IDENTIFICATION AND CHARACTERIZATION OF GENE PRODUCTS OF ORF U90/89 OF HUMAN HERPESVIRUS 6

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Summary. – Human herpesvirus 6 (HHV-6) isolates can be classified into two variants, A and B. Comparison of genomic sequences of these variants has highlighted sequence variability in the region spanning U86 to U100. This region includes the immediate early A (IE-A) locus that was defined as positional homologue of the major IE locus of Human cytomegalovirus (HCMV) with little recognizable sequence homologies. A 3.5 kb transcript, one of the four spliced transcripts identified in the IE-A locus, is derived from the U90/89 ORF encoding the IE1 protein. We expressed six *Escherichia coli* fragments spanning the HHV-6A U90/89 ORF as IE1 fusion proteins. The bacterially expressed fusion protein was used to raise monospecific polyclonal antiserum for detection and identification of the IE1 protein product(s). Using this antiserum we detected 165, 190, and >190 K proteins in HHV-6A- and HHV-6B-infected cells and the 165 K protein in cells transfected with an IE1 cDNA construct. The IE1 proteins exhibited perinuclear and cytoplasmic localization in infected cells. There was a correlation between the expression level of IE1 and the degree of permissiveness for virus growth in various cell lines. In transient expression experiments a 140 bp fragment from the upstream IE-A region was shown to possess promoter activity. The C-terminal region of IE1 delineated by amino acids (aa) 588 to 636 showed a DNA binding activity in Southwestern blot analysis.

Key words: Human herpesvirus 6; immediate early gene; U90/89; U90; IE-A region

Introduction

HHV-6 (the *Human herpesvirus* species, the *Roseolovirus* genus, the subfamily *Betaherpesvirinae*, the *Herpesviridae* family) has been first isolated from the peripheral blood of patients with lymphoproliferative disorders and AIDS where the virus had reactivated (strain GS; Salahuddin *et al*., 1986). Since then several reports have described isolation of similar viruses from patients with AIDS, including the prototype strain U1102 from Uganda (Downing *et al*., 1987), and Z29 from Zaire (Lopez *et al*., 1988). HHV-6 and the closely related HHV-7 are members of the *Roseolovirus* genus. By molecular and biological criteria, HHV-6 isolates can be classified into two main variants: A consisting of GS- and U1102-like isolates and B consisting of Z29-like isolates (Ablashi *et al*., 1993). Primary infection with HHV-6B is causally associated with exanthema subitum, a mild childhood disease (Hall *et al.,* 1994; Yamanishi *et al*., 1988). To date, primary infections with variant A strains have not been specifically associated with any pathological condition. Accumulated recent data indicate that while A and B variants are closely related, they occupy independent biological niches and meet the criteria for classification into distinct species (Dominguez *et al*., 1999; Johnson *et al*., 2000). HHV-6 is CD4+ T lymphotropic and monocyte/macrophage is a possible site for its latency (Kondo *et al*., 1991). Infection of primary T cells or T cell lines *in vitro* causes a cytopathic

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Abbreviations: CBl = cord blood lymphocytes; DME medium = Dulbecco's Modified Eagle's medium; DR_L = left direct repeats; DR_R = right direct repeats; EBV = Epstein Barr virus; HCMV = Human cytomegalovirus; IE = immediate early; MBP = maltose binding protein; MCMV = Murine cytomegalovirus; post inoculation = p.i.; SCMV = Simian cytomegalovirus; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; XLB = *E. coli* strain XL-1 blue

effect characterized by marked "balloon-like" enlargement of infected cells, features typical for all betaherpesviruses (Frenkel *et al*., 1990; Honess, 1984; Lusso *et al*., 1988).

Although based on serological cross-reactivity (Larcher *et al*., 1988), amino acid sequence homologies (Lawrence *et al*., 1990) and cross-reactivity in the T-cell immune response (Yasukawa *et al*., 1993), HHV-6 and HCMV belong to betaherpesviruses, their overall genome structure is different. The HHV-6 genome consists of a unique segment flanked by two tandem repeats and the computer-assisted analysis suggests that it is closest to a progenitor herpesvirus (Karlin *et al*., 1994b). The position and orientation of all conserved genes on the HHV-6 unique region are collinear with the unique long component of HCMV genome (Lawrence *et al*., 1990; Neipel *et al*., 1991).

Gene transcription and expression in herpesviruses are regulated; they follow a well-ordered and characterized pattern. IE (alpha) genes are expressed immediately after virus entry into the cell and their expression does not require *de novo* protein synthesis, the majority of them encode regulatory proteins. The HHV-6 IE-A locus was defined as positional homologue of the major IE locus of HCMV and does not share significant homology with HCMV or HHV-7 or any other known herpesvirus genes (Dominguez *et al*., 1999; Isegawa *et al*., 1999; Kosuge *et al*., 1997; Martin *et al*., 1991; Schiewe *et al*., 1994).

HHV-6, like HCMV and other betaherpesviruses examined (SCMV and MCMV), have local CpG suppression (Dominguez *et al*., 1999; Gompels *et al*., 1995; Honess *et al*., 1989; Karlin *et al*., 1994a) in which only CpG deficiency and matched TG/CA excess in the major IE gene region were observed (Gompels *et al*., 1995). A *trans*-activating function has also been mapped to the IE gene region (Martin *et al*., 1991). In HCMV major IE genes are transcribed from two genetic units designated IE1 and IE2 (Wathen *et al*., 1981). Transcriptional studies on the HHV-6 IE locus have identified four transcripts: two longer ones of 3.5 and 4.7 kb are derived from one strand and two shorter ones are derived from the opposite strand (Schiewe *et al*., 1994). Among these transcripts only the 3.5 kb mRNA can be detected in the presence of cycloheximide (Schiewe *et al*., 1994; F. Neipel, personal communication). Martin (1991) has identified within this region a function capable of *trans*-activating heterologous promoters in transient expression experiments.

In this study, to further characterize the HHV-6 IE-A locus, we expressed the putative cDNA sequences of HHV-6A strain U1102 (Downing *et al*., 1987) corresponding to the 3.5 kb mRNA identified by Schiewe *et al*. (1994) in *E. coli* and in eukaryotic cell lines. The prokaryotically expressed recombinant IE1 proteins were used to examine the DNA-binding activity of IE1 as well as to generate monospecific polyclonal antiserum in rabbits for detection of authentic IE1 proteins in infected cells.

Materials and Methods

Cells and viruses. HeLa and Cos7 cells were grown at 37°C in a 5% CO_2 atmosphere in the Dulbecco's Modified Eagle's medium supplemented with 5% of fetal calf serum. Cord blood lymphocytes (CBLs) were isolated by centrifugation on Ficoll-paque gradients (Biochrom KG). HHV-6A U1102, HHV-6B R104 and HHV-7 RT (Frenkel, 1990) were propagated in CBLs. Suspension cultures of CBLs were grown in the RPMI 1640 medium containing 15% of heat inactivated fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin and 10 µg/ml phytohemagglutinin at 37°C and in 5% CO_2. Prior to infection with HHV-6 or HHV-7, the CBL cultures were stimulated with 20 U/ml and 5 U/ml human recombinant interleukin-2 (Sigma), respectively. The viruses were propagated by co-cultivation of infected and uninfected cells at a ratio of 1:10 (Schiewe *et al*., 1994). To achieve a sufficient amount of infectious cell-free virus, the culture supernatants were processed as described earlier (Schiewe *et al*., 1994). Jurkat, Molt-3, and HL60 cells were cultured in the RPMI 1640 medium containing 10% of heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and were infected with cell-free viruses (Rapp *et al*., 2000).

Oligonucleotides purchased from Eurogentec (Belgium) were used. They were namely X*ba*I5211 (5'-CAATTCCATATTTGTC TAGAAGGGG-3'), *Xho*I5351 (5'-CCACTTCTCGA GAATAGTG GCACTGAA-3'), *Bam*HI258 (5'-AACAACATGGGATCCAAT GATGAAC-3'), *Pst*I978 (5'-AAAGTCCTTACTGCAGAT GTCAGGA-3'), *Pst*I1598 (5'-CCCTGACTGTCT GCA GTTTTCAGTG-3'), *Eco*RI*1986* (5'-TCACCACCAGAAT TCACCCCTTCTA-3'), *Pst*I2742 (5'-AGTAGGGGTGACGT CATTATATTTA-3'), F1 (5'-CGCGGATCCATGGAC AACGA CACAGTGATG-3'), R1 (5'-CGGGAATTCTCGTTGGAGTTA GAGTCAG-3'), F2 (5'-CGCGGATCCATGGCGGCGGTGATC CACAC-3'), and R2 (5'-CGGGAATTCCCGTCATAGTAGCTTA GCTGAAC-3').

DNA amplification and plasmid construction. The exon structure of the IE A locus has been described by Schiewe *et al.* (1994). The IE1 gene consists of 5 exons and 4 introns, while the coding region contains a part of exon 3 (amino acids (aa) 1–20), exon 4 (aa 21–84) and a part of exon 5 (aa 85–941). Plasmid Cdna1x1 contains the full-length cDNA of the putative HHV-6 IE1 sequence (Schiewe *et al*., 1994). In all the following constructs, the fragments containing parts of the IE1 sequence were prepared from the plasmid Cdna1x1 and cloned into the indicated restriction sites of pMAL-c2 (New England Biolabs) for production of proteins fused to the maltose binding protein (MBP). pMA6001 was constructed by cloning the 3,058 bp *Bam*HI/*Xho*I fragment containing the entire IE1 coding sequences (929 aa, excluding the 12 N-terminal amino acids) into the *Bam*HI/*Sal*I sites. pMA6011 was constructed by cloning the 1,874 bp *Bam*HI/*Pst*I fragment (IE1 aa 13–636) into the *Bam*HI/*Pst*I sites. pMA6013 was constructed by cloning the 651 bp *Xmn*I/*Pst*I fragment (IE1, aa 419–636) into the *Xmn*I/*Pst*I sites. pMA6020 and pMA6025 were constructed by cloning the *Bam*HI/*Pst*I 720 bp and 1,340 bp fragments (IE1 aa 13–252 and 13–458, respectively)

generated by PCR with the primer pair *Bam*HI258/*Pst*I978 (annealing temperature of 52°C) and *Bam*HI258/*Pst*I1598 (annealing temperature of 50°C) into the *Bam*HI/*Pst*I sites, respectively. pMA6023 was constructed by cloning the *Eco*RI/*Pst*I 146 bp fragment (IE1 aa 588–636) isolated from a larger fragment of 756 bp generated by PCR with the primer pair *Eco*RI1986/*Pst*I2742 (annealing temperature 52°C) into the *Eco*RI/*Pst*I sites. PCR was performed using Taq polymerase (New England Biolabs). The PCR conditions used were as follows: initial denaturation step at 94°C for 4 mins, 30 cycles of denaturation at 94°C for 1min, annealing at the temperatures described above for 1min, and extension at 72°C for 1min, and final extension step at 72°C for 4 mins. pMA6012 was constructed by cloning the 3,358 bp *Not*I/*Xho*I fragment of Cdna1x1 containing the entire IE1 sequences into the *Not*I/*Xho*I sites of pcDNAI/NEO (Invitrogen). Putative IE1 promoter sequences were amplified from the plasmid p293-6 (nt 136,111–138,300 of the HHV-6 U1102 genome) using the *Xba*I5211 and *Xho*I5351 primers (annealing temperature of 54°C). The 140 bp PCR product was cloned into the *Xba*I/*Xho*I sites of plasmid pIGA-53 (Gelman and Silverstein, 1987) to generate pMA6033. Fragments containing 112 bp and 124 bp EBV sequences (nt 168, 658–168, 763 and 168, 164–168, 269) were amplified by PCR with the primer pairs R1/F1 (annealing temperature 54°C) and R2/F2 (annealing temperature of 54°C), respectively.

Expression and purification of IE1-MBP fusion proteins. Recombinant plasmids were transferred into the *E. coli* strain XLB. For induction of MBP fusion products, cultures were grown at 37°C in LB broth to A_{600} of 0.5 and were induced with 0.8 mmol/l IPTG (Integra Biosciences). These conditions were determined as optimal in preliminary experiments. The induced cells were cultured at 37°C for 4 hrs, harvested, washed in PBS, and stored at -70°C until used. The recombinant proteins were purified from the cell lysates by affinity chromatography according to the manufacturer's instructions (New England Biolabs).

Production of antisera. The full-length prokaryotically expressed IE1 protein was separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)and isolated by electroelution from 8.5% polyacrylamide gels. White New Zealand rabbits were immunized subcutaneously with 500 µg of the isolated protein mixed with complete Freund's adjuvant. Two booster immunizations were carried out 2 weeks apart with the same inoculum but without adjuvant. The animals were sacrificed 1 week after the final boost. Production of the U89 oligopeptide antiserum (CI465) has been described previously (Papanikolaou *et al*., 2002).

Transient expression experiments. pMA6012 in which the HHV-6 IE1 sequences are under control of the major HCMV IE promoter was used in transient expression experiments with HeLa cells. pMA6033 in which the putative HHV-6 IE1 promoter region containing the TATA sequence was cloned upstream of the CAT gene, was used in transient expression experiments with Cos7 cells. The calcium phosphate precipitation method was used for transfection.

Chloramphenicol acetyltransferase (CAT) assay was performed on duplicate cultures. Three different plasmid preparations were used in each experiment. Two days after the transfection the cells were harvested in 100 µl of 0.25 mol/l Tris-HCl pH 7.8 and disrupted by three cycles of freezing and thawing. The cell lysates were clarified by low-speed centrifugation. Fifty µl of the supernatant was added to 80 µl of the CAT reaction mixture consisting of 0.625 mol/l Tris-HCl pH 7.8, 0.85 mg/ml acetylcoenzyme A (Sigma) and 0.025 mCi/ml ^{14}C-labeled chloramphenicol (specific activity 60 mCi/mmole; Amersham). The reaction proceeded at 37°C for 2 hrs and then 1ml of ethyl acetate was added to each sample. Following shaking and a low-speed centrifugation 900 µl of the upper phase was saved and evaporated under vacuum. The dry products were dissolved in 20 µl of ethyl acetate and applied on a 15 mm silica gel of a thin-layer chromatography (TLC) plate. The acetylated derivatives were separated from non-acetylated chloramphenicol by TLC using a chloroform-methanol (95:5) solvent. The separation was visualized by autoradiography (X-Omat, Kodak)**.**

Western blot analysis. Cell lysates were subjected to SDS-PAGE in 8.5% gel and the separated proteins were electroblotted to a nitrocellulose membrane. The blots were probed with the antisera at various dilutions as described in figure legends.

Indirect immunofluorescence assay (IFA). Mock- and virus-infected cells were processed for IFA as described earlier (Takeda *et al*., 1996). Finally, the slides were counter-stained with 10% Evan's-Blue in PBS for 10 mins. The slides were air dried and were mounted with glycerol containing NaN_3.

Southwestern blot analysis. The 140 bp PCR product generated by the primer pair *Xba*I5211/*Xba*I5351 spanning the IE1 promoter region was purified from low melting agarose gels and was then digested with *Xba*I and *Xho*I whose recognition sites occurred within the primers ends. The 112 bp and 124 bp products generated by PCR with primer pairs R1/F1 and R2/F2, respectively, were purified from low melting agarose gels and were then digested with *Nco*I and *Eco*RI whose recognition sites occurred within the primers ends. The resulting DNA fragments were end-labeled with α-[^{32}P]dCTP (specific activity 3000Ci/mmol; Amersham) using the Klenow fragment of DNA polymerase. Total *E. coli* cell lysates expressing various portions of IE1 protein were subjected to SDS-PAGE in 8.5% gel and the separated proteins were electroblotted to a nitrocellulose membrane The blots were probed with the end-labeled DNA as described by Inoue *et al*. (1994) in the presence of 100 µg/ml fragmented salmon sperm DNA (Sigma).

Results

Prokaryotic expression of the IE1 protein and production of IE1 antisera

The coding sequence regions from the putative IE1 gene of HHV-6A U1102 were cloned into the expression vector pMAL-c2 (Maina *et al*., 1988) in order to produce IE1-MBP fusion proteins under control of the inducible *tac* promoter (Fig. 1).

In preliminary experiments *E. coli* strain XLB and 37°C as expression temperature were found as optimal (data not shown). Upon induction with IPTG, the bacteria containing pMA6001, pMA6011, pMA6013, pMA6020, pMA6023 or pMA6025, respectively, expressed fusion proteins of apparent M_r of 150, 135, 94, 100, 55, and 116 K, respectively (Fig. 2).

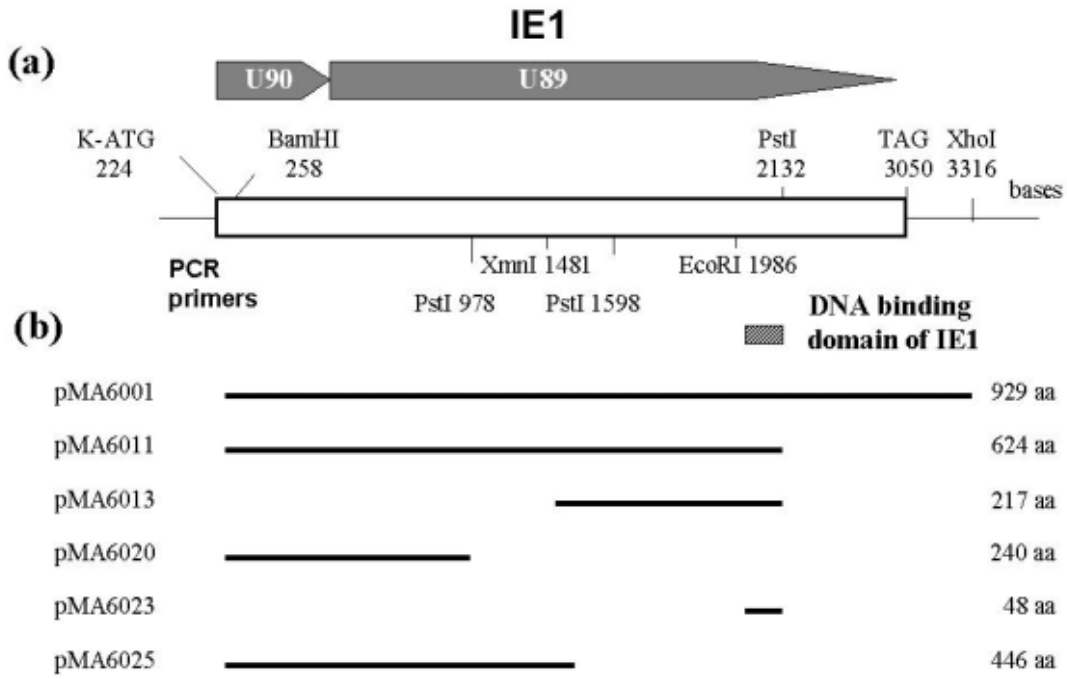

Fig. 1
Schematic map of IE1 cDNA sequences cloned into pMAL-c2

(a) Positions of the translation initiation (K-ATG) and translation termination (TAG); relative positions of restriction sites and PCR primer sites used in the construction of plasmids. (b) IE1 domains for production of MBP fusion proteins.

The identity of these proteins was confirmed by Western blot analysis with a rabbit antiserum specific for the MBP due to the fact that the pMA6013 protein product was produced in traces and could not be easily detected by SDS-PAGE (data not shown). The fusion proteins were purified by affinity chromatography on amylose columns, except for the 150 K protein expressed by pMA6001 and isolated from preparative gels (SDS-PAGE) by electroelution due to insolubility of the chimeric protein. The isolated 150 K protein was used to generate a monospecific polyclonal antiserum in rabbits. The rabbit antiserum was checked for specificity by Western blot analysis against cell lysates prepared from bacteria containing pMA6001, pMA6011, pMA6013, pMA6020, pMA6023, pMA6025 and pMAL-c2 (data not shown). As it was expected the antiserum reacted only with its autologous proteins.

Detection of authentic IE1 proteins in HHV-6 infected CBLs

The authentic IE1 proteins expressed in the HHV-6-infected CBLs were detected by Western blot analysis as products of M_r of 165 K, 190 K, and >190 K with the rabbit IE1 antiserum 6001 (Fig. 3A). Since the proteins detected by the antiserum were significantly larger than the predicted

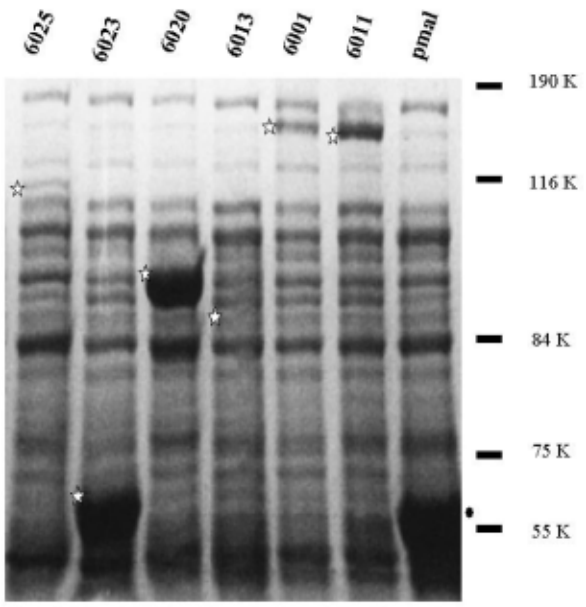

Fig. 2
Prokaryotic expression of HHV-6 IE1 protein

Western blot analysis of lysates prepared from IPTG-induced bacterial cells. The IE1 fusion proteins (stars) and MBP (solid circles). The positions and M_r values of size markers are indicated on the right margin. The fusion protein band of pMA6013 was not readily visible.

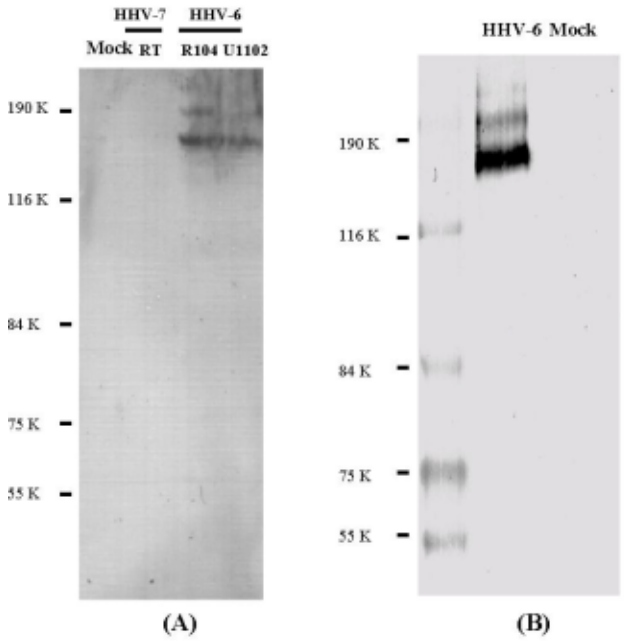

Fig. 3
Identification of the IE1 gene products in HHV-6A- and HHV-6B-infected CBLs

Mock-infected, HHV-6A-, HHV-6B- and HHV-7-infected CBLs were harvested on day 4 p.i. and subjected to Western blot analysis. The immobilized proteins were probed with the IE1 monospecific polyclonal rabbit antiserum 6001 at a dilution of 1:200 (A) and with the IE1 monospecific polyclonal rabbit antiserum CI-465 at a dilution of 1:100 (B). The positions and M_r values of size markers are indicated on the left margin.

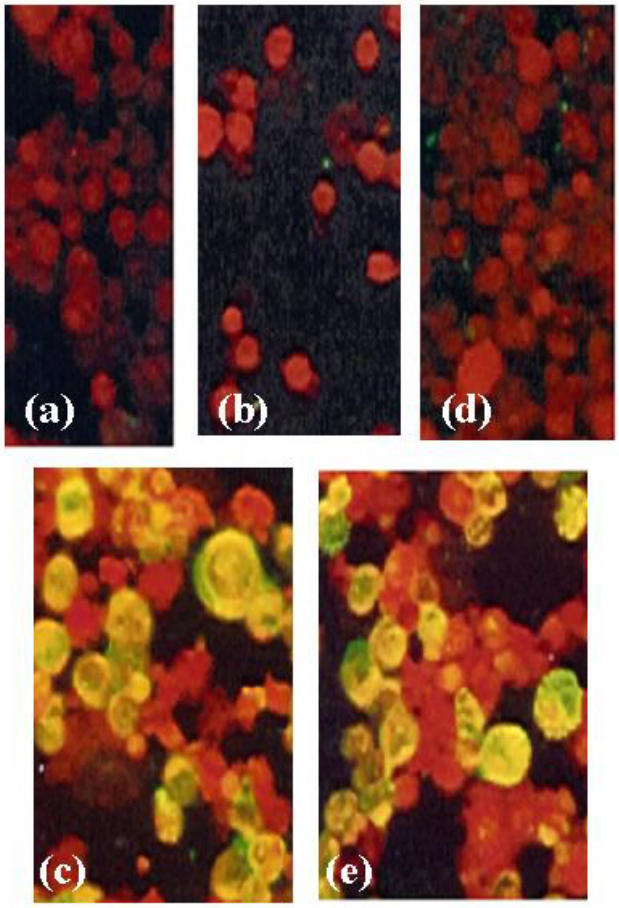

Fig. 4
Localization of the IE1 gene products in the HHV-6-infected CBLs as determined by IFA

Mock-infected (a) and HHV-6A U1102- (b and c), and HHV-6B R104-infected CBLs (d and e) were reacted with a pre-immune antiserum (a, b and d) and with the antiserum CI-465 (c and e) at 1:50 dilution.

one (104.5 K), we next used the antiserum CI-465 that was raised in rabbits against a synthetic polypeptide corresponding to aa 926–941 of U89. This antiserum reacted with exactly the same protein bands (Fig. 3B) as the antiserum 6001. As shown in Fig. 3A, the IE1 antisera reacted with the same proteins in HHV-6A U1102- and HHV-6B R104-infected CBLs. Cross-reactivity was not observed with HHV-7 RT-infected CBLs. Thus the antisera showed specificity for the IE1 locus of both HHV-6 variants.

Localization of IE1 proteins in HHV-6 infected CBLs

As shown in Fig. 4, IE1 in HHV-6-infected CBLs was localized at the nucleus as expected for an IE protein, and at the cytoplasm as well where protein synthesis takes place. There was no apparent difference in localization between HHV-6A and 6B (Figs. 4 and 5). HHV-6 (Z29) IE1 products were detected as early as 1 day post inoculation (p.i.) in the permissive Molt-3 cell line. In contrast, in the non-permissive cell lines Jurkatt (derived from T-cell leukemia) and HL-60 (derived from promyelotic leukemia), expression of IE1 was delayed by 1–3 days (Fig. 5 and Table 1). The IE1 products were detected first as a small granule-like staining in the nucleus and later as diffuse nuclear staining.

Eukaryotic expression of IE1 protein

Functional analysis of a defined eukaryotic polypeptide is greatly facilitated by its production in an appropriate system. The entire IE1 coding sequence was cloned into the eukaryotic expression vector pcDNAI/NEO and used in transient expression experiments with HeLa cells**.** As shown in Fig. 6, in two independent experiments transfected cells expressed two IE1 immunoreactive proteins, one of 165 K and a smaller one which may be a degradation product of IE1.

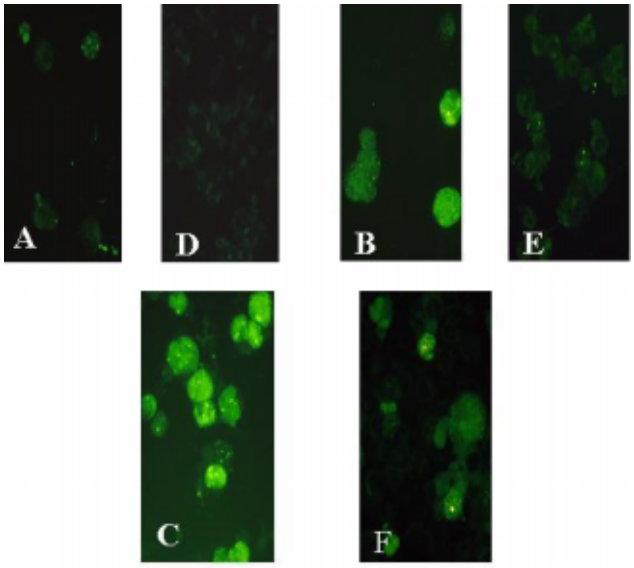

Fig. 5
Localization of the IE1 gene products in Molt-3 and Jurkat cell lines as determined by IFA

Molt-3 (A-C) and Jurkat (E and F) cells were infected with HHV-6B Z29 and the IE1 gene products were reacted with the antiserum CI465 at days 1 (A), 2 (B and E) and 4 (C and F). Uninfected Molt-3 cells (D).

Designation of the promoter regulatory region

In order to provisionally map the promoter of the IE-A region, the upstream region of exon 1 of the IE1 gene (Fig. 7c) was cloned upstream of the CAT gene in pIGA-53 resulting in plasmid pMA6033. High levels of CAT activity were detected in pMA6033-transfected COS-7 cells and the percentage of chloramphenicol acetylation was greater than 50%, thus revealing a strong promoter (Fig. 8).

DNA binding activity of prokaryotically expressed IE1

Cell lysates from bacterial cultures containing plasmids pMA6001, pMA6011, pMA6013, pMA6020, pMA6023, and pMA6025 (Fig. 1) were electrophoretically separated and blotted on nitrocellulose sheets. The blots were probed with a radiolabeled 140 bp DNA fragment spanning the region –131 to +9 of the IE1 promoter (Fig. 7). The experiment was performed in the presence of excess of unlabeled salmon sperm DNA. As shown in Fig. 9, only the fusion protein products encoded by plasmids pMA6001, pMA6011, pMA6013, and pMA6023 bound to the probe specifically, while those encoded by pMA6020 and pMA6025 did not. The amounts of the loaded protein were approximately equal as evidenced by the intensity of the 84 K cross-reacting band that was present in all samples. Similar results were observed when the fusion proteins were probed with radiolabeled

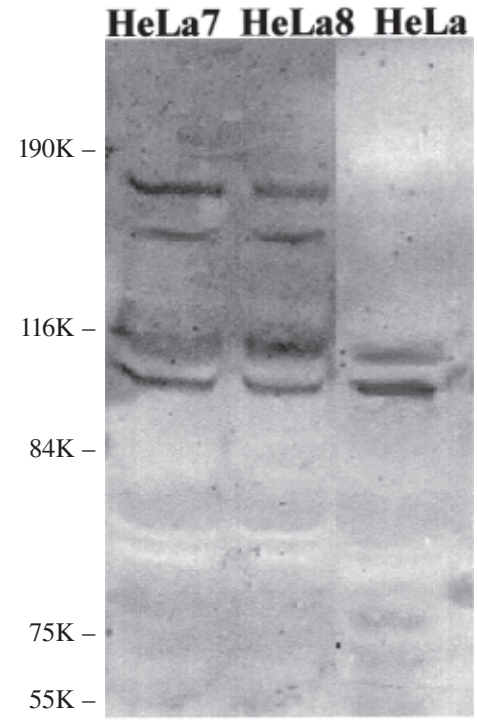

Fig. 6
Transient expression of IE1 proteins in HeLa cells

HeLa cells were transfected with plasmid pMA6012, harvested at 48 hrs p.i., lysed and the lysates were subjected to Western blot analysis. The immobilized proteins were reacted with the IE1 monospecific polyclonal rabbit antiserum 6001 at a dilution of 1:100. HeLa refers to the lysate of non-transfected cells (negative control); HeLa7 and HeLa8 refer to two independent transient transfections. The positions and M_r values of size markers are indicated on the left margin.

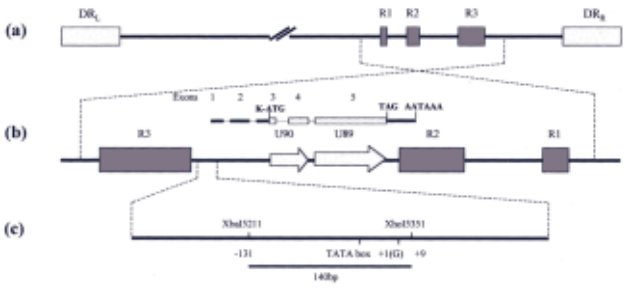

Fig. 7
Putative structure of the regulatory region

(a) Overall arrangement of complete HHV-6 genome showing relative positions of the repeats R1, R2 and R3 and the terminal direct repeats DR_L and DR_R.
(b) Expanded view of the IEA region showing the arrangement of the coding sequence of the IE1 locus.
(c) Expanded view of structure of the putative regulatory region.

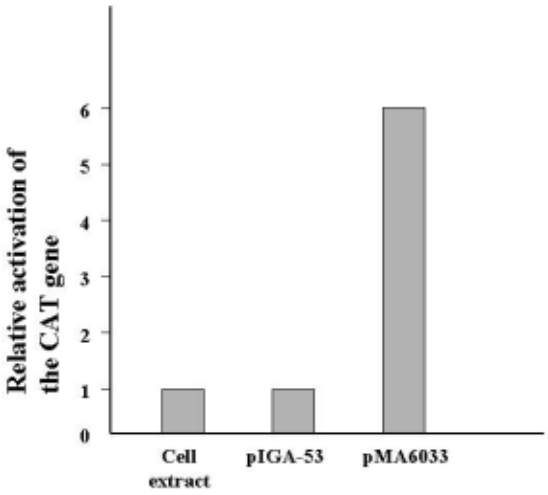

Fig. 8
Relative activity of the IE-A promoter in Cos7 cells

Cos7 cells were transfected with the plasmids indicated and 48 hrs later the cell extracts were prepared for CAT assay. The CAT activity was quantitated by densitometry of autoradiographs. The figure shows average values from two separate experiments. The CAT expression level from the promoter-less pIGA-53 vector was defined as one.

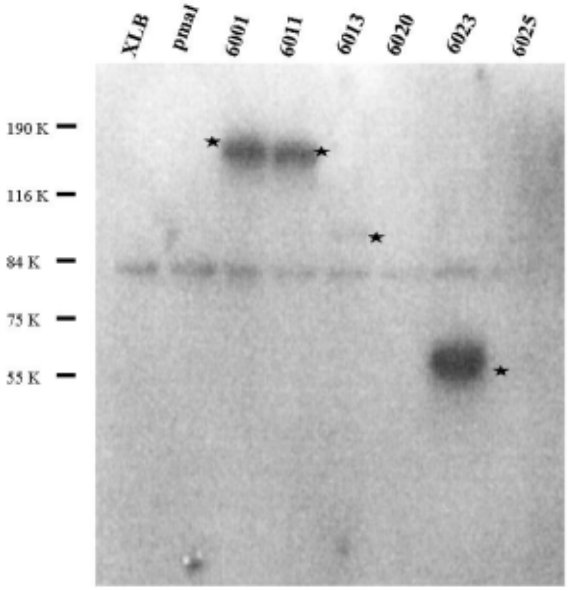

Fig. 9
Southwestern blot analysis of prokaryotically expressed IE1 fusion proteins and identification of the IE1 domains exhibiting DNA-binding activity

Cell lysates from *E. coli* cultures containing plasmid constructs pMA6001, pMA6011, pMA6013, pMA6020, pMA6023 or pMA6025 or the vector plasmid pMAL-c2 were subjected to Southwestern blot analysis. The immobilized proteins were probed with the a[^{32}P]-radiolabeled 140 bp DNA fragment comprising the region of the putative IE1 promoter. The positions of the IE1 proteins exhibiting DNA binding activity are indicated by stars. XLB = *E. coli* strain XL1-Blue control cells. The positions and M_r values of protein size markers are indicated on the left margin.

Table 1. Detection of IE1 (Z29) in various cell lines by IFA with CI 465 antiserum

Days p.i.	Cells infected with HHV-6B (Z29)			
	CBL	Molt-3	Jurkat	HL60
1	++	±	–	–
2	++	++	±	–
4	+++	+++	++	+

CBL, Molt-3, Jurkat and HL60 cells were infected with HHV-6B (Z29) and at the days p.i. indicated were stained with the CI 465 antiserum and subjected to IFA. The relative intensity of staining is indicated as negative (–), very weak (±), positive (+), strongly positive (++) and very strongly positive (+++).

DNA fragments of approximately the same size derived from irrelevant EBV sequences (data not shown).

Discussion

In this study, we extended the characterization of the HHV-6 IE1 gene of the IE-A locus and its products. Comparison of the HHV-6A and -6B nucleotide sequences (Dominguez *et al*., 1999; Isegawa *et al*., 1999) has revealed marked differences in the DR_L-U junction, the left end of U, the region spanning ORFs U86 to U100 and in the DR region. Whereas the highest amount of variability (63.2% nucleotide identity) was observed in the region spanning ORFs U86 to U90, the greatest identity between the variants (96.5%) was observed in the region encoding U94 (the adeno-associated virus type 2 replication homologue; Dominguez *et al*., 1999). In this region, four transcripts have been identified in the presence of cycloheximide (Schiewe *et al*., 1994) from one strand, the 3.5 and 4.7 kb transcripts representing IE1 (U89/90) and IE2 (U86/87), respectively, and from the other strand, the 1.0 and 1.5 kb transcripts representing the spliced products of U91 (Gompels *et al*., 1995; Schiewe *et al*., 1994). Identities in IE1 amino acid sequences between HHV-6A and HHV-6B were 68.4% for exon1, 67.2% for exon2, and 71.5% for exon3 whereas similarities were 73.7%, 70.3%, and 76.7%, respectively (Dominguez *et al*., 1999). Interestingly, while the overall intra-variant sequence variability between HHV-6B strains Z29 and HST was below 2%, in the region of U89/90 the variability was almost 8% (Sunwen *et al*., 1994; Dominguez *et al*., 1999). The variability observed in the IE-A locus is likely to be of importance in determining the biological properties of the virus.

In an earlier study by other investigators (Takeda *et al*., 1996), two immunoreactive IE1 polypeptides of 155 K and 170 K have been detected in HHV-6B (HST)-infected cells. Our finding of 165 K and 190 K products in HHV-6-infected CBLs is basically consistent with their results. Despite the high variability of IE1 between HHV-6A and HHV-6B, our

results show that there is a considerable antigenic conservation between the U90/89 regions of the two variants, since their protein products reacted with the same antiserum (6001) at a similar level. In addition to the 165 K and 190 K products, we also detected a small amount of a product of >190 K in both HHV-6A- and HHV-6B-infected CBLs. Importantly, we succeeded in expression of IE1 cDNA in HeLa cells and detection of the 165 K product with the same antiserum used for the HHV-6-infected CBLs. This finding confirmed that the 165 K product, the major one in the infected cells, was truly an IE1 product, although the apparent M_r was higher than the calculated one of 104.5 K for HHV-6A IE1. Post-translational modifications including phosphorylation, nucleotidylation and ubiquitination are common among herpesvirus regulatory proteins (e.g. Blaho *et al*., 1993; Purves and Roizman, 1992; Hofmann *et al*., 2000; Spengler, 2002).

The origin of the 190 K and >190 K products are currently unknown. It is possible that there is an so far unidentified spliced transcript encoding the proteins containing the amino acid sequence of the C-terminal portion of U89 used for generation of the CI-465 antiserum but not the U86/87 sequence (Papanikolaou *et al*., 2002). An alternative explanation is an additional post-translational modification of the 165 K IE1 in infected cells. We demonstrated that IE1 localizes more to the nucleus and less to the cytoplasm. Importantly, the IE1 products were detected first as a small granule-like staining in the nucleus and later as a diffuse nuclear staining. It is possible that an additional modification triggers shift of the IE1 localization. In HCMV-infected cells IE1 and IE2 early after infection localize to the nucleus but later IE1 is distributed in the plasma membrane and IE2 in the cytoplasm (Tsutsui and Yamazaki, 1991).

To further understand the transactivation function of IE1, we tested the DNA binding of the IE1 protein to its promoter sequence. A typical TATA box has been located 24 nt upstream of the transcription initiation site (+1) of the 3.5 kb IE1 transcript (Schiewe *et al*., 1994). We demonstrated that the 140 bp sequence covering nt –131 to +9 functioned as a strong promoter. We found by Southwestern blot analysis that the bacterially expressed IE1 proteins bound to this 140 bp sequence. In addition, the minimum domain for the DNA binding mapped to the region delineated by aa 588–636. However, the sequence specificity of the DNA binding activity of IE1 is presently unclear, because IE1 bound also to irrelevant EBV sequences. It is possible that the denaturation-renaturation process of IE1 proteins occurring in the Southwestern blot analysis created an artificial non-specific DNA binding domain, e.g. by exposure of a positively charged amino acid stretch. Actually, 18 amino acids of the 48 aa-long putative IE1 DNA-binding domain are basic amino acids. (Lys, Arg and His). Although HCMV IE2 binds directly to its own promoter and down-regulates the promoter activity, HCMV IE1 does not show a DNA-binding activity (Mocarski and Courcelle, 2001). Therefore, it will be essential to confirm the HHV-6 IE1 DNA-binding activity by additional experiments, such as electrophoresis mobility shift assays.

Finally, we demonstrated that HHV-6 IE1 expression levels in cell lines correlated with permissiveness for virus replication, suggesting that HHV-6 IE1 plays a critical role in virus replication, as does HCMV IE1. The reagents developed in this study will be useful for determining the mechanisms of regulation of HHV-6 IE1 expression at the transcriptional and translational levels, for identification of cellular factors that interact with HHV-6 IE1, and for development of diagnostics. It is noteworthy that the antibodies we generated are useful to distinguish the HHV-6 from HHV-7 infection, because they did not cross-react with HHV-7 IE1 homologue.

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References

Ablashi D, Agut H, Berneman Z, Campadelli-Fiume G, Carrigan D, Ceccerini-Nelli L, Chandran B, Chou S, Collandre H, Cone R, Dambaugh T, Dewhurst S, Diluca D, Foa-Tomasi L, Fleckenstein B, Frenkel N, Gallo R, Gompels U, Hall C, Jones M, Lawrence G, Martin M, Montangier L, Neipel F, Nicholas J, Pellet P, Razzaque A, Torrelli G, Thomson B, Salahuddin S, Wyatt L, Yamanishi K (1993): Human herpesvirus-6 strain groups-A nomenclature. *Arch.Virol*. **129**, 1–4.

Arsenakis M, Hubenthal-Voss J, Campadelli-Fiume G, Pereira E, Roizman B (1986): Construction and properties of a cell line constitutively expressing the Herpes simplex virus glycoprotein B dependent on functional a4 protein synthesis. *J. Virol.* **60**, 674–682.

Blaho JA, Mitchell C, Roizman B (1993): Guanylylation and adenylylation of the a regulatory proteins of the herpesvirus simplex virus require a viral b and g function. *J. Virol*. **67**, 3891–3900.

Cherrington JM, Mocarski ES (1989): Human cytomegalovirus ie1 transactivates the á-promoter-enhancer via a 18-base-pair repeat element. *J. Virol*. **63**, 1435–1440.

Dominguez G, Dambaugh TR, Stamey FR, Dewhurst S, Inoue N, Pellett F (1999): Human herpesvirus 6B genome sequence: coding content and comparison with Human herpesvirus 6A. *J. Virol.* **73**, 8040–8052.

Downing RG, Sewankambo N, Serwadda D, Honess RW, Crawford D, Jarrett R, Griffen BE (1987): Isolation of human lymphotropic herpesviruses from Uganda. *Lancet* **2**, 390.

Frenkel N, Schirmer EC, Wyatt LS, Katsafanas G, Roffman E, Danovich RM, June CH (1990): Isolation of a new herpesvirus from human CD4+ T cells. *Proc. Natl. Acad. Sci. USA* **87**, 748–752.

Gelman IH, Silverstein SJ (1987): Herpes simplex virus immediate-early promoters are responsive to virus and cell trans-acting factors. *J. Virol.* **61**, 2286–2296.

Gompels UA, Nicholas J, Lawrence G, Jones M, Thomson BJ, Martin MED, Efstathiou S, Craxton M, Macaulay HA (1995): The DNA sequence of human herpesvirus-6: Structure, coding content, and genome evolution. *Virology* **209**, 29–51.

Hall CB, Long CE, Schnabel KC, Caserta MT, Mclntyre KM, Costanzo MA, Knott A, Dewhurst S, Insel RA, Epstein LG (1994): Human herpesvirus-6 infection in children-A prospective study of complications and reactivation. *N. Engl. J. Med*. **331**, 432–438.

Hofmann H, Floss S, Stamminger T (2000): Covalent modification of the transactivator protein IE2-p86 of human cytomegalovirus by conjugation to the ubiquitin-homologous proteins SUMO-1 and hSMT3b. *J. Virol*. **74**, 2510–2524.

Honess RW, Gompels UA, Barrell BG, Craxton MA, Staden R, Chang Y-C, Hayward G (1989): Deviations from expected frequencies of CpG dinucleotides in herpesviruses DNAs may be diagnostic of differences in the state of their latent genomes. *J. Gen. Virol.* **70**, 837–855.

Honess RW (1984): Herpes simplex and the "herpes comlex": diverse observations and a unifying hypothesis. *J. Gen. Virol*. **65**, 2077–2107.

Inoue N, Dambaugh TR, Rapp JC, Pellet PE (1994): Alphaherpesviruses origin-binding protein homologue by human herpesvirus-6B, a betaherpesvirus, binds to nucleotide sequences that are similar to *ori* regions of alphaherpesviruses. *J. Virol*. **68**, 4126–4136.

Isegawa Y, Mukai T, Nakano K, Kagawa M, Chen J, Mori Y, Sunagawa T, Kawanishi K, Sashihara J, Hata A, Zou P, Kosuge H, Yamanishi K (1999): Comparison of the complete DNA sequences of human herpesvirus 6 variants A and B. *J. Virol*. **73**, 8053–8063.

Johnson G, Nelson S, Petric M, Tellier R (2000): Comprehensive PCR-based assay for detection and species identification of human herpesviruses. *J. Clin. Microbiol.* **38**, 3274–3279.

Karlin S, Doerfler W, Cardon LR (1994a): Why is CpG suppressed in the genomes of virtually all small eukaryoric viruses but not in those of large eukaryotic viruses? *J. Virol.* **68**, 2889–2897.

Karlin S, Mocarski ES, Schachtel GA (1994b): Molecular evolution of herpesviruses: genomic and protein sequence comparisons. *J. Virol.* **68**, 1886–1902.

Kondo K, Kondo T, Okuno T, Takahashi M, Yamanishi K (1991): Latent Human herpesvirus 6 infection of human monocytes/macrophages. *J. Gen. Virol.* **72**, 1401–1408.

Kosuge H, Isegawa Y, Yamanishi K (1997): Nucleotide sequence analysis of a 30-kilobase-pair region of human herpesvirus-6B (HHV-6) genome and strain specific variations in major immediate-early genes. *Virus Res.* **52**, 1–14.

Larcher C, Huemer HP, Margreiter R, Dierich MP (1988): Serological crossreaction of human herpesvirus-6 with cytomegalovirus. *Lancet* **ii**, 963–964.

Lawrence G L, Chee M, Craxton MA, Gompels UA, Honess RW, Barrell BG (1990): Human herpesvirus 6 is closely related to human cytomegalovirus. *J. Virol*. **64**, 287–299.

Lopez C, Pellett P, Stewart J, Goldsmith C, Sandelin K, Black J, Warfield D, Feorino P (1988): Characteristics of human herpesvirus 6. *J. Infect. Dis*. **157**, 1271–1273.

Lusso P, Markham PD, Tschachler E, Veronese FDM, Salahuddin SZ, Ablashi DV, Pahwa S, Krohn K, Gallo RC (1988) : In vitro cellular tropism of human B-lymphotropic virus (human herpesvirus-6). *J. Exp. Med.* **167**, 1659–1670.

Maina CV, Riggs PD, Grandea AG, Slatko BE, Moran LS, Tagliamont JA, McReynolds LA, Guan C (1988): A vector to express and purify foreign proteins in Escherichia coli by fusion to, and separation from, maltose binding protein. *Gene* **74**, 365–373.

Martin MED, Nicholas J, Thomson BJ, Newman C, Honess RW (1991): Identification of a transactivating function mapping to the putative immediate early locus of human herpesvirus 6. *J. Virol*. **65**, 5381–5390.

Mocarski ES, Courcelle CT (2001): Cytomegalovirus and their replication. In Knipe DM, Howley PM (Eds): *Fields Virology*. Fourth ed., Lippincott, Williams &Wilkins, Philadelphia, pp. 2629–2674.

Neipel F, Ellinger K, Fleckenstein B (1991): The unique region of the human herpesvirus 6 genome is essentially collinear with the U(L) segment of human cytomegalovirus. *J. Gen. Virol*. **72**, 2293–2297.

Papanikolaou E, Kouvatsis V, Dimitriadis G, Inoue N, Arsenakis M (2002): Identification and characterization of the gene products of open reading frame U86/87 of human herpesvirus 6. *Virus Res.* **89**, 89–101.

Purves FC, Roizman B (1992): The UL13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein α22. *Proc. Natl. Acad. Sci. USA* **89**, 7310–7314.

Rapp CJ, Krug LT, Inoue N, Dambaugh TR, Pellett PE (2000): U94, the human herpesvirus 6 homolog of the parvovirus nonstructual gene, is highly conserved among isolates and is expressed at low mRNA level as a spliced transcript. *Virology* **268**, 504–516.

Salahuddin SZ, Ablashi CV, Markham PD, Josephs SF, Sturzenegger S, Kaplan M, Halligan G, Biberfield P, Wong-Staal F, Kramarsky B, Gallo R (1986): Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* **234**, 596–601.

Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular Cloning. A Laboratory Manual.* Laboratory Press, Cold Spring Harbor.

Schiewe U, Neipel F, Schreiner D, Fleckenstein B (1994): Structure and transcription of an immediate-early region in the human herpesvirus 6 genome. *J. Virol*. **68**, 2978–2985.

Spengler ML, Kurapatwinski K, Black AR, Azizkhan-Clifford J (2002): SUMO-1 modification of human cytomegalovirus IE1/IE72. *J. Virol*. **76**, 2990–2996.

Sunwen C, Marousek GI (1994): Analysis of interstrain variation in a putative immediate-early region of human herpesvirus 6 DNA and definition of variant-specific sequences. *Virology* **198**, 370–376.

Takeda K, Nakagawa N, Yamamoto T, Inagi R, Kawanishi K, Isegawa Y, Yamanishi K (1996): Prokaryotic expression of an immediate-early gene of human herpesvirus 6 and analysis of its viral antigen expression in human cells. *Virus Res*. **41**, 193–200.

Tsutsui Y, Yamazaki Y (1991): Subcellular distribution of the major immediate early proteins of human cytomegalovirus changes during infection. *J. Gen. Virol*. **72**, 1415–1419.

Wathen MW, Tomsen DR, Stinski MF (1981): Temporal regulation of human cytomegalovirus transcription at immediate early times after infection. *J. Virol*. **38**, 446–459.

Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, Kurata T (1988): Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* **1**, 1065–1067.

Yasukawa M, Yakushijin Y, Furukawa M, Fujita S (1993): Specificity analysis of human CD4+ T-cell clones directed against human herpesvirus 6 (HHV-6), HHV-7, and human cytomegalovirus. *J. Virol*. **67**, 6259–6264.