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Biochemical and Biophysical Research Communications 329 (2005) 11–17
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Transcriptional profiling of human herpesvirus type B (HHV-6B) in an adult T cell leukemia cell line as in vitro model for persistent infection

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Received 7 January 2005
Available online 1 February 2005

Abstract

Human herpesvirus 6 (HHV-6), which is present in more than 90% of the human, is known to cause infectious diseases in immuno-compromised patients, e.g., transplant patients. To clarify the possible role of the pattern of expression of HHV-6 genes in various types of HHV-6B infection, we sought to determine whether or not viral DNA microarray could be used for detailed characterization of viral transcription using a HHV-6B DNA microarray that contains 97 known open reading frames of HHV-6B. A subset of genes are preferentially expressed in persistent infection: U16 (IE-B, transactivator, US22 gene family), U18 (IE-B, homolog to HCMV IE glycoprotein), U20 (glycoprotein), U27 (DNA polymerase processivity transactivator), U82 (gL, gH accessory protein), U83 (chemokine), U85 (OX-2 homology, glycoprotein), U90 (IE-A), and U94 (transactivator), respectively. Although the function of each HHV-6B is not fully understood, our study suggests that comprehensive analysis of HHV-6B transcription is useful not only to clarify the pathogenesis of the virus but also to develop new strategies for anti-viral drugs.

Keywords: Human herpesvirus type 6B; Viral gene expression; DNA microarray; Persistent infection

Human herpesvirus 6 (HHV-6), which is widely spread in the human population at a frequency of more than 90%, was isolated in the blood of patients with AIDS and other lymphoproliferative disorders [1]. Subsequently, the virus was isolated as the causative agent of exanthem subitum [2]. Based on virological analysis, there are two distinct variants (HHV-6A and HHV-6B) [3]. HHV-6A has not been linked to any disease despite its broad tropism, however, HHV-6B is known to cause some infectious diseases in immunocompromised patients, such as transplant patients [4,5]. Resolving the pattern of expression of HHV-6 genes is important to understand the interaction between immune reconstitution and viral reactivation after transplant, however, experimental evidence dealing with comprehensive gene expression analysis of HHV-6 has not been produced.

The analysis of cellular gene expression in response to various stimuli has been given great impetus with the recent development of DNA microarrays. Applied to the study of viruses, this technology enables rapid and global studies of viral gene expression [6,7]. Since human herpesviruses have a promoter rich genome, specific promoter mapping at cognate genes mediates transcript expression during infection. Evidence suggests that the coordinate regulation of expression of viral transcripts must involve the transcriptional machinery of the host cells in vivo [8]. Futhermore, comprehensive viral gene

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.01.090

expression analysis of human herpesvirus simplex revealed that the α27 (U_L54) gene plays a major role in the early decline in cellular gene expression of herpes simplex virus (HSV) infection [9]. Similarly, human cytomegalovirus (CMV)-regulated changes in cellular gene expression have been reported using a global microarray approach [10,11].

We therefore sought to determine whether or not viral DNA microarray technology could enable detailed characterization of viral transcription in HHV-6B infected cells using a HHV-6B DNA microarray that contains 97 known open reading flames (OFRs) of HHV-6B. We also attempted to obtain additional insight into persistent infection of HHV-6B, which could give us a better understanding of the function of HHV-6B gene and its possible roles during virus-mediated immuno-modulation after transplant.

Materials and methods

Virus stock and cell culture. We used an adult T-cell leukemia cell line (TaY) that shows an efficient propagation of the HHV-6B, strain OK, which was provided by Dr. Kikuta (Hokkaido University, Japan) [12]. Viral infection and replication were monitored by light and electron microscopic examination of cytopathic effect, immunofluorescence, and real-time PCR of viral DNA as reported previously [12,13]. The TaY cells were analyzed for a comparative study of gene expression pattern with or without viral infection and a time-course study during viral infection. The chronic infection of HHV-6B (OK) on TaY cells has been maintained for more than 5 years, without adding uninfected TaY cells. This cell line is used as a model for persistent infection. The virus titer produced during a 3-day period of culture consistently reached 1×10^6 $TCID_{50}$ and the detailed characterization of this cell line was reported previously [12]. To identify the possible relationship between protein synthesis and polymerase activity, TaY cells were infected in the presence of 5 μg/ml aphidicolin (APH: Sigma, St. Louis, MO, USA), then, were harvested 24 h after infection. Katata cells with integrated HHV-6B, kindly provided by Dr. Daibata (Kochi University, Japan), were also used as a negative control [14].

Quantification of HHV-6 copy numbers. To monitor viral load, real-time PCR was done using a LightCycler as reported previously [13]. The pair of primers (5′-ACC CGA GAG ATG ATT TTG CG, and 3′-GCA GAA GAC AGC AGC GCG AT) used in this study amplifies a 417-bp-long product corresponding to a part of the coding region of the HHV-6B 101K gene. The cycling condition was 30 cycles at 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min, followed by a 4 min extension at 72 °C.

RNA extraction and labeling of cDNA. Total RNA was extracted from both infected and uninfected cells using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The amount of RNA was measured by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), and then the quality of extracted RNA was checked using a 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA). Label star array kit (Qiagen, Germantown, MD, USA) was used for cDNA labeling according to the supplier's instruction. Briefly, 15 μg of total RNA extracted from cells was denatured at 65 °C for 5 min, and then the denatured template RNA was incubated at 37 °C for 2 h in a reaction mixture containing 2 μM of virus-specific primers as well as control housekeeping gene specific primers (Celonex, Edmonton, Alberta, Canada), 0.02 mM cyanine 3 (Cy3)-labeled UTP or cyanine 5 (Cy5)-labeled UTP (Amersham Biosciences, Piscataway, NJ, USA), RNase inhibitor, and Label Star reverse transcriptase. The cDNA obtained from uninfected TaY cells was labeled with Cy3 as a reference, and cDNA obtained from the HHV-6B infected cells was labeled with Cy5. Labeled cDNA was purified using a MinElute spin column, and finally eluted by 20 μl RNase free water.

Oliginucleotide DNA microarry hybridization. The HHV-6B DNA chip, Viruchip (Celonex, Edmonton, Canada), was made up of triplicate spots of 70-mer oligonucleotide specific probes for each HHV-6B transcription unit (Table 1). Four human genes (Tubilin, G3PDH, Actin, and ribo-S9) were included in order to standardize the amount of applied cDNA and to normalize the signals between samples and references. Seventeen genes of HIV were also printed to exclude non-specific hybridization. Viruchip was prehybridized in buffer containing 5× SSC, 0.1% sodium dodecyl sulfate (SDS), and 1% BSA, then washed three times with sterile water and once with isopropanol. The Viruchip was dried completely by centrifuging at 800 rpm for 3 min. The labeled cDNA was dried by Speed-Vac, then resuspended in hybridization buffer (Sigma–Aldrich, St. Louis, MO, USA) containing 1/100 vol. Salmon testis DNA (Sigma–Aldrich), and then denatured at 95 °C for 5 min. Both Cy3-labeled cDNA and Cy5-labeled cDNA were mixed well and added onto the Viruchip. Hybridization was done at 42 °C for 16 h, and washed with washing buffer containing 2× SSC, 0.1% SDS for 5 min, with buffer containing 0.5× SSC, 0.1% SDS for 3 min, followed by buffer containing 0.5% SSC for 3 min. The entire washing procedure was done at room temperature. Each experiment was done at least three times at different times to confirm reproducibility of the results.

Data analysis and statistic validation. The Virchip was scanned by GenePix 4000B (Axon Instruments, Union City, CA, USA). The scanned data were normalized, verified, and analyzed using the Genomic Profiler software (Mitsui Industrial Knowledge, Tokyo, Japan). First, the value is adjusted by subtraction of background fluorescence of an equivalent area. Analysis was done by taking the median signal of the probe value for each transcript set, and the 75% rank for the total hybridization is calculated. In order to compare data from repeat and time-varying experiments, normalization was also done by the signals obtained from five human housekeeping genes. Microarray data obtained from three independent experiments were then verified in a single file.

Quantitative reverse transcription-PCR. To confirm the result obtained from microarray analysis, quantitative reverse transcription (RT)-PCR was done as reported previously [15]. Briefly, 1 μg of total RNA was used for cDNA synthesis using a Ready-To-Go You-Prime First-Strand Beads and a pd(N)6 Random Hexamer (Amersham Biosciences). The Taqman β-actin kit (Applied Biosystems, Foster City, CA, USA) was also used for normalization of the amount of cDNA used in each PCR. Four microliters of the resulting cDNA was used in each RT-PCR and then, a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used. A 50 μl PCR sample contained SYBR green universal master mix (Applied Biosystems) and 3 pmol of each primer pair as recommended by the manufacturer. The amount of gene expression in each sample was then expressed as a percent of reference with respect to the standard curve.

Results

HHV-6B viral load in the supernatant of HHV-6B infected cells

To confirm viral production in HHV-6B infected TaY cells, real-time quantitative PCR was performed (Fig. 1). Real-time quantitative PCR of HHV-6B

Table 1
HHV-6B genes and control genes spotted on Viruchip

Gene	Comments	Gene	Comments	Gene	Comments
DR1	US22 gene family	U35		U77	Hericase/primase complex
B1		U36		B6	
DR3		U37		B7	
B2 + B1		U38	DNA polymerase	U79	HCMV replication
DR6	US22 gene family	U39		U81	Uraci I-DNA glycosylase
B3		U40	Transport protein	U82	gL, gH accessory protein
B4		U41	Single-strand DNA binding protein	U83	Chemokine
B5		U42	Transcriptional activator	U84	
U2	US22 gene family	U43	Hericase/primase complex	U85	OX-2
U3	US22 gene family	U44		U86	IE-A
U4		U45	Putative dUTPase	U90	IE-A
U7		U46		U91	
U6		U47		B8	
U8	US22 gene family	U48	gH	U94	IE/regulatory gene, latency associated gene
U9		U49	Fusion protein	U95	
U10		U50	Virion protein	U100	gp82/105
U11	Antigenic viral protein, pp100	U51	G-protein-coupled homolog	B9	
U12	Glycoprotein	U52		GAG-1	HIV-1 (negative control)
U13		U52		POL-1	HIV-1
U14		U54	Tegument transactivator, pp65/72K	VIF-1	HIV-1
U16		U55		VPR-1	HIV-1
U17	IE-B	U56	Capsid protein	TAT-1	HIV-1
U18	IE-B, homolog to HCMV IE glycoprotein	U57	Major capsid protein	VPU-1	HIV-1
U19	IE-B	U58		ENV-1	HIV-1
U20	Glycoprotein	U59		REV-1	HIV-1
U21	Glycoprotein	U66	Late spliced gene (U60/66) for DNA packaging	NEF-1	HIV-1
U22	Glycoprotein	U62		GAG-2	HIV-1
U23	Glycoprotein	U63		POL-2	HIV-1
U24		U64		VIF-1	HIV-1
U24A		U65		VPX-2	HIV-1
U25	US22 gene family	U67		VPR-2	HIV-1
U26		U68		ENV-2	HIV-1
U27	DNA polymerase processivity factor	U69	Ganciclovir kinase, phosphotransferase	REV + ENV	HIV-1
U28	Ribonucleotide reductase	U70	Alkaline exonuclease	NEF-2	HIV-1
U29	Major capsid protein	U71		Tubilin	Human gene (housekeeping gene)
U30	Capsid assembly myosin	U73	Origin binding protein	G3PDH	Human gene
U31	Large tegument protein	U72	Integral membrane protein, gM	Actin	Human gene
U32		U74	Hericase/primase complex	ribo-S9	Human gene
U33	Caspid protein	U75			
U34		U76			

revealed that chronically infected TaY cells contain approximately 1×10^6 copies/μl of supernatant. In contrast, Katata did not contain HHV-6B in the supernatant, while DNA obtained from Katata cells contain 1×10^3 copies/μg of DNA. This indicated that HHV-6B chronically infected TaY cells could be used as an ideal model of productive infection, and Katata could be used as that of latent infection. On days 1, 3, and 5 after infection of TaY cells with HHV-6B (OK), the viral loads in the supernatant were increased by degrees, as shown in Fig. 1.

The difference of HHV-6B gene expression patterns between lytic infection and latent infection detected by DNA microarray

To compare the general gene expression pattern in HHV-6B infected cells, we first investigated chronically infected TaY cells and Katata cells. The scattergram clearly shows that a number of genes are expressed in lytic infection but not latent infection (Fig. 2). There was a variation of the gene expression levels that are supposed to be related to lytic infection; A subset of

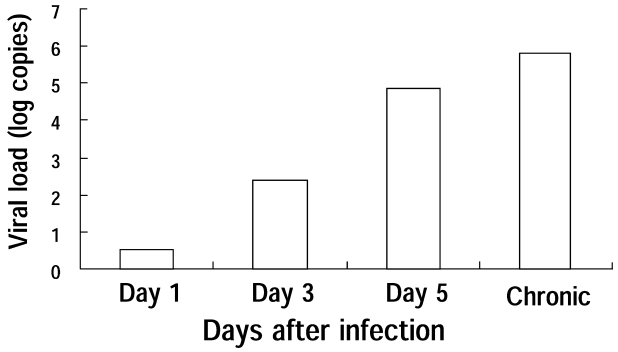

Fig. 1. Changes of viral DNA in the supernatants in TaY. Viral loads are shown on 1 day, 3 days, and 5 days after infection with HHV-6B (OK) and HHV-6B (OK) chronically infected cells.

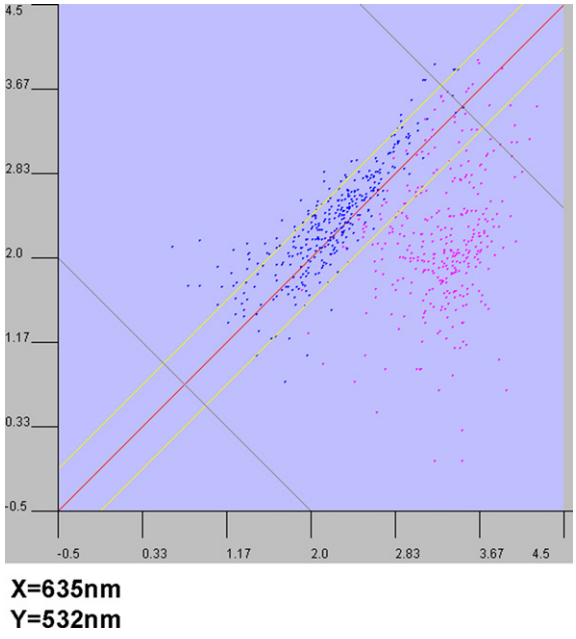

Fig. 2. A scattergram showing HHV-6B productive infection. Plots in pink are genes expressed in HHV-6B chronically infected TaY. Plots in blue are genes expressed in latently infected Katata. Abundant genes were shifted to right as a result of productive infection.

gene showed high signals in chronically infected TaY cells.

Viral DNA polymerase-dependent HHV-6B gene expression

To identify genes which depend on viral polymerase, we examined the inhibition by APH, an inhibitor of both cellular and viral DNA polymerase. In the absence of APH, fluorescent signals of HHV-6B gene were detected in 59 of 97 genes at 24 h after infection, and the remaining gene expression was not detected. Inhibition of HHV-6B gene expression by 24 h of treatment with APH was seen to various degrees (Fig. 3). The gene

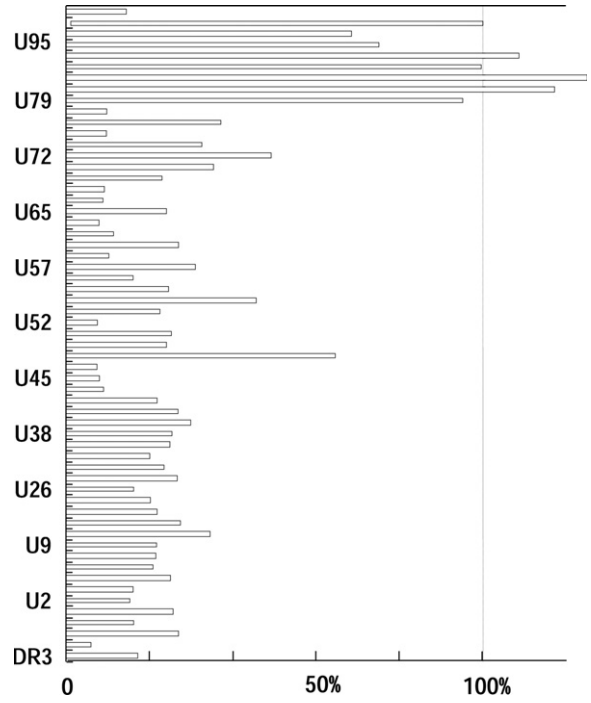

Fig. 3. The ratio of HHV-6B gene expression in APH treated TaY and that in untreated TaY. Cells are harvested 24 h after infection of HHV6B (see [21]).

expression of sample obtained from APH-treated cells was expressed as percentage of controls. The genes, DR3, DR6, B3, B4, B5, U2, U4, U6, U7, U8, U9, U14, U19, U22, U25, U26, U31, U32, U35, U36, U38, U39, U41, U42, U43, U45, U46, U48, U51, U52, U53, U54, U55, U56, U57, U58, U59, U63, U64, U65, U66, U68, U69, U70, U72, U73?, U74, U75, U77, and B9, were inhibited, while genes U79, U82, U83, U86, U90, U95, U100, and B8 were not inhibited, by APH.

Patterns of HHV-6B gene expression as a function of condition of infection

In order to clarify the biological significance of viral genes which were expressed in various intensities, we next compared gene expression patterns in acute and chronically infected TaY cells. Cluster analysis of HHV-6B gene expression revealed that there was a distinct difference in the expression pattern of each gene group in acute and chronic infection of HHV-6B (Fig. 4). The gene expression patterns on day 5 post-infection are similar to those in chronic expression (Fig. 4, lanes 1 and 2), whereas gene expression patterns on day 1 and day 3 of post-infection revealed similar expression patterns (Fig. 4, lanes 3 and 4). Of note is that there was a gene subset that was preferentially expressed in day 5 post-infection and chronic infection (Fig. 4, arrow).

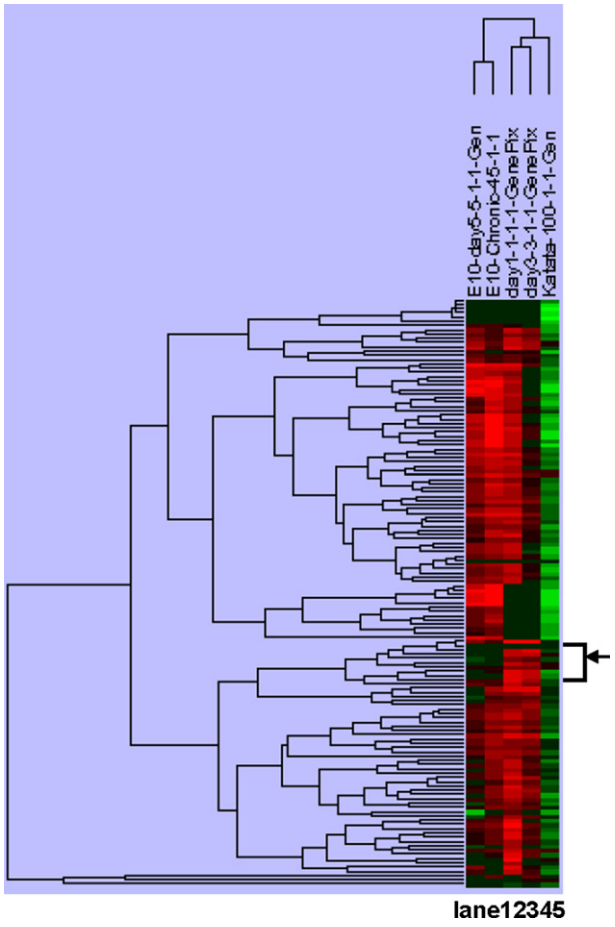

Fig. 4. Cluster analysis in HHV-6B infected cells. The patterns of gene expression in HHV-6B infected cells at various times are shown. Gene expression patterns on day 1 and day 3 of post-infection are different from those on day 5 and chronically infected cells (arrow). Katata is used as a negative control.

Genes preferentially expressed in chronically infected cells

In the current study, we found a subset of genes are preferentially expressed in chronically infected TaY cells as well as on day 5 of after infection, to varying degrees (Fig. 5): U16 (IE-B, transactivator, US22 gene family), U18 (IE-B, homolog to HCMV IE glycoprotein), U20 (glycoprotein), U27 (DNA polymerase processivity transactivator), U34 (possible virion protein), U79, U82 (gL, gH accessory protein), U83 (chemokine), U85 (OX-2 homology, glycoprotein), U90 (IE-A), and U94 (transactivator). The changes of HHV-6B gene expression were more evident in chronically infected cells. It is notable that some of the genes are categorized as immediate-early gene, indicating that the replication of HHV-6B in chronically infected cells is more vigorous than that in the early phase of acute infection. The expression levels of genes U27, U83, and U94 were compatible with the results obtained from real-time RT-PCR (data not shown).

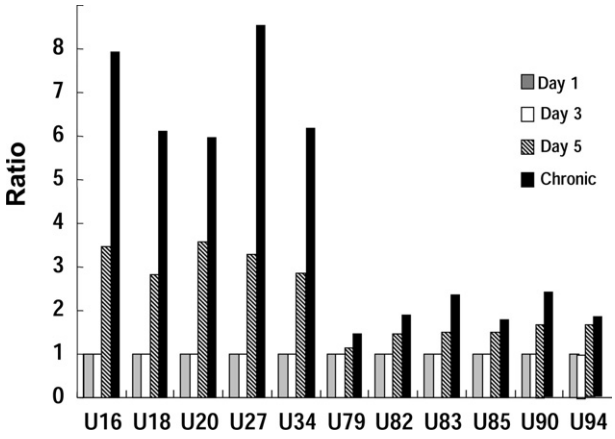

Fig. 5. Gene groups which are up-regulated in chronically infected cells.

Discussion

Comprehensive analysis of viral gene expression appears to be a powerful diagnostic tool for viral infection. So far, two different approaches for viral infection have been made. Microarrays containing specific sequences of more than 100 viruses made it possible to discover an emerging virus, such as SARS [16–18]. In contrast, the comprehensive gene expression analysis of certain viruses allows us to gain more insight into the possible role of the viral transcript in the development of human disease [9,10]. The latter approach accelerates our understanding of the complex genetic process underlying the interaction between virus and the host.

In the current study, we developed an oligonucleotide microarray, Viruchip, to detect HHV-6B gene transcription. Unlike human cellular genes, the evaluation of viral gene transcript detected by microarray is not well established. Several technical difficulties need to be resolved. First, oligonucleotide probes should be highly specific to HHV-6B, because of sequence homology of other herpesviruses and human cellular gene. Second, the ratio of Cy5-labeled sample and Cy3-labeled control must be normalized properly after subtracting the background. Finally, the results obtained from several independent hybridizations should be verified in order to compare the results. To overcome these problems, we scanned the hybridization signals by GenePix 4000B as raw data and then converted data into Genomic Profiler software. All the signals from viral genes were normalized by signals obtained from 4 housekeeping genes. In general, the signal of Cy3-labeled cDNA from uninfected cells is stronger than that of Cy5-labeled cDNA from infected cells, therefore, this procedure was extremely important to normalize the amount of applied cDNA for each microarray. All the normalized data were automatically verified into a single file. The software, a Genomic Profiler, enabled us to validate the data obtained from Viruchip. Our results indicate that

the scattergram could be used for general trends after viral infection, and cluster analysis might be more suitable to compare the condition of infection.

Traditionally, genes related to lytic herpesvirus infection are divided into immediate-early (IE), early (E), and late (L) genes on the basis of their temporal expression and their dependency on other gene products [19]. The IE genes are expressed independent of de novo protein synthesis. The products from these genes are often transcriptional factors and other regulatory proteins. IE gene products are important regulators of E gene transcription, which in turn are involved in DNA replication and metabolism. The L genes are transcribed encoding structural and other proteins involved in virion assembly. In the present study, some discrepancy was seen in the genes inhibited by APH compared with previous reports that have been basically established by Northern blotting or RT-PCR studies [19–21]. Despite some discrepancy, U38, the gene encoding DNA polymerase, was remarkably inhibited, whereas IE genes such as U86 and U90 were not inhibited, indicating that Viruchip is valuable for characterization of viral transcripts. The discrepancy should be resolved by increasing the resolution of microarrays and by carefully characterizing the nature of viral transcription as a function of the cell infected.

It is noteworthy that expression patterns of viral transcript differed in relation to the condition of infection. HHV-6B established a latent infection that may give rise to severe clinical manifestations if reactivated in immuno-compromised host. The HHV-6B chronically infected culture condition somewhat mimics persistent infection occurring in an immunocompromised host. In the current study, we found a subset of genes that were preferentially expressed in the chronically infected cells including immediate early genes and transactivator which may play an important role in the continuous infection of HHV-6B. For instance, the U83 gene which encodes a functional chemokine may modify the host genes [22]. Evidence suggests that the U83 protein is related to an efficient HHV-6B propagation in vivo, by activating and trafficking mononuclear cells to sites of viral replication [22]. An early protein of HHV-6, designated p41, is encoded by the U27 gene and conserved in HHV-6A and HHV-6B [3]. The association between p41 of HHV-6B and human central nerve system diseases, indicated by studies of monoclonal antibodies against p41, is still controversial [23–25], however, our results suggest that transcription of U27 may affect the development of HHV-6B continuous infection.

In conclusion, we validated the use of the oligonucleotide microarray to assess the condition of HHV-6B infection. Although the function of each HHV-6B gene is not fully understood, our study suggests that comprehensive analysis of HHV-6B transcription is useful not only to clarify viral pathogenesis but also to develop new strategies for anti-viral drugs. While its use in the investigation of viral infection is still in the infancy, this emerging technology will improve our understanding of host–pathogen interaction.

Acknowledgments

The authors are indebted to Prof. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of the manuscript. The authors thank Dr. Fariborz Rahbar-Dehghan (Celonex, Inc.), Mr. Kiyokazu Nemoto (NovusGene, Co.), Mr. Tohru Makino (NovusGene, Co.), Ms. Ryoko Hamamura, and Ms. Takako Oda for their help. This work was supported by "High-Tech Research Center" Project for private universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology, 2003–2007), and by "University-Industry Joint Research Project" for private universities: matching fund subsidy from MEXT, 2002–2006.

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