JOURNAL OF VIROLOGY, May 1994, p. 2978–2985
0022-538X/94/$04.00+0

Vol. 68, No. 5

Structure and Transcription of an Immediate-Early Region in the Human Herpesvirus 6 Genome

ULRICH SCHIEWE, FRANK NEIPEL,* DORIS SCHREINER, AND BERNHARD FLECKENSTEIN

Institut für klinische und molekulare Virologie, D-91054 Erlangen, Germany

Received 10 December 1993/Accepted 9 February 1994

The unique segment of the human herpesvirus 6 (HHV-6) genome is essentially collinear to the unique long DNA segment of another betaherpesvirus, the human cytomegalovirus (HCMV). However, the HHV-6 genomic section that is analogous in position to the major immediate-early (IE) locus of HCMV does not exhibit recognizable sequence homologies. The respective HHV-6 region of 5.5 kbp is flanked on one side by 25 to 28 incomplete tandem repeats of 105 to 110 bp that contain, with one exception, a single *Kpn*I restriction site (*Kpn*I repeats). About 250 reiterations of the sequence motif CACATA are located on the other end. We identified two open reading frames of 375 and 2,595 nucleotides, respectively, on one strand. Strand-specific Northern blot analyses with RNA harvested from HHV-6 (strain U1102)-infected HSB-2 cells or cord blood lymphocytes revealed two transcripts of about 3.5 and 4.7 kb in the corresponding orientation. Sequence analyses of the respective cDNA clones and primer extension experiments were used to map the mRNAs. The two transcripts are coterminal and multiply spliced and code for the same putative 104.6-kDa protein, but they are initiated from different promoters. Characterization of smaller cDNA clones and Northern blotting with other strand-specific probes showed that singly spliced mRNAs of 1.0 and 1.5 kb are transcribed from the opposite strand; they could code for a 17.2-kDa polypeptide. Blocking experiments with cycloheximide led to the conclusion that only the 3.5-kb mRNA is synthesized in the absence of protein biosynthesis upon infection with cell-free virus. This identifies a single IE gene of HHV-6 at the genomic position corresponding to the major IE region of HCMV, although the coding content and transcriptional regulation are quite different for these two herpesvirus IE regions.

Human herpesvirus 6 (HHV-6) was first isolated from the peripheral blood of patients with lymphoproliferative disorders (strain GS) (44). Subsequently, numerous strains were obtained from patients with AIDS, including the prototype strains U1102 from Uganda (15) and Z29 from Zaire (33). The known HHV-6 isolates form at least two groups which can be distinguished by molecular and biological criteria. Preliminary classification designates GS- and U1102-like isolates as HHV-6 variant A, while Z29-like viruses are termed HHV-6 variant B (1). Primary infection with HHV-6 variant B is clearly associated with the mild childhood disease exanthema subitum (22, 56, 58). The pathological role of HHV-6 variant A remains to be elucidated. Viral infection may be accompanied by atypical lymphoproliferative reactions mimicking malignant lymphomas (9, 10, 18, 27). The pathological significance of HHV-6 primary infection or reactivation in immunosuppressed patients, such as patients with AIDS, leukemia, or transplants, is under investigation (5, 12, 16, 19, 31). Although HHV-6 and human cytomegalovirus (HCMV) belong to the same subgroup (betaherpesviruses) based on serological cross-reactivity (28) and amino acids sequence homologies (17), their overall genome structures are different (32). Unlike HCMV, the HHV-6 genome is a 142-kbp unique segment flanked by two tandem repeats of 10.5 kbp. However, the position and orientation of all conserved genes on the HHV-6 unique segment are collinear with the unique long component of HCMV (30, 37). In HCMV, a major immediate-early (IE) region is located close to the right end of the unique long segment. Multiply spliced transcripts originate from a strong enhancer/promoter (11, 53). The corresponding proteins have been shown to be regulators of viral gene expression (47). The HHV-6 open reading frames in the equivalent region close to the right genomic end are not homologous to HCMV DNA or any other herpesvirus gene identified so far. Beyond the relative position, however, the region shares some features with the major IE region of HCMV. It exhibits a marked CpG dinucleotide suppression, which is generally taken as evidence for herpesvirus IE genes (23). It was reported that an open reading frame encodes a protein that transactivates heterologous promoters (36) upon transient expression. This putative IE locus of about 5.5 kbp is flanked by approximately 3 kbp of incomplete tandem repeats of 105 to 110 bp that contain, with one exception, a single *Kpn*I restriction site (*Kpn*I repeats) on one side and about 1.5 kb of the reiterated motif CACATA on the other. In this study we analyzed the transcription pattern of this putative IE region in the HHV-6 variant A strain U1102. We identified four mRNAs that originate from three promoters; only one of those reveals the typical characteristics of an IE transcription unit.

MATERIALS AND METHODS

Cells and viral infection. Cord blood lymphocytes (CBL) were isolated by centrifugation on Ficoll-Paque gradients (Pharmacia, Uppsala, Sweden). HHV-6 isolate U1102 (15) was propagated on CBL or on the human T-cell line HSB-2 (ATCC CCL 120.1 CCRF-HSB-2) as described previously (2, 37). Suspension cultures of HSB-2 cells and CBL were grown at 37°C and 7.5% CO_2 in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 100 mg of gentamicin per ml, 350 mg of L-glutamine per ml, and 10 μg of phytohemagglutinin per ml. CBL were stimulated with human recombinant interleukin-2 (20 U/ml; Boehringer, Mannheim,

* Corresponding author. Mailing address: Institut für klinische und molekulare Virologie, Schlossgarten 4, D-91054 Erlangen, Germany. Phone: 49-9131-852763. Fax: 49-9131-852101. Electronic mail address: neipel@imbg.biologie.uni-erlangen.de.

Germany) prior to infection. The virus was propagated by cocultivation of infected and uninfected cells at a ratio of 1:10. Cells and supernatant were harvested at 4 to 5 days after infection with HHV-6 (U1102). To achieve sufficient infectious cell-free virus, cell culture supernatants were concentrated at least 500-fold and were used to infect 1×10^7 to 2×10^7 CBL at a multiplicity of infection of 0.1 to 0.2. Cell-free virus was harvested from CBL 4 to 5 days after infection with HHV-6 (U1102). Cells were disrupted by five freeze-thaw steps (from liquid nitrogen to 37°C), and the debris was pelleted two times at 4°C for 10 min at 3,000 × *g*. Five percent of the virus harvested from a definite number of infected cells was used to infect an equal number of cells. For IE transcription studies, CBL were maintained for 30 min in the culture medium supplemented with 100 μg of cycloheximide (CHX; Sigma) per ml prior to infection. For all other transcription studies, RNA from HSB-2 cells infected for 4 to 5 days was used. The efficiency of infection was enhanced by centrifugation as described previously (40); cells and cell-free lysates from infected cells were spun for 30 min at 1,600 × *g* and 37°C prior to seeding the cultures. At 2 days postinfection, infected cells were not detectable in the cultures. However, after 5 days, 40 to 50% of the cells were HHV-6 positive in an immunofluorescence assay with rabbit sera against the matrix protein p100 (38). Therefore, it is estimated that no more than 1% of the cells are infected in the first round of virus replication.

Isolation of RNA. Total RNA was isolated from HHV-6 (U1102)- or mock-infected cells (HSB-2 or CBL) by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (13). In addition to this protocol, the RNA was precipitated with 5 volumes of 96% ethanol and 0.1 volume of 4 M LiCl. RNA was fractionated by oligo(dT) cellulose (Boehringer) chromatography as described previously (45).

Radioactive labeling of DNA probes. Oligonucleotides (Eurogentec, Seraing, Belgium) were 5′ labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP (3 kCi/mmol; Amersham Buchler, Braunschweig, Germany) according to standard methods (6). Uniform ^{32}P labeling with [α-^{32}P]dATP (3 kCi/mmol; Amersham Buchler) was achieved either by a modification of the PCR (run-off synthesis) as described by Stürzl and Roth (51) or with the random priming kit supplied by GIBCO/BRL.

Northern and Southern blot hybridizations. Sample loading and gel running buffers were prepared by standard methods. RNA and DNA molecules were resolved on 1% agarose gels containing 1× MOPS (0.2 M 3-[*N*-morpholino]propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) and 1.1% formaldehyde and 2.0% agarose gels, respectively. Transfer onto nylon membranes (Hybond-N; Amersham Buchler) was carried out by using a vacuum blotting chamber (Pharmacia/LKB), according to the manufacturer's protocol, with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (45) (Northern [RNA] blot) or with 1.5 M NaCl–0.25 M NaOH (Southern blot). Membranes were subsequently incubated at 80°C for 3 h and at 42°C (Northern blot) or 65°C (Southern blot) for another 3 h in prehybridization buffers. For Northern blot, the prehybridization buffer was a 25 mM sodium phosphate buffer with 5× SSC and 5× Denhardt's solution (45), 0.1 mg of yeast extract tRNA per ml, and 50% formamide. For Southern transfer, a 2× SSC buffer with 5× Denhardt's solution and 0.1 mg yeast extract tRNA per ml was used. For hybridization to oligonucleotide probes, a 20 mM sodium phosphate buffer with 4× SSC, 5× Denhardt's solution, 0.2 mg of yeast extract tRNA per ml, and 0.1% sodium dodecyl sulfate (SDS) was used. Hybridization to the labeled probes or to synthetic oligonucleotide probes was usually carried out overnight at 42°C or room temperature, respectively. Northern blot hybridization buffer was identical to the respective prehybridization solution. Southern blots were hybridized in 5× SSC–5× Denhardt's solution–0.1 mg of yeast extract tRNA per ml–50% formamide–0.1% SDS. For hybridization to oligonucleotide probes, a 40 mM sodium phosphate buffer with 4× SSC, 1× Denhardt's solution, 0.5 mg of yeast extract tRNA per ml, and 0.1% SDS was used. The blots were washed at 65°C (or at room temperature for oligonucleotide probes) in solutions of decreasing ionic strength (4× SSC–0.5% SDS, 2× SSC–0.5% SDS, and 1× SSC–0.5% SDS) for 20 min each prior to autoradiography.

Primer extension analysis. The 5′-labeled oligonucleotides P1 (5′-AGACTGCAACGATATAAGAAA-3′) and P2 (5′-CTTTGGCCCGATATAATTTCCAGTATTAGC-3′) were used for primer extension experiments. Approximately 10^6 counts were mixed with 50 μg of total RNA harvested from 4-day- or mock-infected HSB-2 cells, precipitated with 0.3 M sodium acetate and 5 volumes of 96% ethanol for 30 min at −20°C, and pelleted by spinning (15,000 × *g*) for 30 min in a microcentrifuge at 4°C. The pellets were washed (70% ethanol), briefly dried, and resuspended in 10 μl of 1× reverse transcriptase buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. After 3 min at 90°C and 10 min at 55°C, the samples were slowly cooled to room temperature. A mixture (10 μl) consisting of 0.02 M dithiothreitol, 1 mM deoxynucleoside triphosphate, 35 U of RNA Guard (Pharmacia), 1× reverse transcriptase buffer, and 200 U of Superscript mouse mammary tumor virus reverse transcriptase (GIBCO/BRL) was added. The primer extension reaction was carried out at 40°C for 40 min. RNase digestion and analysis of extension products were done by standard methods (45).

Construction of a cDNA library. RNA from HSB-2 cells infected with HHV-6 (U1102) for 4 days was used as the starting material to construct a cDNA library in the bacteriophage λ-ZAPII. By using a cDNA synthesis kit (Stratagene, La Jolla, Calif.), a library containing approximately 5×10^5 independent clones was constructed. HHV-6 cDNA clones were identified by hybridization to a radioactively labeled probe spanning 4.2 kbp of the putative IE region of HHV-6.

PCR analysis of RNA. RNA [poly(A$^+$) fraction] was transcribed into single-strand cDNA by using Superscript mouse mammary tumor virus reverse transcriptase (GIBCO/BRL) according to the supplier's protocol. Synthesis was initiated by the respective 3′ primer (see below). cDNA single strands were phenol extracted twice and precipitated, and 10% of each was subjected to PCR. The DNA was amplified in a total volume of 100 μl of 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus)–100 μM deoxynucleoside triphosphate–3.75 mM $MgCl_2$–10 mM Tris-HCl (pH 8.3)–50 mM KCl–0.001% gelatin–50 pmol of each primer. The PCR primers were designed according to the known cDNA sequences always flanking an intron, in order to distinguish between amplificates of the cDNAs and contaminating genomic DNA. The first pair of primers was the 5′ primer (P1-5′) GCAAACGAGAACTCTAAAATCT and the 3′ primer (P1-3′) CTGAACTGGCTGTAACTTCTGC. The second pair of primers was the 5′ primer (P2-5′) AGA GAGTCTCATGTGTGATACATC and the 3′ primer (P2-3′) TGGAGATGTCAGGATTGGACATC. Amplification was performed for 30 cycles after one initial denaturation step at 94°C for 2 min. The cycle for primer pair 1 was denaturation for 30 s at 94°C, annealing for 20 s at 60°C, and polymerization for 20 s at 72°C. The cycle for primer pair 2 was denaturation for 30 s at 94°C, annealing for 25 s at 55°C, and polymerization

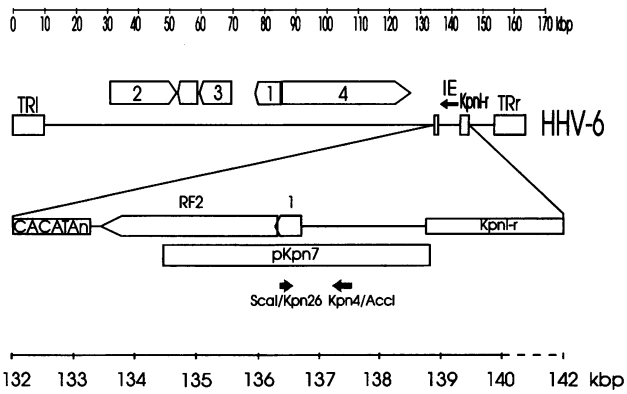

FIG. 1. Localization and structural features of a putative IE locus of HHV-6. Conserved blocks of genes of HHV-6, symbolized by large open arrows above the genome, are collinear in genomic position and orientation to the homologous gene blocks of HCMV. The region in the HHV-6 genome corresponding to the major IE locus of HCMV is designated IE and indicated by a small closed arrow. Repetitive units are shown as boxes. Abbreviations: TR, terminal repeat; KpnI-r, *Kpn*I repeat unit; $CACATA_n$, repetitive sequence motif. The lower part of the figure shows the putative IE region of HHV-6 in more detail. pKpn7, 4.2-kbp *Kpn*I plasmid. Large arrows symbolize the open reading frames RF1 and RF2; single-stranded DNA probes (*Sca*I/*Kpn*26 and *Kpn*4/*Acc*I) are indicated by stippled arrows.

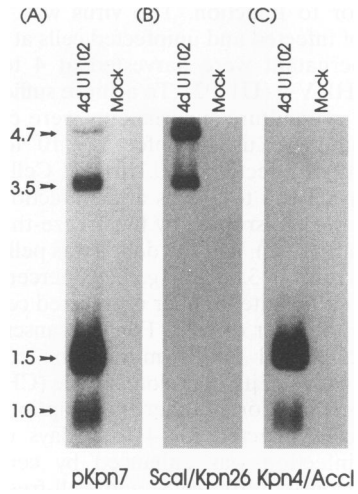

FIG. 2. Transcripts of putative IE region of HHV-6 (U1102). Poly(A^+) RNA (5 μg) harvested from noninfected CBL (lane labeled Mock) or CBL infected for 4 days with HHV-6 (U1102) (lane labeled 4dU1102) were subjected to Northern blot analyses. The sizes of mRNAs (in kilobases) are indicated on the left. (A) By hybridization to the insert of pKpn7, labeled by random priming, four transcripts (4.7, 3.5, 1.5, and 1.0 kb in size) could be detected. (B) The RF1-specific single-stranded DNA probe *Sca*I/*Kpn*26 visualized the 4.7- and 3.5-kb transcripts. (C) *Kpn*4/*Acc*I, a single-stranded DNA probe synthesized in the opposite direction, hybridized to the small transcripts (1.0 and 1.5 kb).

for 25 s at 72°C. Aliquots (20 μl) were analyzed by Southern blotting and hybridized to an appropriate probe.

Sequence analysis. Sequence data were obtained by using the automatic sequencer A373 (ABI, Foster City, Calif.) or a commercial sequencing kit (Pharmacia) based on the dideoxynucleotide termination method described previously (4). The sequence analyses were done by using the Genetics Computer Group (Madison, Wis.) sequence analysis package implemented on a Micro VAX 3500 (Digital Equipment Corporation).

RESULTS

Transcripts of a putative HHV-6 IE locus. The position of a region considered to be a major IE locus of HHV-6 is shown in Fig. 1. The location of this putative IE region was based on the markedly collinear genome organization of HHV-6 and HCMV (30, 37), on a typical CpG depletion (36), and on a transactivating function that has been identified in this region (36). Martin and colleagues who first sequenced the respective genomic section of HHV-6 strain U1102 (HEHS6IELO, GenBank accession no. M73681), described four open reading frames termed RF1 to RF4 (36). We found a C is missing in their sequence at position 1832; by correcting this, their RF1 is interrupted. Therefore, the reading frame termed RF2 in their study is now designated RF1 in this study. In addition, a T is missing in their sequence at position 2851; insertion of this nucleotide results in the fusion of RF3 and RF4 into a single open reading frame, which we designate RF2 (Fig. 1). Downstream (220 nucleotides [nt]) of RF2, we found a characteristic cluster of about 250 tandem reiterations of the sequence motif CACATA. In this way, the presumed IE region of 5.5 kbp is framed by two different stretches of short repetitive motifs (*Kpn*I repeats and CACATA repeats). Northern blot analyses were performed to determine the pattern of transcripts derived from that putative IE region. Initially, the insert of clone pKpn7 was used as a probe (Fig. 1). In the poly(A^+) fraction of RNA harvested from HSB-2 cells infected with HHV-6 (U1102) for 4 days transcripts of four size classes could be detected (Fig. 2A). The most abundant transcript is about 1.5 kb; less abundant ones are 1.0 and 3.5 kb in size. A 4.7-kb transcript was barely detectable. To determine the orientation, single-stranded DNA probes labeled by run-off synthesis were used for further Northern blot experiments. The locations of these probes are indicated in Fig. 1 and Fig. 4. These experiments demonstrated that two mRNA molecules with sizes of 3.5 and 4.7 kb could be traced to RF1 (Fig. 2B) and very likely extended into RF2. The short 1.0- and 1.5-kb mRNAs are transcribed from the opposite strand (Fig. 2C).

Synthesis of a 3.5-kb mRNA under IE conditions. Transcription of virus IE genes does not require de novo protein biosynthesis after infection. Freshly HHV-6-infected CBL were maintained for 6 h in the culture medium supplemented with 100 μg of CHX. Afterwards, total RNA was harvested, poly(A^+) fractionated, and subjected to a Northern blot analysis. Hybridization to a single-stranded DNA probe that recognized the 3.5- and 4.7-kb transcripts in poly(A^+) RNA originating from cells infected for 4 days now resulted in the detection of only one 3.5-kb transcript (Fig. 3A, lane labeled 6hU1102CHX). No transcript of 1.0 or 1.5 kb from the opposite strand could be detected with the appropriate probe (Fig. 3B). This indicated that only the 3.5-kb mRNA, unlike the other three transcripts of the region, is detectable under IE conditions.

Transcript mapping by cDNA sequencing. To map the mRNAs more precisely and to analyze splicing patterns in the IE region, a cDNA library was constructed from HHV-6-infected HSB-2 cells. After multiple screening steps, cDNA clones with sizes of approximately 0.8 to 1.2, 3.3, and about 4 kbp that hybridized in Southern blot analyses to the radioactively labeled pKpn7 insert were selected (data not shown). Sequence analyses of four short cDNA clones (0.8 to 1.2 kbp),

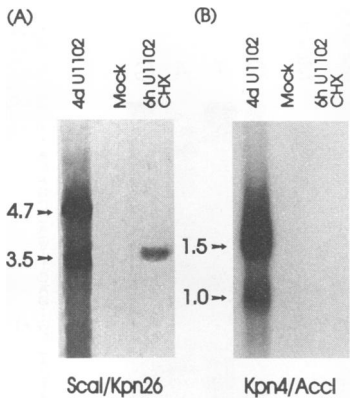

FIG. 3. Mapping of mRNA transcribed without de novo protein synthesis to the putative IE region of HHV-6. One microgram of poly(A^+) RNA from cells infected with HHV-6 (U1102) for 4 days (lane labeled 4dU1102) and 20 μg each of poly(A^+) RNA from mock-infected cells and cells infected with HHV-6 (U1102) for 6 h in the presence of CHX (100 μg/μl) (lane labeled 6hU1102CHX) were separated on the appropriate gel. (A) With CHX, the single-stranded DNA probe *Sca*I/*Kpn*26 detected one transcript (about 3.5 kb) but not the 4.7-kb mRNA. The retardation of the 3.5-kb transcript is due to the use of large amount of RNA from cells infected with HHV-6 (U1102) for 6 h in the presence of CHX. (B) Under IE conditions, probe *Kpn*4/*Acc*I did not hybridize to any transcript. However, with poly(A^+) RNA from cells infected with U1102 for 4 days two strong signals, the 1.0- and 1.5-kb mRNAs, could be observed.

four 3.3-kbp cDNA clones, and five 3.8- to 4.3-kbp cDNA clones indicated four different transcripts (Fig. 4). The 3,295-kb cDNA consists of five exons. The first two exons appear to be untranslated. The ATG sequence at nt 1841, which is located within the third exon, initiates an open reading frame that spans exon 4 and almost the entire exon 5. The stop codon TAG at nt 4859 and the well conserved polyadenylation site AATAAA at nt 5090 were found in all cDNA clones in the 3.3-kbp size class. This cDNA, which most likely corresponds to the single 3.5-kb IE transcript, encodes a putative protein of 941 amino acids with a calculated molecular mass of 104.6 kDa. Homology searches with the algorithm FASTA through all of the GenBank and EMBL data bases revealed no significant amino acids sequence similarities to any known gene product. In particular, the known IE proteins of other betaherpesviruses were not homologous to this putative HHV-6 IE polypeptide. The approximately 3.8- to 4.3-kbp cDNAs code for the same gene product and are coterminal to the 3.3-kbp cDNA but extend by more than 0.5 kb to the 5′ end. They always started upstream of the *Kpn*I repeats and contained four to eight repetitive units, as estimated by gel electrophoresis of an appropriate restriction fragment. In Northern blot analyses however, only one transcript of the respective size class (4.7-kb mRNA) was detectable, indicating that it contains a definite number of *Kpn*I repeats. Thus, very

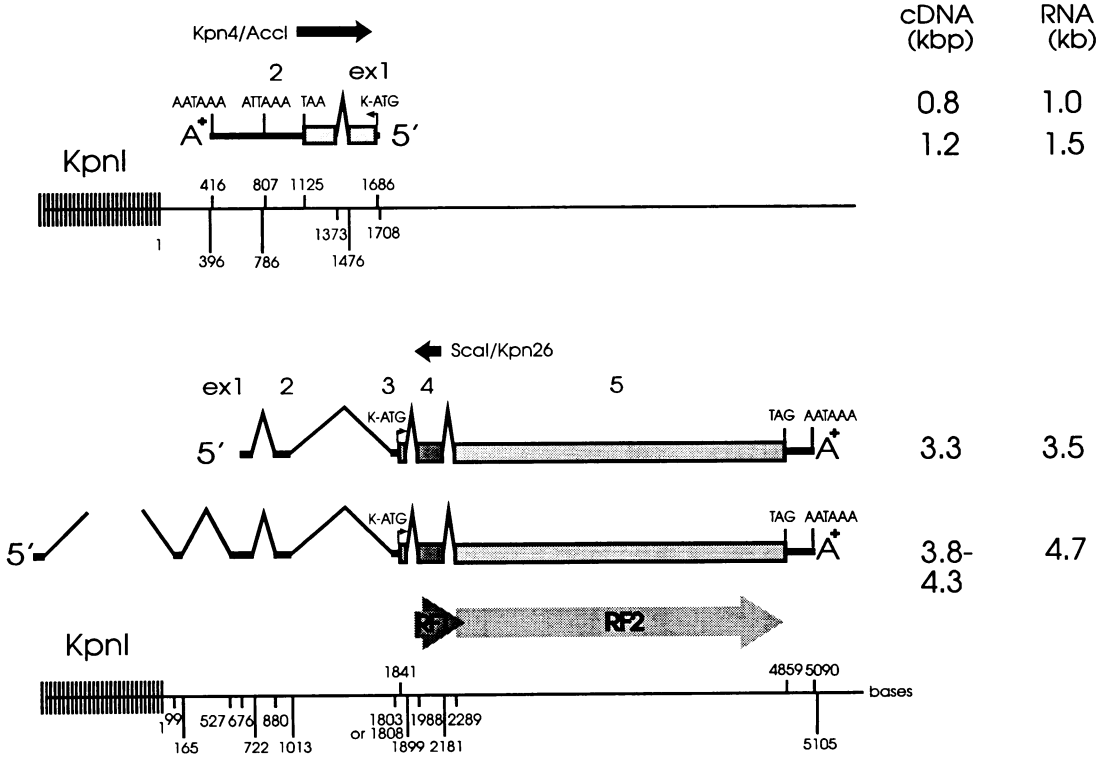

FIG. 4. Organization and transcription pattern of IE region. All exons (ex1 to 5) and introns are drawn to scale. Numbering starts with the first nucleotide of the last *Kpn*I site. The drawing of the cDNAs is inverted relative to the HHV-6 genome. Numbers for splice sites apply to the last nucleotide of exon *n* and the first nucleotide of exon (*n* + 1), and for special sequence motifs, the numbers apply to the first 5′ nucleotide. Sequence motifs for the short 0.8- and 1.2-kbp cDNAs are inverted (to be read from left to right). Note that these two cDNA molecules are represented as one molecule with two polyadenylation sites. Thin lines represent introns; thick lines represent noncoding exons or parts of exons. Stippled boxes represent open reading frames with ATG initiation codons as described by Kozak (26) (K-ATG) and either TAG or TAA termination codons. AATAAA or ATTAAA indicates the location of polyadenylation signals. Open reading frames RF1 and RF2 identified in the genomic sequence are symbolized by stippled arrows. Single-stranded DNA probes (*Kpn*4/*Acc*I and *Sca*I/*Kpn*26) used for Northern blot analyses are indicated by closed arrows. The sizes of cDNA molecules and corresponding mRNAs, identified by Northern blot analyses, are noted on the right.

likely, the cDNA clones must have lost some repeats during the cloning process.

The cDNAs (0.8 and 1.2 kbp) from the shorter transcripts (1.0 and 1.5 kb) of the opposite strand consisted of two exons, forming a single open reading frame of 459 nt (Fig. 4). It codes for a putative 17.2-kDa protein exhibiting no amino acid homology to other known gene products. The two transcripts are collinear and have different termination sites. The 3′ untranslated region of the 1.5-kb transcript comprises 730 nt, while the 1.0-kb mRNA has 340 nt. The longer cDNA revealed the polyadenylation consensus signal AATAAA; but the short cDNA from the less abundant 1.0-kb mRNA contains the less common motif ATTAAA (8).

Identification of two transcriptional start sites located within the IE region. Comparison of cDNA and genomic sequence data have suggested the existence of at least three promoters for the transcription of this region. One must be located upstream of the *Kpn*I repeats (origin of the 4.7-kb transcript), and another seems to be situated downstream of the *Kpn*I repeats (origin of the 3.5-kb transcript) (Fig. 4). The short 1.0- and 1.5-kb mRNAs transcribed from the opposite strand originate very likely from a single third promoter. To confirm this, the transcriptional start sites of the 1.0-, 1.5-, and 3.5-kb mRNAs were determined by primer extension analyses. Total RNA (50 μg) from lytically infected cells was hybridized to the 5′-labeled oligonucleotide primer P1 that was expected to bind to the 3.5- and 4.7-kb mRNAs. A prominent band was observed at a G residue, designated +1 in Fig. 5A. This corresponds to nt 650 (relative to the junction between the *Kpn*I repeats and the IE region as indicated in Fig. 4). A typical TATA box sequence is located 24 nt upstream of this transcription initiation site (with reference to the 3.5-kb mRNA). As indicated above, the promoter of the 4.7-kb mRNA must be located upstream but immediately adjacent to repetitive DNA. Because of this repetitive structure, primer extension experiments failed to reveal a transcriptional start site for the 4.7-kb mRNA. Mapping of the 5′ end of the short (1.0- and 1.5-kb) mRNAs was done with the oligonucleotide P2. This located the main transcription initiation site to a G residue (Fig. 5B), which is nt 1726 of this genomic region (Fig. 4). A minor start site corresponds to an A residue (Fig. 5B) (nt 1722). Canonical TATA and CAAT boxes are situated 26 and 65 nt upstream of the major start site, respectively. These data confirmed that the two 1.0- and 1.5-kb mRNAs originate from a single promoter.

Differential regulation of 3.5- and 4.7-kb transcripts of the IE region. The Northern blot experiments described above indicated that only the 3.5-kb transcript was synthesized when de novo protein synthesis was blocked by CHX, while the 4.7-kb transcript appeared at later phases of the infectious cycle. Apparently, the two transcripts, coding for the same protein, originate from two promoters with differential regulation. To confirm that, a series of RNA PCR experiments were done. One microgram each of poly(A^+)-fractionated RNAs of 4-day-infected HSB-2 cells and 6-h infected CBL treated with CHX were used for the RNA analyses by PCR. The locations of the PCR primers and the 5′-labeled oligonucleotides used for Southern blot analyses are indicated in Fig. 6A. The autoradiography shown in Fig. 6B exhibited no specific amplification product of 189 bp derived from the reverse-transcribed RNA of CHX-treated HHV-6-infected CBL by using primer pair 1 in the PCR (lane labeled 6hU1102CHX). Primer pair 1 was able to detect only the cDNA derived from the 4.7-kb transcript (Fig. 6A). With RNA harvested from 4-day-infected HSB-2 cells (lane labeled 4dU1102) as the starting material, the amplificates from spliced RNA (189 bp) as well as from unspliced RNA or more

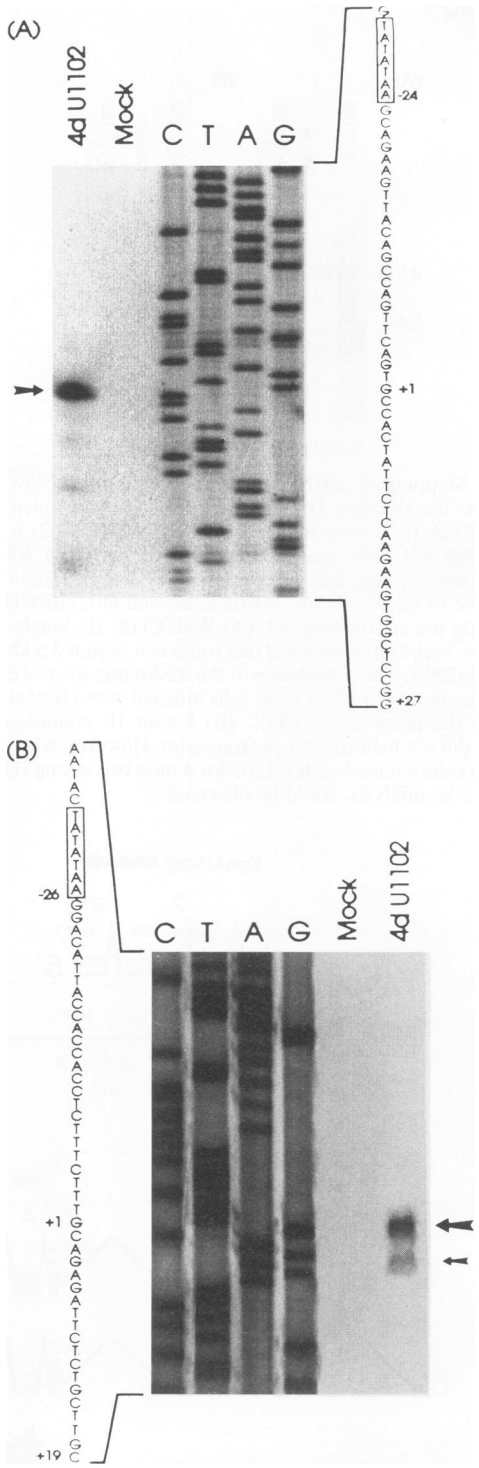

FIG. 5. Start sites of 3.5-kb IE transcript and short 1.0- and 1.5-kb transcripts, identified by primer extension assays. The size of the respective extension product was estimated relative to the homologous sequencing ladder initiated with the same primer. The position of the extension product is indicated by an arrow in reference to the nucleotide designated +1 of the sequencing ladder. (A) The start site of the 3.5-kb IE transcript is positioned at a G residue. A TATA box at position −24 is boxed. Position +27 marks the first 5′ nucleotide of the respective cDNA. (B) The 1.0- and 1.5-kb mRNAs that are transcribed from the opposite strand also start at a G residue. A minor start site at an A residue (+5) is indicated by a small arrow. A TATA box at position −26 is boxed. The C residue at position +19 is the first 5′ nucleotide of the respective cDNAs. Lanes: 4dU1102, cells infected with HHV-6 (U1102) for 4 days; mock, mock-infected cells.

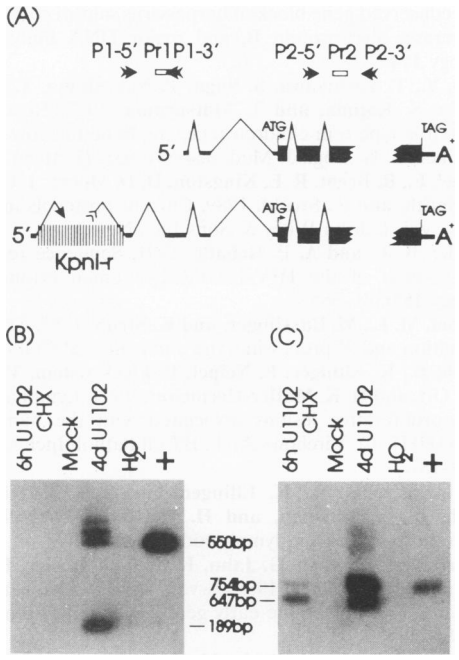

FIG. 6. Detection of 3.5-kb IE transcript by RNA PCR. (A) The positions of both primer pairs (P1-5′ and P1-3′; P2-5′ and P2-3′) are indicated by arrowheads above the drawings of the sequenced cDNA molecules. Oligonucleotide probes Pr1 and Pr2 used for Southern blot analyses are shown as boxes. (B and C) Amplification products are indicated by their sizes. Upper bands (550 and 754 bp, respectively) refer to genomic DNA or unspliced RNA; lower bands (189 and 647 bp) refer to spliced transcripts. For reverse transcription and PCR, RNA from noninfected CBL (lanes labeled Mock) served as a negative control, and RNA from HSB-2 cells infected for 4 days with HHV-6 (U1102) (lanes labeled 4dU1102) served as a positive control. Water instead of cDNA first strands served as a PCR negative control (lanes labeled H_2O), and the plasmid pKpn7 (0.75 fmol) served as a PCR positive control (lanes labeled +). (B) With RNA harvested from 6-h-infected CBL treated with CHX (lanes labeled 6hU1102CHX) as the initial material, primer pair 1 failed to amplify any cDNA sequence. In the lane labeled 4dU1102, amplificates from spliced and unspliced nucleic acids were detectable. Bands migrating above 550 bp are due to mishybridization of prime pair 1 (P1-5′) to repetitive DNA. (C) Primer pair 2 amplified the predicted 647-bp fragment from spliced transcripts, synthesized under IE conditions (lane labeled 6hU1102CHX). In the lane labeled 4dU1102, amplificates from spliced and unspliced nucleic acids are visible. The greatly reduced amount of spliced transcripts detectable under IE conditions compared with the amount of transcripts detectable in cells infected for 4 days is due to the low percentage of cells infected in the first round of virus replication.

likely from genomic DNA (550 bp) could be detected. The high efficiency of the PCR was proven by amplification of 0.75 fmol of the genomic clone pKpn7 (lane labeled +). In contrast to primer pair 1, primer pair 2 amplified a 647-bp fragment derived from spliced RNA, which was harvested from CHX-treated CBL (Fig. 6C, lane labeled 6hU1102CHX). Primer pair 2 hybridized to classes of cDNAs which were derived from the 3.5- and 4.7-kb transcripts (Fig. 6A). A band migrating at 754 bp represented unspliced nucleic acids (Fig. 6C, lanes labeled 6hU1102CHX and 4dU1102). These data conclusively prove that the 3.5-kb mRNA is an IE transcript, originating from a promoter within the IE region, while the large 4.7-kb mRNA is not found in the presence of CHX.

DISCUSSION

The genes of all known herpesviruses that are expressed in the IE stage after infection typically code for regulatory proteins. They coordinate viral gene expression and often transactivate transcription from a variety of homologous and heterologous promoters (34, 35, 39). IE genes are usually defined as those genes that are transcribed in the presence of protein de novo biosynthesis inhibitors, such as CHX. The IE gene regions of various herpesviruses are extremely diverse with respect to the number, genomic position, and orientation of their reading frames and to the respective nucleotide sequences. In contrast to most of the other herpesvirus transcripts, IE mRNAs are frequently spliced. We describe here for the first time the identification of an IE gene locus in the genome of HHV-6. This genomic region is located within *Eco*RI fragment 44 (37) of HHV-6 strain U1102, close to the right end of the unique segment of the virus genome. This is adjacent to a segment of more than 100 kbp in which almost all HHV-6 genes are collinear in position and orientation to the unique long segment of the HCMV genome. Thus, the overall position of this HHV-6 IE region is similar to the major IE gene block of HCMV, but otherwise the genomic region is quite different.

The 5.5-kbp IE region of HHV-6 that we identified here is delineated by two different clusters of short direct repeats (*Kpn*I repeats and CACATA repeats); it is transcribed into at least four spliced mRNAs, two of which (3.5 and 4.7 kb) originate from one strand, while the other two transcripts (1.0 and 1.5 kb) are synthesized from the opposite strand. These short transcripts largely overlap the 5′-untranslated region of the long mRNAs. The longer transcripts code for the same putative 104.6-kDa protein and are coterminal, even though they arise from two promoters. The 3.5-kb mRNA is formed in the presence of CHX (100 μg/ml); the respective promoter is located downstream of the *Kpn*I repeats. The 4.7-kb mRNA, which is not detectable under IE conditions, is initiated by a promoter upstream of the *Kpn*I repeats. Likewise, the short transcripts of opposite orientation do not appear in RNA from infected cells treated with CHX; they arise from a single promoter and code for the same putative 17.2-kDa polypeptide but are terminated differentially. It has been rather difficult to identify the 3.5-kb mRNA under IE conditions, as the infection of lymphocytes with a cell-free HHV-6 inoculum is very inefficient, even when the particles have been concentrated by 2 orders of magnitude. It is estimated that no more than 1% of the CBL are infected in the first round of virus replication. Thus, the apparently low abundance of the 3.5-kb mRNA in Fig. 3A (lane labeled 6hU1102CHX) does not exclude a high rate of synthesis, as seen in some herpesvirus IE genes. Preliminary data with fusion genes containing the promoter and upstream sequences of the 3.5-kb transcription unit indicate strong activity in uninfected lymphoid cells upon transient expression (data not shown).

Martin and colleagues (36) have shown that the carboxy-terminal part of the predicted 104.6-kDa IE protein transactivates gene expression from the long terminal repeat of immunodeficiency viruses. This feature is shared by other herpesvirus IE genes such as the HCMV IE1 and IE2 genes (7, 20, 57) encoding proteins that also induce transcription from a variety of other heterologous promoters (21, 52). Beyond that, we found no similarity in the organization and expression patterns between the IE genes of HHV-6 and HCMV (3, 24, 46, 48–50), even though both viruses belong to the subgroup beta-*Herpesviridae* (43) and possess many homologous genes and antigenic epitopes (38). All betaherpesviruses investigated

in detail so far utilize strong transcriptional enhancer elements to initiate IE gene expression (11, 14, 25, 53). It has been suggested that the *Kpn*I repeats in the HHV-6 IE region may act as a transcriptional IE gene enhancer (36, 54). This, however, seems unlikely in spite of the spatial relationship, with a distance of about 0.5 kb. Transient expression studies with fusion genes did not support this hypothesis (data not shown). Numerous viral enhancers have repeated transcription factor-binding sites, but none of them is a multiply reiterated strict tandem repeat cluster.

It is not unusual that transcription from a herpesvirus IE region is detectable at later times of infection (29, 42, 48, 49). These mRNAs, however, are often different from their cognate IE transcripts. An example is found in HCMV, in which the IE2 gene region codes at IE times for a protein that is able to transactivate early promoters and to repress the major IE enhancer/promoter. The mRNA transcribed at late times from the same region encodes only the carboxy-terminal part. This truncated protein is sufficient to repress the major IE enhancer/promoter but not to transactivate it (41). This is different from that which we observed in HHV-6: IE and later transcripts (3.5 and 4.7 kb) code for the same putative transcriptional transactivator but are initiated from different promoters. There is precedent for this in alphaherpesvirus bovine herpesvirus 1, in which an IE 2.9-kb mRNA and an early 2.6-kb mRNA code for the same gene product which is homologous to ICP0 of herpes simplex virus (55). Since the HHV-6 mRNAs (3.5 and 4.7 kb) originating from two different promoters code for the same protein, the physical separation of these promoters is most likely functionally related to different levels of expression in the time course. In late infection, the small (1.0- and 1.5-kb) HHV-6 mRNAs are most abundant. Because of their antisense orientation relative to that of the 3.5-kb IE transcript, it may be speculated that this contributes to biphasic regulation.

In summary, these data on the structure and transcription of an IE region in HHV-6 should help to elucidate the transcription regulatory principles of this virus and may enable the first steps toward discerning the different stages of the viral cycle, such as productive infection, persistence, or latency. It could be helpful in further clarifying the pathogenic potential of HHV-6 in its natural host, humans.

ACKNOWLEDGMENTS

We thank Thomas Stamminger for critical reading of the manuscript.

This work was supported by Mildred-Scheel-Stiftung and DFG-Graduiertenkolleg RNA-Synthese und -Prozessierung-Vielfalt der Strategien in Pro- und Eukaryonten.

REFERENCES

1. **Ablashi, D. V., H. Agut, Z. Berneman, G. Campadelli-Fiume, D. Carrigan, L. Ceccerini-Nelli, B. Chandran, S. Chou, H. Collandre, R. Cone, T. Dambaugh, S. Dewhurst, D. DiLuca, B. R. J. Franza, B. Fleckenstein, N. Frenkel, R. Gallo, U. Gompels, C. Hall, M. Jones, G. Lawrence, M. Martin, L. Montagnier, F. Neipel, J. Nicholas, P. Pellet, A. Razzaque, G. Torrelli, B. Thomson, S. Salahuddin, L. Wyatt, and K. Yamanishi.** 1993. Human herpesvirus-6 strain groups: a nomenclature. Arch. Virol. **129**:363–366.
2. **Ablashi, D. V., S. Z. Salahuddin, S. F. Josephs, F. Imam, P. Lusso, R. C. Gallo, C. Hung, J. Lemp, and P. D. Markham.** 1987. HBLV (or HHV-6) in human cell lines. Nature (London) **329**:207. (Letter.)
3. **Akrigg, A., G. W. Wilkinson, and J. D. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. **2**:107–121.
4. **Albrecht, J. C., and B. Fleckenstein.** 1990. Structural organization of the conserved gene block of herpesvirus saimiri coding for DNA polymerase, glycoprotein B, and major DNA binding protein. Virology **174**:533–542.
5. **Asano, Y., T. Yoshikawa, S. Suga, T. Nakashima, T. Yazaki, M. Fukuda, S. Kojima, and T. Matsuyama.** 1991. Reactivation of herpesvirus type 6 in children receiving bone marrow transplants for leukemia. N. Engl. J. Med. **324**:634–635. (Letter.)
6. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, vol. 1. John Wiley & Sons Inc., New York.
7. **Biegalke, B. J., and A. P. Geballe.** 1991. Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. Virology **183**:381–385.
8. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: the end is in site! Cell **41**:349–359.
9. **Borisch, B., K. Ellinger, F. Neipel, B. Fleckenstein, T. Kirchner, M. M. Ott, and H. K. Müller-Hermelink.** 1991. Lymphadenitis and lymphoproliferative lesions associated with the human herpes virus-6 (HHV-6). Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. **61**:179–187.
10. **Borisch-Chappuis, B., K. Ellinger, F. Neipel, T. Kirchner, P. Kujath, B. Fleckenstein, and H. K. Müller-Hermelink.** 1989. Human herpesvirus 6 in lymph nodes. Lancet **i**:40–41. (Letter.)
11. **Boshart, M., F. Weber, G. Jahn, K. Dorsch Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
12. **Carrigan, D. R., W. R. Drobyski, S. K. Russler, M. A. Tapper, K. K. Knox, and R. C. Ash.** 1991. Interstitial pneumonitis associated with human herpesvirus-6 infection after marrow transplantation. Lancet **338**:147–149.
13. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
14. **Dorsch Hasler, K., G. M. Keil, F. Weber, M. Jasin, W. Schaffner, and U. H. Koszinowski.** 1985. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**:8325–8329.
15. **Downing, R. G., N. Sewankambo, D. Serwadda, R. W. Honess, D. Crawford, R. F. Jarrett, and B. E. Griffin.** 1987. Isolation of human lymphotropic herpesviruses from Uganda. Lancet **ii**:105. (Letter.)
16. **Drobyski, W. R., W. M. J. Dunne, E. M. Burd, K. K. Knox, R. C. Ash, M. M. Horowitz, N. Flomenberg, and D. R. Carrigan.** 1993. Human herpesvirus-6 (HHV-6) infection in allogeneic bone marrow transplant recipients: evidence of a marrow-suppressive role for HHV-6 in vivo. J. Infect. Dis. **167**:735–739.
17. **Ellinger, K., F. Neipel, L. Foà-Tomasi, G. Campadelli-Fiume, and B. Fleckenstein.** 1993. Identification and characterization of the glycoprotein B homlogue of human herpesvirus type 6. J. Gen. Virol. **74**:495–500.
18. **Ellinger, K., F. Neipel, S. Seidl, B. Borisch-Chappuis, H. K. Müller-Hermelink, and B. Fleckenstein.** 1992. Detection of HHV-6 genomes in lymphoproliferative diseases, p. 209–226. *In* D. V. Ablashi, G. R. Krüger, and S. Z. Salahuddin (ed.), Perspectives in medical virology, vol. 4. HHV-6 epidemiology, molecular pathology and clinical pathology. Elsevier, Amsterdam.
19. **Ensoli, B., P. Lusso, F. Schachter, S. F. Josephs, J. Rappaport, F. Negro, R. C. Gallo, and F. Wong-Staal.** 1989. Human herpes virus-6 increases HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. EMBO J. **8**:3019–3027.
20. **Ghazal, P., J. Young, E. Giulietti, C. DeMattei, J. Garcia, R. Gaynor, R. M. Stenberg, and J. A. Nelson.** 1991. A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic *trans* activation by cytomegalovirus immediate-early proteins. J. Virol. **65**:6735–6742.
21. **Hagemeier, C., S. Walker, R. Caswell, T. Kouzarides, and J. Sinclair.** 1992. The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early protein transactivates heterologous promoters in a TATA box-dependent mechanism and interacts directly with TFIID. J. Virol. **66**:4452–4456.

22. **Hall, C. B.** 1990. Herpes and the rash of roses: a new virus, HHV-6, as a cause of an old childhood disease, roseola. Pediatr. Ann. **19**:517–521.
23. **Honess, R. W., U. A. Gompels, B. G. Barrell, M. Craxton, K. R. Cameron, R. Staden, Y.-N. Chang, and G. S. Hayward.** 1989. Deviations from expected frequencies of CpG dinucleotides in herpesvirus DNAs may be diagnostic of differences in the state of their latent genomes. J. Gen. Virol. **70**:837–855.
24. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD 169. J. Virol. **49**:363–370.
25. **Jeang, K. T., M. S. Cho, and G. S. Hayward.** 1984. Abundant constitutive expression of the immediate-early 94K protein from cytomegalovirus (Colburn) in a DNA-transfected mouse cell line. Mol. Cell. Biol. **4**:2214–2223.
26. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125–8148.
27. **Krueger, G. R., M. Manak, N. Bourgeois, D. V. Ablashi, S. Z. Salahuddin, S. S. Josephs, A. Buchbinder, R. C. Gallo, F. Berthold, and H. Tesch.** 1989. Persistent active herpes virus infection associated with atypical polyclonal lymphoproliferation (APL) and malignant lymphoma. Anticancer Res. **9**:1457–1476.
28. **Larcher, C., H. P. Huemer, R. Margreiter, and M. P. Dierich.** 1988. Serological crossreaction of human herpesvirus-6 with cytomegalovirus. Lancet **ii**:963–964. (Letter.)
29. **Lau, R., G. Packham, and P. J. Farrell.** 1992. Differential splicing of Epstein-Barr virus immediate-early RNA. J. Virol. **66**:6233–6236.
30. **Lawrence, G. L., M. Chee, M. A. Craxton, U. A. Gompels, R. W. Honess, and B. G. Barrell.** 1990. Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. **64**:287–299.
31. **Levy, J. A., A. Landay, and E. T. Lennette.** 1990. Human herpesvirus 6 inhibits human immunodeficiency virus type 1 replication in cell culture. J. Clin. Microbiol. **28**:2362–2364.
32. **Lindquester, G. J., and P. E. Pellett.** 1991. Properties of the human herpesvirus 6 strain Z29 genome: G + C content, length, and presence of variable-length directly repeated terminal sequence elements. Virology **182**:102–110.
33. **Lopez, C., P. E. Pellett, J. Stewart, C. Goldsmith, K. Sanderlin, J. Black, D. Warfield, and P. Feorino.** 1988. Characteristics of human herpesvirus-6. J. Infect. Dis. **157**:1271–1273.
34. **Manet, E., H. Gruffat, M. C. Trescol Biemont, N. Moreno, P. Chambard, J. F. Giot, and A. Sergeant.** 1989. Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional trans-activators. EMBO J. **8**:1819–1826.
35. **Margolis, D. M., A. B. Rabson, S. E. Straus, and J. M. Ostrove.** 1992. Transactivation of the HIV-1 LTR by HSV-1 immediate-early genes. Virology **186**:788–791.
36. **Martin, M. E., J. N. Nicholas, B. J. Thomson, C. Newman, and R. W. Honess.** 1991. Identification of a transactivating function mapping to the putative immediate-early locus of human herpesvirus 6. J. Virol. **65**:5381–5390.
37. **Neipel, F., K. Ellinger, and B. Fleckenstein.** 1991. The unique region of the human herpesvirus type 6 genome is essentially collinear to the UL segment of human cytomegalovirus. J. Gen. Virol. **72**:2293–2297.
38. **Neipel, F., K. Ellinger, and B. Fleckenstein.** 1992. Gene for the major antigenic structural protein (p100) of human herpesvirus type 6. J. Virol. **66**:3918–3924.
39. **Perera, L. P., J. D. Mosca, W. T. Ruyechan, G. S. Hayward, S. E. Straus, and J. Hay.** 1993. A major transactivator of varicella-zoster virus, the immediate-early protein IE62, contains a potent N-terminal activation domain. J. Virol. **67**:4474–4483.
40. **Pietroboni, G. R., G. B. Harnett, and M. R. Bucens.** 1989. Centrifugal enhancement of human immunodeficiency virus (HIV) and human herpesvirus type 6 (HHV-6) infection in vitro. J. Virol. Methods **24**:85–90.
41. **Puchtler, E., and T. Stamminger.** 1991. An inducible promoter mediates abundant expression from the immediate-early 2 gene region of human cytomegalovirus at late times after infection. J. Virol. **65**:6301–6306.
42. **Rawlinson, W. D., and B. G. Barrell.** 1993. Spliced transcripts of human cytomegalovirus. J. Virol. **67**:5502–5513.
43. **Roizman, B., R. C. Desrosiers, B. Fleckenstein, C. Lopez, A. C. Minson, and M. J. Studdert.** 1992. The family herpesviridae: an update. Arch. Virol. **123**:425–449.
44. **Salahuddin, S. Z., D. V. Ablashi, P. D. Markham, S. F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R. C. Gallo.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science **234**:596–601.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Stamminger, T., and B. Fleckenstein.** 1990. Immediate-early transcription regulation of human cytomegalovirus. Curr. Top. Microbiol. Immunol. **154**:3–19.
47. **Stenberg, R.** 1993. Immediate-early genes of human cytomegalovirus: organization and function, p. 330–359. *In* Y. Becker, G. Darai, and E. S. Huang (ed.), Frontiers of virology: molecular aspects of human cytomegalovirus diseases, vol. 2. Springer-Verlag, Berlin.
48. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63**:2699–2708.
49. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56**:665–675.
50. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. **46**:1–14.
51. **Stürzl, M., and W. K. Roth.** 1990. "Run-off" synthesis and application of defined single-stranded DNA hybridization probes. Anal. Biochem. **185**:164–169.
52. **Tevethia, M. J., D. J. Spector, K. M. Leisure, and M. F. Stinski.** 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology **161**:276–285.
53. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.
54. **Thomson, B. J., and R. W. Honess.** 1992. The right end of the unique region of the genome of human herpesvirus 6 U1102 contains a candidate immediate early gene enhancer and a homologue of the human cytomegalovirus US22 gene family. J. Gen. Virol. **73**:1649–1660.
55. **Wirth, U., C. Fraefel, B. Vogt, Č. Vlček, V. Pačes, and M. Schwyzer.** 1992. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3′ coterminal and encode a putative zinc finger transactivator protein. J. Virol. **66**:2763–2772.
56. **Yamanishi, K., T. Okuno, K. Shiraki, M. Takahashi, T. Kondo, Y. Asano, and T. Kurata.** 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet **i**:1065–1067.
57. **Yeung, K. C., C. M. Stoltzfus, and M. F. Stinski.** 1993. Mutations of the human cytomegalovirus immediate-early 2 protein defines regions and amino acid motifs important in transactivation of transcription from the HIV-1 LTR promoter. Virology **195**:786–792.
58. **Yoshiyama, H., E. Suzuki, T. Yoshida, T. Kajii, and N. Yamamoto.** 1990. Role of human herpesvirus 6 infection in infants with exanthema subitum. Pediatr. Infect. Dis. J. **9**:71–74.