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U94, the Human Herpesvirus 6 Homolog of the Parvovirus Nonstructural Gene, Is Highly Conserved among Isolates and Is Expressed at Low mRNA Levels as a Spliced Transcript

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Human herpesvirus 6 variants A and B (HHV-6A and HHV-6B, respectively) encode homologs (U94) of the parvovirus nonstructural gene, *ns*1 or *rep*. Here we describe the HHV-6B homolog and analyze its genetic heterogeneity and transcription. U94 nucleotide and amino acid sequences differ by approximately 3.5% and 2.5%, respectively, between HHV-6A and HHV-6B. Among a collection of 17 clinically and geographically disparate HHV-6 isolates, intravariant nucleotide and amino acid sequence divergence was less than 0.6% and 0.2%, respectively; all 13 HHV-6B isolates had identical amino acid sequences. The U94 transcript is spliced to remove a 2.6-kb intron and is expressed at very low levels relative to other HHV-6B genes, reaching approximately 10 copies per cell 3 days after infection. The mRNA has several small AUG-initiated open reading frames upstream of the U94 open reading frame, a hallmark of proteins expressed at low levels. Consistent with this, the U94-encoded protein was immunologically undetectable in HHV-6B-infected cells. The high degree of sequence conservation suggests that the gene function is nearly intolerant of sequence variation. The low abundance of U94 transcripts and the presence of encoded inefficient translation initiation suggest that the U94 protein may be required only in small amounts during infection.

INTRODUCTION

Human herpesvirus 6 (HHV-6) isolates belong to one of two closely related, yet distinct, variants: HHV-6A and HHV-6B (Ablashi *et al.,* 1991, 1993; Schirmer *et al.,* 1991). Variant A has not been etiologically associated with any disease (reviewed in Braun *et al.,* 1997). Variant B is the etiological agent of exanthem subitum (roseola) and other febrile illnesses in children (Pruksananonda *et al.,* 1992).

HHV-6A and HHV-6B encode several polypeptides with no known counterparts in other herpesviruses. One example is U94, a homolog of the parvovirus replication gene *ns*1 (or *rep*) gene, which was first identified for HHV-6A (Thomson *et al.,* 1991). HHV-6A U94 has 24% amino acid identity to the amino-terminal 490 amino acids of the adeno-associated virus type 2 (AAV-2) Rep 68/78 protein. In a comparison between the genomic sequences of HHV-6A and HHV-6B (Dominguez *et al.,* 1999), we found that the genomes are most divergent in the direct repeats (DR) that bound the unique segment (Fig. 1) and across the 30 kb of sequence at the right end of the unique segment that encodes open reading frames U86-U100. In this region, each open reading frame has less than 90% amino acid identity between the variants, except for U94 (97.6% identity). Nucleotide sequences of HHV-6 isolates are highly conserved, with intravariant heterogeneity seldom exceeding 0.5% (typically 0.1%) and intervariant heterogeneity from gene to gene ranging from less than 2% to 24% (typically 5%) (Dominguez *et al.,* 1999, and reviewed in Braun *et al.,* 1997).

AAV-2 Rep68/78 (Rep_{AAV-2}) is a multifunctional protein essential for AAV-2 DNA replication (reviewed in Berns, 1996; Berns and Giraud, 1996). It is involved in regulation of AAV-2 gene expression and site-specific integration of the AAV-2 provirus into human chromosome 19 (Chiorini *et al.,* 1996; Linden *et al.,* 1996; Surosky *et al.,* 1997). Rep_{AAV-2} can repress heterologous gene expression, including the HIV-1 long terminal repeat promoter; suppress herpes simplex virus-induced SV40 DNA amplification (Heilbronn *et al.,* 1990); and prevent oncogenic transformation by transcriptional suppression of oncogene expression. Biochemical properties of Rep_{AAV-2} include sequence-specific DNA binding to AAV-2 terminal repeats (Ryan *et al.,* 1996), AAV-2 promoter regions (Pereira and Muzyczka, 1997a), and the human chromosome 19 integration site (Weitzman *et al.,* 1994). In addition, it has strand- and sequence-specific endonuclease activity and ATP-dependent helicase activity and interacts with transcription factors (Hermonat *et al.,* 1996; Pereira and Muzyczka, 1997b).

The U94 protein shares some functions with AAV-2 Rep 68/78. Ectopically expressed HHV-6A U94 comple-

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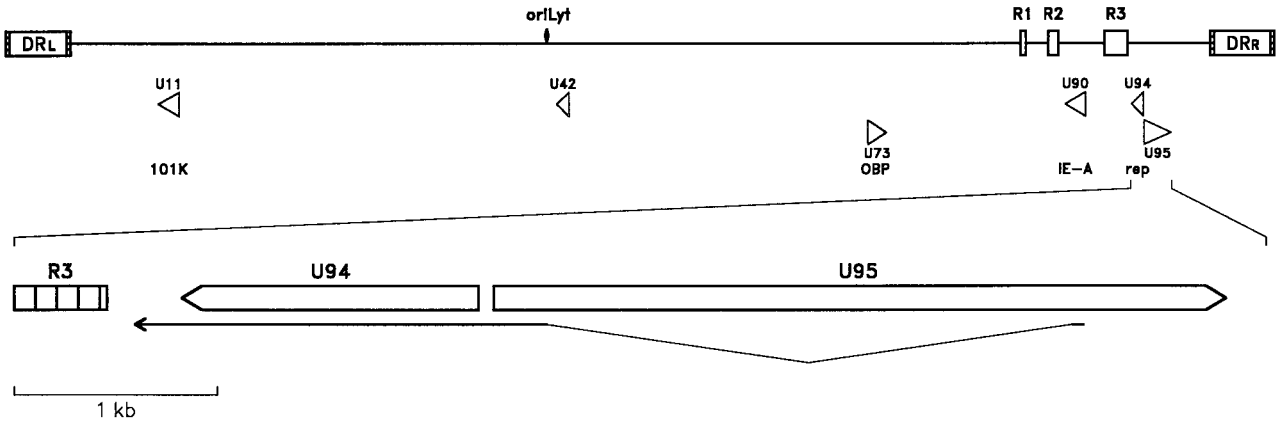


FIG. 1. HHV-6B genes and genomic regions studied in this work. Regions corresponding to the HHV-6A repeat arrays R1, R2, and R3, the left and right terminal direct repeats (DRL and DRR), and the origin of replication are shown. The structure of the spliced transcript that spans U94 is diagrammed below the enlarged U94 and U95 genes.

A

Nucleotide differences among HHV-6B isolates				
Isolate	123	471	750	801
HHV-6B Group 1: Z29	C	C	C	T
HHV-6B Group 2: R-4, R-10, J1-C, J1-E, J1-H	T	A	C	C
HHV-6B Group 3: R-15, R-24, J2-G, J2-H, J2-I, J2-J	T	A	T	C
HHV-6B J1-L	T	A	Y	C
HHV-6A's	T	C	C	C

B

Nucleotide differences among HHV-6A isolates									
Isolate	306	579	714	1062	1065	1093	1167	1215	1218
HHV-6A Group 1: U1102	C	G	C	C	C	C	C	T	G
HHV-6A Group 2: CO-1, CO-7, CO-8	T	A	T	T	A	T	A	C	A
HHV-6B's	C	A	T	T	C	T	A	C	A

Amino acid differences among HHV-6A isolates	
Isolate	389
HHV-6A Group 1: U1102	D
HHV-6A Group 2: CO-1, CO-7, CO-8	E
HHV-6B's	E

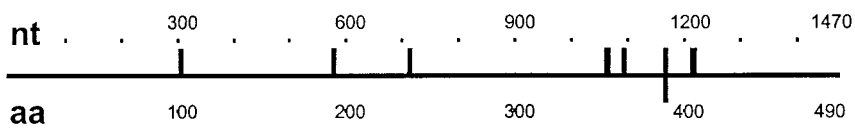


FIG. 2. Nucleotide and amino acid heterogeneity between U94 of HHV-6 isolates. (A) Nucleotide and amino acid differences among HHV-6B isolates. The corresponding sequence of the HHV-6A isolates is shown for reference. (B) Nucleotide and amino acid differences among HHV-6A isolates. The corresponding sequence of the HHV-6B isolates is shown for reference. Although in this representation, HHV-6A group 2 appears to be very closely related to HHV-6B, the complete intervariant comparison shown in Fig. 3 clearly indicates otherwise. For each section, the upper table describes nucleotide positions at which isolates differed and any corresponding amino acid changes. The lower lines are scaled graphic representations of the distribution of nucleotide and amino acid differences. The letters and number following the HHV-6A or HHV-6B designation indicate the origin of each isolate as described in Materials and Methods.

Nucleotide differences between HHV-6B and HHV-6A isolates

Isolate	21	33	76	120	123	139	153	156	306	310	314	344	381	456	471	492	540	579	609	627	656	657	661	663	714	750	751	771
HHV-6B Group 1 (Z29)	A	T	G	T	**C**	C	T	A	C	T	C	C	T	A	**C**	T	A	A	C	T	C	C	A	A	T	C	G	A
Group 2	A	T	G	T	T	C	T	A	C	T	C	C	T	A	A	T	A	A	C	T	C	C	A	A	T	C	G	A
Group 3	A	T	G	T	T	C	T	A	C	T	C	C	T	A	A	T	A	A	C	T	C	C	A	A	T	**T**	G	A
HHV-6A Group 1 (U1102)	G	C	A	C	T	T	C	G	C	C	T	G	C	G	C	C	G	**G**	T	C	T	T	G	G	**C**	C	A	G
Group 2	G	C	A	C	T	T	C	G	**T**	C	T	G	C	G	C	C	G	A	T	C	T	T	G	G	T	C	A	G

Amino acid differences between HHV-6B and HHV-6A isolates

Isolate	26	47	105	115	219	221	251
HHV-6B Group 1 (Z29)	V	P	T	T	T	R	V
Group 2	V	P	T	T	T	R	V
Group 3	V	P	T	T	T	R	V
HHV-6A Group 1 (U1102)	M	S	I	S	I	G	I
Group 2	M	S	I	S	I	G	I

Nucleotide differences between HHV-6B and HHV-6A isolates (continued)

Isolate	777	801	820	846	886	888	891	913	982	987	1011	1062	1065	1086	1093	1101	1167	1197	1215	1218	1236	1302	1306	1359	1413	1422	1461
HHV-6B Group 1 (Z29)	A	**T**	T	A	G	A	C	T	A	G	T	T	C	C	T	T	A	G	C	A	C	A	A	T	C	A	A
Group 2	A	C	T	A	G	A	C	T	A	G	T	T	C	C	T	T	A	G	C	A	C	A	A	T	C	A	A
Group 3	A	C	T	A	G	A	C	**T**	A	G	T	T	C	C	T	T	A	G	C	A	C	A	A	T	C	A	A
HHV-6A Group 1 (U1102)	G	C	G	G	C	G	T	C	G	A	C	**C**	C	T	**C**	A	**C**	A	**T**	**G**	T	C	C	C	T	G	G
Group 2	G	C	G	G	C	G	T	C	G	A	C	T	**A**	T	T	A	A	A	C	A	T	C	C	C	T	G	G

Amino acid differences between HHV-6B and HHV-6A isolates (continued)

Isolate	274	296	328	389	436
HHV-6B Group 1 (Z29)	S	E	R	E	I
Group 2	S	E	R	E	I
Group 3	S	E	R	E	I
HHV-6A Group 1 (U1102)	A	Q	G	**D**	L
Group 2	A	Q	G	E	L

FIG. 3. Nucleotide and amino acid differences between HHV-6B and HHV-6A isolates. The upper table describes nucleotide positions at which isolates differed and any corresponding amino acid changes; unique nucleotides and amino acids are bold. The lower lines are scaled graphic representations of the distribution of nucleotide and amino acid differences. The group numbers following the HHV-6A or HHV-6B designation are described in Figure 2.

ments DNA replication of Rep-deficient AAV-2 (Thomson *et al.,* 1994), regulates gene expression in cotransfection assays (Araujo *et al.,* 1995), and can prevent oncogenic transformation, possibly through a mechanism involving transcriptional regulation (Araujo *et al.,* 1997). Thus U94 has retained some of the biochemical activities of its parvovirus counterpart. Recently, transcripts spanning U94 were identified by reverse transcription–polymerase chain reaction (RT-PCR) as the only HHV-6B transcripts detected in latently infected peripheral blood mononuclear cells obtained from healthy adult donors (Rotola *et al.,* 1998). In addition, cell lines containing the HHV-6B U94 gene were refractory to productive infection by HHV-6A (Rotola *et al.,* 1998).

In this report, we describe genetic relationships in U94 among 17 HHV-6A and HHV-6B isolates, detected the U94 transcript in HHV-6B-infected cells by RT-PCR, and mapped its termini. To characterize the transcriptional regulation of the gene, the absolute amount of its transcript was compared with those of HHV-6B genes predicted to belong to various kinetic classes. The expression of these same genes was examined in the absence of *de novo* protein synthesis.

RESULTS

Genetic heterogeneity of U94 among HHV-6 clinical isolates

As a guide for future functional studies, genetic heterogeneity among U94 sequences was analyzed to assess global sequence variation in the gene and to identify regions that might be critical for U94 biochemical function. The U94 nucleotide sequence was determined from 12 low-passage HHV-6B clinical isolates and compared with that of HHV-6B(Z29) (Fig. 2A). The nucleotide sequences of all of the HHV-6B clinical isolates except J1-L belonged to one of three groups that differed at most by four nucleotides in pairwise comparisons. U94 of the 12 HHV-6B clinical isolates each had greater than 99.7% nucleotide and 100% amino acid identity with HHV-6B(Z29) U94. The nucleotide at position 750 of HHV-6B J1-L appears to be a mixture of C and T. Although not included in our analysis, the recently published HHV-6B(HST) U94 sequence (Isegawa *et al.,* 1999) differs from HHV-6B(Z29) at four nucleotide positions, with no amino acid differences.

The U94 nucleotide sequences from three HHV-6A

isolates from Cologne (Germany) were identical, differing from HHV-6A(U1102) by nine nucleotides (99.4% identity), with one resulting conservative amino acid difference (99.8% identity) (Fig. 2B). At seven of the nine positions where the HHV-6A isolates varied, the nucleotide was the same among the German HHV-6A isolates and all of the HHV-6B isolates.

As expected, there were considerably more nucleotide and amino acid differences in pairwise intervariant comparisons than in intravariant comparisons (Fig. 3). The 17 U94 sequences clearly partitioned into two variant-specific categories. In addition, within each variant, there was further segregation that corresponded to the geographical origin of the isolates, although the number of isolates studied and the high conservation preclude firm conclusions.

All HHV-6A isolates have a V-to-M substitution at amino acid 26. This sequence difference leads to a third possible translational start in HHV-6A U94 mRNA, GCCCCAUGG, that more closely corresponds to the Kozak consensus sequence (Kozak, 1989, 1991) for efficient translation initiation than two upstream AUG codon environments in this ORF. However, as shown later, we found no evidence in an *in vitro* translation system that this initiation codon is used.

U94 mRNA expression in HHV-6B(Z29)-infected cells

U94 expression was not detected in HHV-6B(Z29)-infected lymphocytes by either Northern blotting (G. Dominguez and P.E.P., unpublished data) or by immunoblotting or immunofluorescence assays with anti-peptide antibodies reactive with U94 protein expressed in recombinant baculovirus-infected insect cells (data not shown). In addition, of 60 human serum specimens tested, none had detectable antibodies to U94 in an immunoblot assay that used baculovirus-expressed U94 as antigen (data not shown). Because of the apparent low-level expression of U94, we assayed its transcription by RT-PCR. U94-specific amplification by RT-PCR was obtained only from mRNA isolated from HHV-6B(Z29)-infected cells (Fig. 4, lane 8). Hybridization of DNA from the reactions shown in Fig. 4 with a radiolabeled oligonucleotide (U94 probe, Table 1) confirmed the specificity of the amplification (data not shown). Similar results were obtained with mRNA isolated from HHV-6B(Z29)-infected umbilical CBLs (data not shown).

U94 transcript mapping

To verify that the mRNA detected above originated as U94 transcripts and to define the boundaries of the transcripts for the purpose of identifying regulatory elements, the 5′ termini were mapped by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). The nucleotide sequence of the largest DNA band (representing the farthest upstream U94 5′ terminus detected) indicated that the U94 transcript is spliced, the

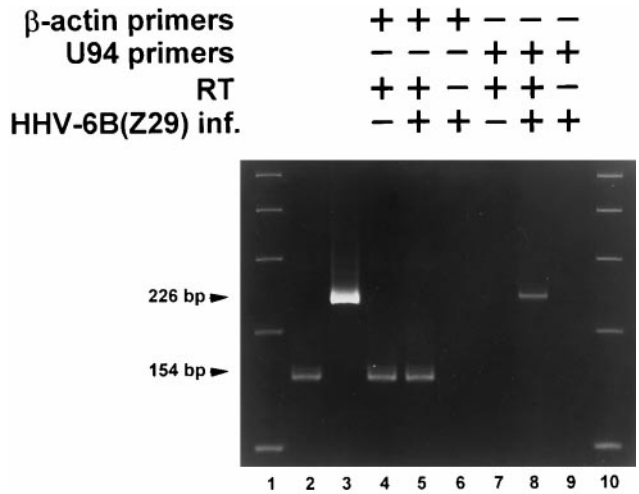


FIG. 4. RT-PCR detection of U94 transcripts. mRNA was extracted 3 days after HHV-6B(Z29) infection of Molt-3 cells. RT-PCR was conducted as described in Materials and Methods for QC RT-PCR. Lanes 1 and 10 contain 100-bp DNA ladder molecular weight standards (GIBCO BRL). Lane 2 contains the PCR product of a reaction using human β-actin sequences as primers (Inoue *et al.,* 1994) and mock-infected Molt-3 cell cDNA as template. Lane 3 contains the PCR product of a reaction using primers U94RTP5 and U94RTP3 (Table 1) and a nucleocapsid preparation of HHV-6B(Z29) DNA as template. Lanes 4–9 are RT-PCRs performed under the indicated conditions: reverse transcriptase (RT); mock-infected (−), or HHV-6B(Z29)-infected (+) Molt-3 cells.

2.6-kb intron extending from nucleotide positions 147512–144904. Of the 13 largest RLM-RACE amplimers cloned and sequenced, nine mapped to a single site (nucleotide position 147567) at an appropriate distance (25 nucleotides) downstream from a possible TATA box (Fig. 5). To confirm the existence of the U94 splice site, primers bracketing the splice site (primers 122 and 124, Table 1) were used for either PCR amplification of HHV-6B(Z29) nucleocapsid DNA or for RT-PCR of mRNA extracted from HHV-6B(Z29)-infected CBLs. The sizes of the observed amplimers were in accordance with the predicted size of the intron (data not shown). Although we did not experimentally assess their use, the splice sites are present in the HHV-6A(U1102) sequence.

The U94 3′ transcript terminus was mapped by RLM-RACE (Liu and Gorovsky, 1993) instead of the standard RACE technique because of a homopolymeric stretch of adenylic acid residues located 45 bp downstream from the U94 reading frame. A single polyadenylation site was identified at nucleotide position 142891 (Fig. 5). The DNA sequence surrounding the polyadenylation site contains consensus features of mRNA 3′ cleavage and polyadenylation signals (Fig. 5); notably, the sequence AATACA was found instead of the canonical AATAAA polyadenylation signal. Using the 3′ RLM-RACE technique, we found products with poly(A)$^+$ tails 6–10 residues long. We do not know whether the U94 poly(A)$^+$ tail lengths are

TABLE 1

Oligonucleotides Used in the Study

Gene	Primer	Primer sequence[a]	Coordinates[b]
U11	U11out5	5′-AGATGGATTTGAAAGCGGCAG-3′	22536–22517
	U11out3	5′-ACATCACGACGCGATCACTG-3′	19955–19974
	U11mut	5′-TAGAAAGAATTCCTGCAGTTAGTGATCCTC-3′	20391–20362
	U11mutrc	5′-GAGGATCACTAACTGCAGGAATTCTTTCTA-3′	20362–20391
	U11RTP5	5′-AGCCGAATTTACAAATCTGA-3′	20519–20500
	U11RTP3	5′-CGGAGACTGCACAGAATC-3′	20306–20323
U42	U42out5	5′-AAGCAAACATGTATCCTCGT-3′	71661–71642
	U42out3	5′-ACGAGAAGGTTATGACGACG-3′	69839–69858
	U42mut	5′-ACCTAACATCGATAAAAACA-3′	70353–70334
	U42mutrc	5′-TGTTTTTATCGATGTTAGGT-3′	70334–70353
	U42RTP5	5′-CATGCAGCTATTCAACAAGT-3′	70467–70448
	U42RTP3	5′-ATCTGGTATTTTGTTGTCGG-3′	70280–70299
U73	U73out5	5′-GTGTCTACACGACTGGAGGC-3′	109605–109624
	U73out3	5′-AATCGGTACTCTGTAGATCT-3′	112034–112015
	U73mut	5′-AGGCCTCGATATCAAGGCGG-3′	111858–111877
	U73mutrc	5′-CCGCCTTGATATCGAGGCCT-3′	111877–111858
	U73RTP5	5′-GTGACGGGTGTGTTTTCTAT-3′	111638–111657
	U73RTP3	5′-CAACTTCACTAACGGAAAGG-3′	111935–111916
U90	U90out5	5′-TGGATTTTTTTAATTGGTGC-3′	137692–137673
	U90out3	5′-ACATCTAGGTTTCATCTAGC-3′	134984–135003
	U90mut	5′-TAAACACTGCAGAGCCAAAC-3′	135448–135329
	U90mutrc	5′-GTTTGGCTCTGCAGTGTTTA-3′	135329–135448
	U90RTP5	5′-GCAAAACAGACTTGGAAGAC-3′	135436–135417
	U90RTP3	5′-TTCGGCTGTTACAAGATTTT-3′	135231–135250
U94	U94out5	5′-CACAAACGGTATGTTTTCCA-3′	144575–144556
	U94out3	5′-CAGGGTGAGTTATCAGCGTC-3′	142995–143014
	U94mut	5′-ATGGAATTCTAGACGGATCT-3′	143493–143474
	U94mutrc	5′-AGATCCGTCTAGAATTCCAT-3′	143474–143493
	U94RTP5	5′-CTTTTGACCCTATATTAGCAGG-3′	143637–143616
	U94RTP3	5′-ATGTCTTTCCACCACAAGAC-3′	143415–143434
	U94probe	5′-ATCCCGTTACATGGAATATTACACGGATCT-3′	143503–143474
	081	5′-TTCTCGAGTGCAAAAAAAATCCGTAGAT-3′	144442–144462
	080	5′-TTCTCGAGCCAAAAATCATCACTTGGA-3′	144530–144548
	122	5′-TTACTAGTGATCAAAACCGCGAATCACT-3′	144862–144881
	124	5′-TTGGTACCCACAGGAGAACCACCATG-3′	147617–147600
	U94PCR5	5′-TTCGTCACACGTGGACGAGATT-3′	144738–144717
	U94PCR3	5′-GTTGGATCCGTCTTATAATGTGATGTC-3′	143011–143029

[a] Underlined nucleotides were altered to generate restriction sites except for those in U90out3, which are underlined to indicate HHV-6A(U1102)-derived nucleotides.

[b] Coordinates are from the HHV-6B(Z29) genome, GenBank accession number AF157706.

truly this short or if short poly(A)$^+$ tails are preferentially amplified by 3′ RLM-RACE.

Quantification of HHV-6B(Z29) mRNA levels during lytic infection

U94 mRNA expression levels were determined both absolutely and relative to other HHV-6B(Z29) transcripts by using quantitative-competitive (QC) RT-PCR. The reference transcripts studied were chosen because of their predicted kinetic properties. U11 is predicted to be expressed as a late gene and encodes the 101-kDa virion protein (Pellett *et al.,* 1993). U42 encodes a homolog of the herpes simplex virus type 1 (HSV-1) α27 gene (Sekulovich *et al.,* 1988) and the HCMV UL69 gene (Winkler *et al.,* 1994); the HSV-1 counterpart is an α or immediate-early (IE) gene, whereas HCMV UL69 is expressed as a delayed-early gene (Winkler *et al.,* 1994). U73 encodes the origin binding protein and would be predicted to be expressed as an early gene (Inoue *et al.,* 1994). HHV-6B(Z29) U90 corresponds to the HHV-6A IE gene U89 (Schiewe *et al.,* 1994; Dominguez *et al.,* 1999). The genomic locations of these genes are shown in Fig. 1. mRNA expression was initially studied for each of these genes in a manner similar to that shown in Fig. 4 for U94 (Inoue *et al.,* 1994, data not shown). In each case, the oligonucleotide PCR primers in the RT-PCR amplified a single band specific to HHV-6B(Z29)-infected cells.

Several items deserve mention before describing the QC RT-PCR results. (1) The competitive templates were designed to resemble in length and content the mRNA

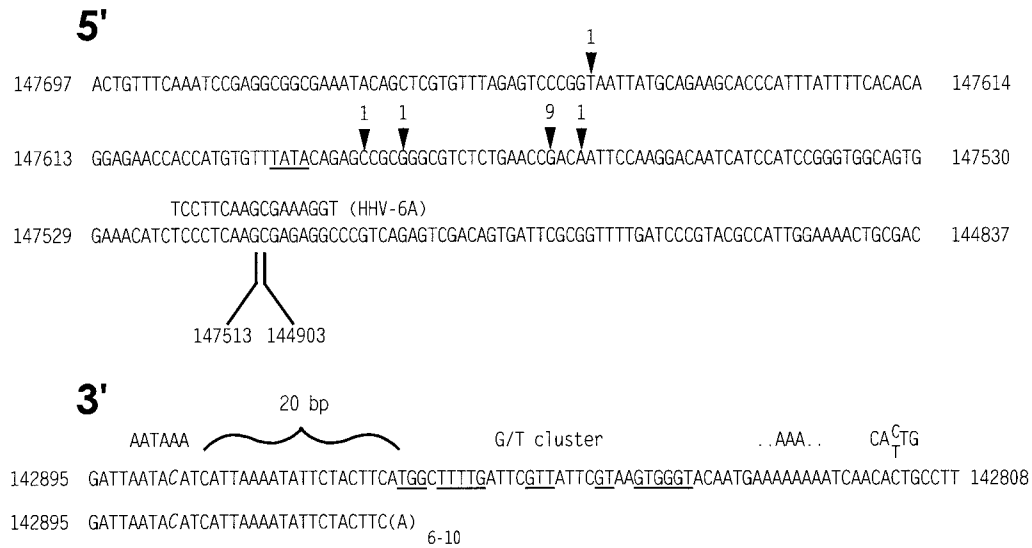


FIG. 5. U94 transcript mapping by RLM-RACE. (A) 5′ RLM-RACE products that mapped upstream of the splice site. Arrows with numbers indicate the number of sequenced oligo-cap products that mapped to a particular site. The underlined region indicates a potential "TATA box" promoter element. Coordinates for the splice site of the U94 transcript are indicated, and the corresponding HHV-6A(U1102) sequence is shown. (B) Polyadenylation site of the U94 transcript as determined by 3′ RLM-RACE. Consensus features of 3′ cleavage and polyadenylation signals are shown above the sequence (Birnstiel *et al.,* 1985). The bottom line of sequence represents the 3′ RLM-RACE products that contained poly(A)$^+$ tails (ranging in length from 6 to 10 residues). The C that varies from the fourth adenylic acid residue found in the canonical AATAAA element is highlighted, and the G/T cluster is underlined.

species predicted from ORF lengths and sequences. (2) The amplification method is limited in that it measures only steady-state mRNA levels and is unable to distinguish the strandedness of transcription across the amplified locus. The latter is because random hexamer primers were used at the cDNA synthesis step to ensure that uniform pools of cDNAs were available for amplification with the gene-specific primers. (3) The QC-RT PCR experiment described in Fig. 6A describes events that occurred in a single infected-cell culture. All of the assays at a particular time point were conducted using the same preparation of mRNA. Results obtained with the initial 10-fold and subsequent 2-fold dilution series were internally consistent, as were independent repeat anal-

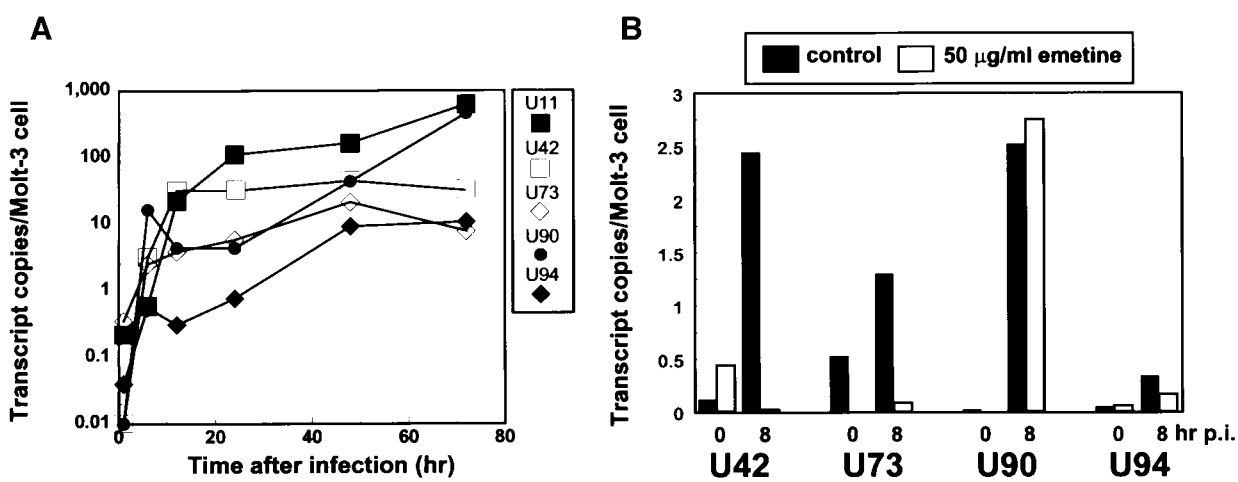


FIG. 6. Time course of transcript accumulation and dependence on *de novo* protein synthesis. (A) Transcript kinetics. U11, U42, U73, U90, and U94 mRNA levels were measured by QC RT-PCR at various times after synchronized infection of Molt-3 cells. The lower limit of quantitation was 0.01 transcript copy/cell (approximately 10^4 transcript copies/reaction). Transcript copies/cell values are based on the total number of cells used for RNA extraction and do not directly reflect the percent of cells infected. (B) Effect of protein synthesis inhibition on synthesis of U42, U73, U90, and U94 mRNAs. Molt-3 cells were treated with 50 μg/ml emetine before HHV-6B(Z29) infection as described in Materials and Methods. mRNA was extracted at 0 and 8 h p.i., and transcript copy numbers were determined by QC RT-PCR. Transcript copies/cell values are based on the total number of cells used for RNA extraction and do not reflect percent infectivity.

yses on several of the samples (data not shown). (4) The results shown in Fig. 6A are consistent with independent experiments that followed slightly different time courses (data not shown).

As shown in Fig. 6A, mRNA levels for the HHV-6B(Z29) genes studied varied by as much as two orders of magnitude by 74 h postinfection (p.i.). U11, which encodes an abundantly expressed structural gene, was expressed at the highest levels in comparison to the other genes as early as 26 h p.i., reaching approximately 600 copies per cell by 74 h p.i. of Molt-3 cells. For U42, a putative HHV-6 transcriptional regulator, mRNA levels increased sharply (approximately 10-fold) within the first 14 h of infection and then remained at approximately 30 copies per cell for the 14- to 74-h time course. U73 transcripts, which encode a protein involved in DNA replication, were among the least abundant. Levels of these transcripts increased steadily to a maximum of approximately 20 copies per cell at 50 h p.i. and declined to less than 10 copies per cell by 74 h p.i. Typical of an IE gene, U90 mRNA levels increased rapidly to approximately 16 copies per cell during the first 8 h of infection, decreased approximately 4-fold during the next 6 h, and then increased during the remainder of the time course to reach a maximal level of approximately 400 copies per cell by 74 h p.i. U94 mRNA levels remained below approximately 1 copy per cell until 50 h after infection, reaching a maximum of approximately 10 copies per cell at 74 h after infection. It should be noted that only mRNA levels were measured by QC RT-PCR and not protein levels, although in other experiments that used similar materials, immunoblots showed high levels of U11 protein (Pellett *et al.,* 1993), moderate levels of U42 and U90, and undetectable levels of U73 and U94 (N.I., C. Ko, and P.E.P., unpublished data).

Effect of protein synthesis inhibition on HHV-6B(Z29) mRNA synthesis

We studied the dependence of U94 transcription on *de novo* protein synthesis. As was done earlier for transcript quantification, several genes expected to represent different kinetic classes were included as controls.

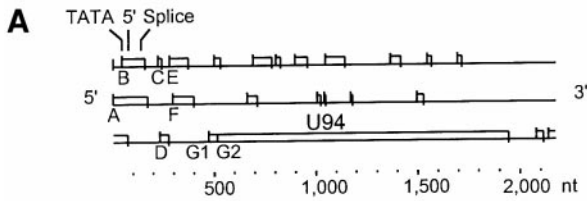
U90 transcripts accumulated to the same levels in either the absence or presence of *de novo* protein synthesis (Fig. 6B), extending to HHV-6B the results of Schiewe *et al.* (1994), who reported that HHV-6A(U1102) U89 is transcribed in the presence of cycloheximide. In the presence of emetine, U42 transcripts did not accumulate, indicating that U42 is not expressed as an IE gene in Molt-3 cells. In contrast, HSV-1 α27 is expressed as an IE gene (Sekulovich *et al.,* 1988). The HCMV homolog of this gene, UL69, is expressed as an early-late gene (Winkler *et al.,* 1994), suggesting that HHV-6B U42 is more closely related to HCMV UL69 than to α27 in its expression kinetics. U73 transcripts did not accumulate under conditions of inhibited protein synthesis, which is consistent with the presumption that as a gene involved in DNA synthesis, it would not be expressed as an IE gene (Fig. 6B). For U94, the results were equivocal due to the low copy numbers ($<$1 transcript per cell) of U94 mRNA extracted from control cells at 8 h p.i. (data not shown). Therefore, unlike the other HHV-6 transcripts examined, the expression kinetics of U94 could not be definitively classified.

Coding content of the HHV-6B(Z29) U94 mRNA sequence and its translation initiation environment

Figure 7A shows the open reading frames present in the mRNA species deduced from the mRNA mapping studies. The mRNA sequence differs from most herpesvirus mRNAs and mRNAs that encode efficiently expressed genes by the presence of several small AUG-initiated ORFs upstream from U94. When the sequences surrounding the potential translation initiation codons are compared with the Kozak consensus sequence for efficient translation initiation (Kozak, 1989), two sites (G1 and G2) at or near the 5′ end of U94 do not appear to qualify as efficient sites, and none of the others fully match the consensus (Fig. 7A). The combination of multiple upstream AUG-initiated small ORFs and a suboptimal translation environment for the expressed protein is commonly seen for genes that function near the beginning of regulatory cascades or whose expression at high levels might be deleterious to the cell (Kozak, 1991). Two of the upstream AUG-initiated small ORFs are not present in HHV-6A, but they are replaced with two not represented in HHV-6B (not shown). Further, as described earlier, a third downstream start codon only in the HHV-6A U94 ORF has a well conserved Kozak consensus sequence.

We compared the preference of codon use for translation initiation between HHV-6A and HHV-6B *in vitro* in a coupled transcription-translation system. Major products of approximately 54 and 56 kDa were present in reactions programmed with plasmids encoding full-length U94 derived from HHV-6A(U1102) and HHV-6B(Z29) in addition to a low-passage clinical isolate of each variant (Fig. 7B, lanes 2–6). These protein mobilities correspond to those predicted from G1 and G2 start codon use in Fig. 7A. The largest product predicted to initiate from the G1 codon has an identical mobility to baculovirus-expressed U94 (data not shown). There was no evidence of a lower-mobility product initiating from the third in-frame start codon in the reactions programmed with HHV-6A-derived U94 (Fig. 7B, lanes 2–3).

To determine whether the smaller protein present in all lanes might be due to C-terminal proteolysis or premature translation termination, we evaluated the products of a coupled *in vitro* transcription translation reaction programmed with a plasmid encoding a C-terminally trun-



ORF	Initiation Context	aa in ORF	Genome Coordinate
A	GuaAuuAUGc	23	147642
B	aCCACCAUGu	38*	147602
C	uCCAguAUGa	6	144816
D	cCGcCgAUGu	13	144805
E	cguAaaAUGa	31	144759
E	aaaAugAUGu	30	144756
F	GCuGCgAUGu	31	144743
G1	aaCGguAUGu	490	144565
G2	uauAuCAUGu	472	144511
Kozak	GCCRCCAUGG		

* ORF crosses splice site

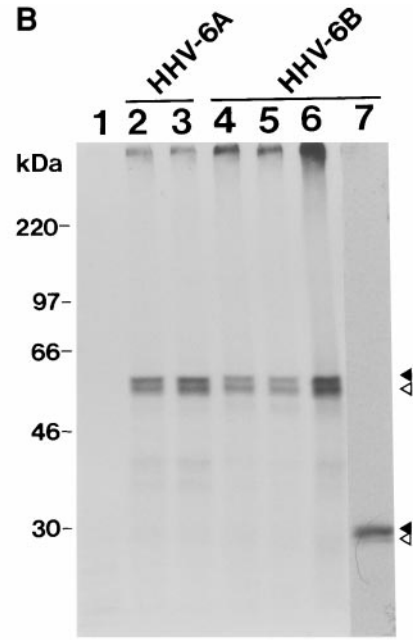


FIG. 7. Translation initiation environment and codon use of U94 mRNA. (A) Coding capacity and translation initiation environment of the spliced U94 mRNA. The upper panel shows AUG-initiated open reading frames in each of the three translational reading frames of the longest mRNA species mapped. The location of a putative TATA box, the most abundant 5′ end, and the splice site are indicated. The lower panel shows the initiation codon context of the upstream and U94 potential initiation codons for ORFs A though G (G is U94) relative to the Kozak consensus sequence for efficient translation initiation environments (Kozak, 1989). The scale coordinates are relative to the 5′ end of the longest cDNA species. (B) *In vitro* expression of HHV-6 U94. Coupled transcription-translation reactions programmed without added DNA (lane 1) or with plasmids encoding full-length U94 derived from HHV-6A (isolates U1102 and CO-1, lanes 2 and 3) and HHV-6B (isolates Z29 and R-24, lanes 4 and 5 and pH6Z-4201, lane 5) or C-terminally truncated HHV-6B U94 (pH6Z-4204, lane 7) were incubated in the presence of ^{35}S-methionine. One tenth of the labeled protein from each reaction was mixed with loading buffer, analyzed by SDS–PAGE, and detected by fluorography. Expected bands corresponding to protein products derived from the G1 and G2 codons are indicated with closed and open symbols, respectively. Lane 7 was exposed for a shorter time period to enable better resolution of the two products.

cated U94. Truncated proteins of approximately 27 and 29 kDa were expressed, consistent with translation initiation from the G1 and G2 start codons (Fig. 7B, lane 7). A second C-terminal mutant with a different deletion also gave the predicted truncated products (data not shown). Mutagenesis of the second start codon is required to address the minor possibility of N-terminal proteolysis.

DISCUSSION

In this work, we studied sequence heterogeneity of HHV-6 U94 genes and transcriptional regulation of HHV-6B U94. We found (1) high intervariant and intravariant sequence conservation, including 100% identity among 13 HHV-6B isolates from three continents; (2) that U94 transcripts are spliced; (3) that U94 transcripts are expressed at very low levels during infection; and (4) that the environment for translation initiation appears to have been designed for inefficient translation. In addition to the similarity of U94 with its parvovirus homologs, these properties support the hypothesis that the U94 protein has a role in gene regulation.

The variant-specific classification of HHV-6 isolates as HHV-6A or HHV-6B is based on their differential ability to grow in some cell types, patterns of reactivity with panels of monoclonal antibodies, and epidemiology (Ablashi *et al.,* 1993). These properties are rooted in the genetic divergence revealed by heterogeneity of restriction endonuclease restriction patterns and nucleotide sequences. It was relevant to examine geographically distinct isolates of each variant for genetic heterogeneity in U94, a gene unique to HHV-6A and HHV-6B among herpesviruses. There was high conservation at both the nucleotide and amino acid levels. Based on U94 amino acid sequences, the isolates studied segregated into variant-specific groups consistent with previous classifications based on markers such as restriction endonuclease profiles, reactivity with monoclonal antibodies, and cell tropism (Pellett *et al.,* 1990; Ablashi *et al.,* 1991; Pruksananonda *et al.,* 1992). The high degree of genetic conservation of U94 suggests that it provides a critical function in the HHV-6 life-cycle nearly intolerant of sequence variation. The existence of easily distinguished variant versions of the U94 gene, however, further supports the consideration of HHV-6A and HHV-6B as separate entities that have followed divergent evolutionary pathways.

Transcription mapping demonstrated that U94 transcripts are spliced during an HHV-6B infection. The U94 splicing signals are conserved between HHV-6A and HHV-6B. Splicing of herpesvirus transcripts is not common, occurring most often in transcripts encoding regulatory proteins. Herpesvirus mRNA splicing is a potential target for transcriptional regulation by viral gene products or by infection-induced modification of the host cell's splicing mechanism. It will be interesting to determine whether there are variant-specific differences in U94 transcript splicing and expression kinetics.

To identify promoter/regulatory elements that might control the expression of U94, the 5′ end of its transcript was mapped. The 5′ terminus is at a distance from the ORF because of a 2.6-kb intron present in the 5′ leader sequence. The most abundant 5′ terminus mapped 25 nucleotides downstream from a TATACA sequence that potentially encodes a TATA box. The sequence upstream from the TATA sequence contains potential binding sites for several transcription factors, including SP1, PEA3, and AP-2. Further experiments will be needed to confirm this 5′ region as the true U94 promoter.

At most time points after infection, U94 transcripts were the least abundant of the five genes studied. A negative regulatory mechanism of U94 expression may be used to maintain the low abundance of U94 mRNA. The low transcript levels, coupled with the apparently inefficient translation initiation environment, are consistent with our inability to detect U94 protein. Our results suggest that the U94 protein may be required in only small amounts during infection.

Mirandola *et al.* (1998) published a study of HHV-6 transcription. Our results differ from theirs in several ways. Of the genes studied in common, Mirandola *et al.* found that U42, U73, U89, and U94 are expressed as IE genes, in comparison with our observations that U90 (previously identified as U89) is expressed as an IE gene, U42 and U73 expression is dependent on *de novo* protein synthesis, and U94 was expressed at levels too low to allow unambiguous determination of its kinetic class. The differences in the results may have resulted from the use of qualitative PCR versus quantitative PCR. In our work, levels of expression were measured by QC RT-PCR, with the results being expressed as copies per cell. In the study by Mirandola *et al.* (1998), results were obtained by visual inspection of ethidium bromide-stained gels. Perhaps more importantly, Mirandola *et al.* (1998) used 35 cycles of PCR to amplify their RT products; we used the fewest number of cycles that produced readily detectable PCR products to remain in the exponential phase of amplification. It is possible that small numbers of "leaky" transcripts were amplified to the point of visibility by the end of 35 amplification cycles. For better or worse, our results do not require the development of any new paradigms for herpesvirus gene regulation; in our hands, the DNA replication (U73) and structural (U11) genes studied required *de novo* protein synthesis for their expression, as do such genes in other herpesviruses. U42, which is homologous to HCMV U69 and the HSV-1 α27 gene, was not expressed in the absence of *de novo* protein synthesis; this behavior is similar to its more closely related HCMV counterpart than to its more distantly related HSV homolog.

The U94 transcript is spliced and expressed at low levels and contains a 5′ mRNA upstream environment that is likely to result in inefficient U94 translation. This indicates that U94 expression is tightly regulated. Numerous questions surround the function of U94, which is a homolog of the parvovirus *ns*1 (rep) protein, a protein with well defined transcriptional regulatory properties. Several promoters can be regulated by both $Rep_{AAV\text{-}2}$ and U94, indicating that these proteins share at least some functional properties. These proteins have numerous similarities at the amino acid level in regions required by $Rep_{AAV\text{-}2}$ for NTP binding, helicase, and endonuclease activity (Walker *et al.,* 1997a, 1997b; Davis *et al.,* 1999). This suggests that U94 may have domains required for DNA binding and helicase activity. Such activities are likely to play a role in its regulatory function and also suggest possible roles in DNA replication and perhaps rare occurrences of HHV-6 genomic integration (Daibata *et al.,* 1998; Morris *et al.,* 1999). The lack of a U94 homolog in the otherwise closely related HHV-7 indicates U94 may have a significant role in HHV-6 pathogenesis.

MATERIALS AND METHODS

Viruses and cell culture

Fifteen low-passage HHV-6 clinical isolates from four distinct geographic regions were used for U94 heterogeneity analysis. The commonly studied isolates, HHV-6A(U1102) and HHV-6B(Z29), originated in Africa (Uganda and Zaire, respectively) (Downing *et al.,* 1987; Lopez *et al.,* 1988). HHV-6A isolates CO-1, CO-7, and CO-8 were from Cologne, Germany (Ablashi *et al.,* 1991), and were gifts from Dr. Bala Chandran. HHV-6B isolates R-4, R-10, R-15, and R-24 were obtained in Rochester, NY, and were gifts from Dr. Caroline Hall (Pruksananonda *et al.,* 1992). HHV-6B isolates J1-C, J1-E, J1-H, and J1-L (Pellett *et al.,* 1990) were obtained from Japanese exanthem subitum patients in the vicinity of Osaka, Japan, and were gifts from Dr. Koichi Yamanishi. HHV-6B isolates J2-G, J2-H, J2-I, and J2-J were obtained from Japanese exanthem subitum patients in northern Japan and were gifts from Dr. Kazuo Yanagi. Viruses were cultured as previously described (Black *et al.,* 1989).

For mRNA studies, HHV-6B(Z29) was propagated in human umbilical cord blood lymphocytes (CBLs) as previously described (Black *et al.,* 1989) or in Molt-3 cells that were cultured in the same medium as that used for CBLs except no phytohemagglutinin or inter-

leukin-2 was used. Cells were infected with a cell-free virus stock at a multiplicity of infection (m.o.i.) of 0.15. After 2 h, inoculation medium was replaced with fresh medium. Typical infectivities at 72 h p.i. were 23–33% for Molt-3 cells and 12–17% for CBLs as determined by immunofluorescence with a monoclonal antibody against HHV-6 p41 (Advanced Biotechnologies, Inc., Columbia, MD).

Plasmid construction and sequence analysis

For the study of U94 genetic heterogeneity among clinical isolates, HHV-6 DNA was prepared as previously described (Straus *et al.,* 1981; Kadakia *et al.,* 1996; Lindquester *et al.,* 1996). A 1735-bp product containing the 1470-bp U94 ORF was amplified using primers U94PCR5 and U94PCR3 (Table 1). Proofreading polymerases were used to reduce possible errors arising during PCR. To decrease contamination risks, PCR reactions were prepared in a biological safety cabinet that had been UV irradiated for several hours before use, located on a different floor from the template preparation area.

Amplified DNA fragments were blunt-ended by *Pfu* DNA polymerase (Stratagene, La Jolla, CA) treatment and ligated to *Srf*I-digested pCR-Script according to the manufacturer's recommendations (Stratagene) and then cycle sequenced using fluorescent dye terminators (Applied Biosystems, Foster City, CA). The sequence of both strands was determined with an overall 4-fold redundancy. Sequences were analyzed using GCG Version 9.0 (GCG, Madison, WI). Unique nucleotide sequence changes were confirmed by resequencing the original amplified material as well as by sequencing a second clone. The integrity of this approach was verified by independently reproducing the previously determined U94 nucleotide sequences of HHV-6A(U1102) (Thomson *et al.,* 1991) and HHV-6B(Z29) (Dominguez *et al.,* 1999).

For use in the *in vitro* translation analysis, pH6Z-4201 and pH6Z-4204 were constructed as follows. pH6Z-204 containing the HHV-6B(Z29) *Hin*dIII-C fragment (Lindquester *et al.,* 1996) was digested with *Xba*I and *Kpn*I. The *Xba*I–*Kpn*I fragment was inserted between the *Xba*I and *Kpn*I site of pBluescript II KS(+) (Stratagene) to construct pH6Z-4201. pH6Z-4201 was digested with *Mlu*I, treated with T4 DNA polymerase in the presence of all four dNTPs, and re-ligated to construct pH6Z-4204. This results in the generation of a frameshift mutation at amino acid 246 and an in-frame termination codon at amino acid 253.

RNA extraction

Total RNA was extracted from 10^7 viable cells (as determined by trypan blue exclusion) with guanidinium thiocyanate–phenol (RNAzol B; CINNA/BIOTECX Laboratories International Inc., Friendswood, TX). mRNA was purified from total RNA on an oligo(dT) matrix (QuickPrep Micro mRNA Purification Kit; Pharmacia, Piscataway, NJ) and then stored at −70°C.

Transcript mapping

The 3′ end of the U94 transcript was determined by RLM-RACE (Liu and Gorovsky, 1993). Briefly, 1 μg of mRNA extracted from CBLs 7 d p.i. was DNase treated (RQ-1; Promega, Madison, WI) for 50 min at 37°C. DNase was inactivated at 90°C for 5 min. Phosphorylated SK oligonucleotide (Stratagene) was ligated to the RNA sample with 100 U of T4 RNA ligase (New England BioLabs, Inc., Beverly, MA). First-strand cDNA synthesis was performed using 2 pmol of the reverse complement of SK oligomer (RCOFSK) and 200 U of reverse transcriptase (RT) (SuperScript II; GIBCO BRL, Gaithersburg, MD). The initial round of PCR was performed with 1/50th of the RT reaction as template, *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT), and primers U94RTP5 (Table 1) and RCOFSK. Cycling conditions were 93°C for 1 min, 48°C for 1 min, and then 72°C for 2 min, for a total of 35 cycles. For the second round of PCR, 1/100th of the primary PCR was used as template with U94mut (Table 1) and RCOFSK as primers. Cycling conditions were identical to the initial round of PCR except only 30 cycles were used. Bands were eluted from a 2% agarose gel, cloned into pBluescript KS(−), and then sequenced as described earlier.

The 5′ end of the U94 transcript was mapped by oligo-capping (Maruyama and Sugano, 1994). One microgram of mRNA was DNase treated as described for 3′ end mapping, incubated with 1 U of thermolabile phosphatase (HK phosphatase; Epicentre, Madison, WI), heat-inactivated, and then extracted with phenol and chloroform. After centrifugally dialyzing twice, the RNA sample was treated with 5 U of tobacco acid pyrophosphatase (Epicentre), extracted with phenol and chloroform, and then dialyzed centrifugally. Gel-purified RNA oligonucleotides transcribed from *Eco*RI-linearized pBluescript KS(−) (MEGAscript; Ambion, Austin, TX) were ligated to the RNA sample with 100 U of T4 RNA ligase (New England BioLabs) and then incubated with 200 U of RT and 2.5 μM random hexamers (GIBCO BRL). The initial round of PCR was performed with one fifth of the RT reaction as template and with primers 081 (Table 1) and SK. Thermal cycling conditions were 93°C for 1 min, 50°C for 1 min, and then 72°C for 1 min, for a total of 30 cycles. Second-round PCR was performed with one fifth of the initial round PCR products as template and with primers 080 (Table 1) and SK. Thermal cycling conditions were identical to the initial round of PCR. Amplimers were purified, cloned, and sequenced as described earlier.

Quantitative competitive RT-PCR (QC RT-PCR)

Templates for the production of competitor RNAs were prepared by the introduction of restriction site mutations into U11, U42, U73, U90, and U94 genes by recombinant PCR (Higuchi *et al.,* 1988). The resulting PCR products containing the restriction site mutation (listed in Table 1) were cloned into pBluescript KS(−) by T-vector ligation (Marchuk *et al.,* 1991). Linearized competitor plasmids were *in vitro* transcribed at 25°C (MEGAscript), yielding predominantly full-length transcripts as determined by agarose gel electrophoresis of samples treated with formaldehyde and ethidium bromide (Liu and Chou, 1990).

Quantification of mRNA was done in two steps (Ramakrishnan *et al.,* 1994). The first used a 10-fold dilution series of the competitor template in the presence of a constant amount of infected-cell mRNA and was intended to provide an approximation of the abundance of an RNA; the second used a 2-fold dilution series centered around the approximate value to obtain a more precise value. Briefly, competitor RNAs were suspended in 7 μg/ml yeast tRNA (Sigma Chemical Co., St. Louis, MO). Infected-cell and competitor RNA dilutions were then treated with DNaseI for 1 h at 37°C and 6 min at 90°C and then immediately placed on ice. RT reactions were performed on the products of the DNaseI reactions with random hexamer primers (GeneAmp RNA PCR kit; Perkin–Elmer Cetus, Norwalk, CT). PCRs were performed according to the kit protocol with the addition of 1 μl of [α-^{32}P]dCTP (3000 Ci/mmol) and primer sets designated by the RTP5 and RTP3 suffixes in Table 1. Thermal cycling parameters were 2 min at 95°C for 1 cycle, 1 min at 95°C, and 1 min at 60°C for 20–27 cycles and then 7 min at 60°C for 1 cycle. The fewest number of cycles that produced readily detectable PCR products were used to remain in the exponential phase of amplification and to avoid heteroduplex formation. Aliquots of 15 μl of each coamplified RT-PCR were removed and digested with the appropriate restriction endonuclease (Table 1) overnight at 37°C to allow discrimination of infected-cell RT-PCR products from mutant competitor RT-PCR products. Products were then separated by electrophoresis on an 8% polyacrylamide gel and quantified (Betascope 603 Blot Analyzer, Betagen, Mountain View, CA). Controls consisted of an RT-PCR in which RT was absent from the reaction and an RT-PCR with mock-infected cell mRNA as template. Equivalence points were computed as described previously (Piatak *et al.,* 1993), taking into account the dCTP content of the amplimers.

Protein synthesis inhibitor treatments

Various concentrations of the protein synthesis inhibitors emetine, cycloheximide, or anisomycin (Mackem and Roizman, 1981) were tested to determine optimal conditions for protein synthesis inhibition in HHV-6B(Z29)-infected Molt-3 cells. Because treatment with 50 μg/ml emetine had the least residual protein synthesis (8%) and the least effect on cell viability, these conditions were used in subsequent experiments.

In vitro translation

U94 protein was expressed *in vitro* in coupled transcription-translation reactions (Promega) programmed with 2 μg of plasmid DNA. U94 from HHV-6A isolates U1102 and CO-1 and HHV-6B isolates Z29 and R-24 was expressed from plasmids used for the genetic heterogeneity study. ^{35}S-Labeled *in vitro*-translated products were mixed with an equal volume of loading buffer containing 2% SDS and 1.8 M 2-mercaptoethanol and separated on a 10% SDS–polyacrylamide gel. Gels were visualized by fluorography as described previously (Inoue *et al.,* 1994).

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