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Characterization of the immediate-early 2 protein of human herpesvirus 6, a promiscuous transcriptional activator

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Abstract

In the present work we report the cloning of a full-length cDNA encoding the immediate-early (IE) 2 protein from human herpesvirus 6 (HHV-6) variant A (GS strain). The transcript is 4690 nucleotides long and composed of 5 exons. Translation initiation occurs within the third exon and proceeds to the end of U86. Kinetic studies indicate that the 5.5-kb IE2 mRNA is expressed under IE condition, within 2–4 h of infection. IE2 transcripts from both variants A and B are expressed under similar kinetics with IE2 transcripts accumulating up to 96 h postinfection. Although several large transcripts (>5.5 kb) hybridized with the IE2 probe, suggesting multiple transcription initiation sites, a single form of the IE2 protein, in excess of 200 kDa, was detected by Western blot. Within cells, the IE2 protein was detected (8–48 h) as intranuclear granules while at later time points (72–120 h), the IE2 protein coalesced into a few large immunoreactive patches. Transfection of cells with an IE2 expression vector (pBK-IE2A) failed to reproduce the patch-like distribution, suggesting that other viral proteins are necessary for this process to occur. Last, IE2 was found to behave as a promiscuous transcriptional activator. Cotransfection experiments in T cells indicate that IE2 can induce the transcription of a complex promoter, such as the HIV-LTR, as well as simpler promoters, whose expression is driven by a unique set of responsive elements (CRE, NFAT, NF-kB). Moreover, minimal promoters having a single TATA box or no defined eukaryotic regulatory elements were significantly activated by IE2, suggesting that IE2 is likely to play an important role in initiating the expression of several HHV-6 genes. In all, the work presented represents the first report on the successful cloning, expression, and functional characterization of the major regulatory IE2 gene/protein of HHV-6.

Introduction

Human herpesvirus 6 (HHV-6) primary infection in infants is associated with intense fever and often followed by the development of a maculopapular rash known as *exanthem subitum* (Yamanishi et al., 1988). More serious consequences, including meningitis, encephalitis, and hepatitis have also been reported to occur following HHV-6 infection (Asano et al., 1990, 1992; Huang et al., 1991; Ishiguro et al., 1990; Knox et al., 1995). In adults, HHV-6 has been, and is still considered, a potential cofactor in AIDS disease progression (for a review see Lusso and Gallo, 1995). One key factor fueling this hypothesis is the ability of HHV-6 to modulate HIV-1 infection in vitro (Carrigan et al., 1990; Di Luca et al., 1991; Ensoli et al., 1989; Geng et al., 1992; Horvat et al., 1989, 1991; Zhou et al., 1997). Several genomic segments or open reading frames (ORFs) capable of efficiently transactivating the HIV-LTR promoter have been identified (Geng et al., 1992; Gravel et al., 2002; Horvat et al., 1991; Martin et al., 1991; Nicholas and Martin, 1994; Zhou et al., 1997).

The HHV-6 linear genome is composed of a large unique segment (~144 kb) flanked by two directly repeated units of 8.7 kb. Within the unique segment are three regions of repeated sequences termed R1-R3. These repeat regions are clustered within or around the immediate-early locus A (IE-A) of HHV-6 which encodes two major IE proteins, IE1

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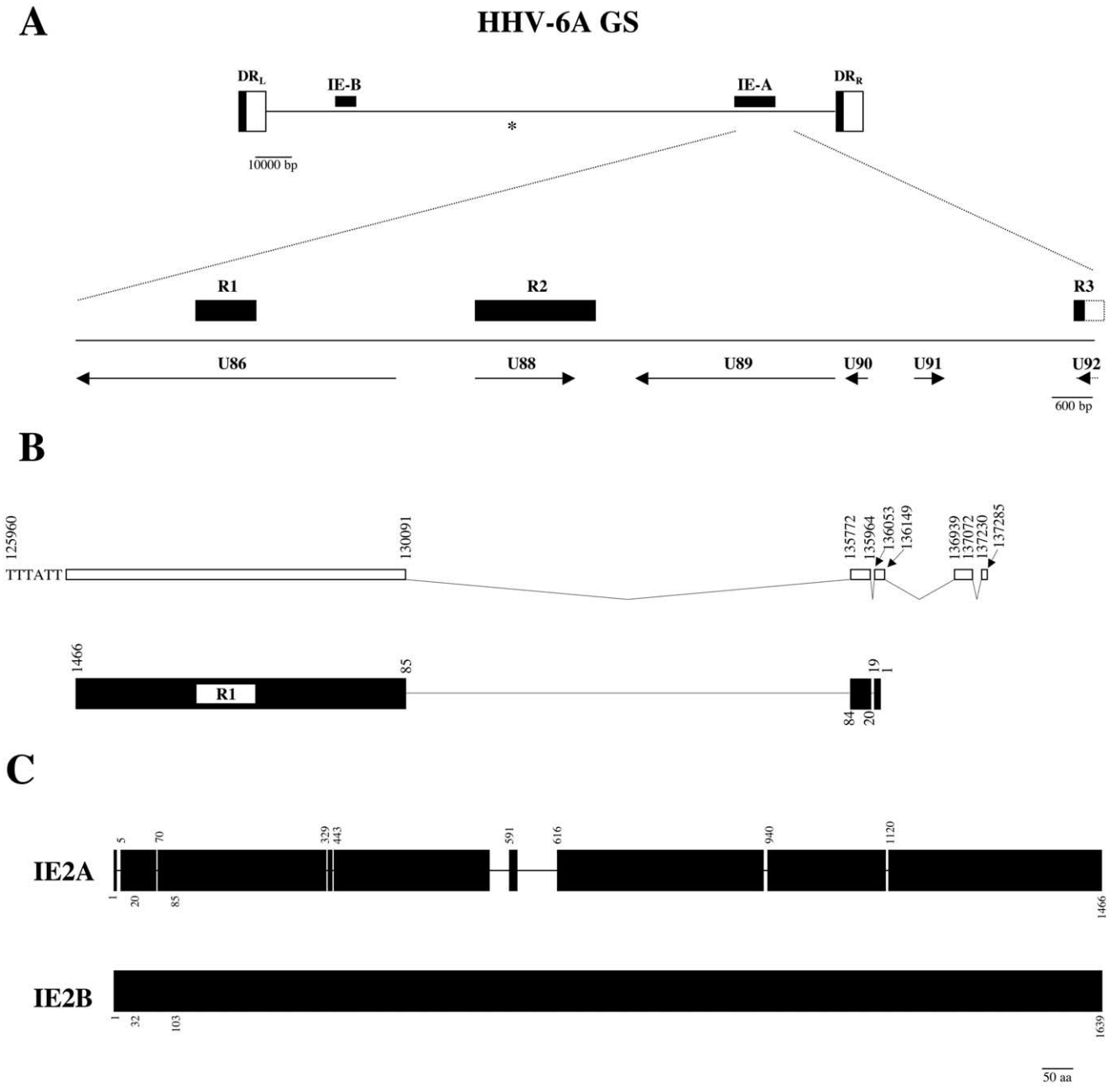
and IE2. R1 is located within the U86 coding region, R2 lies between U86 and U89, and R3 is located upstream of the IE1 gene. R3 (*Kpn*I repeat region), the largest of the repeat regions, consist of 24–28 segments of 104–107 nucleotides depending of the HHV-6 variants and isolates (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999). Within the repeated units of R3, consensus binding sites for transcription factors such as PEA3, NF-κB, and AP-2 were identified, suggesting that this region may act as potential *cis*-acting elements governing the expression of the IE-A locus (Dominguez et al., 1999; Martin et al., 1991; Thomson and Honess, 1992). However, a recent report suggests that the R3 region acts as a strong enhancer for the expression of the U95 immediate-early gene in an NF-κB-dependent manner (Takemoto et al., 2001). Only a very limited number of studies on genes encoded from the major IE-A have been reported. Three studies have detailed the *IE1* gene organization for HHV-6 variants A and B and the relative transactivation ability of the IE1 protein (Gravel et al., 2002; Martin et al., 1991; Schiewe et al., 1994). The other major protein encoded within the IE-A locus, the IE2 protein, has yet to be characterized. Genomic sequence analysis indicates that the HHV-6 U86 is a positional homologue to the human cytomegalovirus (HCMV) UL122 (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Nicholas, 1994). Alignments of the translated products from HHV-6 U86 and HCMV UL122 indicate that both protein differ considerably, especially in the first half of the protein, where no identity can be detected. The greatest amino acid similarity (44%) is observed in C-terminal portion of both proteins (Nicholas, 1994). HCMV UL122 is expressed under IE conditions and encodes an 86-kDa protein, IE86, whose biological functions include transactivation of heterologous promoters (Hagemeier et al., 1992; Hermiston et al., 1987; Iwamoto et al., 1990; Klucher et al., 1993; Lukac et al., 1994), repression of its own promoter (Chiou et al., 1993; Hermiston et al., 1990; Huang and Stinski, 1995; Jupp et al., 1993; Liu et al., 1991; Macias and Stinski, 1993; Pizzorno and Hayward, 1990; Wu et al., 1993), an association with the viral DNA replication compartment (Ahn et al., 1999), and its ability to block cell cycle progression (Murphy et al., 2000) and modulate apoptosis (Chiou et al., 2001; Zhu et al., 1995). From sequence analysis and comparison with HCMV, it is hypothesized that both the *IE1* and the *IE2* genes of HHV-6 share a common promoter and a 5′ mRNA organization with transcription initiation originating either downstream or upstream of the R3 region depending on whether the transcripts have IE or late kinetics of expression, respectively (Schiewe et al., 1994). To our knowledge, only one article has reported the protein encoded by U86 as a transcriptional activator of heterologous promoter (Flamand et al., 1998). Within the HHV-6 IE2 primary amino acid sequence lie approximately 48 imperfect repeats of SSRA, SSRD, and SSKA (R1 region), whose significance and presence with the IE2 protein remain to be determined (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Nicholas, 1994). In the present work we report the cloning of a complete cDNA sequence encoding HHV-6 IE2 from variant A (GS strain) and kinetics of mRNA and IE2 protein expression and provide data on the promiscuous nature of IE2 in promoting transcriptional activation of promoters containing several distinct regulatory elements.

Results

Isolation of a full-length IE2 transcript

The HHV-6 linear genome with location of the two IE loci is presented in Fig. 1A. In addition, details of IE locus A, with ORFs and repeated regions, are also shown. A cDNA library from HHV-6 variant A (GS strain)-infected HSB-2 cells was screened for IE2 mRNA, using a U86 probe. After two screens, a 4690-nucleotide-long transcript made up of 5 exons was isolated. A diagram of IE2 mRNA organization and splicing junctions with the corresponding genomic coordinates referenced according to the HHV-6 U1102 sequence (GenBank X83413) is presented in Fig. 1B. The transcript initiates at position 137285, between U91 and the R3 region, and progresses in the reverse orientation up to the end of U86. A total of 5 exons make up the mature mRNA with 4 small exons originating upstream of U89 and the fifth exon corresponding to U86. Our IE2 cDNA extends, at the 5′ region, by 7 nucleotides the HHV-6 variant A IE1 transcript previously reported by Schiewe et al. (1994). Although primer extension is needed to identify the precise IE2 transcription initiation site, it very likely matches the IE1 transcription start site (Schiewe et al., 1994), located 18 nucleotides upstream of our IE2 cDNA sequence. The 5′ regions (exons 1–4) of IE1 and IE2 transcripts are very similar with minor differences likely attributable to the varying strains of HHV-6 variant A studied (GS and U1102). A putative TATA box is located at position 137326, 41 nucleotides upstream of our cloned IE2 transcript. The IE2 coding sequence is contained within exons 3 to 5 with the first in-frame ATG codon located at position 136111 in the middle of exon 3 (Fig. 1B). All introns have typical splicing donor and acceptor sites following the GT . . . AG rule. A polyadenylation signal is located 21 nucleotides downstream of the stop codon. The variant A IE2 protein is 1466 amino acids long and has an estimated molecular weight of 165 kDa with an isoelectric point of 9.8. Within the carboxy-terminal half of the protein lays the R1 region, containing a repeated (>40 times) tetrapeptide sequence (SSRA > SSRD > SSKA > SRKA) as initially described following partial HHV-6 genomic sequence analysis (Nicholas, 1994). Compared to the R1 region of U1102, the GS R1 region contains an extra 36 nucleotides, translating into an additional three tetrameric-repeated peptide motif. The IE2 sequence from the GS strain is 94 and 92% identical to the DNA and amino acid



	IE2A (U1102)	IE2A (GS)	IE2B (Z29)	IE2B (HST)
IE2A (U1102)	100%	92%(92%)	85%(56%)	85%(61%)
IE2A (GS)		100%	85%(64%)	85%(62%)
IE2B (Z29)			100%	98%(91%)
IE2B (HST)				100%

sequence of HHV-6 variant A U1102 strain, respectively (Fig. 1C). The sequence is much more divergent from variant B, with identities of 85 and 64% at the DNA and amino acid levels, respectively (Fig. 1C). The IE2 protein from variant A is 167 amino acids shorter that its counterpart form variant B, with the largest difference occurring between amino acids 590 and 616 of variant A IE2 protein.

Kinetics of IE2 mRNA expression

The cloned IE2 cDNA originates within the major IE-A locus. To analyze the kinetics of IE2 transcription during the course of an infection, HSB-2 cells were infected with HHV-6 variant A (GS strain) for varying periods of times, after which total RNA was extracted and analyzed by Northern blot using a DNA probe internal to U86. The results obtained (Fig. 2A) indicate that a transcript of approximately 5.5 kb could be detected by 4 h of infection. The intensity of the 5.5-kb IE2 transcript increased gradually, up to 96 h, at which time the experiment was terminated. At least four additional transcripts of size ranging between 8 and 15 kb (approximation) with kinetics of expression similar to that of the 5.5-kb mRNA were detected. In addition, the 8 to 15-kb transcript intensities are similar to that of the 5.5-kb message. The 5.5-kb transcript corresponds to our isolated cDNA (4690 nt) plus the poly(A) tail and a few additional nucleotides in the 5′ region. The structures of the larger transcripts have yet to be determined.

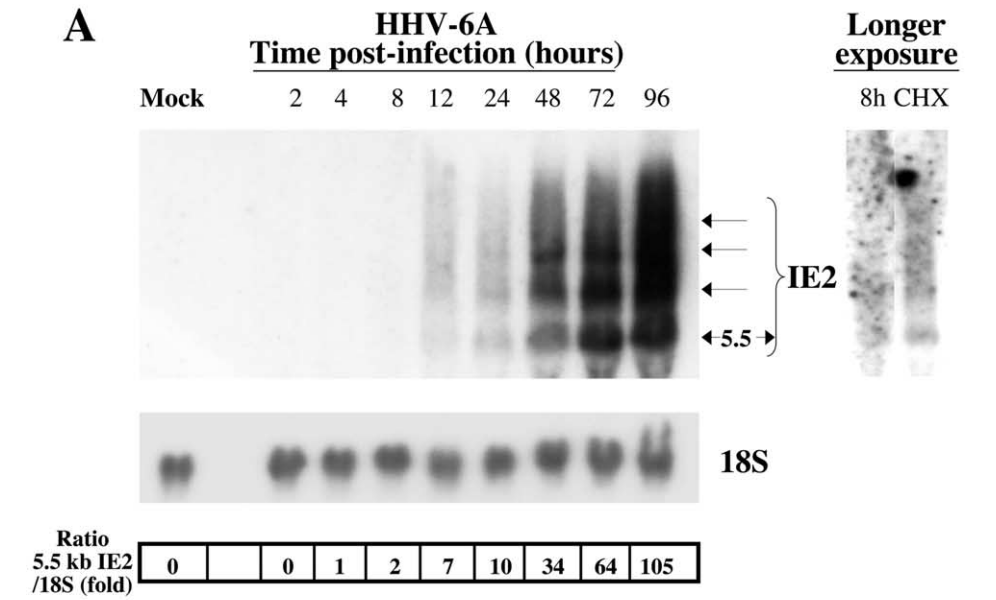
We next studied whether IE2 is expressed under IE conditions, in the presence of a protein synthesis inhibitor. HSB-2 cells were treated with cycloheximide (CHX) 30 min prior to and during the 8-h-long infection with HHV-6 variant A and total RNA was extracted and analyzed by Northern blot. At 8 hours, in the absence of CHX, IE2 expression was weak and a longer film exposure was necessary to detect this transcript (Fig. 2A, right panel). The IE2 transcript was equally expressed in the absence or in the presence of CHX, confirming that it belongs to the IE class of genes. For comparison purpose, we analyzed IE2 transcript expression in MOLT-3 cells infected with HHV-6 variant B (Fig. 2B). The kinetics of IE2 mRNA expression was similar for both variants to IE2 mRNA expression by 2–4 h with increasing expression over time. However, two major differences were observed. First, in the course of the variant B infection, the 6.3-kb IE2 message, analogous to the 5.5-kb IE mRNA of variant A, was much more abundantly expressed relative to the larger IE2 transcripts. In fact, the 6.3 kb IE2 message was at least three times more abundantly expressed than any of the larger IE2 transcripts. Second, in the presence of CHX, the 6.3 kb IE2 mRNA accumulated to a much greater level (sixfold) relative to that of infected and untreated cells (compare Figs. 2A and B).

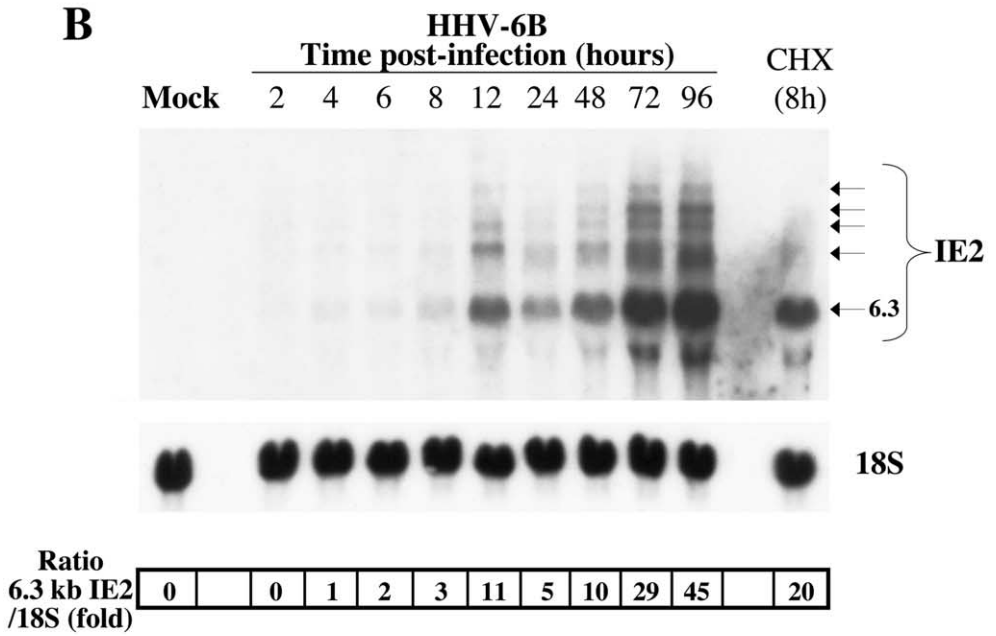
Last, the IE2 mRNA expression kinetics was compared to that of IE1, the other major transcript originating form the IE-A locus. RNA extracted during the same experiments presented in Fig. 2A was hybridized with a U89 probe, specific for the IE1 mRNA. The results obtained (Fig. 2C) indicate that IE1 is transcribed sooner than IE2 with initial detection by 2 h of infection. In addition, IE1 mRNA expression reached near to maximal expression by 12 h, while IE2 mRNA steadily increased up to 96 h. Interestingly, at 24 h postinfection, a twofold decrease in IE1 mRNA expression was consistently observed, suggesting that the *IE1* gene may be negatively regulated during this period. Similar results were observed with variant B (Gravel et al., 2002). The IE1 mRNA increase at 48–72 h is the likely result of secondary infections. Finally, IE1 mRNA could easily be detected in the presence of CHX, with a threefold greater expression than that observed in infected and untreated cells.

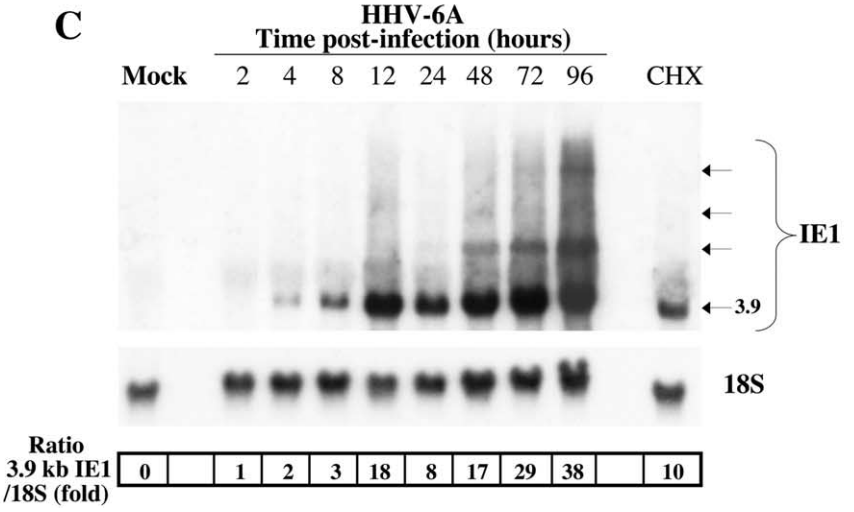
Kinetics of HHV-6 IE2 protein expression

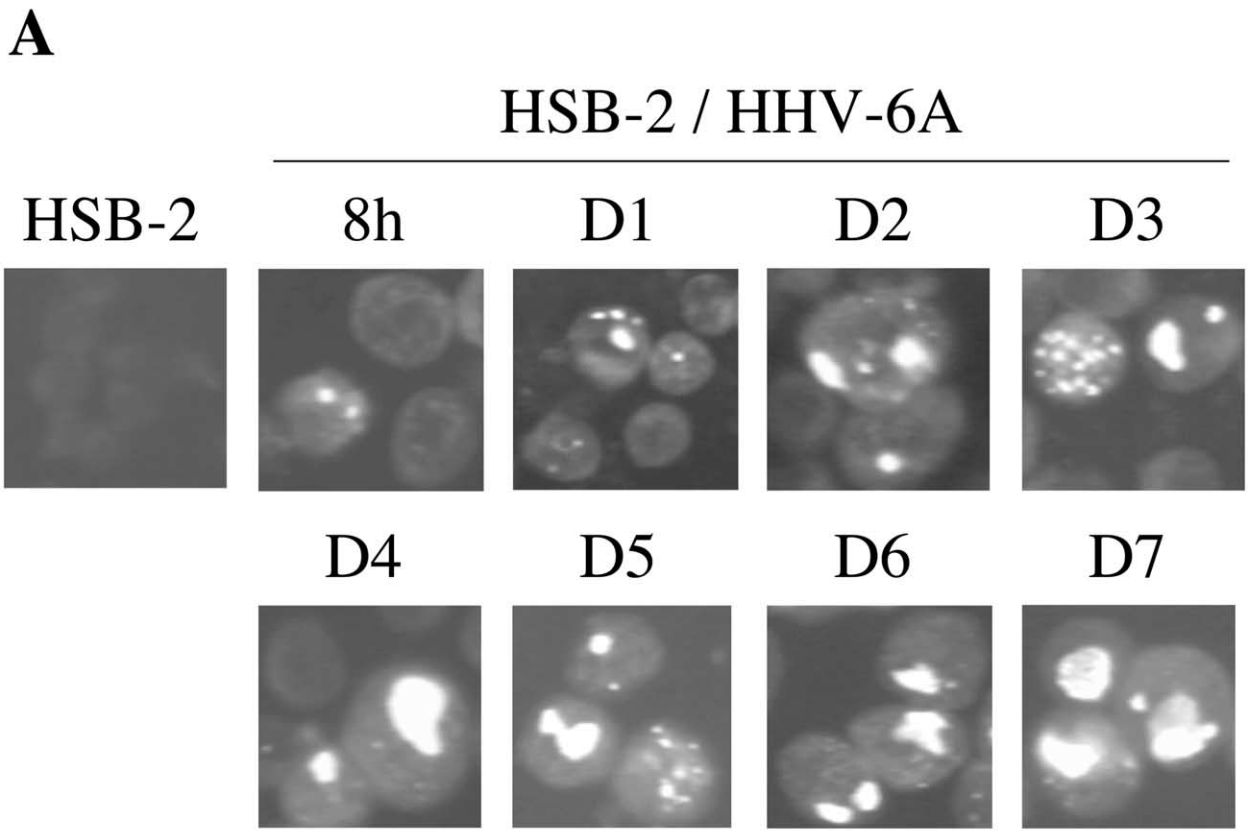
To our knowledge, detection of the HHV-6 IE2 protein has never been documented. Recombinant GST-IE2 protein is unstable and rapidly degraded in bacteria (data not shown) and this may explain in part the difficulty in generating a suitable immunogen for specific anti-IE2 antibody generation. To alleviate this problem, a branched synthetic peptide, whose sequence is conserved (14 out of 15 residues) between both HHV-6 variants, was synthesized and used to hyperimmunize New Zealand rabbits. Purified anti-IE2 IgG were first used to detect IE2 protein in HHV-6 variant A-infected HSB-2 cells by immunofluorescence. The earliest time point at which we could detect IE2 was 8 h postinfection (Fig. 3A). IE2 was present in the nuclei of infected cells as small granules whose number varied from 2 to 5. By Days 1 through 3 postinfection, IE2 was found in a mixture of small and medium-size granules. From Day 4

Fig. 1. (A) HHV-6 genome and details of the major IE locus A. The upper drawing represents the entire HHV-6 genome with the position of the two major IE loci (IE-A and IE-B) depicted as black rectangles. An asterisk identifies the origin of replication. Below is an enlarged view of the IE locus A and surroundings with open reading frames represented by arrows. The three major internal repeated regions (R1-R3) are presented as black rectangles. (B) Mapping of the IE2A transcript and protein sequences. A schematic representation of the full-length IE2 cDNA (GS strain) with genomic coordinates referenced according to the published HHV-6 (U1102 strain) (GenBank X83413). The IE2 transcript is made up of 5 exons with the first in-frame ATG codon located at position 136111 in the third exon. The fourth exon is spliced in frame with U86 and ends with a stop codon followed by a poly(A) signal. A line diagram of the IE2A protein with numbers indicating amino acid position is also presented. (C) Stick diagrams of IE2 protein from HHV-6 variants A and B. The numbers below the lines represent the amino acid number at the start of individual coding exons. The proteins were aligned for maximal similarity with gaps (unrelated sequences) drawn as thin lines. The overall nucleotide and amino acid identities, in parenthesis, between IE2 proteins from prototypic strains from the A variant (GS and U1102) and B variants (Z29 and HST) are also presented.


A
HHV-6A
Time post-infection (hours)
Longer exposure
Mock 2 4 8 12 24 48 72 96
8h CHX
IE2
5.5
18S
Ratio 5.5 kb IE2 /18S (fold)
0 0 1 2 7 10 34 64 105


B
HHV-6B
Time post-infection (hours)
Mock 2 4 6 8 12 24 48 72 96
CHX (8h)
IE2
6.3
18S
Ratio 6.3 kb IE2 /18S (fold)
0 0 1 2 3 11 5 10 29 45 20


C
HHV-6A
Time post-infection (hours)
Mock 2 4 8 12 24 48 72 96 CHX
IE1
3.9
18S
Ratio 3.9 kb IE1 /18S (fold)
0 1 2 3 18 8 17 29 38 10



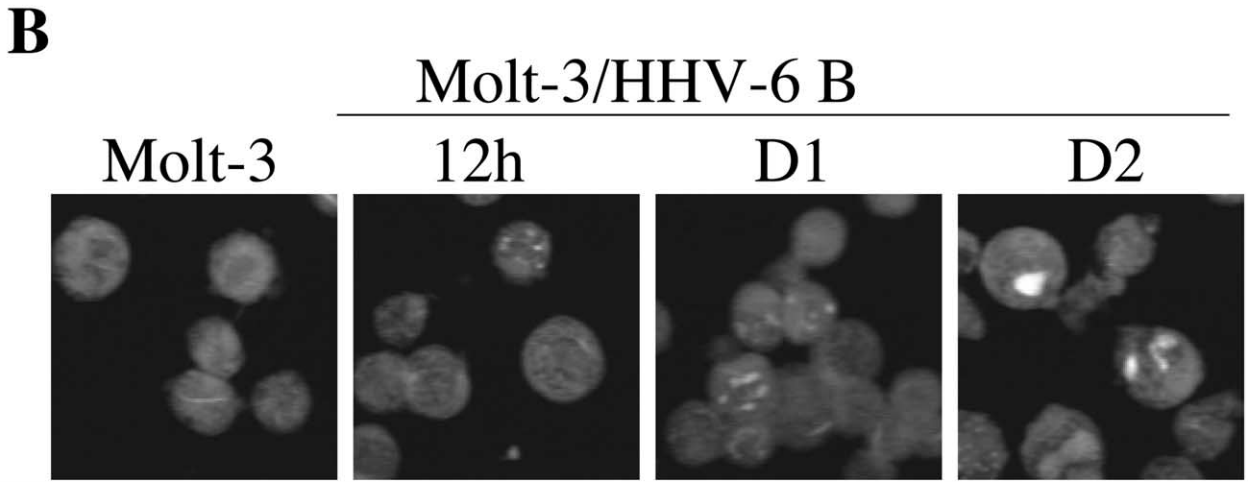


Fig. 3. (A) Kinetics of IE2A protein as detected by immunofluorescence. HSB-2 cells were infected with variant A of HHV-6 (GS strain) for varying periods of time and processed for immunofluorescence using anti-IE2 antibodies as described under Materials and Methods. (B) Kinetics of IE2B protein as detected by immunofluorescence. Same as in (A) with the exception that MOLT-3 cells were infected with HHV-6 variant B (Z29 strain).

Fig. 2. (A) Kinetics of IE2A mRNA expression. HSB-2 cells were infected with variant A of HHV-6 (GS strain) and total RNA isolated at varying time after infection was analyzed by Northern blot using a probe corresponding to a fragment of U86. In the right panel, infection was performed in the presence of CHX for 8 h before Northern blot analysis of IE2 mRNA. IE2 and 18S hybridization signals were determined, normalized, and compared with the 4-h time point, arbitrarily set at 1. (B) Kinetics of IE2B mRNA expression. MOLT-3 cells were infected with HHV-6 variant B (Z29 strain) and IE2 transcripts detected as described in (A). IE2 and 18S hybridization signals were determined, normalized, and compared with the 4-h time point, arbitrarily set at 1. (C) Kinetics of IE1A mRNA expression. HSB-2 cells were infected with variant A of HHV-6 (GS strain) and total RNA isolated at varying time after infection was analyzed by Northern blot using a probe corresponding to a fragment of U89. Arrows on the side of the figures point to the various transcripts detected. IE1 and 18S hybridization signals were determined, normalized, and compared with the 2-h time point, arbitrarily set at 1.

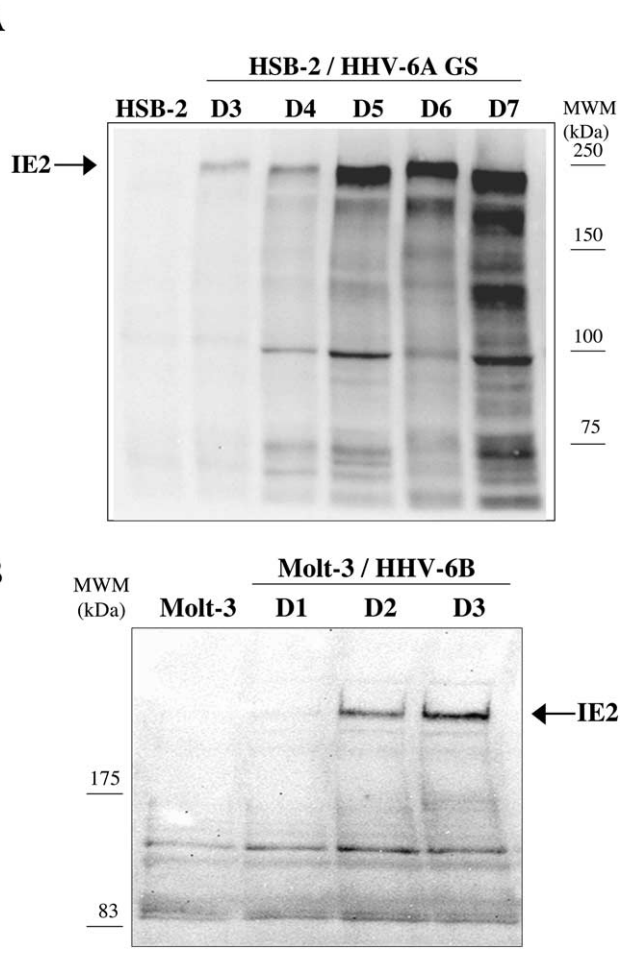


Fig. 4. (A) Kinetics of IE2A protein as detected by Western blot. HSB-2 cells were infected with HHV-6 variant A (GS strain) for varying periods of time and IE2 protein detection made by Western blot analysis using anti-IE2 antibodies as described under Materials and Methods. (B) Kinetics of IE2B protein as detected by Western blot. Same as in (A) with the exception that MOLT-3 cells were infected with HHV-6 variant B (Z29 strain).

postinfection and onward, the IE2 granules reached maximal size with a tendency to fuse into one large patch. By Day 7 postinfection, the vast majority of infected cells displayed a single large accumulation of IE2 protein that occupied up to one-third of the nuclear space. A similar experiment was conducted using MOLT-3 cells infected with variant B (Fig. 3B). The overall IE2 distribution followed closely that of variant A with IE2 initially (up to Day 1) present as small nuclear dots that eventually fused together forming an IE2 patch by the second day postinfection.

Western blot characterization of HHV-6 IE2 protein

The HHV-6 variant A IE2 protein has, in the absence of posttranslational modifications, a calculated molecular weight of 165 kDa. Since no published records exist on the electrophoretic profile of HHV-6 IE2 protein, we proceeded to analyze the kinetics of IE2 expression by Western blots. Total protein extracts from uninfected and HHV-6 variant A-infected HSB-2 cells were electrophoresed and transferred to a PVDF membrane. The blots were reacted with the anti-IE2 antibodies and a single protein, with an estimated molecular weight of 220 kDa, could be detected on the third day postinfection (Fig. 4A). IE2 expression gradually increased up to the fifth day postinfection where it remained stable up to Day 7. Whether the lower-molecular-weight proteins reacting with the antibodies represent degraded or splice variants of IE2 remains to be determined. Considering that the smaller IE2 reacting proteins are observed only during late infections, much beyond a single replicative cycle, when cytopathic effects are pronounced, it is anticipated that they represent proteolytic cleavage products. For comparative purpose, a similar experiment was conducted in MOLT-3 cells infected with a B variant of HHV-6 (Fig. 4B). Much like the results presented in Fig. 4A, a single large IE2 protein of approximately 225 kDa is detected on the first day postinfection. IE2 expression continued to increase on the second and third day postinfection.

IE2 is a promiscuous transcriptional activator

The function of HHV-6 IE2 is currently unknown. In a previous study, we reported that the genomic segment containing U86 could transactivate the CD4 promoter, highlighting the possibility that IE2 acts as a transcriptional modulator (Flamand et al., 1998). To further analyze this possibility, an expression vector coding for a full-length IE2 from variant A was used to determine the ability of this protein to transactivate promoters driven by different regulatory elements. Before doing so, we first tested whether our vector, pBK-IE2A, was able to express the IE2 protein. 293T cells were transfected with the pBK control plasmid or with the pBK-IE2A vector and processed 2 days later for immunofluorescence assay. Compared to lymphoid cell lines, 293T cells were transfected with much greater efficiency and were chosen for IE2 detection by immunofluorescence. The results obtained (Fig. 5A) clearly show that our anti-IE2 antibodies react against a nuclear protein in pBK-IE2A-transfected cells but are unreactive toward pBK-transfected control cells. IE2 is diffusely dispersed throughout the nucleus, in contrast to its dense patchy distribution in infected cells (Fig. 3).

Having determined that the IE2 protein is expressed following transfection with the pBK-IE2A vector, we proceeded to study the ability of this viral protein to activate various promoter constructs in MOLT-3 T lymphocytes, a cell type susceptible to HHV-6 infection. The first promoter studied was the HIV-LTR. Transfection of MOLT-3 cells with pBK-IE2A led to a dose–response activation of the HIV-LTR promoter with a maximal activity (18-fold) recorded with 4 μg of pBK-IE2A vector (Fig. 5B). As control, cells were transfected with an empty pBK vector whose activity was arbitrarily set at 1. The DNA quantities were

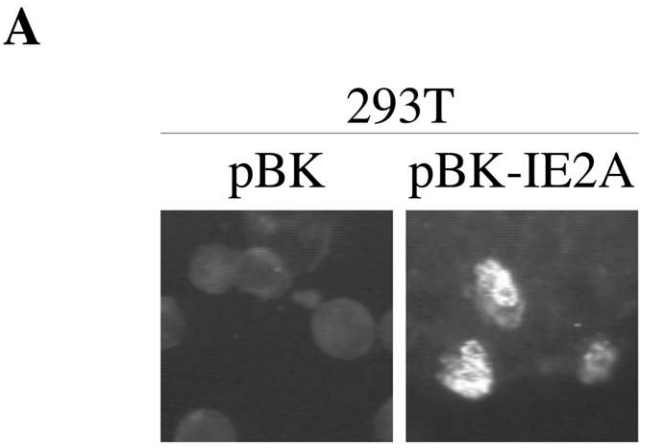


Fig. 5. (A) Transfection of 293T cells with pBK-IE2A expression vector. 293T cells were transfected with an expression vector for IE2A (pBK-IE2A) and processed for immunofluorescence using an IE2 rabbit antiserum. (B) IE2 acts as promiscuous transcriptional activator. MOLT-3 T cells were electroporated with various luciferase reporter plasmids (4 μg) and varying quantities (2–8 μg) of pBK-IE2A expression vector. The plasmid DNA levels were kept constant at 12 μg for each condition by the addition of empty pBK vector. The 0-μg value, arbitrarily set at 1, represents cells transfected with the reporter plasmid (4 μg) and 8 μg of pBK control vector. The data represent the normalized mean ± standard deviation (SD) (in fold induction) of three independent experiments performed in duplicate. The bottom right graph represents MOLT-3 transfection efficiencies in relation to increasing doses of pBK-IE2A. A GFP expression vector (4 μg) was cotransfected with 0, 2, 4, or 8 μg of pBK-IE2 vector. DNA levels were kept constant at 12 μg by the addition of empty pBK vector. Forty-eight hours after electroporation, cells were analyzed for GFP expression by flow cytometry. Transfection efficiencies were computed relative the pBK-IE2A 0 μg values, arbitrarily set at 100%. Results represent the mean ± SD of three independent transfection experiments.

kept constant at 12 μg by the addition of control pBK vector. In addition to the HIV-LTR, we generated a reporter construct containing the HHV-6 IE promoter spanning −554 to +82 relative to the transcription start site identified previously (Schiewe et al., 1994). HHV-6 IE2 also transactivates, in a dose-dependent manner, its own promoter (Fig. 5B). The HIV-LTR and the HHV-6 IE promoters are complex and contain multiple *cis*-acting regulatory elements that can influence transcription. To gain a better understanding of the promoter elements responsive to IE2, we used vectors whose reporter genes are by a single class of transcription factors. These include the CRE-LUC reporter, driven by the cyclic AMP responsive element binding proteins (CREBs), the pNFAT-LUC plasmid, regulated by the nuclear factor for activated T-cell (NFAT) proteins, and the NF-κB-LUC reporter, activated by the members of the NF-κB family of proteins. All of these promoters were significantly activated by IE2. The CRE-LUC and NFAT-LUC reporter showed the strongest activation with a 20-fold activation relative to the pBK control vector. A 4-fold activation of the NF-κB-LUC reporter was observed with 4 μg of pBK-IE2A effector plasmid. In most cases, the 8 μg dose of pBK-IE2A caused a reduction in promoter activity, suggesting that in MOLT-3 cells maximal activation is reached with effector plasmid doses ranging between 4 and 8 μg. Last, we studied the ability of IE2A to activate minimal promoters. The promoters studied include the pTATA-LUC with a TATA box as the only recognizable regulatory element and the pGL3 basic plasmid, which lacks typical eukaryotic promoter regulatory elements. Once again, cotransfection of pBK-IE2A with the two minimal promoter constructs led to a dose–response activation of the luciferase reporter gene. To confirm that the transactivation by IE2 is real and does not simply reflects an increase in transfection efficiency, were carried out experiments in which MOLT-3 cells were cotransfected with a constant amount of GFP-expressing vector (4 μg) along with varying quantities (0–8 μg) of pBK-IE2. DNA levels were brought up to 12 μg by the addition of empty pBK vector. Forty-eight hours postelectroporation, MOLT-3 cells were analyzed by flow cytometry and the number of GFP+ cells was determined. The results (last graph of Fig. 5B) indicate that at 0, 2, and 4 μg of pBK-IE2A, the number of GFP+ cells was similar, indicating that transfection efficiencies were roughly equal. However, cells transfected with 8 μg of pBK-IE2A were found to consistently have a reduced number of GFP+ cells. Considering that the total DNA quantities are identical (12 μg) in each samples, this effect is likely attributable to IE2. One possibility is that HHV-6 IE2 causes a cell cycle blockade, preventing the transfected cells to divide in a manner analogous to that reported by the HCMV IE2 protein (Murphy et al., 2000). Current work is in progress to address this issue. In summary, these results indicate that the IE2A protein of HHV-6 is capable of activating multiple promoters having no regulatory elements in common, suggesting that IE2A likely modulates transcription through interaction with the basic cellular transcription machinery.

Discussion

The major IE locus A of HHV-6 contains two large ORFs, U89 and U86, coding in part for the IE1 and IE2 proteins, respectively. We recently reported on the characterization of HHV-6 variant B IE1 transcript and protein (Gravel et al., 2002). In the present article we report the cloning of a complete IE2 cDNA and transcript organization and provide data on the kinetics of expression and the biological activities of the IE2 protein from HHV-6 variant A. The mature IE2 mRNA 5′ region is essentially identical to the 5′ end of the IE1 mRNA previously described (Schiewe et al., 1994). In fact, the first 4 small exons are the same with minor sequence variations attributable to the differing HHV-6 A variant isolates studied (GS versus U1102). For both IE1 and IE2, translation is presumed to initiate in the middle of exon 3. In the IE2 mRNA, the splice donor site of exon 4 is identical to that of the IE1 transcript and joins, in-frame, a splice acceptor site located 47 nucleotides upstream of the first ATG codon of ORF U86. The

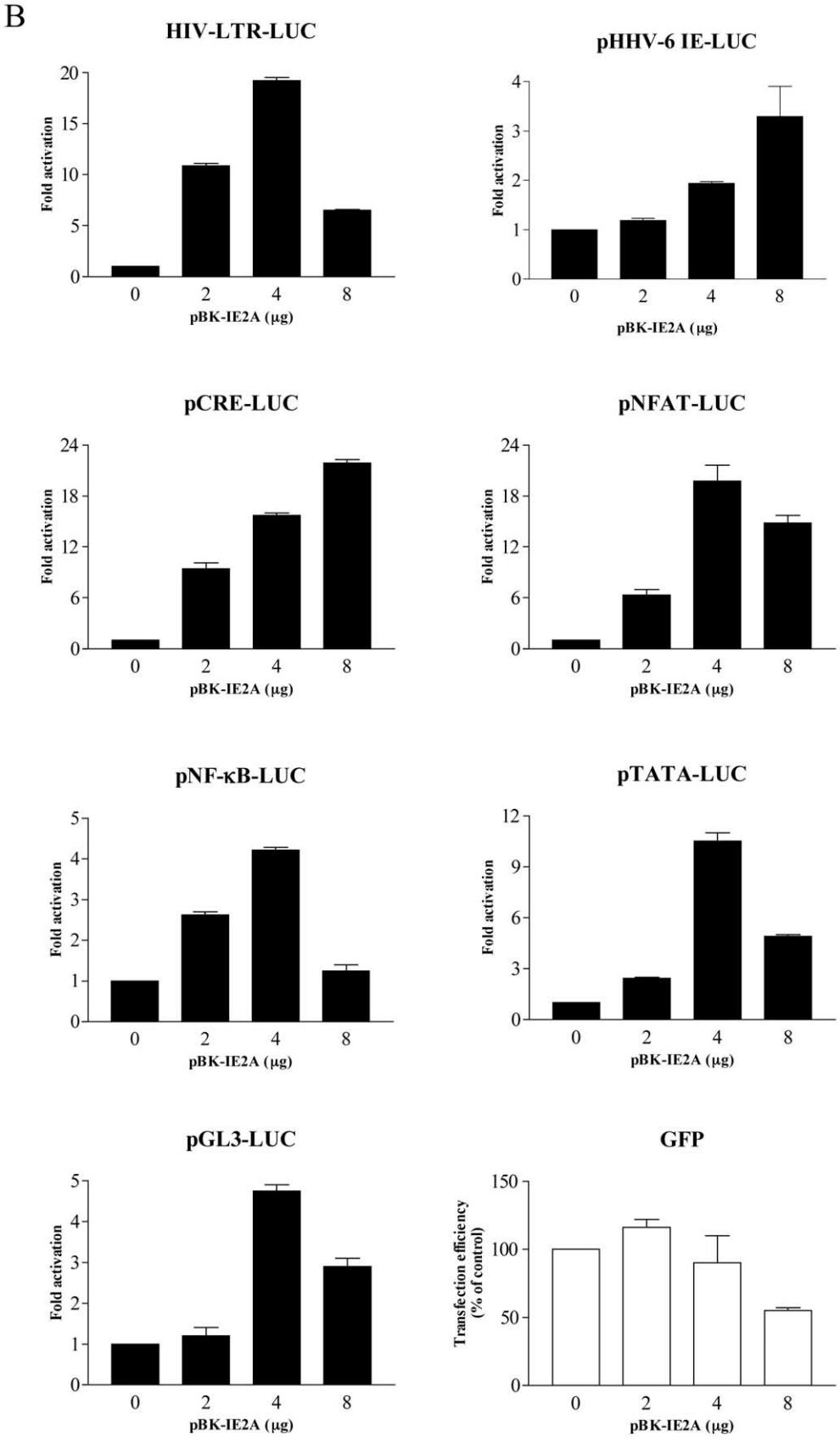


Fig. 5 (*continued*)

IE2 transcript includes all of U86 and terminates with a poly(A) signal. In terms of expression, the 5.5-kb IE2 transcript can be detected in the presence of CHX, confirming its expression under IE kinetics. IE2 mRNA is expressed after the IE1 message and continues to accumulate throughout the infectious cycle in contrast to IE1 mRNA, which remains constant after 12 h of infection. Both HHV-6 variants A and B had similar IE2 kinetics. Large transcripts hybridizing with the IE2 probe accumulated during late-stage infection, suggesting multiple transcription initiation sites as previously proposed for the *IE1* transcripts (Schiewe et al., 1994). It is worth noting that the abundance of the larger IE2 transcripts, relative to the 5.5- and 6.3-kb *IE2* mRNAs from variants A and B, respectively, differ considerably. In the case of variant A, the larger IE2 transcripts accumulated with kinetics and abundance similar to those of the 5.5-kb IE2 mRNA. For variant B, IE2 expression was heavily skewed in favor of the 6.3-kb transcript, suggesting that transcriptional regulation of IE2 mRNAs differ between HHV-6 variants. Interestingly, a recent article reported the U86 ORF to be part of latency-associated transcripts in HHV-6 variant B-infected monocytes (Kondo et al., 2002). Such transcripts were present as two distinct species. Type II transcript originated between exons 2 and 3 of the productive infection IE2 transcription start site while type I initiated 9.8 kb upstream of the productive IE2 transcript (Kondo et al., 2002, and present study). IE2 protein is apparently not translated from these latency-associated transcripts, suggesting that viral replication may be suppressed at the point of the translation of major IE proteins during latency (Kondo et al., 2002). These HHV-6 latency-associated transcripts were present during the productive phase of infection, albeit at very low levels, minimizing their potential roles during productive infection.

Protein sequence analysis indicates that the IE2 protein is approximately 165 kDa in size and contains several putative PKC and CaKII phosphorylation sites, suggesting that IE2 may be a phosphoprotein. HHV-6 IE2 protein shares limited amino acid similarities with the HCMV IE2 protein. Translated sequence analysis indicates that the R1 region, composed of 46 imperfect tetrameric amino acid repeated motifs (SSRA [31], SSRD [8], SSKA [5], SSRT [1], SSRE [1]), is present within the variant A (GS strain) IE2 protein. For comparison, the R1 region from the Z29 B variant contains 48 tetrameric repeated motifs, 42 of which have the SSRA sequence. The functional significance of these reiterated sequences remains undetermined. In terms of protein, infection of cells by both HHV-6 variants led to the detection of a single specie of IE2 having a molecular mass in excess of 200 kDa. This result is somewhat different than that observed during HCMV infection where spliced variants of IE2 are produced (Stenberg et al., 1985). Within infected cells, HHV-6 IE2 was first detected as intranuclear granules, which coalesced into a single large immunoreactive patch by 72 h postinfection. These IE2 patches are reminiscent of the HCMV IE2 patches observed in infected cells (Ahn et al., 1999). HCMV IE2 is known to be required for efficient viral DNA replication and to be associated with the viral replication compartments. Work is in progress to address whether HHV-6 IE2, in a manner analogous to HCMV IE2, is associated with the viral DNA replication complex. A basic isoelectric point of 9.8 for HHV-6 IE2 is consistent with a potential for this protein to interact with DNA. IE2 expression, in the absence of any other HHV-6 proteins was observed as a diffuse nuclear staining. Thus, the inability to reproduce the patchy appearance observed during late-stage infection is likely attributable to the failure in recruiting viral factors, such as polymerase processivity factor (U27) and the single-stranded DNA binding protein (U41), for the generation of functional replication DNA compartment.

It is currently unknown whether HHV-6 IE2 can negatively regulate its own expression in a manner similar to that of HCMV IE2 (Cherrington et al., 1991; Liu et al., 1991; Pizzorno and Hayward, 1990). HCMV IE2 is reported to bind to a *cis* repression signal (crs) located between that TATA box and the transcription start site (Lang and Stamminger, 1993, 1994; Macias and Stinski, 1993). This crs site overlaps an initiator sequence (Carcamo et al., 1991; Javahery et al., 1994; Smale and Baltimore, 1989). Interestingly, such an initiator sequence (Py Py A N T/A Py Py) is present adjacent to the transcription start site (-10 to -4) of HHV-6 *IE* genes and whether HHV-6 IE2 protein interacts with this region to negatively modulate its own expression remains to be determined. Our IE2 mRNA kinetics data indicate that IE2 expression continues to increase with time and does not appear to be turned off, even at times when large quantities of IE2 protein are detected. This suggests that IE2 does not appear to directly suppress its own transcription. Our transactivation assay results using the HHV-6 IE promoter (-554 to $+82$), which indicate that IE2 activates its own promoter (Fig. 5B), are in support of such affirmation.

The IE2 promoter/enhancer has yet to be fully characterized. A study from Schiewe et al. (1994) has mapped the transcription start site of the *IE1* gene, which more than likely is the same for the *IE2* gene, 27 nucleotides downstream of the TATA box. It has been speculated that the R3 region could function as an enhancer for MIE genes (Dominguez et al., 1999; Martin et al., 1991; Thomson and Honess, 1992). The R3 region consists of 24 copies of repeated 104- to 107-bp units with abundant binding sites for cellular transcription factors (AP-2, NF-κB, PEA3). A recent report indicates that the R3 region plays an important role for *U95* gene expression, an IE gene located downstream of the R3 region in orientation opposite the *IE2* gene (Takemoto et al., 2001). Whether the R3 region plays a role in the regulation of HHV-6 *IE2* gene expression remains to be determined.

One common feature of HHV-6 and HCMV IE2 proteins is their ability to promiscuously transactivate heterologous promoters. So far, HHV-6 IE2 transactivated all promoters tested. These include complex promoters such as HIV-LTR,

HHV-6 IE, and more simple promoters driven by unique responsive elements (NFAT, NF-κB, CRE), or a minimal promoter containing only a TATA box. The ability of IE2 to activate multiple promoters having no regulatory element in common suggests that IE2 can interact with basic cellular machinery and positively modulates transcription. This is not unique to HHV-6, as several viruses rely on host factors to govern the expression of their genes. In fact, HCMV IE2 is reported to interact with host factors such as TFIIB, TFIID, P/CAF, and CBP, all of which lead to the transcriptional activation of specific HCMV-responsive elements. (Bryant et al., 2000; Caswell et al., 1993; Schwartz et al., 1996; Sommer et al., 1994). Our current difficulties in expressing full-length IE2 in bacteria or immunoprecipitating this viral protein from infected or transfected cells have so far prevented us from studying the physical interactions between IE2 and cellular transcription factors.

The results presented in this article represent the first molecular and biological characterization of the IE2 protein of HHV-6. Considering that the major IE locus A is among the most divergent region between variants, a thorough comparative analysis is warranted to determine whether some of the differing biological properties among variants could be ascribed to differences in IE2 proteins functionalities. The IE2 transactivator likely plays a crucial role in the regulation of HHV-6 gene expression and the importance of this protein in the replicative cycle awaits the generation of specific IE2 mutants of HHV-6.

Materials and methods

Cell culture and virus preparation

The HSB-2 and MOLT-3 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma–Aldrich Canada, Oakville, Ontario) and M-plasmocin (InvivoGen, San Diego, CA) to prevent mycoplasma contamination. The 293T cell line was cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum and M-plasmocin. The GS strain of HHV-6A was propagated on HSB-2 cells and the Z29 strain of HHV-6B, on MOLT-3 cells, as previously described (Gravel et al., 2002). The infectivity titer of both viruses was determined after 24 h of infection with various dilutions of HHV-6, by immunofluorescence. Infected cells were fixed in acetone for 10 min at −20°C, air-dried, and incubated with Alexa 488-labeled anti-IE1 antibodies for 1 h at room temperature. Three 5-min PBS washes were done before slides were mounted with glycerol and examined under a fluorescent Leitz Aristoplan microscope (Leica, Richmond Hill, Canada). The HHV-6 titers were then determined by calculating the percentage of IE1-positive cells. The HHV-6A titer was determined to be 2×10^7 infectious particles per milliliter, while the HHV-6B titer was 6×10^6 infectious particles per milliliter.

cDNA library construction, screening, and sequencing

To obtain a cDNA library representative of the majority of HHV-6 transcripts, RNA was isolated from HSB-2 cells infected with HHV-6 variant A under three different conditions: under IE condition using a 7-h infection in the presence of 10 μg/ml CHX, under early condition using a 2-day infection in the presence of 100 μg/ml phosphonoacetic acid (PAA), and under late condition using a 3-day infection in the absence of inhibitors. Total RNA was isolated using the Trizol reagent (Invitrogen, Burlington, Ontario), mRNA was obtained using the poly(A) Tract kit (Promega, Madison, WI), and a λ ZAP express cDNA library was constructed according to the manufacturer's guidelines (Stratagene, La Jolla, CA). A 1.8 kb *Eco*RI fragment obtained from the central part of the U86 gene from HHV-6A, strain U1102, was used to screen the cDNA library. A full-length IE2A was obtained after two successive rounds of hybridization with the U86 fragment. The transcript was completely sequenced on one strand using an automated ABI Prism sequencer (Perkin–Elmer, Boston, MA). Sequence analyses of IE2 from different variants and isolates of HHV-6 were performed using BLAST programs (Altschul et al., 1990, 1997).

Kinetics of IE1 and IE2 mRNA expression in HHV-6-infected cells

HSB-2 cells were infected with HHV-6A (GS strain) for 2 to 96 h, at a MOI of 0.1. At indicated times, total RNA was isolated from 10×10^6 cells using the Trizol reagent. Total RNA was also isolated from 10×10^6 cells infected with HHV-6A for 8 h in the presence of 10 μg/ml cycloheximide, to determine the presence of *IE1* and *IE2* genes under IE conditions. Ten micrograms of total RNA was migrated on a 1% agarose–formaldehyde gel, transferred onto nylon membranes, and probed for *IE1* and *IE2* genes, as previously described (Gravel et al., 2002). Membranes were probed for the presence of the *IE1* gene using a *Kpn*I–*Bgl*II fragment from the U89 region of HHV-6A (U1102 strain), while membranes were probed for the presence of *IE2* gene using a *Eco*RI fragment derived from the U86 region of HHV-6A (U1102 strain). MOLT-3 cells were infected with HHV-6B (Z29 strain) for different periods of time, as previously described (Gravel et al., 2002).

Anti-IE2 antibody generation

A branched peptide of 15 amino acids (IESSYRESRNTNRGY) having 93% identity between both HHV-6 variants was synthesized using the FastMoc chemistry. The purified peptide, directed against the N-terminal part of the full-length IE2 protein (GS strain: amino acids 212–226), was resuspended in PBS. One milligram of peptides was emulsified in complete Freund's adjuvant and injected in-

tramuscularly into New Zealand rabbits. After seven immunizations, rabbits were bled by cardiac puncture and IgG immunoglobulins were purified using a HiTrap Protein G Sepharose HP affinity column (Amersham Biosciences, Baie d'Urfé, Quebec). Anti-peptide IgG was labeled using the Alexa-Fluor 488 protein labeling kit for fluorescence analysis (Molecular Probes, Eugene, OR, USA).

Kinetics of IE2 protein expression in HHV-6-infected cells

HSB-2 cells were infected with HHV-6A (GS strain) at a MOI of 0.03 for periods of time ranging from 4 h to 7 days and MOLT-3 cells were infected with HHV-6B (Z29 strain) at a MOI of 0.02 for 12 h to 3 days. At indicated times, 3×10^6 cells were prepared for immunofluorescence (IFA) and 10×10^6 cells were prepared for western blot analysis. For IFA, cells were washed twice in PBS before 1.5×10^5 cells/well were deposited onto 10-well microscope glass slides (ICN Pharmaceuticals, Costa Mesa, CA). When dry, they were fixed in cold acetone (−20°C) for 10 min. The slides were then incubated with HHV-6 Alexa 488-labeled rabbit anti-IE2 antibody in PBS for 1 h at room temperature. Slides were washed three times in PBS, mounted with glycerol/PBS before examination, and photographed on a fluorescent Leitz Aristoplan microscope using a Dage MTI black and white cooled camera (8 bits) and Bioquant True Color Windows 98 software (R&M Biometrics, Inc., Nashville, TN). For Western blot analysis, cells were washed in PBS and lysed in 1 ml of lysis buffer for 30 min on ice, as described (Gravel et al., 2002). Samples were sonicated briefly and centrifuged at 18,000*g* for 10 min. Soluble proteins from the supernatants were electrophoresed through a 6% SDS–PAG, transferred to polyvinylidene fluoride (PVDF) membranes, and blotted for HHV-6 IE2 protein using a rabbit anti-IE2 antibody for 1 h at room temperature. After three 10-min washes with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies for 1 h. The blots were washed with TBST and the proteins were visualized with enhanced chemiluminescence (Perkin–Elmer) using a PhosphorImager system (Fuji Medical Systems, Stamford, CT).

Transient DNA transfection of 293T cells

293T cells were seeded into six-well plates at 3×10^5 cells per well the day before transfection. Cells were transfected with 2 μg of pBK control vector or pBK-HHV-6 IE2A expression vector using the ExGen transfection reagent (MBI Fermentas, Flamborough, Ontario). Forty-eight hours posttransfection, cells were trypsinized, washed three times in PBS, and 3.5×10^5 cells per well were deposited onto 10-well microscope glass slides and processed for immunofluorescence as described above.

Luciferase reporter assay

Two, four, or eight micrograms of pBK-HHV-6 IE2A expression plasmid was cotransfected with 4 μg of different luciferase reporter plasmids in 1×10^7 MOLT-3 cells by electroporation (250 V, 960 μFd). The DNA levels were kept constant at 12 μg by the addition of empty pBK vector. The cells were harvested 2 days after transfection, resuspended in 0.2 ml of cell lysis buffer (Promega), and incubated at room temperature for 30 min with gentle agitation. Aliquots of 20 μl were analyzed for the luciferase activity using a MLX luminometer (Dynex Technologies, Chantilly, VA). Protein quantities were assessed for all samples and luciferase activity recorded was normalized accordingly.

Nucleotide sequence accession number

The sequence described in this article has been deposited under GenBank Accession No. AY037931.

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