Mapping of human herpesvirus 6 immediate–early 2 protein transactivation domains

Andru Tomoiu, Annie Gravel, Louis Flamand *

Laboratory of Virology, Rheumatology and Immunology Research Center, Centre de Recherche du CHUL and Faculty of Medicine, Laval University, 2705 Laurier Blvd., Room T1-49, Québec, Qc, Canada G1V 4G2

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Abstract

The immediate–early 2 (IE2) protein of human herpesvirus 6 (HHV-6) is a potent transactivator of multiple cellular and viral promoters. Deletion mutants of HHV-6 variant A IE2 allowed us to map functional transactivation domains acting on complex and minimal promoter sequences. This mapping showed that both the N-terminal and C-terminal domains of IE2 are required for efficient transactivation, and that deletion of the C-terminal (1397–1466) tail of IE2 drastically reduces both transactivation and the intranuclear distribution of IE2. Moreover, we determined that the ATF/CRE binding site within the HHV-6A polymerase promoter is not required for efficient transactivation by IE2, whereas the R3 repeat region of the putative immediate–early promoter of HHV-6A is responsive to and positively regulated by IE2. These results contrast sharply to that of human cytomegalovirus (HCMV) IE2, which down-regulates its promoter. Our characterization of HHV-6 IE2 transactivating activity provides a better understanding of the complex interactions of this protein with the viral and cellular transcription machinery and highlights significant differences with the IE2 protein of HCMV.

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Introduction

Human herpesvirus 6 (HHV-6) is a betaherpesvirus initially isolated from human immunodeficiency virus (HIV)-infected individuals and from patients suffering from lymphoproliferative disorders (Salahuddin et al., 1986). Two HHV-6 variants (A and B) have been identified. Although they share a high degree of homology for the majority of their genes (95–99%), these two variants are considered distinct infectious agents in view of their biological properties (such as gene expression and splicing pattern, in vitro cell tropism, reactivity to monoclonal antibodies), tissue distribution and disease association (Ablashi et al., 1991). The greatest genetic variability between the two HHV-6 variants is observed within the immediate–early 1 and 2 genes (60–70% amino acid identity), variations that may account for the observed differing biological properties. HHV-6B is the etiologic agent of the childhood disease roseola or exanthema subitum (Yamanishi et al., 1988). Links between HHV-6 infection and other pathologies such as meningencephalitis (Ishiguro et al., 1990), organ transplant rejection (Carrigan et al., 1991; Yoshikawa et al., 1991) and AIDS (Knox and Carrigan, 1995; Saito et al., 1995) have also been suggested. Evidence for a co-factorial role of HHV-6A in AIDS come from several observations, including that co-infection of T cells with HIV and HHV-6 leads to the activation of HIV long terminal repeat (LTR)-directed viral gene expression and accelerates cytopathic effects (Lusso et al., 1989).

Several HHV-6 gene products have been identified as potential transcriptional activators. Among them, products of open reading frames (ORFs) DR7 (Thompson et al., 1994a, 1994b), U16 (Geng et al., 1992), U27 (Zhou et al., 1994), U90/U86 (Gravel et al., 2003), U89 (Martin et al., 1991) and U94 (Thompson et al., 1994a, 1994b) were found to transactivate the HIV LTR promoter in vitro. ORFs U86 and U89 are comprised within the HHV-6 immediate–early A (IE-A) locus. IE-A
includes two genetic units termed IE1 and IE2, corresponding to ORFs U90/U89 and U90/U86, respectively (Fig. 1A). We have previously characterized the IE1 variant B (Gravel et al., 2002) and IE2 variant A (Gravel et al., 2003) proteins translated from spliced transcripts of the IE-A locus.

It has been reported that the IE2 protein is a potent transcriptional activator of heterologous promoters (Flamand et al., 1998; Gravel et al., 2003). Moreover, cotransfection experiments in T cells indicated that IE2 variant A can induce the transcription of a complex promoter such as the one present in the HIV LTR, as well as simpler promoters, whose expression is driven by a unique set of responsive elements (CRE, NF-AT, NF-κB) (Duprez et al., 1999; Gravel et al., 2003). Finally, the C-terminal domain encompassing the final 436 residues of HHV-6A IE2 was shown to bind a DNA fragment containing the transcription initiation site, TATA box and upstream sequence of the putative IE-A promoter (Papanikolaou et al., 2002).

Transcriptional activators must possess at least two functional domains: a DNA binding domain that allows attachment of the transactivator to its target sequence within a gene promoter, and an activation domain that promotes the transcription of the target genes. For HHV-6A IE2, the activation and DNA binding domains have not been defined yet, but clues pertaining to the nature of its functional domains could perhaps be deduced from studies of human cytomegalovirus (HCMV) immediate–early protein IE2. HCMV like HHV-6 is a betaherpesvirus and shares limited amino acid sequence similarities, immunological cross-reactivity and overall gene organization with HHV-6 (Lawrence et al., 1990; Neipel et al., 1991; Yasukawa et al., 1993). HCMV gene UL122, encoding for protein IE2, is a positional homologue of HHV-6 ORF U86 (Nicholas, 1994) and corresponds to the C-terminal portion of HHV-6 IE2. The similarity between HCMV IE2 and the carboxy-terminal region of HHV-6 IE2 is 45% (Nicholas, 1994). HCMV IE2 is a 86-kDa protein whose biological functions are well defined and include transactivation of heterologous promoters (Pizzorno et al., 1988), repression of its own promoter (Hermiston et al., 1990), association with the viral DNA replication compartment (Ahn et al., 1999), blocking of cell cycle progression (Wiebusch and Hagemeier, 1999) and modulation of apoptosis (Zhu et al., 1995).

Mapping studies have revealed that HCMV IE2 contains two distinct acidic activation domains, one at the N terminus and one at the C terminus (Fig. 1B). Both domains are required for transactivation of most target reporter genes within the context of wild-type IE2 (Pizzorno et al., 1991). The activator domains of HCMV IE2 do not seem to fall within the conserved region with HHV-6A IE2. However, minimal dimerization and DNA binding domains have been identified in the HCMV IE2 region having significant similarity with HHV-6 (Chiu et al., 1993). In the present work, we used deletion mutants to map functional transactivation domains of HHV-6A IE2 using complex and minimal promoter sequences. This mapping allowed us to determine that both the N-terminal and C-terminal domains of IE2 are required for efficient transactivation, and that deletion of the C-terminal (1397–1466) tail of IE2 drastically reduces both transactivation and the nuclear patchy distribution of IE2. Moreover, we determined that the ATF/CRE binding site in the HHV-6A polymerase promoter is not required for efficient transactivation of the promoter by IE2, whereas the R3 repeat region of the putative immediate–early promoter of HHV-6A strongly enhances IE2 transactivation of this promoter. This and future characterization of IE2 should provide a better understanding of this complex viral protein. Furthermore, this study underscores important functional differences between HCMV and HHV-6A IE2 proteins.

Results

Mapping of HHV-6A IE2 domains required for transactivation

The main function currently known of HHV-6A IE2 is to promiscuously promote transcriptional activation (Gravel et al., 2003). IE2 being a large protein, we generated various deletion mutants in order to determine which domains are essential for transactivation. We arbitrarily divided IE2 into three major
domains: the N-terminal region (amino acids 1–720), the R1 repeat region (amino acids 721–944) and the C-terminal region (amino acids 945–1466). We generated constructs lacking either one or more of the large domains or smaller portions of the protein for a more detailed mapping (Fig. 2A). All of the IE2 constructs have an N-terminal 6xHis tag for detection purposes.

The various constructs encoding for IE2 deletion mutants were transfected into Molt-3 or HEK293T cells together with luciferase reporter plasmids. Because promoter consensus sequences could have an influence on which IE2 domains are required for efficient transactivation, complex (multiple transcription factor binding sequences) and minimal (single known binding sequence) viral and cellular promoter reporters were tested. The complex promoters tested were pLTR-Luc (a luciferase reporter plasmid driven by the HIV LTR promoter), pHV-6-Pol-Luc (driven by the HHV-6A polymerase promoter), pHHV-6 IE-Luc (HHV-6A immediate–early A locus putative promoter) and P2-1900 (driven by the human COX-2 promoter). The minimal promoters tested were pCRE-Luc (a reporter plasmid encoding for a luciferase transcript under control of a cyclic AMP response element), pNFκB-Luc (under control of multiple κ enhancer elements), pNFAT-TA-Luc (regulated by nuclear factor for activated T cell consensus sequences), pAP1-Luc (driven by multiple copies of Activator Protein 1 enhancer element) and pTA-Luc (having a minimal TA promoter, TATA box, as the only recognizable regulatory element). All of these promoters are significantly activated by IE2, as reported by Gravel et al. (Gravel et al., 2003) and this study.

We first performed transfection of promoter constructs in Molt-3 T cells, a cell line efficiently infected by HHV-6. Wild-type IE2 strongly transactivated the HIV-1 LTR promoter construct (Fig. 2B). Surprisingly, none of the IE2 mutant constructs had transactivating activity similar to wild-type IE2, suggesting that the full-length protein is required for efficient transactivation. Transfection efficiency being relatively low in T cell lines, it was difficult to obtain consistent results with many of the reporters tested. For these reasons, we switched to HEK293T cells, which can be transfected more efficiently. Our results indicate that transactivation of the HIV-1 LTR promoter by wild-type IE2 and IE2 mutants follow closely what was observed in Molt-3 T cells, suggesting no major differences between the two cell lines. Results (Fig. 3) show that the wild-type IE2 construct is able to strongly transactivate all of the reporter constructs, including a minimal promoter comprised of a TATA box. The N-terminal (IE2 2-1719) or C-terminal (IE2 945–1466) domains have no significant transactivating activity by themselves on any of the tested promoters. Likewise, mutants lacking either the N-terminal (IE2 714–1466) or the C-terminal (IE2 2-1030) domains are unable to transactivate most reporter constructs. The only exception to this is the HHV-6A IE promoter who is significantly transactivated by the IE2 2-1131 and 2-1030 mutants.

On the other hand, deletion of the central R1 repeat region inhibited full transactivation of the reporters without completely aboliishing the activity of IE2. None of the promoters were activated by IE2 ΔR1 as strongly as by wild-type IE2. Residual transactivation activity (more than 20% of IE2 wild-type) was observed with many (HHV-6A Pol, HHV-6A IE, LTR, COX-2, NF-AT, TATA) but not all of the promoters (CRE, NF-κB, AP1). On the whole, the results suggest that the complete IE2 protein is required for successful transactivation of all tested promoters in HEK293T cells.

Interestingly, deletion of a short C-terminal stretch of 70 amino acids (1397–1466) drastically reduced IE2 activity on the whole array of reporter constructs, either minimal or complex. This region is not capable of transactivation by itself, as we have shown that the C-terminal (945–1466) domain of IE2 is transcriptionally inactive. In the light of these results, we further investigated the impact of this small deletion on IE2 activity.

Expression of the IE2 mutants

The low transactivating activity of all IE2 deletion mutants could potentially be caused by poor expression or defective
intracellular localization. In order to determine whether the IE2 mutants are properly expressed upon transfection in the HEK293T cell line, we analyzed their relative expression by western blotting using anti-His and anti-IE2 antibodies. As shown in Fig. 4, all of the mutants are detected just as strongly or better than wild-type IE2. The enhanced expression of some mutants relative to wild-type IE2 can be explained by the shorter length of the encoded transcripts and proteins that lead to increased transcriptional and translational efficiencies. Mutant IE2 2-1030 could not be detected by the P6H8 anti-
IE2 mAb because this antibody binds to a region located between amino acids 1030–1131 (Tomoiu et al., unpublished results). Therefore, expression levels cannot account for the lower transactivating activity of the IE2 mutants.

**Nuclear distribution of the IE2 mutants**

Because IE2 needs to reach the nucleus in order to act as a transactivator, we had to confirm whether the inactive mutants were properly transported to the nucleus. A computer search of nuclear localization sequences using PSORT II (Nakai and Horton, 1999) has revealed four recognizable putative NLS in HHV-6A IE2. Monopartite four-residue pattern sequences are present at 355–358, 1046–1051 and 1210–1215, including potential overlapping sequences. A bipartite putative NLS is also detected at residues 423–439. To test the functionality of these putative sequences we cloned in frame with GFP residues 423–440 (KKHICRSVQKKKRRS, putative NLS residues italicized) from the N-terminal domain and 1207–1215 (KKKCKKKKPR) of the C-terminal domain of IE2. These constructs were transfected into HEK293T cells and fluorescence assessed by microscopy. Both GFP-IE2 NLS fusion proteins displayed intense and exclusively nuclear localization patterns, as opposed to the diffuse, cytoplasmic distribution of the control GFP protein (data not shown). We have therefore identified at least two sequences from HHV-6A IE2 that act as functional NLS and are most likely required for the proper nuclear targeting of IE2.

We subsequently analyzed the intranuclear localization of the different IE2 mutants by immunofluorescence. Transfected HEK293T cells were prepared for microscopy and probed using an anti-His antibody. In HHV-6A-infected cells, IE2 shows a punctate pattern that evolves into large nuclear patches at 24–48 h post-infection (Gravel et al., 2003). In transfected HEK293T cells, wild-type IE2 also shows a patchy nuclear pattern (Fig. 5). IE2 mutants 2-1030 and 2-1131, lacking the C-terminal putative NLS at residues 1207–1215, have a diffuse distribution that appears to be mostly cytoplasmic, probably due to impaired nuclear shuttling of the mutant proteins. Unexpectedly, the IE2ΔR1 construct (lacking the R1 region) also shows a diffuse distribution even though it retains the C-terminal NLS. It would thus appear that both the C-terminal NLS and the R1 repeat domain are important for proper nuclear localization of IE2. However, the diminished transactivation activity of some mutant IE2 proteins such as IE2 2-1396 and IE2 2-1289 does...
not correlate with impaired nuclear import, as these retain the C-terminal NLS and their localization pattern is still nuclear.

Interestingly, the IE2 2-1396 C-terminal tail mutant has a visibly different punctate nuclear distribution. Unlike wild-type IE2, IE2 2-1396 does not form large patches but numerous small dot-like structures. Moreover, all of the mutants lacking the C-terminal (1397–1466) tail also lose the patchy distribution of the wild-type protein. In contrast, the IE2 714–1466 construct (lacking the N-terminal region but not the C-terminal tail) does show the patchy distribution of wild-type IE2. Because IE2 714–1466 has very little transactivating activity, it appears that transactivation and patchy nuclear distribution of IE2 are not correlated.

Transactivation of HHV-6A promoters

Because we have shown that IE2 is able to transactivate both its own promoter and the viral polymerase promoter, we sought
to further characterize the consensus sequences required for efficient transactivation. The HHV-6A Pol promoter has been characterized before (Agulnick et al., 1994), and its only recognizable transcription factor binding sequence is an ATF/CRE-binding site. We looked into the importance of the ATF/CRE site for IE2 transactivation by generating a mutant of the pHHV-6 Pol-Luc reporter in which the ATF site was rendered non-functional by directed mutagenesis. Fig. 6A shows that transactivation of the pHHV-6 PolΔATF-Luc reporter is weaker than that of wild-type Pol promoter. However, because basal expression level of the mutant promoter is 10-fold lower than that of the wild-type promoter, the overall relative transactivation of pHHV-6 PolΔATF-Luc is slightly stronger (105-fold) than pHHV-6 Pol-Luc (62-fold). Therefore, the ATF/CRE binding site is dispensable for IE2 transactivation of the HHV-6A polymerase promoter. Interestingly, because the Pol promoter is TATA-less (Agulnick et al., 1994), there must exist other unidentified binding elements that promote IE2 transactivation of the viral promoter.

The HHV-6A immediate–early locus A promoter has been tentatively identified as a sequence of circa 4 kbp between ORFs U90 and U94 (Martin et al., 1991). Putative AP1 transcription factor binding sites have been identified in this sequence. Another important element of the promoter is the R3 region, composed of 25–30 copies of approximately 105 bp repeat units containing putative transcription factors AP2, NF-κB and PEA3 binding sites. For HHV-6B, it has been shown that the R3 repeats strongly enhance transcription of gene U95, downstream of the IE promoter and on the opposite side of R3 from the IE-A locus (Takemoto et al., 2001). However, the R3 region has no significant effect on transactivation by HHV-6A IE1, a IE-A protein encoded downstream of R3 on the reverse strand (Martin et al., 1991). IE2 itself was shown to weakly transactivate an IE-A promoter construct lacking the R3 repeats (Gravel et al., 2003). We looked into the relevance of the R3 repeat region for IE2 transactivation by generating a pHHV-6 IE-Luc construct encoding for the full 4-kbp putative IE-A promoter upstream of the luciferase gene. IE2 transactivation of pHHV-6 IE-Luc was compared to that of pHHV-6 IEΔR3-Luc, lacking the repeat region. Fig. 6B shows that full-length IE promoter is much more efficiently transactivated by IE2 than the promoter lacking the R3 region. This suggests that the AP2, NF-κB and PEA3 binding sites may be important for IE2 transactivation of what likely constitutes its own promoter.

**Discussion**

The IE2 protein of HHV-6A is able to functionally transactivate diverse promoters (Gravel et al., 2003; Martin et al., 1991). To better characterize the IE2 transactivation domains, we generated deletion mutants of various regions of the protein and tested them for activation of both complex and minimal promoter reporter constructs. Both the N-terminal and the C-terminal domains of IE2 are required for efficient transactivation of the tested reporters. The R1 SSRA/SSRD repeat region is dispensable for transactivation on all complex and many minimal promoters tested, although its absence...
reduces IE2 activity by at least 2-fold. The exact role of R1 in the transactivation of promoters by IE2 is as yet unclear, but its strong basic charge and unstructured sequence suggest the ability to non-specifically bind DNA and thus participate in the attachment of IE2 to the promoter binding sites. Alternatively, the R1 deletion may have an influence on global IE2 folding and mask NLS sequences, change its ability to bind promoter regions or recruit transcription factors.

The significant transactivating activity of deletion mutants IE2 2-1131 and 2-1030 on the putative HHV-6A IE promoter was observed for this promoter only. An explanation for this residual activity may be linked to the numerous repeats (28 copies) of AP2, NF-κB and PEA3 binding sequences present within this promoter. It is possible that these IE2 mutants exhibit basal transactivating potential on minimal promoters but when the number of regulatory elements is high, a cumulative effect can be detected. This, however, does not explain why similar results are not observed with the IE2 2-1396 and IE2 2-1289 mutants. Perhaps differences in protein folding of the various mutants are responsible for the obtained results. It is of note that even if these mutants are less efficiently transported to the nucleus, as seen by immunofluorescence, because of a missing NLS, cell fractionation assays have shown that a significant fraction of these mutant proteins are still imported to the nucleus and should be available for transactivation (data not shown). The N-terminal NLS possibly plays a role in the import of mutants missing the C-terminal NLS.

The sequence similarity between the C-terminal domains of HHV-6A IE2 and HCMV IE2 could entail similar functions for the two domains. The IE2 1397–1466 region aligns with residues 486–568 of HCMV’s 579 amino acid IE2 protein (Fig. 1B). The HHV-6A IE2 C-terminal tail includes a short motif that is highly conserved among all beta herpesviruses, namely, residues 1397–1417 (486–506 for HCMV IE2) (Chiou et al., 1993). This motif forms part of the required transactivation, autoregulation, dimerization and DNA binding domains of HCMV IE2 (Chiou et al., 1993; Pizzorno et al., 1991). The conservation of this element in HHV-6A may indicate an important role in the IE2 protein activity. Accordingly, we hypothesize a role in dimerization and DNA binding for the C-terminal tail of HHV-6A IE2. Indeed, it has been shown that the C-terminal domain of IE2 demonstrates some DNA binding activity (Papanikolou et al., 2002). The nuclear distribution of wild-type IE2 suggests that there is some form of nuclear aggregation or polymerization of the protein that is not present with truncated IE2 2-1396. However, an unambiguous proof that the punctate nuclear pattern of IE2 is linked to its ability to bind DNA or to form protein dimers remains to be produced.

Interestingly, the 544–579 residues of HCMV IE2 have been defined as an acidic activator domain (Pizzorno et al., 1991). Similarity between HCMV IE2 544–579 and the corresponding HHV-6A IE2 residues (1456–1466) is virtually non-existent, so we can surmise that HHV-6A IE2 lacks the C-terminal acidic activator domain present in its HCMV homolog. Mammalian one-hybrid tests with the C-terminal tail of HHV-6A IE2 did confirm that this protein fragment cannot act as a transcriptional activator (data not shown).

Another important functional difference between the IE2 proteins of HHV-6A and HCMV is here underscored. Although it has been shown that HCMV IE2 is able to downregulate its promoter through binding to a cis repression signal near the transcription start site (Cherrington et al., 1991; Hermiston et al., 1990; Pizzorno et al., 1988), HHV-6A IE2 upregulates the putative viral IE promoter in our system. Moreover, in infected cells the HHV-6A IE2 transcript is increasingly expressed throughout the whole infectious process (Gravel et al., 2003). Thus, downregulation of its own transcript does not appear to be a characteristic of HHV-6A IE2. This may in part be explained by the fact that the HCMV cis repression signal is not conserved in HHV-6A. Indeed, although HCMV and HHV-6 share high levels of genetic identity, both viruses have found different ways to exploit similar genetic elements. Table 1 presents a summary of phenotypical and functional differences between HCMV and HHV-6 variants A and B.

Although the ATF/CRE binding site within the HHV-6A polymerase promoter is required for transactivation of the viral polymerase promoter upon infection (Agulnick et al., 1994), its absence does not reduce transactivation of the polymerase promoter by IE2 (this study). A similar observation was made about the human CD4 promoter. HHV-6A infection can induce CD4 gene transcription via an ATF/CRE binding site within the CD4 promoter, but IE2 transactivates equally well the wild-type promoter and a mutant CD4 promoter lacking the ATF/CRE binding site (Flamand et al., 1998). This suggests that other viral proteins are likely to contribute to the CD4 and HHV-6A polymerase transcriptional activation.

Our analysis of IE2 activation of multiple promoters failed to pinpoint specific transcription binding sequences unconditionally required for efficient transactivation. IE2 is able to transactivate via the TATA box and the basal transcriptional machinery but can also activate promoters that are TATA-less (such as the HHV-6A polymerase promoter) through yet unidentified transcription factor binding sites. Although the array of promoters efficiently transactivated by IE2 in vitro is extensive, this does not imply that IE2 will also activate all of those cellular promoters in vivo. Availability of a specific promoter is determined by chromatin remodeling, and transcription factors have access to only a fraction of the cellular promoters at any moment. Interestingly, we have demonstrated that upon HHV-6A infection, IE2 localizes in the vicinity of promyelocytic leukemia-associated nuclear bodies (PML-NB) (Tomoiu et al., unpublished results). PML-NB have a role in chromatin dynamics and are thought to participate in the repression of gene expression, as reviewed in (Everett, 2006). Upon HHV-6 infection, the nuclear PML-NB distribution is altered (Gravel et al., 2002), and a consequence of this alteration may be greater chromatin availability to viral transactivators such as IE2.

Promoter transactivation by HHV-6A IE2 is possibly the result of several non-exclusive mechanisms of action. First, the IE2 protein may bind directly to transcription factor binding sites within promoters and recruit transcriptional units; second, the IE2 protein may act as a co-activator by interacting with other transcription factors via protein–protein interaction and...
favor transcriptional activation; third, the IE2 protein could affect post-translational modifications, such as phosphorylation or ubiquitination, leading to increased or more sustained activity of transcription factors; and fourth, IE2 may activate the expression of other genes involved in transactivation processes (Wang et al., 1994). The precise mechanisms by which IE2 transactivates promoters remain unknown. The ability of IE2 to transactivate multiple promoters having distinct transcription factor responsive elements could suggest that the HHV-6A IE2 protein does not bind a specific DNA consensus sequence directly, but rather interacts with regulatory proteins common to many transcriptional units. However, the existing evidence that the C-terminal domain of HHV-6A IE2 is able to bind the putative IE-A promoter (Papanikolaou et al., 2002) and that its homologue HCMV IE2 also has a DNA binding domain (Chiou et al., 1993) suggests that HHV-6A IE2 acts as a transcriptional enhancer through DNA binding followed by the activation or recruitment of transcriptional unit complexes. More characterization of the functional domains of IE2 is required for a better understanding of the mechanisms underlying the complex relationship between the viral protein and its host transcriptional machinery.

Materials and methods

Virus and cell lines

The HSB-2 and Molt-3 leukemia human T cell lines were cultured in MegaCell RPMI-1640 medium supplemented with 10% foetal bovine serum (Sigma-Aldrich Canada, Oakville, ON) and M-plasmocin (InvivoGen, San Diego, CA) to prevent mycoplasma contamination. HEK293T human epithelial kidney cell line was cultured in MegaCell Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 3% foetal bovine serum and M-plasmocin, and passaged every two days. HHV-6A (GS strain) was propagated in HSB-2 cells as previously described (Flamand et al., 1991).

Plasmid generation

For the transactivation assays, complete (except for the initial methionine) wild-type HHV-6A IE2 was PCR amplified from pBK-IE2A (Gravel et al., 2003) (forward primer 5′-agttaccGGA GCC AGC AAA ACC-3′; reverse primer 5′-ctcgagTTA ACA TTT TGA AAG TGT AC-3′; adapters not homologous with viral sequence are indicated by lower script, KpnI and XhoI restriction sites are italicized), TOPO-ligated into pCR4 cloning vector (Invitrogen, Carlsbad, CA) then subcloned in-frame into KpnI/XhoI digested pcDNA4/His-MaxA vector (Invitrogen) to yield pcDNA4-IE2.

In order to map the transactivation domains of IE2, the following constructs were generated by single-base mutagenesis following the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) instructions. Wild-type plasmid pcDNA4-IE2 was mutated to pcDNA4-IE2 2-1396 using stop-codon mutagenic primer 5′-CAT GAT CTA TTT ACG TGA CAT TCG GAC GTA AAG-3′ and its complementary oligonucleotide (mutated base is italicized). Constructs pcDNA4-IE2 2-1289 and pcDNA4-IE2 2-1131 were also generated by single-base mutagenesis of pcDNA4-IE2 using, respectively, primers 5′-GAG AGC ATT TGA ATA CTA ACA TAT -3′ and 5′-CAG ATT CAA AAC AC -3′, and their complementary oligonucleotides.

Table 1

Summary of phenotypical and functional differences between HCMV, HHV-6A and HHV-6B

<table>
<thead>
<tr>
<th>Function</th>
<th>HCMV</th>
<th>HHV-6A</th>
<th>HHV-6B</th>
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<tbody>
<tr>
<td>Pathogenesis</td>
<td></td>
<td></td>
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<tr>
<td>In vitro cell tropism</td>
<td>Skin or lung fibroblasts, endothelial cells</td>
<td>T lymphocytes, monocytes, neural cells (oligodendrocytes, astrocytes, neurons)</td>
<td>T lymphocytes, monocytes</td>
</tr>
<tr>
<td>Primary infection</td>
<td>Usually asymptomatic, mononucleosis</td>
<td>Asymptomatic? (encephalitis, meningitis, multiple sclerosis?)</td>
<td>Exanthema subitum, febrile seizures</td>
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<tr>
<td>Congenital infection</td>
<td>Neurological damage</td>
<td>Unknown consequences</td>
<td>Unknown consequences</td>
</tr>
<tr>
<td>Immunocompromised hosts</td>
<td>Organ damage and failure</td>
<td>Unknown</td>
<td>Encephalitis, graft versus host disease, delayed platelet engraftment</td>
</tr>
<tr>
<td>Virus–cell interactions</td>
<td>Host genome integration</td>
<td>Yes (infrequent)</td>
<td>Yes (infrequent)</td>
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<tr>
<td>Effect on PML-NB</td>
<td>Dispersal</td>
<td>Limited aggregation</td>
<td>Aggregation</td>
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<td>IE2 protein</td>
<td>Transactivation</td>
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<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Autoregulation</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Transrepression</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
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</table>
TGT AAG GGT AC-3′ (EcoRV and XhoI restriction sites are italicized). The PCR amplicon was TOPO-ligated into pCR4 cloning vector then subcloned in-frame into EcoRV/XhoI digested pcDNA4/HisMaxA vector. Construct pcDNA4-IE2 2-719 was created by PCR amplification of the 5′ segment of IE2 from the pBK-IE2A template, using forward primer 5′-agttgacca GGA GCC AGC AAA ACC-3′ and reverse primer FLAL259 5′-cggata ATG GTG CAA CTT CTA CTC AG-3′ (KpnI and EcoRV restriction sites are italicized). Resulting DNA was TOPO-ligated into pCR4 cloning vector then subcloned in-frame into KpnI/EcoRV digested pcDNA4/HisMaxA vector. The same amplicon was subcloned in-frame into KpnI/EcoRV digested pcDNA4-IE2 945–1466 to yield construct pcDNA4-IE2 ΔR1 (2–719+945–1466). Finally, deletion mutant pcDNA4-IE2 714-1466 was generated by PCR amplification on the pBK-IE2A template, using forward primer 5′-agttgacca GGA TAA AAG TTG CAC CAT G-3′ and reverse primer 5′-ctcaggg TTA ACA TTA TTA AAG TGT AC-3′ (KpnI and XhoI restriction sites are italicized). Amplicon was TOPO-ligated into pCR4 cloning vector then subcloned in-frame into KpnI/Xhol digested pcDNA4/HisMaxA vector.

 Luciferase reporter construct pHVH-6 Pol-Luc was generated by PCR amplification on genomic DNA from HHV-6A-infected HSB-2 cells, using forward primer 5′-tatggttcc GAA GCT AAA ATT CCT ACK AAT ACR CR-3′ and reverse primer 5′-egagcggag CAC GCT TCT TCT ACA TTA CTC CT-3′ (KpnI and SmaI restriction sites are italicized). Amplicon was TOPO-ligated into pCR4 cloning vector then subcloned into KpnI/SmaI digested pGL3-Basic vector (Promega, Madison, WI). ATF site mutant construct pHHV-6 PolΔATF-Luc was generated by directed mutagenesis on the pHHV-6 Pol-Luc template using mutagenic primers 5′-GGG TAT GGC TGT TAA CAG TGG GCA AG-3′ and its complementary oligonucleotide (mutated base are italicized). Construct pHHV-6 IE was generated by PCR amplification of the putative IE-A promoter sequence from pRS6 (kindly provided by Dr. M. E. D. Martin) (Martin et al., 1991) using forward primer 5′-TTG ACT TAC CAG ACT GCA ACG-3′ and reverse primer 5′-TTC CTA CCC AAG CGG GTT AG-3′. Resulting DNA was TOPO-ligated into pCR4 cloning vector, EcoRI digested and ligated into pcDNA3.1 (Invitrogen), extracted using XhoI and HindIII, and ligated into Xhol/HindIII digested pGL3-Basic vector.  

Transfections and luciferase assays

Transfections of HEK293T were performed using the calcium phosphate precipitation procedures. Cells were plated at 200 000 cells/well (6-well plate) the day prior to transfection. Cells were transfected with 1 μg of reporter plasmid and up to 7 μg of expression vector per well and brought to a total of 8 μg of DNA per well for each condition with the pcDNA4 control plasmid. Cells were lysed 48 h after transfection. Transactivation was evaluated using luciferase reporter constructs, coding for the luciferase gene driven by various promoters. Luciferase activity was measured on an MLX Microtiter plate luminometer (Dynex Technologies, Chantilly, VA). The values obtained are means of 3 distinct experiments performed in duplicate and were normalized for protein concentration in each sample, as determined by a BCA colorimetric assay (Pierce, Rockford, IL).

Molt-3 cells were transfected by electroporation using a Gene Pulser apparatus and capacitance extender (Bio-Rad Laboratories, Hercules, CA). Briefly, 18 μg total DNA was added to 10² cells in 400 μL RPMI medium. Cells were pulsed at 0.25 kV with a 960-μF capacitance in a 0.4-cm gap electroporation cuvette (Bio-Rad Laboratories). Cells were transferred into 10 mL culture medium and lysed after 48 h of growth. Transactivation of reporter constructs was assayed as described above.

The following luciferase reporter plasmids have been tested for IE2 transactivation. Constructs pCRE-Luc, pNFκB-Luc, pNFAT-TA-Luc, pAPI-Luc and pTA-Luc were purchased from Clontech (Mountain View, CA). Construct pLTR-Luc was kindly provided by Dr. M. Tremblay; P2-1900 as previously described (Iniguez et al., 2000) was kindly provided by Dr. M. Fresno); pHV6-IEΔ3-Luc was previously described (Gravel et al., 2003). Generation of pHV6-IE1-Luc, pHV6-Pol-Luc and pHV6-PolΔΔATF-Luc was detailed above.

Western blotting

For western blot analysis, cells were washed in PBS, lysed in an appropriate volume of Laemmlli buffer and boiled. Samples were electrophoresed through an SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membranes and blotted for 1 h at room temperature. After three 10-min washes with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were then washed with TBST and the proteins were visualized with enhanced chemiluminescence (Perkin-Elmer, Boston, MA) using a PhosphorImager system (Fuji Medical Systems, Stamford, CT). Primary antibodies used were P6H8 mouse anti-HHV-6A IE2 as previously described (Arsenault et al., 2003), mouse anti-His (Amersham Biosciences, Baie d’Urfé, QC) and mouse anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence

HEK293T cells were transfected for 48 h, fixed with cold (−20 °C) acetone and incubated with the antibodies described hereafter. IE2 expression was detected with a mouse anti-His monoclonal antibody (Amersham Biosciences), followed by a rabbit anti-mouse antibody coupled to the Alexa 568 red dye (Molecular Probes, Eugene, OR). Nuclei were stained by incubating the cells with a 1:1000 solution of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) nuclear acid stain for 10 min. Images were captured by a CoolSNAP HQ camera mounted on an Olympus BX-51 upright microscope (Olympus America, Melville, NY) using a 40× Uplan Apo objective and 568 nm (red dye, His) and 461 nm (blue dye, DAPI) filters, and processed with ImagePro 4.5.1 software (Media Cybernetics, Silver Spring, MD).
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References


terminal repeat in an Sp1 binding site-dependent manner. J. Virol. 68 (3), 1706–1713.


