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Identification of a Transactivating Function Mapping to the Putative Immediate-Early Locus of Human Herpesvirus 6†

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Sequencing studies have indicated that the unique component of the human herpesvirus 6 (HHV-6) genome and the unique long segment of the human cytomegalovirus genome are genetically colinear. Of particular interest is the identification of a region of local CpG dinucleotide suppression in the genome of HHV-6, a feature conserved in the genomes of human cytomegalovirus, murine cytomegalovirus, and simian cytomegalovirus, and a characteristic of the major immediate-early loci of these viruses. Adjacent to this region in HHV-6 are approximately 30 copies of a 103- to 108-bp sequence element, which contains consensus binding sites for the transcription factors AP2 and NFκB, in addition to a single *Kpn*I recognition site. Together, these *Kpn*I repeat units may compose an immediate-early enhancer, analogous to those found in the cytomegaloviruses. We present the sequence of this region of HHV-6 and demonstrate that a transactivating function is encoded by this region. We have used polymerase chain reaction to synthesize fragments containing open reading frames and 5′ sequences with or without the upstream *Kpn*I repeat units. Effector plasmids containing these HHV-6 coding and 5′ sequences were able to effect activation of heterologous promoter-chloramphenicol acetyltransferase (CAT) constructs, including adenovirus E3-CAT and E4-CAT, human T-cell lymphotropic virus type I long terminal repeat (LTR)-CAT, and human immunodeficiency virus LTR-CAT, in cotransfection experiments in Vero cells and peripheral blood lymphocytes. Furthermore, we have identified the major open reading frame (RF4; 2.3 kb) as being essential for activation, and we have shown that the NFκB, SP1, and TATA box motifs in the human immunodeficiency virus LTR are all required for full induction of the promoter by the HHV-6-encoded transactivator.

Human herpesvirus 6 (HHV-6) is a recently identified herpesvirus, first isolated following the in vitro cultivation of peripheral blood lymphocytes (PBLs) from six patients with lymphoproliferative disorders, two of whom were also infected with human immunodeficiency virus (HIV) (48). The genome of HHV-6 consists of a largely unique sequence of 141 kbp with direct repeats of approximately 10 kbp at the termini. We have recently constructed the restriction endonuclease map of the genome of HHV-6 strain U1102 (40), and this has provided a basis for the further characterization of the genetic organization of the HHV-6 genome. Previous sequencing studies have indicated that the organization of coding features in the genome is colinear with the unique long segment of the genome of human cytomegalovirus (HCMV) (30). Furthermore, this has been confirmed and extended by subsequent sequencing studies (11a, 39a, 57a, 57b). This sequencing has allowed us to predict the likely location of the viral immediate-early (IE) regulatory genes.

The IE genes are the first genes to be transcribed in productive herpesvirus infections and are essential for the expression of delayed early genes, which are transcribed prior to viral DNA replication, and late gene expression. Thus, the IE gene products act as transactivators of expression from homologous promoters and, in addition, have been shown experimentally to transactivate heterologous viral and cellular promoters (46). One such promoter is the HIV long terminal repeat (LTR), which has been shown to be transactivated by the IE genes of several herpesviruses, including HCMV (9, 39, 41, 45), Epstein-Barr virus (29, 37), pseudorabies virus (59), and herpes simplex virus (41, 42) by *tat*-independent mechanisms.

HHV-6 is highly prevalent in the population, and virus has been isolated from the saliva of healthy adults (4, 18, 32, 44) and persists in peripheral blood of a majority of seropositive individuals (15). Furthermore, HHV-6 has a predominantly $CD4^+$ T-lymphocyte tropism both in vivo and in vitro (35, 56) which, together with its ability to coinfect $CD4^+$ T lymphocytes with HIV and to transactivate the HIV enhancer (12, 22, 34), has implicated HHV-6 as a cofactor in the progression of HIV infection to AIDS. More recently Lusso et al. (33) have shown that HHV-6 infection upregulates the expression of CD4, the major membrane receptor for HIV type 1 (HIV-1) (8), and therefore has the potential to expand the range of HIV-1-susceptible cells. Thus, HHV-6 may act as a cofactor in HIV infection either by expanding the range of susceptible cells and/or by regulating the expression of the HIV-1 genes. The gene product(s) involved in this transactivation is yet to be characterized, although the regulation has been reported to be mediated either directly or indirectly through the NFκB sites in the HIV enhancer region (34).

With a view to identifying the HHV-6 IE genes, our interest has focused on a region near the right end of the unique component of the HHV-6 genome corresponding precisely with the location of the major IE genes in HCMV. This region shares with betaherpesviruses HCMV, murine cytomegalovirus (MCMV), and simian cytomegalovirus (SCMV) both the feature of local relative CpG dinucleotide suppression (21) and a complex array of repetitive sequences (5, 14, 26). We have sequenced the putative IE gene locus of HHV-6 and have identified open reading frames (ORFs) and a putative enhancer of 3 kb, comprising *Kpn*I repeat units,

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† We dedicate this paper to the memory of Bob Honess.

upstream of these ORFs. We show that effector plasmids containing this region transactivate a variety of heterologous promoter-chloramphenicol acetyltransferase (CAT) constructs, including an HIV LTR-CAT target in transient expression assays, and that the major 2.3-kb ORF, RF4, is required for this function. Our studies using HIV LTR mutants have identified those sequences in the HIV LTR enhancer important for transactivation by the HHV-6 IE gene product(s).

MATERIALS AND METHODS

Cells. Vero cell monolayers (Flow Laboratories, Inc., Irvine, United Kingdom) were grown at 37°C in Dulbecco modified Eagle medium containing antibiotics (penicillin and streptomycin; 10 and 20 μg/ml, respectively) and 10% newborn calf serum. PBLs were isolated by centrifugation on Ficoll-Paque gradients (Pharmacia) and grown at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin). PBLs, for use in transfection, were stimulated with both phytohemagglutinin (5 μg/ml) and human recombinant interleukin-2 (20 U/ml; Boehringer Mannheim).

Growth of virus and viral DNA preparation. All experiments reported here employed a Ugandan isolate (U1102) of HHV-6 isolated by R. G. Downing (11). The propagation of the virus and subsequent purification of viral DNA for use in polymerase chain reactions (PCRs) were as previously described (40).

DNA sequencing. The isolation of the HHV-6 strain U1102 clone pHD12 (*Hin*dIII-C) and the PCR product from which the sequence presented in this report was obtained have been described elsewhere (40). The nucleotide sequence of sonicated DNA was determined from single-stranded template by using the dideoxynucleotide chain termination method (2, 49) or from the cloned DNA by using double-stranded sequencing methods (60). Sequence data were assembled by using the computer programs DBAUTO and DBUTIL (52, 53) and analyzed for the presence of ORFs longer than 300 nucleotides by using the program ANALYSEQ (54).

PCR. Oligonucleotides for use as primers in PCR were synthesized with an automated DNA synthesis machine (ABI 380B). The sequences (capital letters represent viral sequences) of the primers used were as follows: RPT3, 5′ acacgaaTTCCAAAATTGGGAATCATGTGT 3′; RK1, 5′ acacgaaTTCTCGGAAAATTAAAGGTCA 3′; RK2, 5′ acac gaaTTCCTACCCAAGCGGGTTAGA 3′; RF1A, 5′ acacgg atccACATCTAGGTTTCATCTAGC 3′; RF1B, 5′ acacaagc ttTTAAACATGTGACATATAAC 3′. The positions of the primers used in PCR, with the exception of RK2, which lies in the unique sequence at the right end of the *Kpn*I repeat units, are annotated in Fig. 2. PCR was performed with 200 ng of U1102 DNA as the template and 1 μM (final concentration) of each primer in a reaction mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.01% gelatin, 250 μM each deoxynucleoside triphosphate, and 5 U of *Taq* polymerase (Amplitaq; Perkin Elmer Cetus) in a final volume of 100 μl. Initial denaturation at 94°C for 6 min was followed by 25 cycles of denaturation (94°C; 1 min), annealing (55°C; 1 min), extension (65°C; 12, 10, or 5 min for 8.6- to 7.5-, 5.4-, or 2.6-kb products, respectively). These primers allowed the specific synthesis of the required products with a yield of approximately 500 ng to 1 μg per reaction. The products were extracted with phenol-chloroform and chloroform. Fragments were then digested with the appropriate restriction enzyme, gel purified (57), and cloned into the appropriate vectors.

HHV-6 U1102 effector gene constructs. Genetic manipulations described below were performed as described by Maniatis et al. (38). Vectors used in cloning experiments were pUC19 and pBC12/CMV/IL-2 (7). The primer pair products of RPT3/RK2 (7.5 to 8.6 kb) and RPT3/RK1 (5.4 kb) were designed with terminal *Eco*RI sites and cloned into pUC19. The RPT3/RK1 product was cloned into pUC19 to create pK28. The products synthesized with primers RPT3 and RK2 showed some size variation. Two different clones, pR56 and pR47, were isolated from the RPT3/RK2 product and differ only in the number of *Kpn*I repeat units present (pR56, 7 copies; pR47, 28 copies), as confirmed by partial digestion with *Kpn*I. The primer pair RF1A/RF1B product (2.6 kb) was cloned as a *Hin*dIII-*Bam*HI fragment into pBC/CMV/IL-2 to give pBC-ORF. pR56.ml was created by inserting an *Eco*RV linker (8-mer) at the unique *Bst*EII site following *Bst*EII digestion of pR56 and end repair. Linker insertion was confirmed by digestion and double-stranded sequencing with a USB Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio) according to the manufacturer's instructions.

Heterologous target and effector genes. pLTR-CAT and pTAT were gifts from A. Akrigg. The construct pLTR-CAT contains the entire LTR of HIV-1, the *cat* gene from SV2-CAT (16), and the polyadenylation-termination sequences from the HCMV major IE gene (1) cloned into a modified pUC18 vector. The HIV LTR-CAT plasmids containing mutations in the κB, TATA, κB+TATA, or Sp1 sites (gifts from G. Nabel) have previously been described (42). pU3R-I, which contains the LTR (nucleotides 1 to 670) of human T-cell lymphotropic virus type I (HTLV-I) linked to CAT, has been described by Sodroski et al. (51). The plasmid pE4-CAT1 contains the adenovirus early E4 promoter (nucleotides −224 to +33 relative to the transcription initiation site) and pE4-CAT2 is identical except for mutations in each of the three ATF binding sites (43). The adenovirus early E3 promoter-CAT plasmid, pE3-CAT, has been described by Weeks and Jones (58). pMLP-CAT contains adenovirus major late promoter sequences between −260 and +33 (relative to transcription initiation site) cloned upstream of the *cat* gene and was a gift from C. Goding.

DNA transfection and transient CAT expression assays. Plasmid DNA was purified on cesium chloride-ethidium bromide gradients (3). Monolayer cultures were transfected essentially as described by Gorman et al. (16). Vero cell monolayers were split 1 day prior to transfection. Calcium phosphate precipitates were applied for 10 h prior to changing the medium, and cells were harvested 48 h after changing the medium. The cell extracts were quantitated for protein concentration, and equal amounts of protein were assayed for CAT activity as described by Gorman et al. (16).

PBLs were transfected by using the DEAE-dextran method essentially as described by Hammarskjöld et al. (17). PBLs were taken directly from 3-day-old phytohemagglutinin–interleukin-2-stimulated cultures. Briefly, PBLs were washed twice with 10 ml of TD (140 mM NaCl, 25 mM Tris, 5 mM KCl, 0.5 mM Na_2HPO_4 [pH 7.5]) and resuspended in 1.5 ml of TD containing 50 μg of DNA and DEAE-dextran (500 μg/ml/10^7 cells). After a 40-min incubation at 37°C, cells were suspended in 10 ml of TD, washed once with medium, and incubated at 37°C in 10 ml of growth medium until harvest. Cells were harvested 48 h posttransfection and assayed for CAT activity as described by Gorman et al. (16).

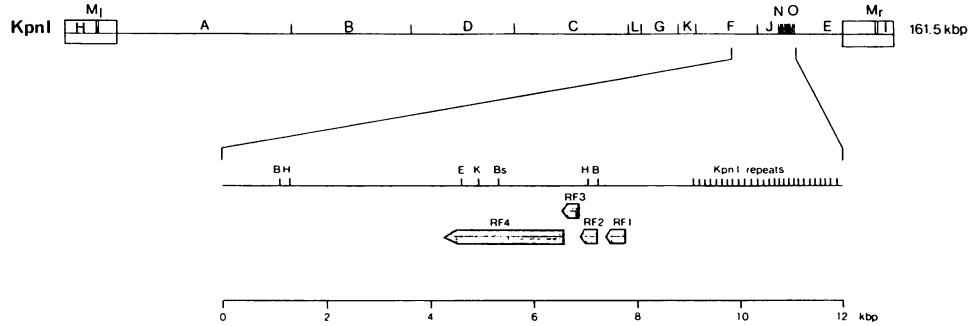


FIG. 1. The *Kpn*I restriction endonuclease map of the genome of HHV-6 strain U1102. M_l, a fragment in the direct left repeat; M_r, a fragment in the direct right repeat. Below the genome is an expanded restriction map of the region sequenced. B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I. *Kpn*I repeat units, described previously (40), are indicated. Positions of four leftward ORFs are illustrated in shaded boxes and are designated RF1, RF2, RF3, and RF4.

The percentage of chloramphenicol converted to acetylated forms was quantitated by liquid scintillation spectroscopy.

Nucleotide sequence accession number. The sequence of the putative IE region of the HHV-6 genome reported in this article will appear in EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M73681.

RESULTS

Sequence analysis of the putative IE locus. The position of the region sequenced at the right end of the unique segment of the HHV-6 genome strain U1102 is illustrated in Fig. 1. The principle features of the DNA sequence of 5,900 nucleotides within this region are illustrated in Fig. 2. The translations of the ORFs RF1 (1668 to 2045), RF2 (2203 to 2574), RF3 (2547 to 2879), and RF4 (2846 to 5146) are shown above the nucleotide sequence. The deduced translation products of the ORFs shared no significant amino acid sequence similarities with the IE products of the betaherpesviruses HCMV, MCMV, and SCMV. The IE proteins encoded by these previously sequenced IE genes do not themselves share extensive similarity, although large glutamic acid-rich domains in their carboxy-terminal regions, a feature not shared by RF4, have been reported (28, 55). However, this region in HHV-6 exhibited local CpG dinucleotide suppression (Fig. 3), which is a characteristic feature of the beta herpesvirus major IE gene loci (21). The CpG dinucleotide suppression is accompanied by an increase in TpG and CpA dinucleotides, indicating exposure of this region to DNA methylation. This feature is not shared by the sequences immediately upstream of the reading frames (Fig. 3) or by any other sequenced regions of the HHV-6 genome (30, 39a, 57b).

The positions of two overlapping putative AP1 binding sites between nucleotide positions 593 to 603 (TGACTGA and TGAGTCA) are indicated on the sequence (Fig. 2). Upstream of the first ORF are 25 to 30 copies of a repeat unit containing a single *Kpn*I site. The sequences of three complete repeat units are presented (Fig. 2, nucleotides 64 to 375). Repeat units vary in length from 103 to 108 bp and contain a perfect consensus sequence for the binding site of the cellular transcription factor AP2 (25) and a 9 of 10 match (GGGTCTTTCC) of the consensus sequence recognized by the NFκB family of transcription factors (GGGRATYYCC) (31). There are only minor sequence variations in each of the copies of the repeats. By analogy with the presence of multiply repeated transcription binding sites in the HCMV and SCMV major IE enhancers (5, 24, 26, 36), it is possible that the HHV-6 *Kpn*I repeat units perform a similar function.

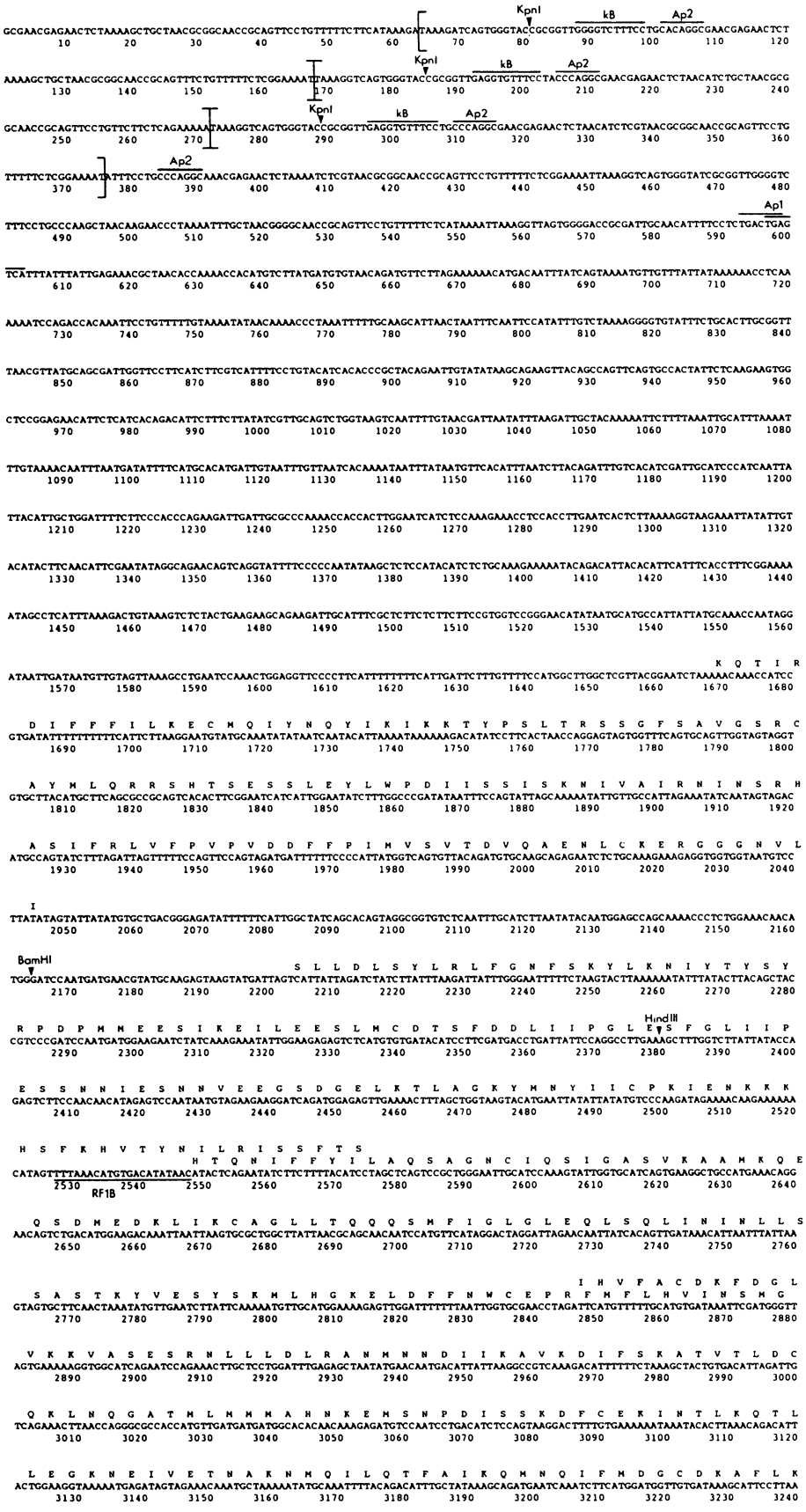
Because of the position of ORFs colinear with HCMV IE genes, the relative local CpG dinucleotide suppression and the presence of upstream sequences with enhancerlike features, we investigated the possibility that *trans*-regulatory functions were encoded by this region.

Transactivation of heterologous promoters. Effector plasmid pR56, which contains the four ORFs (Fig. 1), the 5′ sequences, including seven copies of the *Kpn*I repeats, and 800 bp of 3′ sequences, was used in initial experiments. To determine the effects of gene products encoded by this plasmid on HIV-I LTR-directed transcription, pR56 (1 to 30 μg) was cotransfected into Vero cells with 1 μg of the target plasmid pLTR-CAT. The results of these experiments demonstrated that pR56 transactivated pLTR-CAT, with an optimal induction of eightfold observed with 20 μg of effector plasmid (Fig. 4a).

Effector construct pR56 (40 μg) was cotransfected with pLTR-CAT (20 μg) into PBLs by using DEAE-dextran. Expression was stimulated approximately eightfold over basal expression (Fig. 4b). Therefore, pR56 encodes a transactivator(s) which is functional in both epithelial and lymphoid cells. Because of the relative ease of calcium phosphate transfections and the higher basal levels of expression, further experiments were all performed in Vero cell monolayers.

To determine whether pR56 could activate transcription from other heterologous promoters, effector plasmid pR56 was cotransfected into Vero cells with the target plasmids pLTR-CAT, pE4-CAT1, pE4-CAT2, pE3-CAT, pMLP-CAT, or pU3R-I (Fig. 5). pR56 induced levels of activity of between 2.5- and 9.5-fold for all these heterologous promoters, and similar levels of activation were obtained in numerous other experiments (not shown). pE4-CAT1 and pE4-CAT2, which has a mutation in each of the three ATF binding sites, were both responsive to pR56, demonstrating that the ATF site is not required for induction.

Identification of those regions necessary for transactivation in coding and noncoding sequences. To establish whether the largest ORF was essential for the transactivating function, we created a plasmid, pR56.ml, which contains a frame-shift mutation in RF4. This was created by inserting a linker in the *Bst*EII site (Fig. 1) in pR56, and the mutation was verified by sequencing. CAT activity was dramatically reduced when pR56.ml was cotransfected with the HIV LTR construct,


KpnI
kB
Ap2
Ap1
BamHI
HindIII
RF1B

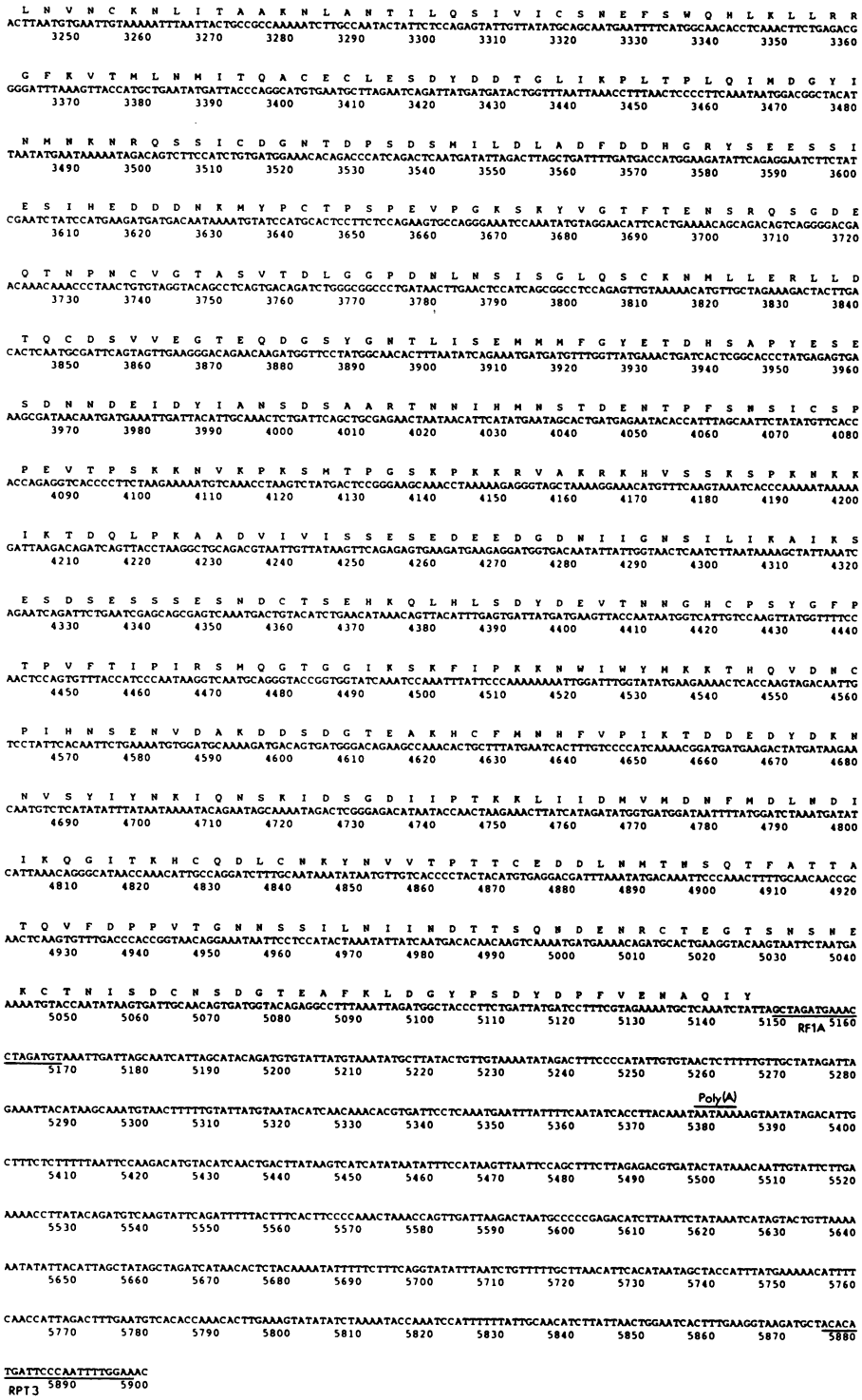


FIG. 2. Sequence of 5,900 bp across the putative IE region, reading from right to left (approximately, nucleotide positions 9400 to 3500) as shown in Fig. 1, with the translation products of the major ORF RF4 and minor ORFs RF3, RF2, and RF1. The DNA sequence includes the fragments *Kpn*I-J, 1.4 kb of *Kpn*I-K, and three complete copies of the *Kpn*I repeat units. Positions of the recognition sites for selected restriction endonucleases; the polyadenylation signal; AP1, NFκB, and AP2 consensus sequences; and the oligonucleotide primers used in PCR are annotated. The position of the PCR primer RK2 (not shown) is approximately 3 kb upstream of the position of RK1. The repeat units, with internal *Kpn*I sites, are in brackets.

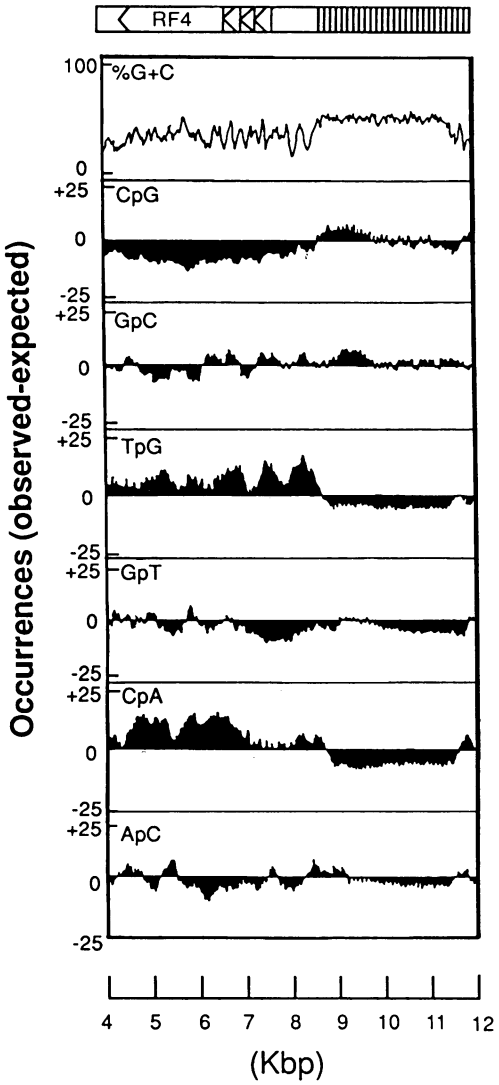


FIG. 3. Frequency of occurrence of selected dinucleotide pairs corresponding to the region of the genome of HHV-6 expanded in Fig. 1. The major ORFs within this region are indicated, together with a plot of the mean percent G+C content. The other panels show the difference between the observed and expected number of occurrences (observed-expected) of the dinucleotides CpG, GpC, TpG, GpT, CpA, and ApC in a 401-nucleotide sliding window with datum points plotted every 5 nucleotides and with plots scaled from +25 to −25. A significant deficiency of CpG doublets occurs between 4 and 9 kb and is accompanied by an excess of TpG and CpA.

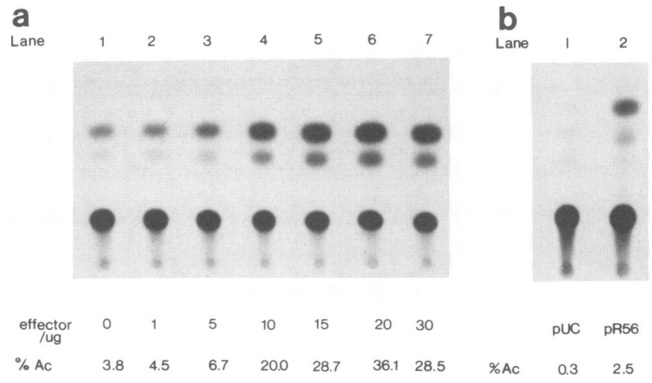


FIG. 4. (a) CAT activity induced in cotransfections of pLTR-CAT (1 μg) with increasing amounts of the effector plasmid, pR56 (as indicated), which contains the putative IE sequences. Percent acetylations (% Ac) are indicated below. Transfections were performed in Vero cell monolayers as described in Materials and Methods. Amounts of DNA used in transfections were made up to a total of 31 μg with pUC18. Fold inductions were 1.2, 1.8, 5.3, 7.5, 9.5, and 7.5 for lanes 2 to 7, respectively. (b) Induction of pLTR-CAT (20 μg) expression by pR56 (40 μg) in PBLs. Cells were transfected by the DEAE-dextran method, as described in Materials and Methods.

demonstrating that the largest ORF was essential for efficient transactivation (Fig. 6a).

The region encoding the transactivator was defined more clearly by using pBC-ORF, which contains reading frames RF3 and RF4, under the control of the HCMV major IE promoter/enhancer (see Materials and Methods). The amount of pBC-ORF required for optimal activation of pLTR-CAT was determined from the results of dose-response experiments (1 μg; data not shown). pBC-ORF (1 μg) was cotransfected with pLTR-CAT (1 μg) into Vero cells, and cell extracts were assayed for CAT activity. pBC-ORF induced expression of CAT significantly above basal levels (Fig. 6a). Therefore, the sequences spanning RF3 and RF4 are sufficient for the transactivating function.

To examine the potential effect of the *Kpn*I repeat units on the expression from the effector plasmids, levels of transactivation by pR47 (28 repeats), pR56 (7 repeats), and pK28 (0 repeats) were compared. All plasmids contain the same sequences, including the AP1 sites, downstream of the repeats. The results shown in Fig. 6b demonstrate that at approximately equimolar amounts of effectors pR56, pR47, and pK28 (0.35, 0.28, and 0.28 pmol, respectively), comparable levels of expression were induced in transient expression assays in Vero monolayers, suggesting that the minimal promoter elements are present in pK28 and that the *Kpn*I repeat units are not essential for expression in these cells. Since the level of activation, when equimolar amounts of effector plasmid were used, was greater with pK28 than that induced by pR47, the *Kpn*I repeats may in fact have a modest negative effect on expression. However, this repetitive region containing multiple NFκB and AP2 binding sites may act as an enhancer of HHV-6 IE gene expression in T cells permissive for HHV-6 productive infections.

Identification of the sequences within the HIV-1 LTR required for HHV-6 transactivation. To identify functional regions within the HIV LTR enhancer likely to be important for HHV-6 transactivation, we tested the set of site-directed mutants that were previously used to identify the location of *cis*-acting regulatory sequences in the HIV LTR required for transactivation by HSV-1 and adenovirus E1A (42). The activities of the mutants were tested in Vero cells by cotransfection with pR56. HIV-1 encodes its own potent transactivator, *tat*, and this allowed us to use an effector plasmid, pTAT, as a positive control and for comparative purposes in these experiments. Figure 7 illustrates the levels of CAT activity induced by pR56 (20 μg) or pTAT (5 μg) when cotransfected with the wild-type plasmid pLTR-CAT or constructs with mutations in the κB sites, TATA box, Sp1 sites, or κB+TATA. A decrease in the constitutive expression of CAT was observed with the mutants (Fig. 7). The results we obtained are clearly in agreement with those

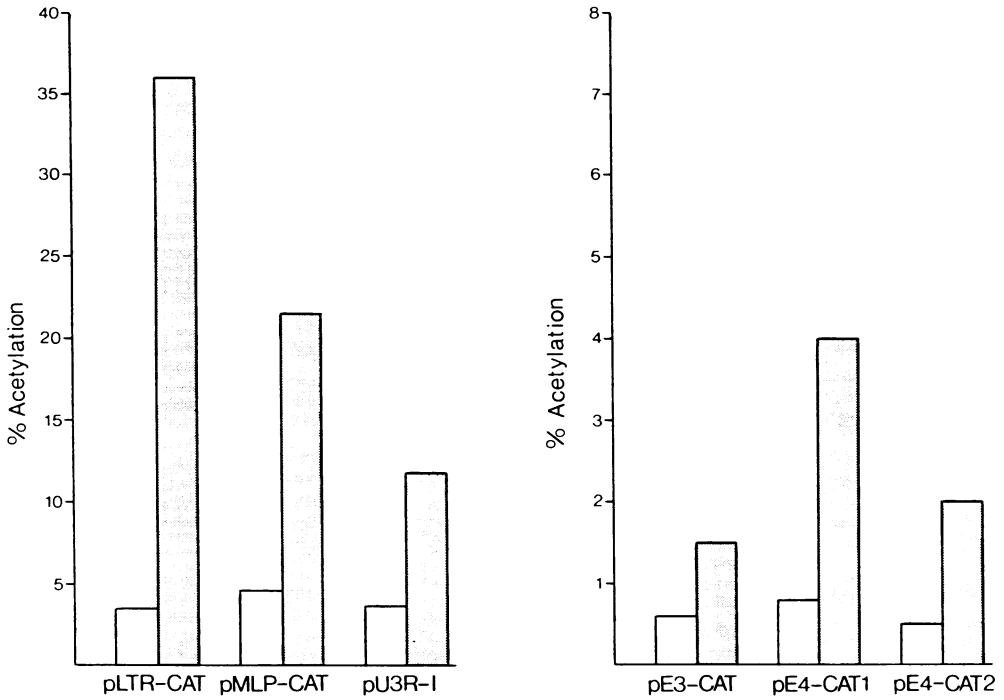


FIG. 5. Induction of the heterologous promoters pLTR-CAT (1 μg), pMLP-CAT (1 μg), pU3R-I (1 μg), pE3-CAT (1 μg), pE4-CAT1 (1 μg), and pE4-CAT2 (1 μg) by effector plasmid pR56 (20 μg). Cotransfections were performed in Vero cell monolayers as described in Materials and Methods.

previously published, which demonstrated the importance of the Sp1 sites and TATA box motifs in *tat*-induced gene expression in both transient expression assays and by using provirus constructs (13, 19, 20, 27, 47). The levels of expression induced by gene products encoded by pR56 were reduced in comparison to wild-type responses. pR56 activation of the mutant κB LTR-CAT construct was reduced (4.3- to 2.7-fold), but the most dramatic effects on expression were observed with the other mutated target plasmids, with activity reduced to near basal levels. The effect of the κB mutation on the relative induction of LTR-CAT by pTAT and pR56 is particularly noteworthy since there is a specific effect on activation by pR56, suggesting that the HHV-6 transactivator acts, at least in part, by a transcriptional mechanism.

DISCUSSION

The conservation of the general organization of gene blocks within the HHV-6 genome has identified HHV-6 as likely to be a member of the betaherpesvirus subfamily. Furthermore, current sequence data suggest that the unique segment of the HHV-6 genome and the unique long component of the HCMV genome are colinear (11a, 30, 39a, 57a, 57b), and this has allowed us to localize the region of HHV-6 most likely to encode the IE genes. Sequencing of the region near the right end of the HHV-6 genome identified ORFs corresponding precisely with the location of HCMV major IE genes, and this region is depleted in CpG dinucleotides, a feature shared by the IE genes of the betaherpesviruses (21). Upstream of the ORFs is an array of repetitive elements (103 to 108 bp) which have potential binding sites for the cellular transcription factors NFκB and AP2, which are capable of activating transcription in response to raised levels of cyclic

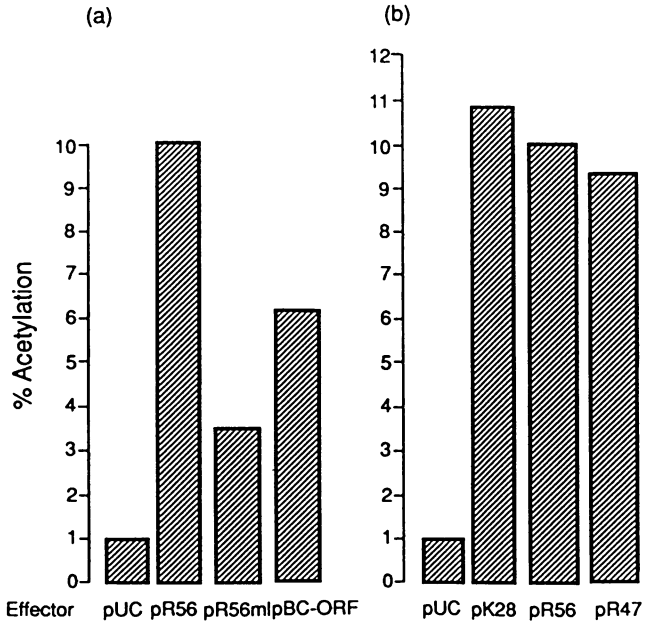


FIG. 6. Determination of the regions necessary for transactivation in either coding (a) or noncoding (b) sequences. (a) Effects on LTR-CAT expression of effector plasmids pR56 (20 μg); pUC18 (20 μg); pR56.ml (20 μg), containing a frameshift mutation in RF4; and pBC-ORF (1 μg), containing reading frames RF3 and RF4. (b) Effects of the number of copies of *Kpn*I repeat units on the induction of expression from pLTR-CAT. pK28 (15 μg), pR56 (20 μg), and pR47 (20 μg), with 0, 7 and 28 copies, respectively, of the repeat units, as determined by partial digestion, were cotransfected into Vero cells with pLTR-CAT (1 μg) as described in Materials and Methods. The optimal amounts of each effector shown here were determined from dose-response experiments (not shown). Total amounts of DNA used in transfections was made up to 21 μg with pUC18.

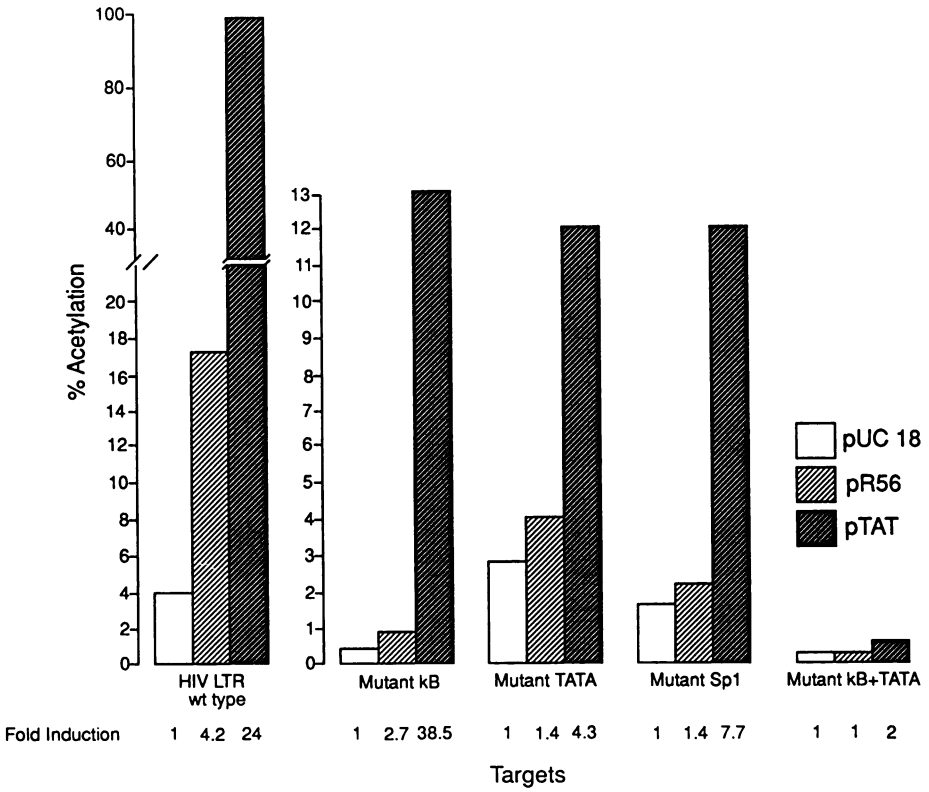


FIG. 7. Stimulation of HIV-CAT promoter variants by pR56. pR56 (20 μg) or pTAT (5 μg) was cotransfected with wild-type or mutant LTR plasmids (1 μg) into Vero cell monolayers, as described in Materials and Methods. Basal levels of expression were determined with pUC18 as a control. Total transfected DNA was made up to 21 μg with pUC18. The fold stimulations obtained are indicated below.

AMP (25). The NFκB elements identified in HHV-6 have a pyrimidine rather than a purine at position 4 of the consensus sequence (31). The effect which this substitution has on factor binding has not yet been established, but high copy number may compensate for potentially low-affinity binding. It is possible that this region may constitute an enhancer, since it shares many features with the IE regulatory regions of HCMV, SCMV, and MCMV (5, 6, 10, 26, 36). For, although the specific organization of these regions differ, all contain multiple binding sites for cellular transcription factors within reiterated sequences. HCMV and SCMV contain NFκB and cyclic AMP response elements (5, 6, 24, 36).

The usual method for detecting IE viral transcripts in herpesvirus infections employs protein synthesis inhibitors which allow the accumulation of IE mRNAs while blocking synthesis of delayed-early and late messages. However, our present inability to achieve efficient cell-free infections means that this method is not practicable. Herpesvirus IE genes encode transactivators of homologous and heterologous promoters (46), and we have used this property to provide functional evidence that the right-end sequence encodes an IE gene(s).

By using PCR we have synthesized and cloned fragments containing the ORFs from this region with or without the repeat units. The effector plasmids encoded transactivators which enhanced expression from a variety of heterologous promoters. Although the adenovirus E4 and E3 promoters and the HTLV-I LTR have ATF binding sites as common *cis*-acting regulatory sequences, mutations in these binding sites in the E4 promoter did not affect induction of CAT activity. Contrary to other findings (12), the gene product(s) described here transactivate the HTLV-I LTR. This may be because of the different cell types used in these experiments and may therefore be a consequence of the availability of relevant cellular transcription factors.

We have found that the carboxy-terminal region of the largest ORF is required for the transactivation of heterologous promoters and that reading frames RF3 and RF4 (contained between nucleotides 2547 and 5146) are sufficient for the transactivating function. The region (*Kpn*I repeats) upstream of these ORFs contains potential binding sites for the cellular transcription factors NFκB and AP2. Although the presence of repeat units in the effector constructs did not appear to influence expression from these plasmids in our experiments in Vero cells, it is still possible that they may confer positive and/or negative regulatory effects on gene expression in a cell-specific manner. Since HHV-6 replicates only in activated T cells and T-cell activation induces levels of active NFκB (50), the presence of multiple binding sites for NFκB implies that this is probably one of the cellular transcription factors likely to play a role in IE gene regulation. The role of the *Kpn*I repeats as regulatory elements in a number of cell types is currently being investigated.

In view of the possible role of HHV-6 as a cofactor in the disruption of latency and activation of HIV replication, we have investigated the responsiveness of the HIV LTR enhancer to HHV-6-encoded products. In order to identify those sites in the LTR responsive to transactivation by HHV-6, site-specific promoter mutants were used in cotransfections with the HHV-6 effector plasmid, pR56. Transactivation occurs by a *tat*-independent mechanism, as has been observed with other herpesviruses (39, 41, 42).

However, although no single specific *cis*-acting regulatory sequence has been found to be involved in induction by HCMV or herpes simplex virus (9, 39, 41, 42, 45), the adenovirus transactivator, E1A, appears to require the TATA box element (42), and Sp1 sites have been implicated as the responsive elements for the pseudorabies virus IE protein (59). We have found that the TATA box, Sp1, and κB sites all play a role in the inducibility of the HIV-1 enhancer by the HHV-6 putative IE gene products. These data suggest that activation is mediated, at least in part, at the transcriptional level, although posttranscriptional effects may also be involved.

Ensoli et al. (12) demonstrated that the region −103 to −48 of the HIV LTR was critical for transactivation by HHV-6 and that HHV-6 induced binding of NFκB to the LTR. The apparent disparity between our results and those of Ensoli et al. (12) may be attributed to the fact that these researchers were using mock- and virus-infected cells in transfections and therefore the possibility that other viral transactivators are playing a role cannot be excluded. We have also used these mutants in superinfection studies and found that transactivation with infected cells was NFκB independent (data not shown). It is also true, however, that the use of different cell lines may have a qualitative effect on the ability of HHV-6 IE transactivators to function.

Although Horvat et al. (23) identified two regions of the HHV-6 (strain GS) which strongly transactivate the HIV LTR, these fragments do not map to this locus of HHV-6 (strain U1102) (40). Our studies are the first to identify reading frames of an HHV-6 transactivating gene, and our studies have mapped this gene to a region of the genome possessing noncoding features characteristic of the IE regions of other beta herpesviruses. Our findings linking the local CpG dinucleotide suppression with a transactivating function provide further evidence that the unique component of HHV-6 and the unique long component of HCMV are colinear and that HHV-6 is closely related to members of the beta herpesvirus subfamily. We have shown that a gene product(s) from this locus transactivates the HIV-1 enhancer and therefore may play a role in the regulation of HIV replication. Lusso et al. (33) have recently shown that HHV-6 is involved in the upregulation of CD4 expression, thus expanding the range of HIV-1-susceptible cells. It has been suggested that this function is mediated by the IE genes. Therefore, the IE gene products may have dual functions in their role as a cofactor in the control of HIV infection.

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