the transcription start site.

Interestingly, the HCMV DNA polymerase promoter, which otherwise has no sequence homology with the HHV-6 polymerase promoter (18), contains an ATF-like motif, TGA CGTCG, at approximately the same position (−85 bp) as the HHV-6 promoter ATF site, TGA CGTCG (−70 bp). Whether the HCMV ATF-like site is virally inducible has yet to be determined. The only human herpesvirus DNA polymerase promoter which has been previously mapped by deletion studies is that of Epstein-Barr virus (8). The results implicate two upstream regions of 140 and 101 bp (beginning at −853 and −239 relative to the transcription start site, respectively) as important for promoter induction. One of these Epstein-Barr virus promoter regions contains a potential SP1-binding site, but the elements within these regions responsible for activity have not been defined.

In summary, the HHV-6 DNA polymerase promoter contains a single upstream ATF site which is a strong positive regulatory element and which is capable of interacting specifically with cellular DNA-binding proteins. Mutation of this element greatly reduces promoter activity and abolishes protein binding. The ATF site, in the absence of any other polymerase promoter sequences, is capable of conferring HHV-6 inducibility to a heterologous basal promoter. This further demonstrates the primary importance of the ATF-binding site for promoter function.

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