Microarray-based determination of the lytic cascade of human herpesvirus 6B

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The lytic gene expression of several members of the human herpesvirus family has been profiled by using gene-expression microarrays; however, the lytic cascade of roseoloviruses has not been studied in similar depth. Based on the complete DNA genome sequences of human herpesvirus 6 variant A (HHV-6A) and variant B (HHV-6B), we constructed a cDNA microarray containing DNA probes to their predicted open reading frames, plus 914 human genes. Gene-expression profiling of HHV-6B strain Z29 in SupT1 cells over a 60 h time-course post-infection, together with kinetic classification of the HHV-6B genes in the presence of either cycloheximide or phosphonoacetic acid, allowed the placement of HHV-6B genes into defined kinetic classes. Eighty-nine HHV-6B genes were divided into four different expression kinetic classes: eight immediate-early, 44 early, 33 late and four biphasic. Clustering of genes with similar expression profiles implied a shared function, thus revealing possible roles of previously uncharacterized HHV-6B genes.

INTRODUCTION

Human herpesvirus 6 (HHV-6) is a betaherpesvirus, first isolated in 1986 from individuals with lymphoproliferative disorders (Salahuddin et al., 1986). Primary infection occurs in early childhood (Hall et al., 1994), when it can cause febrile illnesses including exanthem subitum (ES) (Yamanishi et al., 1988; Zerr et al., 2005). Following initial infection, HHV-6 establishes lifelong latency in the host, with monocytes (Kondo et al., 1991) and bone-marrow progenitor cells (Luppi et al., 1999) being suggested as the sites of latency. HHV-6 reactivation in immunocompromised hosts can be pathogenic, particularly in the transplant setting, and has been associated with encephalitis, bone-marrow suppression and graft rejection (Clark & Griffiths, 2003).

Two variants of HHV-6 exist (-6A and -6B), each with distinct genetic, antigenic and biological properties (Pellett & Black, 1996; Schirmer et al., 1991). HHV-6A is not associated consistently with disease, but HHV-6B is the cause of ES in childhood (Dewhurst et al., 1993; Yamanishi et al., 1988; Zerr et al., 2005). Publication of the complete DNA genome sequences of HHV-6A and -6B (Domínguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999) confirmed the close relatedness of the two variants.

The ArrayExpress accession numbers for the microarray data generated in this paper are E-MEXP-2129 and E-MEXP-2130.

Supplementary methods, figures, tables and references are available with the online version of this paper.

However, significant sequence variations observed at the right end of the unique region, plus the existence of variant-specific open reading frames (ORFs), may explain the differential properties described for HHV-6A and -6B (Clark, 2000).

As HHV-6 replicates predominantly in CD4+ T cells in vitro and in vivo (Lopez et al., 1988; Lusso et al., 1988), there is interest in the regulation of viral gene expression in its target cell type. We have previously described the use of DNA microarrays for expression profiling of two gammaherpesviruses, Kaposi’s sarcoma-associated herpesvirus (Jenner et al., 2001) and murine gammaherpesvirus 68 (Ahn et al., 2002), and demonstrated the functional predictions of uncharacterized viral genes and transcription from intergenic regions. By adopting the same methodology, we report here the characterization of the global transcription programme of HHV-6B.

METHODS

Virus and cells. HHV-6B (Z29) (Lopez et al., 1988) was grown and analysed in the CD4+ cell line SupT1 (see Supplementary Methods, available in JGV Online, for details). Cell-free virus inoculum was prepared by using the freeze–thawing method as described by Miranda et al. (1998) with modifications (Supplementary Methods). HHV-6 infectivity was determined as described by Gravel et al. (2002) with modifications. Briefly, three independent infections were performed whereby 5 × 10⁶ SupT1 cells were exposed to 1 ml virus inoculum for 1 h, washed and incubated at 37 °C for 60 h. Infected cells were fixed in cold acetone for 10 min, air-dried
and reacted with murine anti-gp116 antibodies (Advanced Biotechnologies) for 40 min at 37 °C. After three 5 min washes in PBS, cells were reacted with fluorescein isothiocyanate-conjugated anti-mouse antibodies (Sigma) for 40 min at 37 °C. This was followed by another three 5 min washes in PBS, and the slides were mounted and observed under an Olympus fluorescent microscope. The mean percentage of gp116-positive cells at 60 h post-infection (p.i.) was approximately 32 %. As 32 % of the starting number of cells mounted and observed under an Olympus fluorescent microscope, followed by another three 5 min washes in PBS, and the slides were observed under an Olympus fluorescent microscope.

**Virus infection.** SupT1 cells (5 × 10⁶) were suspended in HHV-6B (Z29) inoculum to give an m.o.i. of 0.5. After 1 h adsorption at 37 °C, cells were washed twice with PBS and resuspended in complete medium at a concentration of 5 × 10⁵ cells ml⁻¹. The infected cells were incubated in 5 % CO₂ at 37 °C and harvested at 6, 12, 24, 36, 48 and 60 h p.i. For the classification of immediate-early (IE) genes, SupT1 cells were first treated with cycloheximide (CHX, 200 μg ml⁻¹; Sigma) for 1 h prior to infection. The infection and subsequent incubation were carried out in the continual presence of CHX and the infected cells were harvested at 6 h p.i. For the classification of early (E) and late (L) genes, the infected cells were cultured in the presence of phosphonoacetic acid (PAA, 500 μg ml⁻¹; Sigma) and harvested at 36 h p.i. All cells were lysed in TRIzol (Invitrogen), frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction.

**RNA preparation and cDNA labelling.** Both procedures were described as performed previously (Jenner et al., 2001; Supplementary Methods). Cy5-labelled cDNA was mixed 1:1 with Cy3-labelled cDNA synthesized from a custom-made universal reference containing pooled mRNA from nine different cell lines in the following percentages: U937 and HeLa, each at 21.1%; Ramos and SSCEM, each at 12.6%; HuH7, 8.4%; MRC5, 4.2%; SupT1 infected chronically with HHV-7, 10%; with HHV-6A, 5%; and with HHV-6B, 5%. This universal reference provides a controlled Cy3 signal in the majority of the array probes for calculating the expression ratios (Cy5/Cy3), especially for the HHV-6 genes (expression ratios derived from infected versus mock-infected samples are not reliable, due to the absence of viral RNA in the latter). As the same universal reference was used in each array hybridization, data normalization between different arrays could be achieved.

**HHV-6 array.** DNA fragments of approximately 300 bp were amplified from the 5’ end of predicted ORFs of HHV-6 and from genomic regions (>300 bp) that are predicted to be non-coding and devoid of long stretches of repetitive elements for the intergenic probes. All primers (see Supplementary Table S1, available in JGV Online) were derived from the published sequence for HHV-6A (U1102; GenBank accession no. NC_001664) (Gompels et al., 1995) and HHV-6B (Z29; accession no. NC_000898) (Dominguez et al., 1999) by using the Primer 3.0 program (Rozen & Skaltsky, 2000). Primers were selected to avoid amplifying regions where the predicted ORFs overlap (see Fig. 6 for the location of individual HHV-6B ORFs and intergenic probes). The majority of the primers support the amplification of both HHV-6A and -6B; for these ORFs, the HHV-6B (Z29) DNA was used as template. Variant-specific primers were designed and the respective DNA [HHV-6A (U1102) or HHV-6B (Z29)] was used for PCR amplification of array elements for ORFs that exhibited low sequence identity (see Supplementary Table S1, available in JGV Online). All PCR products were cloned into the pGEM-T Easy vector (Promega) and their sequences were verified. These clones were used for generating the HHV-6 array probes by PCR using common vector primers flanking the cloning site. In addition, 914 array probes to host genes were PCR-amplified from the IMAGE lymphochip clone set (MRC Genservice). All PCR products were analysed by gel electrophoresis. Amplicons were purified and concentrated by using a Montage PCR filter plate (Millipore) and the DNA was resuspended in betaine buffer [3 x SSC (0.3 M NaCl+0.03 M sodium citrate) + 1.5 M betaine; Sigma] at approximately 200 ng ml⁻¹.

**Microarray fabrication, hybridization, scanning and data analysis.** The DNA microarrays for HHV-6 were constructed and used as described previously (Wilson et al., 2007). Briefly, both the host and HHV-6 array probes were spotted onto aminoanilide slides (Schott) in triplicate. The array slides were post-processed and hybridized with a Cy5/Cy3 mixture for 20 h. Microarrays were washed, dried and scanned. Data were extracted from microarray image files by using GENEPIX PRO 4.0 software (Axon Instruments). The signal-to-noise ratios for individual spots were calculated by dividing the spot signal by the local background. The log₂ median of ratios (Cy5/Cy3) was filtered to remove all flagged data and spots with signal-to-noise ratios of <2 in both the Cy3 and Cy5 channels. The median expression ratio from the triplicate spots of each array probe was used for subsequent analysis. Microarray data of all samples were assembled into a matrix, filtered for genes present in all the arrays and median-centred by Cluster (Eisen et al., 1998) to normalize across genes and samples. To unmask the temporal order of the viral genes, microarray data of all the host genes were removed from the normalized matrix and readjusted by Cluster to reorganize the viral genes. Data were grouped by average linkage hierarchical clustering, using the uncentred Pearson correlation coefficient as the similarity metric. The ordering of the nodes produced by clustering data from 11 arrays (or 13 including two arrays for sample PAA) was first determined in Cluster by using a one-dimensional self-organizing map with the number of nodes set to n. The clustered data were visualized by using TreeView (Eisen et al., 1998). The microarray data are publicly available under ArrayExpress accession numbers E-MEXP-2129 and E-MEXP-2130.

**RT-PCR.** The primers used for RT-PCR are listed in Supplementary Table S1. Oligonucleotide dT-primed cDNA, prepared by using a Sensiscript kit (Qiagen), was used to PCR-amplify the gene of interest. PCRs were performed by using a HotStar Taq kit (Qiagen). Each reaction comprised 1 × PCR buffer, 200 μM each dNTP, 0.4 μM forward and reverse primers, 2.5 U HotStar Taq DNA polymerase and either the cDNA or no-RT control. After an initial denaturation at 95 °C for 15 min, conditions for each PCR cycle were 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. The cycle was repeated 30 times for time-course profile and PAA analysis or 35 times for CHX analysis, all with a final extension at 72 °C for 10 min in a thermal cycler (Hybaid).

**RESULTS**

**RNA-free virus inocula.**

The virus inocula used in this study were prepared by lysing a large number of HHV-6B (Z29)-infected SupT1 cells. RNA released into this inoculum upon cell lysis could interfere with the subsequent microarray and RT-PCR analyses, especially at early time points, and was therefore removed by RNase digestion. To confirm the absence of residual viral RNA, SupT1 cells were exposed to the virus inocula at 4 °C and harvested immediately for RNA extraction (sample 0 h p.i.). The detection of transcripts for β-actin, but not for four HHV-6 genes (U27, U48, U90 and U95), confirmed that this treatment was sufficient to remove cell-free HHV-6 RNA in the virus inocula (see Supplementary Fig. S1, available in JGV Online).
Specificity of the HHV-6 microarray

The microarrays constructed in this study contained probes to 116 predicted HHV-6 ORFs (both -6A and -6B), ten intergenic regions and 914 host genes. Triplicate hybridizations of RNA prepared from mock-infected SupT1 cells and labelled with Cy5 compared with the universal reference showed Cy5 signals for 17 viral probes (B3, B4, B5, B9, DR2, DR3, DR4, DR5, DR6, L1, U36, U61, U66, U68, U71, U86 and U98), suggesting the potential for cross-hybridization with host transcripts (data not shown). The Cy3-labelled universal reference RNA, which contained RNA from different cell lines including HHV-6-infected cells, gave no or low signals for 11 HHV-6 probes (B1, U1, U2, U18, U24, U58, U62, U90A, U91A, U94 and U95; data not shown). Spots of compromised quality (either missing or cross-hybridizing) were excluded from subsequent microarray analysis of the HHV-6B time-course. Pairwise comparison of the data from these three arrays obtained a mean Pearson correlation coefficient of 0.921 (data not shown), indicating that the HHV-6 arrays are highly reproducible.

Expression profiling of HHV-6B

The HHV-6 arrays were used to obtain a transcription profile of HHV-6B gene expression in SupT1 cells over a 60 h time-course of the virus lytic cycle. Other studies on HHV-6 transcription kinetics have demonstrated that 60 h is sufficient for expression of genes in all kinetic classes (Mirandola et al., 1998; Oster & Hollsberg, 2002), but insufficient for the release of progeny viruses from infected cells (Black et al., 1989), thereby minimizing the confounding effect on expression analysis due to secondary infection. Hybridizations of HHV-6 arrays were performed in duplicate using RNA sampled at 6, 12, 24, 36, 48 and 60 h p.i. (except for 48 h p.i., where only one sample was available). As each array has three spots per gene, this gives six data points per gene for each time point (three spots × two samples for each time point, except for the 48 h p.i. sample, which has three data points), which were averaged for subsequent analysis.

This array analysis profiled the expression patterns of 85 HHV-6 ORFs and eight intergenic regions (Fig. 1). Transcripts originating from seven intergenic regions (G17/18, G41/42.1, G41/42.2, G86/90.2, G86/90.3, G91/B8 and G100/B9) were confirmed by RT-PCR (see Supplementary Fig. S2, available in JGV Online) and their implications are considered in the Discussion. Clustering of genes and time points shows a clear temporal order in viral gene expression, with the early time points (6–24 h p.i.) being more similar to each other than the later times (36–60 h p.i.). Profiles of the replicates for each time point were highly similar and clustered in pairs as expected.

Based on the temporal order, all viral genes and intergenic regions could be divided broadly into three clusters: early, delayed-early and late. Genes in the early cluster were transcribed at high levels that are typical of herpesvirus immediate-early (IE) and early (E) genes. Indeed, the cluster contained genes such as U41, U42, U73, U90 and U91, which have been reported to be IE or E genes (Mirandola et al., 1998; Rapp et al., 2000; Schiewe et al., 1994). Transcription of genes in the delayed-early cluster peaked between 12 and 24 h p.i. and involved two known E genes, U51 and U69 (Menotti et al., 1999; Oster & Hollsberg, 2002), together with genes proposed to be involved in DNA replication, such as U27 and U28 (Pellett & Dominguez, 2001). The late cluster consisted of genes expressed only at times after 24 h p.i. Among this group of genes, U11, U22, U23, U47 and U100 have been described previously as L genes (French et al., 1999; Mirandola et al., 1998; Oster & Hollsberg, 2002).

Although these data showed good agreement with the known temporal order of many HHV-6 genes, the kinetics of some remained poorly defined, especially for the early cluster, which contains a mixture of IE and E genes. To better define the transitions between IE-to-E and E-to-L genes, viral gene expression in cells treated with CHX (protein synthesis inhibitor) and PAA (DNA synthesis inhibitor) was analysed for the classification of IE and E/L genes, respectively.

Analysis of HHV-6B IE transcription

To define genes that were transcribed at early times (6–12 h p.i.) into the IE class, we used RT-PCR to compare the effect of CHX treatment on gene expression, as IE genes are transcribed independently of de novo protein synthesis. The RT-PCR conditions employed were optimized for the specific detection of HHV-6 IE genes (Fig. 2a and Supplementary Fig. S1). In all, 36 ORFs and five intergenic regions were examined for IE classification at 6 h p.i. following CHX treatment (Fig. 2b). Transcripts derived from 12 of the 41 genomic regions tested were detected in the CHX-treated sample. Detection of B7, U79 and G91/B8 (which corresponds to the 5′ untranslated region of U90) under IE conditions is in agreement with previously published data (Oster & Hollsberg, 2002; Schiewe et al., 1994; Taniguchi et al., 2000). Several previously unclassified transcripts including B2, U85, G86/90.2 and G86/90.3 were also detected under IE conditions. In contrast, previous studies have classified B3, U18, U39, U42, U73 and U81 as IE genes (Mirandola et al., 1998; Oster & Hollsberg, 2002), but their transcription in the presence of CHX was not detected here. As transcription of B3 was inhibited by PAA, it was reclassified here as an L gene. The other five genes (U18, U39, U42, U73 and U81) were assigned as E genes here, on the basis that their transcripts were insensitive to PAA treatment in this study (Fig. 3).

Transcripts of B1 and B5 were detected in the CHX-treated sample. However, the IE classification of B1 was complicated by its significant overlap with the 5′ end of DR3. Thus, a new set of primers (DR3B) was designed to amplify the 3′-end region that is unique to DR3, to determine whether the two ORFs were regulated independently. RT-
PCR results showed that, in contrast to B1, DR3B amplicons were not detected in the presence of CHX, suggesting that B1 and DR3 have different transcription kinetics. The IE classification of B2 offered further support to our evaluation. B2 is located immediately after B1 in the HHV-6B genome and both ORFs are transcribed in the same orientation. The expression of B1 and B2, but not DR3, in the CHX sample suggested that the former two were co-regulated IE genes.

**Fig. 1.** Lytic replication of HHV-6B. Progressive changes in viral transcription over time are shown in the gene-expression map, with rows representing the individual viral genes and columns representing the samples collected at the corresponding time points. Gene-expression ratios are colour-coded: black represents genes that were expressed at the baseline level (log2 median expression ratio=0), expression ratios above zero are in red and those below are in green. The colour intensity indicates the degree of deviation from zero, e.g. increasing expression ratios are shown in progressively richer shades of red. The dendrograms illustrate the relationship between each node, with the distance between branch points representing the degree of similarity. The dendrograms at the top and on the left indicate the similarity of expression patterns between samples and genes, respectively. Hence, samples or genes that have similar expression patterns are grouped closer together, e.g. the replicates of 12 h p.i. are immediately next to each other, whereas those with dissimilar patterns are grouped further apart, e.g. 6 vs 60 h p.i. are at opposite ends of the dendrogram. The division of viral genes into three main clusters [early (red), delayed-early (green) and late (blue)] is apparent.

**Effect of PAA on HHV-6B gene expression**

To distinguish between E and L genes, SupT1 cells were infected with HHV-6B and cultured for 36 h in the presence of PAA (PAA36). The expression of early genes is totally insensitive to PAA treatment, whereas any gene that was inhibited significantly by PAA was assigned as an L gene. Cluster analysis showed that the 6 and 12 h p.i. and PAA-treated samples formed a distinct group compared with the 24–60 h p.i. samples (Fig. 3). As the PAA samples were more similar to those at 6 and 12 h p.i., this shows that the PAA treatment was effective in restricting expression to IE and E genes.

Introduction of the data from the PAA-treated sample led to a minor alteration in the ordering of clusters. The kinetic classification of most genes remained unchanged compared with those in Fig. 1, except for ten genes. A subset of genes comprising B6, U21, U83, U99 and G100/B9 were previously assigned to the early cluster, probably due to the detection of relatively high levels of their transcripts at 6 h p.i. However, these genes were clearly inhibited by PAA, resulting in a switch from the early cluster (Fig. 1) to the late cluster (Fig. 3). Transcription of these genes exhibited an apparently biphasic pattern of expression with peaks at both IE and at L time points, a feature explored in greater depth later. The other five genes affected by a change in classification were U14, U40, U81, U29 and U52. PAA treatment demonstrated that the transcription of L-cluster genes U14, U40 and U81 (Fig. 1) was unaffected by PAA, and these genes are now in the E cluster (Fig. 3). Conversely, U29 and U52 were previously in the delayed-early cluster (Fig. 1), but are now in the L cluster (Fig. 3), as expression of both genes was inhibited by PAA treatment.
To confirm this classification, we validated certain genes in the E and L clusters by RT-PCR. Transcription of five genes (U12, U27, U51, U69 and U73) in the E cluster was comparable in both the PAA-treated and untreated samples (Fig. 4). Similar analysis demonstrated the reduction in expression of nine of ten genes in the L cluster (DR1, U11, U32, U43, U48, U50, U56, U57 and U72; U38 being the only exception). RT-PCR validated the classification of 14 of 15 genes (93%).

Expression profiling and PAA analysis of the ORFs excluded from the array analysis

As not all HHV-6 genes were represented in the array analysis, we used RT-PCR to profile the expression patterns of a further 19 ORFs and two intergenic regions (Fig. 5, left panel). One E gene (U73) and an L gene (U57) classified by microarray and validated by RT-PCR were included in the RT-PCR profiling as controls. All genes could be divided...
into three groups, equivalent to early, delayed-early and late as defined by microarray (Fig. 1), based on two criteria: the time point at which the corresponding transcript was first detected and that at which transcription peaked. Transcripts of the ORFs in the early group (B1, U94, U95, B5 and B9) were detected readily at 6 h p.i. and the level of their transcription remained constant throughout the time-course. For the delayed-early group (U6, U18, U33, U34, U36, U62 and U71), the corresponding transcripts were detected readily at 12 h p.i. and remained constant thereafter. Transcription of most ORFs in the late group (B4, B8, U9, U66, U68 and G86/90.1) was detected weakly.

Fig. 3. Lytic replication of HHV-6B with PAA treatment. The description for the gene-expression map and the dendrograms is the same as in the legend to Fig. 1. The viral genes are divided into IE + E (red), E (green) and L (blue) clusters. The results show that PAA treatment was effective in inhibiting the expression of L genes.

Fig. 4. Effect of PAA treatment on HHV-6B gene expression. The transcripts examined and the size of their amplicons (in bp) are labelled. E and L indicate ORFs that were assigned as E or L genes by microarray analysis, respectively. Except for the U38 gene, RT-PCR results are in agreement with the kinetic classifications by microarrays. RNA, Purified mRNA; cDNA, reverse-transcribed mRNA; M, 100 bp ladder; 36, HHV-6B-infected cells harvested at 36 h p.i.; PAA, PAA-treated, HHV-6B-infected cells harvested at 36 h p.i.
around 12 h p.i. DR6 and B3 were the exceptions, in that their transcripts were detected weakly at 6 h p.i.; however, both ORFs were duplicated in the viral genome and therefore the early detection may not be a true reflection of their transcription levels at 6 h p.i. The key feature linking all ORFs in this late group is that their transcription peaked at or after 24 h p.i. To test our RT-PCR-based classification, we analysed the effect of PAA inhibition of transcription. All genes in the early and delayed-early groups were insensitive to PAA treatment, whereas all genes (except U9) in the late group were expressed at a reduced level in the presence of PAA (Fig. 5, right panel). Taken together, the kinetic classification of E and L transcription based on gene-expression patterns alone (without PAA) by microarray [compare Figs 1 and 3; 42/52 (80.8%)] or RT-PCR [Fig. 5; 18/19 (94.4%)] analyses is highly reliable. However, the use of chemical inhibitors is necessary to refine the classification of genes expressed at transition points between the E and L classes, e.g. U9.

**Genes with biphasic transcription**

The array analysis identified a subset of genes (B6, U21, U83, U99 and G100/B9) that appeared to be expressed in two distinct phases during infection. Relatively high levels of transcripts were detected at IE times (6 h p.i.) and late times (48–60 h p.i.), but were at minimal levels between the two peaks (12–36 h p.i.). In light of the presence of relatively high levels of transcripts at 6 h p.i., all genes in the biphasic cluster were analysed for IE transcription except for U99 (the primers are 6A-biased). Three of the four genes tested (B6, U21 and G100/B9) were present in the sample treated with CHX (Fig. 2b). Although U83 transcripts were undetectable in this study, the IE expression of U83 has been described previously (French et al., 1999). In addition, the PAA-induced downregulation of B6, U21 and U83 observed in this study was in agreement with previously published data (French et al., 1999; Oster & Hollsberg, 2002).
Gene expression of B9 as profiled by RT-PCR showed that transcripts were detected at IE times and that its second peak in expression at late times was dependent on DNA replication (Fig. 4). The expression of B9 transcripts in the presence of CHX was confirmed by RT-PCR (data not shown). Taken together, these genes appeared to be expressed in two distinct phases; therefore, they were assigned as biphasic genes.

Transcription kinetics of HHV-6 genes according to the array and RT-PCR data in this study are summarized in Fig. 6 and compared with data from other studies involving HHV-6A (U1102) (Yao et al., 2006) and HHV-6B (PL-1) (Oster & Hollsberg, 2002) in Supplementary Table S2.

**DISCUSSION**

Here, we have mapped the expression patterns of the HHV-6B lytic cascade over a 60 h time-course p.i. By using both microarrays and RT-PCR, of the 97 unique ORFs annotated for the HHV-6B (Z29) genome (Dominguez et al., 1999), the expression pattern and kinetics of 92 HHV-6B ORFs (except DR3, U2, U24, U58 and U86) and ten intergenic regions were determined. Together with the analysis of HHV-6B transcription in the presence of inhibitors of either protein synthesis (CHX) or DNA replication (PAA), these data were used to further refine and validate the kinetic classification.

Consistent with other chemical or microarray time-dependent cluster analysis of the herpesvirus lytic cascade (Ahn et al., 2002; Chambers et al., 1999; Honess & Roizman, 1973; Jenner et al., 2001), the HHV-6 genes can be clearly classified as IE, E and L genes. Initial attempts to designate HHV-6B IE genes by microarray was complicated by low or no signals from the viral probes due to the low levels of viral transcript present in the CHX-treated samples (data not shown). IE transcription was therefore analysed by RT-PCR. Although it offers no structural information about the transcript, the identification of transcripts that are insensitive to the CHX block provides useful leads for further studies. The sensitivity of RT-PCR means that non-IE genes may become detectable in the CHX-treated sample; the PCR conditions were therefore optimized to avoid spurious IE classification. No transcription of DNA replication or structural genes was ever detected in the presence of CHX; hence, the optimized RT-
PCR conditions were suitable for the specific detection of IE transcripts. HHV-6B IE genes include B1, B2, B5, B7, U79, U85, U90 and U95. The function of these genes is unclear except for U90, which encodes the viral transactivator IE1 (Schiewe et al., 1994). U85 and U95 have been implicated in modulation of the immune response and apoptosis, respectively (Shiratori et al., 2005; Yeo et al., 2008). B1, B2, B5 and B7 are unique to HHV-6B among all human herpesviruses (Dominguez et al., 1999). It is believed that virus-specific genes evolve to help the virus to adapt to its specific biological environment (Davison et al., 2002). The IE classifications of these genes suggest that they may perform functions that are important for facilitating initial infection. An unexpected observation was the absence of U94 expression after CHX treatment, as it facilitates initial infection. An unexpected observation was et al.

B1, B2, B5 and B7 are unique to HHV-6B among all human herpesviruses (Davison et al., 2008). B1, B2, B5 and B7 are unique to HHV-6B among all human herpesviruses (Dominguez et al., 1999). It is believed that virus-specific genes evolve to help the virus to adapt to its specific biological environment (Davison et al., 2002). The IE classifications of these genes suggest that they may perform functions that are important for facilitating initial infection. An unexpected observation was the absence of U94 expression after CHX treatment, as it has been reported to be an IE gene (Mirandola et al., 1998; Oster & Hollsberg, 2002). The U94 RT-PCR repeated with the primer set described by Mirandola et al. (1998) confirmed the lack of expression, suggesting that this discrepancy is not PCR primer-dependent. Forty-four HHV-6B genes were classified as E genes, including seven genes encoding viral proteins that are thought to be involved in nucleotide metabolism and DNA replication (U27, U41, U69, U70, U73, U74 and U81). The classification of five E genes (U7, U8, U9, U42 and U51) is in agreement with previously reported data (Menotti et al., 1999; Oster & Hollsberg, 2002; Rapp et al., 2000; Taniguchi et al., 2000). Transcription of 33 HHV-6B genes was shown to be inhibited by the viral DNA synthesis inhibitor PAA; therefore, they were classified as L genes. These include 19 genes encoding proteins that are required for virus assembly: six viral glycoproteins (U22, U23, U47, U48, U72 and U100), five tegument proteins (U11, U30, U31, U50 and U65), four capsid proteins (U29, U32, U56 and U57), three virion-associated proteins (U46, U49 and U54) and the viral protease (U53) for capsid processing. It is important to note that L genes can be subdivided further into two classes: leaky-late and true-late genes. In the presence of PAA, the former are still transcribed, but at a much-reduced level, whereas the latter are silenced completely. The delineation of these 33 L genes into leaky-late and true-late genes is beyond the scope of this study; however, we predict that DR1 and B4 are true-late genes, based on the complete lack of transcripts in the presence of PAA (Figs 4 and 5).

Four HHV-6B genes (B6, B9, U21 and U83) were classified here as biphasic genes where the transcripts were detectable under IE conditions, but atypically for IE genes, their maximal expression was dependent on DNA replication. Apart from U83, the transcripts of B6, B9 and U21 were detected by RT-PCR in the presence of CHX. However, U83 transcripts in CHX-treated cells infected with HHV-6 have been reported previously (French et al., 1999). The PAA inhibition of transcription of B6, U21 and U83 observed in this study was consistent with previously published data (French et al., 1999; Oster & Hollsberg, 2002; Zou et al., 1999). It is possible that these biphasic transcripts are controlled by different promoters, as reported for the biphasic regulation of the HHV-6 IE region (Schiewe et al., 1994). Alternatively, the expression profile for the biphasic genes may be a result of the confounding presence of virion-associated RNAs, akin to that observed in virions from other human herpesviruses (Bechtel et al., 2005; Bresnahan & Shenk, 2000; Sciortino et al., 2001). Similarly, these virion-associated transcripts are characterized by their detection at early times during infection and are transcribed maximally only at the time of viral assembly (Bechtel et al., 2005; Bresnahan & Shenk, 2000). The functions of two HHV-6B biphasic genes have been reported: U21 encodes a protein that interferes with major histocompatibility complex class I surface expression (Glosson & Hudson, 2007), whilst U83 encodes two isoforms of viral chemokines capable of chemotaxing leukocytes and may therefore aid virus spread by recruiting uninfected cells during primary infection (Dewin et al., 2006).

We detected and classified ten intergenic transcripts: the IE class includes G86/90.2, G86/90.3 and G91/B8; the E class includes G17/18, G41/42.1 and G41/42.2; the L class includes G83/B4, G86/90.1 and G96/97; G100/B9 exhibits biphasic kinetics. Although there are no obvious ORFs in these intergenic regions, the possibility that they encode untranslated but functional RNA should be considered. Interestingly, we analysed the probe sequences covering all ten intergenic regions by using Mireval (Ritchie et al., 2008), a computational tool for microRNA (miRNA) prediction, and detected the presence of miRNA precursor-like hairpin structure for G41/42.2, G86/90.2 and G100/B9 (data not shown).

A microarray analysis of HHV-6A gene expression has been described previously using different methodologies and experimental conditions (Yao et al., 2006). The microarrays employed by Yao et al. (2006) contained PCR-amplified, full-length HHV-6 ORFs as array probes and were biased towards the HHV-6A genome. For the eight HHV-6A IE genes described, only U84 was analysed for IE transcription in the presence of CHX by RT-PCR. Two of eight IE genes described by Yao et al. (2006) were also classified as IE genes in this study. It is well-recognized that the biological properties of the HHV-6 variants are distinct, and it has been hypothesized that the difference in IE-gene expression may be responsible for the differences (Oster & Hollsberg, 2002). As approximately 30 % of the ORFs were assigned in a combined IE/E grouping by Yao et al. (2006), it is difficult to compare the kinetics of these genes with the results of this study. However, there was a strong agreement on the classification of L genes between the two studies (25 of 29 ORFs analysed, 86.2 %).

Transcription kinetics of 35 ORFs of HHV-6B (PL-1) have been studied by using real-time PCR (Oster & Hollsberg, 2002) and, although the kinetics of two genes (U2 and U58) were not determined here, expression kinetics of two-thirds of the remaining 33 genes were in agreement.
The comparison of kinetic classifications of HHV-6 genes between different studies showed that, despite the use of different methodologies, viral variants and strains, the consistent classification of U90 and U95 as IE genes (Isegawa et al., 1998; Schiewe et al., 1994; Takemoto et al., 2001), of U41, U51 and U69 as E genes (Isegawa et al., 2008; Mirandola et al., 1998) and of U22 and U100 as L genes [no detectable transcription in the presence of PAA as reported by French et al. (1999) and Mirandola et al. (1998)] suggests that these represent ideal markers for determining the stages of HHV-6 lytic replication.

In summary, this study has provided information on the possible function of some uncharacterized genes, based on their associations with genes of known function, that should provide useful leads to facilitate further research into the functional genomics of HHV-6B.

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