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U94 of human herpesvirus 6 is expressed in latently infected peripheral blood mononuclear cells and blocks viral gene expression in transformed lymphocytes in culture

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ABSTRACT **Human herpesvirus 6 (HHV-6) like other herpesviruses, expresses sequentially immediate early (IE), early, and late genes during lytic infection. Evidence of ability to establish latent infection has not been available, but by analogy with other herpesviruses it could be expected that IE genes that regulate and transactivate late genes would not be expressed. We report that peripheral blood mononuclear cells of healthy individuals infected with HHV-6 express the U94 gene, transcribed under IE conditions. Transcription of other IE genes (U16/17, U39, U42, U81, U89/90, U91) was not detected. To verify that U94 may play a role in the maintenance of the latent state, we derived lymphoid cell lines that stably expressed U94. HHV-6 was able to infect these cells, but viral replication was restricted. No cytopathic effect developed. Furthermore, viral transcripts were present in the first days postinfection and declined thereafter. A similar decline in the level of intracellular viral DNA also was observed. These findings are consistent with the hypothesis that the U94 gene product of HHV-6 regulates viral gene expression and enables the establishment and/or maintenance of latent infection in lymphoid cells.**

Human herpesvirus 6 (HHV-6) infection is commonly acquired within the first 2 years of life (1). Primary infection is associated with exanthem subitum (2) and nonspecific febrile illness in infants (3). After primary infection, the virus persists in the host and up to 90% of healthy adult blood donors harbor HHV-6 DNA in peripheral blood mononuclear cells (PBMCs) (4). More than 90% of healthy adults also show serologic evidence of past infection (5), and approximately 5% of the healthy adult population has anti-HHV-6 IgM antibody, suggesting that the virus may periodically reactivate, without clinically apparent sequelae (6).

HHV-6 is frequently isolated from immunosuppressed individuals, such as HIV-infected individuals, transplant recipients, and patients with autoimmune diseases. In these cases, viral infection can be associated with severe outcomes, including interstitial pneumonitis, encephalitis, bone marrow suppression, and graft failure (7–13). Active (productive) HHV-6 may be also associated with renal allograft rejection (14, 15).

Several indirect lines of evidence support the view that HHV-6 is maintained in a latent state in the peripheral blood of most healthy adults. First, a large number of healthy individuals harbor detectable amounts of HHV-6 DNA in their PBMCs (16), even though infectious virus cannot be isolated from the blood of these persons, and levels of viral DNA remain below the limit of detection. Second, treatment with phorbol esters induces virus replication from cultured human macrophages isolated from HHV-6 seropositive adults (17). Third, *in vitro* infection of PBMC specimens from HHV-6 seropositive adults with HHV-7 results in the active, lytic replication of endogenous HHV-6B (18). Nonetheless, direct experimental evidence for a bona fide viral latent state, as opposed to a state of low-level persistent viral replication, has not as yet been forthcoming (19). Here we report that we have identified a potential hallmark of HHV-6 latency that may play a role in the establishment of latent HHV6 infection in human PBMC. Relevant to this report are the following.

Herpesviruses cause both productive and latent infections. In productive infection, the infected cells express most, if not all, viral genes sequentially. The regulatory immediate early (IE) or α genes are expressed first, followed by early (β) and, lastly, by late or γ genes. HHV-6 genes transcribed under IE conditions, that is, in the absence of prior protein synthesis, are U16/17, U39, U42, U81, U89/90, U91, and U94 ORFs (20). In the process of viral replication, the cell dies. HHV-6 is closely related to HHV-7 and to the human cytomegalovirus (HCMV) (21). Recent sequence analysis of the HHV-6 and HHV-7 genomes has revealed that all but two HHV-6 genes have HHV-7 counterparts (22). One of the two exceptions is the U94 ORF of HHV-6, which has no known counterparts in any of the other human herpesviruses, is expressed under IE conditions, and encodes a product (RepH6) that is homologous to the adeno-associated virus type 2 *rep* gene product (RepAAV-2) (23). *rep* AAV-2 encodes four overlapping proteins, which are required for AAV-2 DNA replication and for modulating the expression of homologous and heterologous genes. The function(s) of RepH6 are unknown, although available evidence suggests that this protein may have a regulatory role in the viral life cycle. Furthermore, RepH6 exhibits at least conservation of function with respect to its AAV counterpart, since it can complement the replication of Rep-defective AAV-2 mutants (24). RepH6 also suppresses transformation by H-ras and inhibits transcription from HIV type 1 long terminal repeat (25).

In latent infection, only a small subset of viral genes are expressed, usually in the absence of prior infected cell protein synthesis, and the cell survives. In the case of herpes simplex virus (HSV), the only viral transcripts that are detectable during viral latency are the latency-associated transcripts (LATs) (26). Similarly, latent infection of hematopoietic pro-

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Abbreviations: PBMC, peripheral blood mononuclear cell; HHV, human herpesvirus; HSV, herpes simplex virus; IE, immediate early; LAT, HSV latency-associated transcript; RT-PCR, reverse transcription–PCR.

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genitor cells by HCMV is characterized by the expression of sense and antisense transcripts from the immediate early region 1 (27). In the present study, we show that the U94 ORF is transcribed in freshly isolated PBMC from HHV-6 infected adults, whereas other viral mRNA species are not detected in this cell compartment. Furthermore, T cell lines that stably express HHV-6 U94 are permissive for HHV-6 infection, but viral replication is restricted. Collectively, these findings support the hypothesis that U94/*repH6* may have a function in the regulation of HHV-6 latency.

MATERIALS AND METHODS

Virus and Cells. JJhan cells were grown in suspension at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. Cell-free viral inocula were obtained by pelleting 250 ml of cell cultures infected with HHV-6A (strain U1102) exhibiting complete cytopathic effect (CPE). Infected cells, resuspended in 2 ml of fetal calf serum supplemented with RNase (Boehringer Mannheim, 50 μg/ml), were disrupted by four cycles of freezing in liquid nitrogen and thawing at 37°C. The resulting inoculum was completely free of living cells, as checked by microscopic observation and cultivation, and also was analyzed by reverse transcription–PCR (RT-PCR) (both for β-actin and for a panel of viral mRNAs) to ensure that RNA was completely absent. Infection then was performed by resuspending 10^7 cells in the resulting viral inoculum. After 1 hr of adsorption at 37°C the cells were diluted with fresh medium to attain a final concentration of 5×10^5 cells/ml.

Plasmids. The U94 expression plasmid, pSR2ph, was derived by inserting U94 ORF into the pSRα1Neo expression vector (28). This vector contains a selective neomycin-resistance cassette, together with a polylinker and a hybrid-promoter element comprising the simian virus 40 early promoter, together with the R-U5 segment of the human T cell leukemia virus type 1 long terminal repeat (29). U94 was amplified by PCR from the R-1 strain of HHV-6B (29), using standard amplification conditions with oligodeoxynucleotide primers (PH1, 5′-aacgggaagcttATGTTTTCCATAATA-AATCCGAGTG; PH2, 5′-gatgcatgctcgagTATAAAATTT-TCGGAACCGTG; underlined residues denote restriction sites introduced for cloning purposes, uppercase letters indicate viral sequences, and sequences in bold represent translational start and stop codons for the U94 ORF). The U94 ORF initially was cloned in pKS (Stratagene) and then subcloned into pSRα1Neo.

Transfection and Cloning. pSR2ph and pSRα1Neo were transfected in JJhan cells by electroporation (Gene Pulser, Bio-Rad). Cells were resuspended in serum-free medium (5×10^6 cells/0.8 ml) and placed in 0.5-cm-wide cuvettes. Twenty micrograms of plasmid was pulsed at 250 mV and 960 μF. After electroporation, cells were seeded in 96-well culture dishes at a concentration of 10^4 cells per well. Transformants were selected in medium containing 1 mg/ml of the neomycin analogue G418 (Sigma), and medium was changed once a week. After 30 days of selection, 3 of 96 wells transfected with pSR2ph and 5 of 96 wells transfected with pSRα1Neo contained viable cells. Cell lines were obtained after further dilution (10^3 cells per well) by seeding cells into new 96-well culture plates in the presence of G418.

Extraction of Nucleic Acids. Buffy coats were collected from 36 healthy blood donors, and PBMCs were purified on Ficoll gradients. Aliquots corresponding to 5×10^6 cells were frozen at −70°C until nucleic acid extraction was performed.

DNA from PBMCs was extracted as described previously (30), using proteinase K digestion, phenol and phenol-chloroform extractions, and ethanol precipitation. Particular care was taken to avoid contamination of the samples, and blank reactions, consisting of the extraction mixture alone, were interspersed with every third experimental sample (so as to control for possible cross-contamination of DNA samples). DNA concentration was determined by reading optical density at 260 nm.

RT. Samples positive for HHV-6 genomic DNA by PCR analysis were analyzed for HHV-6 gene expression. mRNA was extracted with mRNA Isolation Kit (Boehringer Mannheim) as indicated by the manufacturer. Aliquots of mRNA corresponding to 6×10^5 cells were stored at −70°C in ethanol/sodium acetate until utilized for RT and PCR amplification. Immediately before reverse transcription, the mRNA pellet extracted from 6×10^5 cells was rinsed with 75% ethanol and resuspended in water treated with diethyl pyrocarbonate. First-strand cDNA synthesis was carried out with cDNA Cycle Kit (Invitrogen) following the manufacturer's recommendations, with random hexamer primers. Five different RT reactions were performed separately for each sample. cDNA subsequently was pooled, purified by Centricon30 microconcentrators (Amicon, MA), and stored in aliquots at −70°C until utilized for PCR amplification. The complete absence of contaminating DNA within these RNA samples was verified by conducting PCR amplification on sample aliquots that were not submitted to reverse transcription. Experimental PCR analysis was conducted on cDNA equivalent to 1.5×10^5 cells, using primers specific for human β-actin (20) and for HHV-6 genes (see below) (20).

PCR Analysis. To determine which samples harbored HHV-6 genomic DNA sequences, 1 μg of DNA (corresponding to 1.5×10^5 cells) was analyzed by nested PCR by using primers amplifying different genomic regions (U31, U39, U94) (20) (Fig. 1). Samples harboring HHV-6 DNA were analyzed by nested RT-PCR for seven HHV-6 mRNAs: U16/17, U39, U42, U81, U89/90, U91, and U94. All primers were derived from the published HHV-6 sequence (21). The primers and conditions employed in the first round of PCR, as well as the expected size of amplified fragments resulting from DNA and cDNA amplification, have been described recently (20). To increase sensitivity of detection, nested PCR was developed for all HHV-6 genes tested in this study. Primers' sequences, amplification products, and PCR conditions for the nested reactions are shown in Table 1.

Aliquots of single-step PCRs were added to a solution containing 400 nM specific internal primers (200 nM in the case of U31)/1.5 mM $MgCl_2$ (3 mM in the case of U16/17 and U39, and 2 mM in the case of U89/90)/200 μM dNTPs/1 unit of AmpliTaq DNA polymerase (Perkin–Elmer) in the buffer supplied by the manufacturer. PCR products were run on 2–3% agarose gels, according to the expected fragment size, and UV was visualized after ethidium bromide staining. Due care was taken to avoid sample-to-sample contamination: different rooms and dedicated equipment were used for mRNA extraction and processing, for PCR set-up and gel analyses, all pipette tips had filters for aerosol protection and all experimental samples were interspersed with blank reactions. All PCRs for HHV-6 transcripts had similar sensitivities: 10-fold dilutions of DNA extracted from HHV-6 infected cells similarly were amplified by all primers for nested PCR.

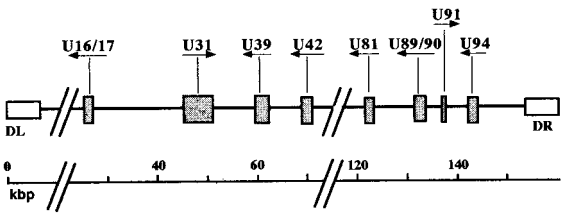

FIG. 1. Schematic representation of the HHV-6 genome, showing the physical location of the ORFs analyzed in this study. Arrows indicate the direction of transcription. U16/17, U91, and U89/90 correspond to spliced transcripts.

Table 1. Primers and conditions for amplification of HHV-6 genes (nested reaction)

ORF	Primer sequence (3′ → 5′)	Amplimer DNA	Size cDNA, bp	PCR conditions
U16/17	AGAACTGCAAATCGTTCCG CGTAGAACAGAAGACCGGC	401	315	1 min at 94°C, 1 min at 55°C, 1 min + 3 sec/cycle at 72°C for 35 cycles; 10 min at 72°C
U39	GGCATAAGGAAACATCATGT GTAGAGCAAACGTTTTCGAT	353	—	
U31	GGAGAATCTTGTAAGTATATGGTC CTCGGACTCATAGATCTCATACTG	659	—	1 min at 92°C, 1 min at 58°C, 1 min at 72°C for 10 cycles; 1 min at 92°C, 1 min at 58°C, 1 min + 3 sec/cycle at 72°C for 20 cycles; 10 min at 72°C
U42	AGGTTTACCGCAGAGTTGCC CAAAACAACGCATCCGAGAC	404	—	1 min at 94°C, 1 min at 61°C, 1 min +3 sec/cycle at 72°C for 35 cycles; 10 min at 72°C
U89/90	TCGCACCAATTAAAAAAATCC TCAAAGAAATATTGGAAGAGAGGTC	531	424	
U91	CAGACATTACACATTCATTTCA(C/G) CAACTGCACTGAAACCACTAC	385	283	
U94	CCCATTGGAACTGTGGTCT TTCAGGGCTATGCACTCACC	388	—	
U81	AAATTGGTGGACAACGATAG ATTCTGGAATCGAGCACTCT	184	—	1 min at 94°C, 1 min at 57°C, 1 min + 3 sec/cycle at 72°C for 35 cycles; 10 min at 72°C

RESULTS

Characterization of Lymphoid Cells Stably Transformed with U94/repH6. JJhan T cells were transfected with plasmid pSR2ph, which contains the intact U94 and a selectable neomycin-resistance cassette. After two rounds of limiting dilution, and selection in the presence of G418, three cell lines (RepCl2, RepCl3, RepCl4) were derived. The cell lines had the same morphology and growth characteristics as the parental cell line, and no toxicity was evident. Several cell lines transformed with the vector sequences alone were obtained similarly, and one of them (N1) was used as control in all subsequent experiments. Southern blot analysis of DNA isolated from these cell lines revealed the presence of U94 DNA in all Rep-cell lines (as expected), at a level of approximately 1 copy per cell (Fig. 2*A*). Cellular DNA was digested with *Hin*dIII, so as to excise the U94 ORF from the vector, and the DNA then was hybridized to a radiolabeled plasmid DNA probe. These experiments revealed the presence of high-molecular-weight DNA fragments, indicating that the Rep expression plasmid had undergone integration into host cell chromosomes. The presence of *rep*-specific mRNAs within transfected cells was confirmed by RT-PCR analysis (Fig. 2*B*). mRNA was stably maintained for >2 months in continuous culture in the absence of selection (data not shown).

HHV-6 Infection of Rep-Expressing Cell Lines. Rep-expressing cell lines, parental cells, and the control line N1 were infected with cell-free HHV-6A (U1102), since HHV-6B strains do not grow litically in JJahn cells (16). After adsorption with viral inocula, cells were washed extensively and collected at different times postinfection (0, 7, 22, and 28 days). Control cell lines (JJhan and N1) showed cytopathic effects within 7 days of infection and were completely lysed by 18 days postinfection. In contrast, the Rep-expressing cell lines did not show any evidence of cytopathic effect and were regularly split twice a week at a 1:10 ratio. Viral replication was assessed by immunofluorescence and by RT-PCR detection of viral transcripts. In addition, the presence of viral genomic DNA was investigated by PCR for the HHV-6 U31 ORF, and a semi-

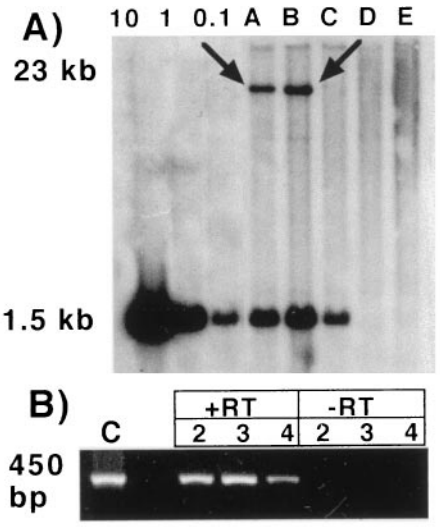

FIG. 2. (*A*) Southern Blot of DNA extracted from Rep-transformed cell lines and hybridized to radiolabeled U94 DNA probe. The numbers 10, 1, and 0.1 indicate lanes containing, respectively, 10, 1, and 0.1 copy equivalents of U94 DNA. Rep-expressing lines RepCl2, RepCl3, and RepCl4 are shown in lanes A, B, and C, while the control cell line, N1, and parental JJhan cells are shown in lanes D and E. DNA was digested with *Hin*dIII to excise U94 from the vector as a 1.5-kb band. (*B*) Presence of U94 transcripts in Rep-expressing cell lines (2, 3, 4). RNA (no initial reverse transcription step) and cDNA (+RT) were amplified by PCR and analyzed by agarose gel. Lane C represents a control amplification reaction, performed by using 10,000 DNA molecules of U94 template.

quantitative assessment of DNA copy number was achieved by serial 10-fold dilutions of cellular DNA.

Rep-expressing cell lines and control cells were infected by HHV-6A with essentially equivalent efficiency, as evidenced by the amount of viral DNA detected in infected cells immediately after infection (Fig. 3). At 7 days postinfection, after two cell passages, control cells (JJhan and N1 cell lines) harbored approximately 10-fold more DNA than the Rep-expressing cell lines, in spite of the fact that fewer cells were present, due to cell lysis. Within 18 days of viral infection, the control cells were completely lysed. In contrast, the Rep-expressing cell lines did not show any cytopathic effect or decreased rate of cell growth. At 22 days postinfection, the level of viral genomic DNA in the Rep-expressing cell lines had declined by roughly 100-fold, and this level of viral DNA continued to decline by 28 days postinfection (Fig. 3).

Indirect immunofluorescence analysis (IFA), using the HHV-6-specific 9A5D12 mAb (31), at 7 days postinfection revealed the presence of p41 early viral antigen in the majority of JJahn and N1 infected cells. However, less than 1% of the Rep-expressing cell population exhibited detectable antigen expression at this time point, and at later times no antigen-positive cells could be detected (data not shown).

HHV-6 gene expression in HHV-6-infected Rep-expressing and control lines also was examined by RT-PCR, searching the presence of HHV-6 transcripts associated with lytic viral replication: ORFs U42, U91, U16/17, and U89/90 are α genes (20), and U41 (major DNA-binding protein) and U100 (gp82/105, structural glycoprotein) are, respectively, β and γ genes. The cell-free infecting viral inoculum was tested for the presence of these transcripts and was found negative. Fig. 4 shows the results obtained for U42, U91, and U16/17. The same results were obtained for all the other transcripts (data not shown). Viral mRNAs were readily detected at 7 days after infection both in control cells and in Rep-expressing lines (Fig. 4). The detection of spliced RNA transcripts (U91, U16/17) also rules out the possibility of DNA contamination in RNA samples (Fig. 4). Despite this initial evidence of viral transcription, the levels of viral mRNAs in the Rep-expressing cell lines declined to almost undetectable levels within 22 days postinfection (Fig. 4).

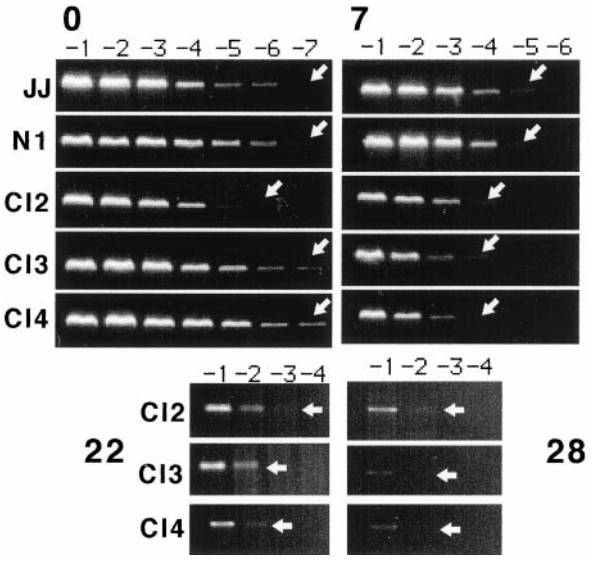

FIG. 3. Semiquantitative analysis of viral DNA in cell lines infected with cell-free HHV-6. DNA was extracted from Rep-transformed cell lines (Cl2, Cl3, Cl4), parental cells (JJ), and N1 control cell line at various time points after viral infection [either immediately postinfection (0), or at 7, 22, and 28 days postinfection]. Serial 10-fold dilutions of cellular DNA (starting at 0.1 μg) were analyzed by PCR, using primers specific for the viral U31 ORF. The arrows point to the highest dilution of template DNA yielding a positive signal upon PCR amplification.

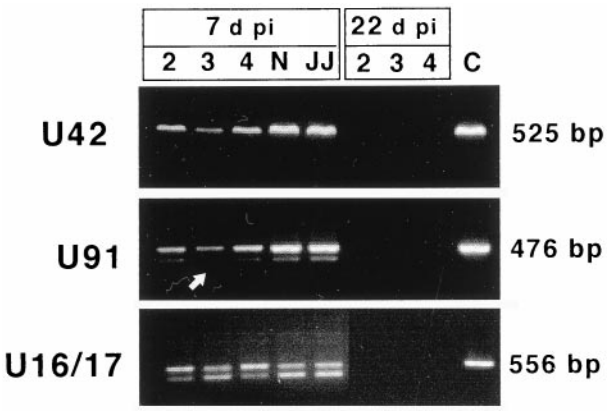

FIG. 4. Analysis of HHV-6 transcription in Rep-expressing cell lines infected with cell-free viral inoculum. RNA extracted from Rep-expressing cell lines 2, 3, and 4 and from parental JJahn cells (JJ) and control N1 cells was analyzed by RT-PCR, using primers specific for the viral ORFs U42, U91, and U16/17. U91 and U16/17 are spliced mRNAs. The arrow points to a faint band that reproduced poorly in the print.

Detection of Transcripts Associated with *in Vivo* Persistence of HHV-6. To identify the viral transcripts associated with *in vivo* viral persistence, DNA was extracted from PBMCs of 36 healthy blood donors, and 1.5×10^5 cells were screened by PCR for the presence of HHV-6 genomic DNA. Twelve samples (33%) were positive with three different sets of primers, amplifying viral DNA fragments from different genomic regions (U31, U39, U94) (Fig. 1). mRNA extracted from these DNA-positive samples was analyzed by RT-PCR to determine whether a panel of selected HHV-6 (U16/17, U39, U42, U81, U89/90, U91, and U94, also known as *rep*) genes were being transcribed (Fig. 1). All these transcripts belong to the IE transcriptional class and are normally detected during all phases of lytic infection (20). The sensitivity of detection was similar for all of the PCRs; results of nested PCR for U16/17, U42, and U94, using serial 10-fold dilutions of DNA extracted from HHV-6 infected cells are shown in Fig. 5.

Having verified the sensitivity of our PCR assay we analyzed the reverse-transcribed RNA samples. Single-step PCR amplification of cDNAs did not reveal the presence of detectable levels of viral transcripts. A nested round of amplification was required to visualize the cDNAs (Table 2 and Fig. 6). Of the 12 samples analyzed, 8 (67%) were positive for U94/repH6 mRNA. No other viral transcript was detected. Furthermore,

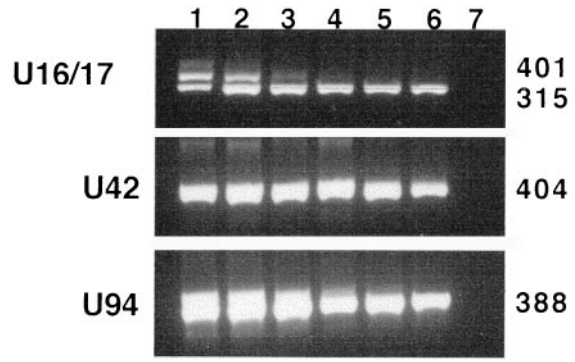

FIG. 5. Results of a reconstruction experiment, comparing the sensitivities of the various PCRs. DNA was extracted from HHV-6-infected cells, and serial 10-fold dilutions were analyzed by nested PCR, using primers specific for the viral ORFs U16/17, U42, and U94. The size of the PCR products produced is expressed in base pairs.

Table 2. HHV-6 transcripts in PBMCs of healthy donors

Sample	HHV-6 DNA	mRNA						
		U94	U16/17	U39	U42	U81	U89/90	U91
1	+	+	−	−	−	−	−	−
2	+	−	−	−	−	−	−	−
3	+	+	ND	−	ND	−	−	ND
4	+	+	−	−	−	−	−	−
5	+	−	−	−	−	−	−	−
6	+	+	−	−	−	−	−	−
7	+	−	−	−	−	−	−	−
8	+	+	−	−	−	−	−	−
9	+	+	−	−	−	−	−	−
10	+	+	−	−	−	−	−	−
11	+	−	−	−	−	−	−	−
12	+	+	−	−	−	−	−	−
Total	12	8						

ND, not done.

no residual DNA contamination of RNA samples was detected by analyzing the same amount of mRNA, without the initial reverse transcription reaction. Finally, PCR positivity was progressively lost upon analyzing decreasing numbers of cells (Table 3). These observations suggest that the amount of U94 mRNA in the positive samples was very low. Since our nested U94 PCR assay consistently detects <20 target molecules (data not shown), this suggests that only very few PBMCs harbor U94 transcripts (not more than one cell out of 1,500)—although precise quantitation of transcript levels is not possible.

DISCUSSION

By analogy with other herpesviruses, it is expected that HHV-6 persists in postconvalescent humans in latent state, most likely within T lymphocytes (19). However, evidence of true latent infection characterized by persistent presence of viral DNA in the absence of lytic-phase viral replication has not been described. Although *in vitro* reactivation of endogenous HHV-6 was obtained from monocytic cells and in PBMC cultures by phorbol esters (17) or by superinfection with HHV-7 (18), the experimental systems employed in these studies do not necessarily rule out the possibility that viral "reactivation" was simply the enhancement of an ongoing, but low-level, state of viral replication. Therefore, the theoretical possibility of a persistent, chronic state of HHV-6 infection has not yet been formally dismissed.

To examine whether HHV-6 indeed might be capable of establishing a state of true viral latency, we analyzed the PBMCs from healthy adult individuals with detectable levels of viral genomic DNA in their blood for the presence of viral RNA transcripts by highly sensitive RT-PCR. We focused our attention on transcription of seven IE genes, reasoning that their presence is a necessary requisite for productive viral replication. Of the seven transcripts studied, only U94 was present at detectable levels in the RNA specimens from healthy HHV-6-positive adults.

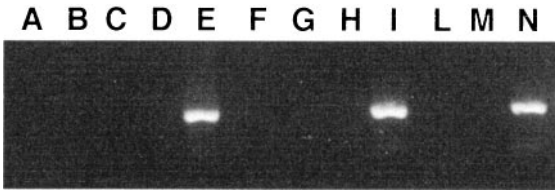

FIG. 6. Presence of U94 mRNA in PBMCs from healthy, HHV-6-positive, adult blood donors. RNA was extracted from PBMCs from healthy individuals harboring HHV-6 DNA sequences and analyzed by RT-PCR by using primers specific for the U94 ORF (lanes C–F and I–N). Control reactions (to test for possible DNA contamination of RNA specimens and/or contamination of PCRs) are shown in lanes A and G (extraction blanks) and B and H (blank reactions).

Table 3. Relative abundance of U94 mRNA in PBMCs from healthy donors

Sample	No. of cells analyzed by RT-PCR			
	150,000	60,000	30,000	15,000
1	1/1	2/2	2/2	0/4
3	ND	2/2	2/2	1/4
4	1/1	2/2	1/2	0/4
5	1/1	2/2	1/2	0/4
8	1/1	0/2	ND	ND
9	1/1	0/2	ND	ND
10	1/1	0/2	ND	ND
12	1/1	0/2	ND	ND

Results are shown as no. positive reactions/no. analyzed aliquots. ND, not done.

Relevant to the significance of this finding are the following. First, the PBMC samples subjected to our studies did not appear to harbor defective viral genomes inasmuch as the DNA analyses indicate that they contain sequences from different regions of the viral genome.

Second, we exclude the likelihood that we missed IE mRNAs inasmuch as all of the nested RT-PCR assays had similar sensitivities and most of the IE RNAs are made in abundant quantities during productive infection.

Third, the possibility that our RNA samples might be contaminated with residual DNA is ruled out, since no PCR product was detected upon amplification of RNA samples that were not subjected to reverse transcription.

Fourth, U94 mRNA previously has been shown to be expressed only at very low levels during productive viral replication (19). Thus, its detection, in the absence of other viral IE transcripts, is particularly significant.

The simplest explanation of this finding is that U94 mRNA may be associated with an *in vivo* state of viral latency in the PBMC compartment. Our findings do not allow us to identify the cell type(s) that might harbor latent virus. Conceivably, these could include circulating monocytes, as previously proposed by Kondo *et al.* (17), or T cells (perhaps long-lived cells such as memory T cells). Further studies will be required to address this question.

The hypothesis that U94 may be involved in regulating the outcome of viral infection is supported by our *in vitro* findings. Cell lines that constitutively express this viral transcript remain readily susceptible to HHV-6 infection, as shown by detection of viral DNA and transcripts for several days after infection, but viral replication is repressed, and the expression of viral genes (of α, β, and γ transcriptional classes) is down-regulated. Indeed, the HHV-6-infected cells continued to actively divide, no CPE was apparent, viral antigens were not expressed, and viral mRNAs and DNA became diluted and were progressively lost. Therefore, Rep-expressing cell lines supported an abortive or, more appropriately, a "restrictive" infection (32).

It is extremely unlikely that the lack of HHV-6 production was due to a clonal artifact, resulting in a preferential selection of nonpermissive cells, since the same result was observed with all three Rep-positive cell lines but not with the control cell line, N1, which was derived by using the same drug-selection regimen and the same parental plasmid vector. It is therefore reasonable to suppose that the expression of U94/RepH6 restricted viral infection. U94 is expressed at very low levels during lytic-phase viral replication (19), and our results support the hypothesis that overexpression of U94 may indeed be restrictive for viral growth, as was suggested previously by Braun and colleagues (19).

In summary, both *in vivo* and *in vitro* evidences point to the same conclusion: HHV-6 U94 is a latency-associated transcript. In this context, it is intriguing to note that the HSV-1 LATs recently have been shown to repress viral replication and to inhibit viral immediate-early gene expression in neuronal cells (33). In the case of HSV-1, it is proposed that suppression of the viral lytic cycle may promote the establishment of viral latency (33). It is reasonable to propose that HHV-6 U94 might have an analogous function.

This report describes a specific HHV-6 gene with a role in viral latency. HHV-6 is the only herpesvirus encoding a Rep-like function, while latency is a general feature of this family of viruses. Therefore, it is possible, and even likely, that some other yet-unidentified viral transcripts may play a role in latency—not least because several latency-associated transcripts have been described for human cytomegalovirus (27), which is rather closely related to HHV-6. At the moment the function of U94 is unknown, although at least two possibilities present themselves. First, the U94 gene product might function as a sequence-specific DNA-binding protein (like its AAV counterpart), to allow latent-phase replication of the viral genome. In functional terms, this would make U94 the equivalent of the Epstein–Barr nuclear antigen type-1 (34). However, that viral DNA is rapidly lost from U94-expressing cell lines makes this hypothesis somewhat difficult to accept. A second possibility is that the U94 gene product is a repressor of the viral IE genes, as is the case for the HSV-1 LATs (33). This hypothesis is also consistent with the fact that the HHV-6 U94 gene product is a potent transcriptional regulator, which has been reported to suppress the expression of several genes (25).

In conclusion, we would like to comment briefly on a possible application for the findings reported here. HHV-6 can be an important opportunistic pathogen in immunosuppressed patients and, particularly so, in bone marrow transplant recipients (8). In these cases, rapid diagnosis of viral infection is important to establish prompt antiviral treatment. Serologic analysis is of little value, since HHV-6 is a ubiquitous agent, and immunohistochemical examination of tissue biopsies can be applied only when the virus has already induced disease. Therefore, PCR often is used to detect HHV-6 DNA—even though this assay cannot discriminate between latent and active/reactivated viral infection. Quantitative PCR is cumbersome and, in addition, cannot distinguish between a low-level productive infection and an increased load of latently infected cells in immunocompromised patients (8). The present results suggest that the detection of U94 mRNA, in the absence of other viral transcripts, might be suggestive of a state of latency, while the detection of lytic cycle transcripts would be indicative of active infection. Thus, an RT-PCR assay might allow for relatively simple differentiation between latent and active viral infection. This could have considerable clinical utility in the posttransplant setting.

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