Analysis of Individual Human Trigeminal Ganglia for Latent Herpes Simplex Virus Type 1 and Varicella-Zoster Virus Nucleic Acids Using Real-Time PCR

RANDALL J. COHRS,¹ JESSICA RANDALL,¹ JOHN SMITH,¹ DONALD H. GILDEN,^{1,2}
CHRISTINE DABROWSKI,³ HARJEET VAN DER KEYL,³ AND RUTH TAL-SINGER^{3*}

Departments of Neurology¹ and Microbiology,² University of Colorado Health Sciences Center, Denver, Colorado 80262, and Department of Molecular Virology and Host Defense, SmithKline Beecham Pharmaceuticals, Collegeville, Pennsylvania 19426³

Received 29 June 2000/Accepted 22 September 2000

Herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) establish latent infections in the peripheral nervous system following primary infection. During latency both virus genomes exhibit limited transcription, with the HSV-1 LATs and at least four VZV transcripts consistently detected in latently infected human ganglia. In this study we used real-time PCR quantitation to determine the viral DNA copy number in individual trigeminal ganglia (TG) from 17 subjects. The number of HSV-1 genomes was not significantly different between the left and right TG from the same individual and varied per subject from 42.9 to 677.9 copies per 100 ng of DNA. The number of VZV genomes was also not significantly different between left and right TG from the same individual and varied per subject from 37.0 to 3,560.5 copies per 100 ng of DNA. HSV-1 LAT transcripts were consistently detected in ganglia containing latent HSV-1 and varied in relative expression by >500-fold. Of the three VZV transcripts analyzed, only transcripts mapping to gene 63 were consistently detected in latently infected ganglia and varied in relative expression by >2,000-fold. Thus, it appears that, similar to LAT transcription in HSV-1 latently infected ganglia, VZV gene 63 transcription is a hallmark of VZV latency.

Herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) are neurotropic alphaherpesviruses that are endemic in the human population. Under normal conditions, both viruses are acquired early in life: HSV-1 often as an inapparent asymptomatic infection of the mouth and lips (38) and VZV as childhood chickenpox (1). Following primary infection, both viruses establish latent infections in sensory ganglia. Reactivation of latent HSV-1 typically results in localized epithelial eruptions (cold sores) which shortly resolve with few, if any, consequences. Reactivation of VZV involves the skin innervated by one to three dermatomes lasting for weeks (shingles) and the excruciating pain can persist long after skin lesions are cleared (reviewed in reference 18). In the normal population, the frequency of HSV-1 reactivation wanes with age, while VZV reactivation occurs usually once and is associated with the elderly (32). The differences in the frequency and severity of HSV-1 and VZV reactivation may be attributed to a number of factors that are yet to be elucidated. Some possible explanations that have been proposed include the cell type harboring latent virus, the virus burden during latency, and the virus genes transcribed during latency, including interactions with host factors (5, 6, 12, 18, 23, 30). The neuronal site of HSV-1 latency has been firmly established in both human and animal models (31). Lacking an animal model, the site of latent VZV has been shown to be predominantly neuronal in human ganglia removed at autopsy; however, nonneuronal satellite cells are also implicated as a reservoir of latent VZV (11, 15; and reviewed in reference 19).

Latent virus DNA burden has been shown to be a major determinant in the frequencies of HSV-1 and HSV-2 reactivation (17, 29). By extension, latent VZV DNA copy number would be expected to be a factor in the frequency of virus reactivation. Early studies using Southern blot analysis of high-molecular-weight DNA extracted from human trigeminal ganglia (TG) detected HSV-1 DNA in 9 of 20 individuals at a virus DNA burden of 0.01 to 0.1 copy per ganglionic cell (7). Taking into account the neuronal site of HSV-1 latency and a 1:100 frequency of neuronal cells to satellite cells in the human TG (16), the present initial study detected 1 to 10 copies of HSV-1 DNA per neuron. The quantity of HSV-1 DNA present during latent infection in animal models has been determined. Latently infected rabbits were found to contain, on average, 16.8 copies of HSV-1 DNA per 100 cells (9), while latently infected mice contained on average 50 copies of virus per 100 cell equivalents (10). At the single cell level, the HSV-1 DNA burden was shown to range from 10 to 1,000 copies per individual latently infected mouse neuron (29). The amount of VZV DNA present in human ganglia was determined to be 6 to 28 copies per 100,000 ganglionic cell equivalents (21). At the single-cell level, VZV DNA was found at a rate of two to five copies per 100 neuronal cells (15). Although it has been shown that both HSV-1 and VZV can reside in the same human ganglia during latency (18, 21); until recently, the simultaneous virus burden has not been determined. Using the currently most sensitive and accurate method to determine virus DNA copy number, real-time fluorescence PCR, Pevenstein et al. (27) detected approximately 2.9×10^3 copies of HSV-1 and 0.2×10^3 copies of VZV DNA per 10^5 human TG cells. When the satellite cell VZV DNA burden was taken into account, these researchers concluded that similar amounts of HSV-1 and VZV DNA were present per latently infected cell.

* Corresponding author. Mailing address: SmithKline Beecham Pharmaceuticals, 1250 South Collegeville Rd., UP1455, Collegeville, PA 19426. Phone: (610) 917-6869. Fax: (610) 917-4171. E-mail: Ruth_Tal-singer-1@sbphrd.com.

TABLE 1. Clinical history

Subject	Age (yr)	Sex ^a	Immediate cause of death	Time (h) between death and autopsy
1	87	M	Alzheimer's	24
2	49	F	Respiratory failure: veno-occlusive disease (peripheral vascular disease)	17
3	66	F	Congestive heart failure, respiratory arrest	18
4	83	M	Pneumonia	22
5	77	M	Brain metastases	18
6	73	M	Multiorgan failure; pancreatitis	29
7	85	M	Sudden cardiac arrest	20
8	50	M	Metastatic pancreatic cancer	19
9	67	M	Arteriosclerosis	15
10	84	F	Parkinson's and Alzheimer's	16
11	73	M	Ruptured thoracic aneurysm; coronary artery disease; hypertension	26
12	58	M	Lymphoma	9
13	63	M	Chronic hepatitis with cirrhosis	17
14	60	M	Pulmonary fibrosis with pulmonary hypertension	12
15	47	F	Endocarditis; hypertrophic cardiomyopathy	8
16	52	M	Sepsis	25
17	57	M	End-stage liver disease	12

^a M, male; F, female.

The lytic pattern of virus gene transcription differs substantially from the latent program. A single HSV-1 transcriptional unit has been consistently detected in human ganglia and in animal models. The unstable 8.5-kb primary transcript maps to a region antisense with respect to ICP0 and ICP4 from which stable introns of 2.0 and 1.5 kb are spliced and accumulate in nuclei of latently infected neurons (25, 33, 37, 39, 40). In the mouse model of HSV-1 latency, low levels of acute immediately and early transcripts such as ICP4, ICP27, and ICP6 have been detected (14, 35, 36), but the presence of these lytic viral transcripts in latently infected human ganglia has not been documented.

The transcriptional pattern of latent VZV is complex. Although the HSV-1 and VZV genomes share a great deal of homology and most of the open reading frames (ORFs) are colinear, VZV lacks a significant portion of the long inverted repeat (IR_L). Since the major HSV-1 transcript detected in both human and animal models during latency maps within the IR_L (37), it would not be surprising if the mechanism by which VZV maintains latency is different from that of HSV-1. Supporting this assertion is the finding that, unlike HSV-1, multiple VZV gene transcripts have been detected in latently infected human ganglia (2–4, 22). However, due to the low abundance of virus transcripts, previous analysis of latently

TABLE 2. Oligonucleotides

Target	Name	Type ^a	Sequence (5' to 3')	5' Location
HSV-1	LAT	F	CCC ACG TAC TCC AAG AAG GC	6652; 119719
	LAT	R	AGA CCC AAG CAT AGA GAG CCA G	6581; 119745
	LAT	Probe	CCC ACC CCG CCT GTG TTT TTG TG	6626; 119790
	UL39 (ICP6)	F	ATA GCC AAT CCA TGA CCC TGT ATG	89661
	UL39 (ICP6)	R	GGG TGG AGG CTG GGA GG	89723
	UL39 (ICP6)	Probe	CAC GGA GAA GGC GGA CGG GA	89686
	UL44 (gC)	F	GAT GCC GGT TTC GGA ATT C	96738
	UL44 (gC)	R	CCC ATG GAG TAA CGC CAT ATC T	96780
	UL44 (gC)	Probe	ACC CGC ATG GAG TTC CGC CTC	96758
	ICP27 (UL54)	F	CGC CAA GAA AAT TTC ATC GAG	114766
	ICP27 (UL54)	R	ACA TCT TGC ACC ACG CCA G	114829
	ICP27 (UL54)	Probe	CTG GCC TCC GCC GAC GAG AC	114790
VZV	Gene 21	F	TGT TGG CAT TGC CGT TGA	32816
	Gene 21	R	ATA GAA GGA CGG TCA GGA ACC A	32880
	Gene 21	Probe	CTG CTT CCC CAG CAC GTC CGT C	32835
	Gene 29A	F	GGC GGA ACT TTC GTA ACC AA	52951
	Gene 29A	R	CCC CAT TAA ACA GGT CAA CAA AA	53017
	Gene 29A	Probe	TCC AAC CTG TTT TGC GGC GGC	52973
	Gene 63A	F	CGC GTT TTG TAC TCC GGG	119179; 110718
	Gene 63A	R	ACG GTT GAT GTC CTC AAC GAG	110778; 119119
	Gene 63A	Probe	TGG GAG ATC CAC CCG GCC AG	119160; 110737
Cellular	GAPDH	F	CAA GGT CAT CCA TGA CAA CTT TG	
	GAPDH	R	GGC CAT CCA CAG TCT TCT GG	
	GAPDH	Probe	ACC ACA GTC CAT GCC ATC ACT GCC	
			A	

^a F, forward primer; R, reverse primer.

TABLE 3. Quantitative analysis of HSV-1 DNA in individual human TG

Subject	TG location	HSV-1 DNA copy no.									
		LAT		UL44		UL54		Per 100 ng		Per subject	
		Avg	SEM	Avg	SEM	Avg	SEM	Avg	SEM	Avg	SEM
1	Left	60.8	19.3			71.8	5.1	66.3	15.1		
	Right	54.5	20.5			78.0	6.4	66.2	19.2	66.3	17.3
2	Left	135.0	65.0			36.5	0.5	85.8	67.4		
	Right	0.0	0.0			0.0	0.0	0.0	0.0	42.9	64.1
3	Left	127.5	2.5			233.9	9.7	180.7	53.7		
	Right	132.5	7.5			178.2	10.6	155.4	24.6	168.0	43.6
4	Left	115.0	15.0			131.0	0.0	123.0	13.3		
	Right	87.5	7.5			107.2	4.6	97.4	11.7	110.2	17.9
5	Left	0.0	0.0			0.0	0.0	0.0	0.0		
	Right	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0
6	Left	0.0	0.0			0.0	0.0	0.0	0.0		
	Right	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0
7	Left	157.5	32.5			256.3	12.6	206.9	55.2		
	Right	322.5	227.5			132.6	2.6	227.5	186.8	217.2	138.1
8	Left	9.9	5.1			45.8	4.5	27.9	18.6		
	Right	210.0	130.0			138.6	8.7	174.3	98.8	101.1	102.1
9	Left	0.0	0.0			0.0	0.0	0.0	0.0		
	Right	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0
10	Left	41.3	0.8			122.5	1.6	81.9	40.6		
	Right	185.0	60.0			138.1	17.6	161.5	50.0	121.7	60.5
11	Left	9.3	0.8			36.6	2.6	22.9	13.8		
	Right	114.0	71.0			96.6	7.5	105.3	51.2	64.1	55.7
12	Left			212.4	57.1	194.8	14.0	203.6	42.5		
	Right			255.0	40.4	315.1	68.8	285.0	63.9	244.3	67.9
13	Left			338.1	113.5	120.6	3.6	229.4	135.2		
	Right			134.4	24.9	115.6	1.1	125.0	20.0	177.2	109.8
14	Left			0.0	0.0	0.0	0.0	0.0	0.0		
	Right			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	Left			135.5	35.3	392.1	11.0	263.8	131.0		
	Right			329.3	87.4	539.0	275.1	434.2	229.4	349.0	205.3
16	Left			455.4	151.8	738.7	330.6	597.1	293.7		
	Right			691.3	35.6	826.4	18.7	758.8	73.3	677.9	228.8
17	Left			0.0	0.0	0.0	0.0	0.0	0.0		
	Right			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

expressed VZV genes has required pooling ganglia from multiple individuals. Inherent in these experiments is the chance of virus reactivation within one or more ganglia confounding the results.

Since both HSV-1 and VZV can establish a latent or reactivated infection within the same ganglia, we investigated individual trigeminal ganglia removed at autopsy from unselected individuals for the footprints of both herpesviruses. Our real-time PCR data indicate that VZV DNA is present in all ganglia tested; however, in contrast to HSV-1, the VZV viral load is highly variable within this population. Real-time reverse transcription-PCR (RT-PCR) of virus transcripts associated with latency demonstrated that HSV-1 LAT and VZV gene 63 ranged in relative expression within individual ganglia from 500- to 2,000-fold, respectively. These observations are consistent with the DNA copy number in our samples which may represent the variability in viral load within the adult population.

MATERIALS AND METHODS

Tissue collection. Both left and right trigeminal ganglia were removed from the subjects who at autopsy did not show cutaneous signs of recent herpesvirus infection. Table 1 shows the age, sex, cause of death, and time between death and autopsy of the 17 individuals included in this study. Using fresh instruments for each sample, we cleaned the nerve roots and flash froze them in liquid nitrogen. All samples were stored at -70°C .

Nucleic acid extraction. Individual ganglia were powdered under liquid nitrogen. Approximately 50 mg of the powdered tissue was removed for DNA ex-

traction (DNeasy Tissue Kit; Qiagen, Valencia, Calif.). Total RNA was extracted from the remaining tissue (200 to 800 mg) (TRI Reagent; Molecular Research Center, Cincinnati, Ohio).

RT. RNA was digested with RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 45 min at 37°C , followed by a 5-min incubation at 70°C . DNA (50 μl) was generated from 4 μg of total RNA by using the Superscript Preamplification Kit (Life Technologies/Gibco-BRL, Grand Island, N.Y.), priming with oligo(dT) and random hexamers as described previously (35, 36).

Real-time PCR analysis (fluorescence-based simultaneous amplification and product detection). Reactions were performed in 50- μl volumes containing $2\times$ TaqMan Universal PCR Master Mix (Perkin-Elmer, Norwalk, Conn.) and appropriate amounts of cDNA (10% for detection of viral transcripts, 2% for detection of cellular transcripts) or 100 ng of DNA. Reactions also contained a 200 nM concentration of TaqMan primers and a 200 nM concentration of TaqMan probe. Primer pairs and probes described in Table 2 were designed using Primer Express software (Perkin-Elmer) and analyzed in a 96-well optical plate. Probes were labeled at the 5' end with the fluorescent reporter dye Fam and at the 3' end with fluorescent quencher dye Tamra (Synthegene, Houston, Tex.) to allow direct detection of the PCR product. Real-time PCR amplification and detection were performed using ABI 7700 Sequence Detector (PE Biosystems). Relative copy numbers were calculated from a standard curve generated in each individual assay using PCR standards. Standard curves were highly reproducible. As a negative control, each plate contained a minimum of three wells lacking template. Each sample was analyzed in duplicate. The copy numbers for each individual gene transcript were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels by dividing the copy number obtained from standard curves to that obtained for GAPDH.

Real-time virus DNA PCR standards. Purified HSV-1 (SC-16) or VZV (strain Scott) viral DNA was serially diluted in 10 ng of human genomic DNA (Clontech, Palo Alto, Calif.) per μl . The virus DNA was diluted such that 2 μl of the sample contained 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , or 10^0 of either HSV-1 or VZV DNA. For quantitation of GAPDH, standard curves were generated using serial

TABLE 4. Quantitative analysis of VZV DNA in individual human TG

		VZV DNA copy no.									
Subject	TG location	Gene 21		Gene 29		Gene 63		Per 100 ng		Per subject	
		Avg	SEM	Avg	SEM	Avg	SEM	Avg	SEM	Avg	SEM
1	Left	6.4	6.4			43.4	2.8	24.9	23.0		
	Right	31.2	31.2			66.9	58.4	49.0	81.0	37.0	39.8
2	Left	199.8	29.4			771.3	142.9	485.5	175.2		
	Right	563.2	139.0			2,894.0	105.6	1,728.6	1,420.6	1,107.1	1,057.9
3	Left	56.7	14.3			108.3	13.8	82.5	21.5		
	Right	99.4	26.4			67.3	14.6	83.3	242.1	82.9	28.1
4	Left	519.8	121.0			710.8	217.5	615.3	282.6		
	Right	244.0	58.9			430.5	10.9	337.2	143.9	476.3	211.2
5	Left	701.7	67.3			972.9	177.8	837.3	422.2		
	Right	166.7	0.7			280.1	21.9	223.4	37.1	530.3	337.9
6	Left	212.9	4.5			335.4	81.2	274.2	111.9		
	Right	143.6	7.4			294.0	59.7	218.8	1,768.8	246.5	89.6
7	Left	3,762.1	489.5			4,291.1	1,687.5	4,026.6	1,542.3		
	Right	2,504.3	560.0			3,684.4	34.8	3,094.3	1,668.9	3,560.5	1,129.9
8	Left	347.3	39.0			730.0	2.9	538.6	285.6		
	Right	166.7	67.3			184.8	39.4	175.7	202.0	357.2	230.6
9	Left	567.4	82.9			962.1	312.4	764.7	226.7		
	Right	887.1	48.6			1,259.6	308.6	1,073.3	285.3	919.0	333.4
10	Left	893.9	26.5			1,219.4	63.3	1,056.7	328.3		
	Right	573.3	53.5			862.8	10.3	718.1	354.8	887.4	233.0
11	Left	153.4	0.8			219.2	0.4	186.3	294.6		
	Right	627.8	300.1			718.3	9.8	673.0	326.1	429.7	288.7
12	Left	67.8	31.8	86.2	62.6	78.1	45.6	82.6	45.6		
	Right	159.0	44.5	38.7	21.3	60.0	66.5	79.2	66.5	80.9	57.0
13	Left	79.0	79.0	40.2	38.4	93.0	56.3	66.4	56.3		
	Right	309.7	6.2	50.4	34.1	78.5	117.0	132.5	117.0	99.4	97.6
14	Left	126.5	47.3	111.0	33.8	133.0	40.1	121.7	40.1		
	Right	68.5	68.5	49.0	58.4	100.5	59.2	69.3	59.2	95.5	57.0
15	Left	1,031.8	418.1	143.9	41.0	830.0	548.6	593.6	548.6		
	Right	1,160.5	606.2	421.0	138.7	980.0	484.7	792.0	484.7	692.8	527.1
16	Left	102.0	28.8	150.3	72.8	127.0	60.9	129.8	60.9		
	Right	198.5	198.5	73.5	51.1	200.0	131.4	145.3	131.4	137.6	102.7
17	Left	159.0	0.0	72.6	28.9	22.5	50.0	70.3	50.0		
	Right	247.7	83.1	134.0	125.5	109.5	112.6	159.5	112.6	118.3	99.8

dilutions of human genomic DNA. All samples in duplicate were subjected to TaqMan PCR with each primer set to generate standard curves and to evaluate relative primer sensitivity.

Standards for quantitative RT-PCR. Polyadenylated VZV gene 21, 29, and 63 transcripts were individually synthesized, quantitated, diluted, and reverse transcribed, and the cDNA yield was quantitated by real-time PCR. To construct the plasmid templates for the in vitro synthesis of polyadenylated VZV transcripts, restriction endonuclease (RE) fragments containing each ORF were inserted into pAlter-1 (Promega, Madison, Wis.). Plasmid DNA was extracted, alkali-denatured, and annealed to synthetic oligonucleotides containing unique RE sites in apposition to the initiation and termination codons of the VZV ORF. DNA was synthesized from the annealed oligonucleotide primers with T4 DNA polymerase, and the gaps were sealed with T4 DNA ligase. The newly synthesized DNA was transformed into Es1301 *mutS* component *Escherichia coli* and plasmids were screened for the inserted RE sites by RE digestion and agarose gel electrophoresis. The plasmids were then shuttled to the JM109 strain of *E. coli* for extended propagation. The DNA sequence was obtained to verify the fidelity of all constructs.

Each VZV ORF was directionally shuttled into pSP64 poly(A) (Promega) between the SP6 promoter and the [A]₃₀ tract. Extracted plasmid DNA was linearized downstream from the polyadenosine track, and SP6-dependent RNA transcripts were synthesized. After DNase (Gibco-BRL) digestion, transcripts were extracted by affinity chromatography. Each reaction yielded a single, discrete transcript of the expected size upon denaturing agarose gel electrophoresis. The concentration of each transcript, determined by measuring the optical density at 260 nm, was used to calculate the total number of each transcript synthesized. Since HSV-1 LAT is nonpolyadenylated in vivo, pSP64 was not used as the base plasmid for the in vitro synthesis of HSV-1 LAT transcripts. Instead, pLAT (the generous gift of Roderick Smith, University of Colorado Health Sciences Center), which contains 1.2 kb of the major HSV-1 LAT coding sequences, was used to synthesize T3 transcripts. Following linearization of pLAT and in vitro T3-dependent transcription, the RNA was digested with DNase, extracted, resolved on denaturing agarose gels, and quantitated as described above. A stock

solution was prepared which contained 10⁷ copies per µl of polyadenylated VZV gene 21, 29, and 63 transcripts and nonpolyadenylated HSV-1 LAT transcripts. Dilutions of from 10⁶ to 10¹ copies of each transcript per 10 µl of nuclease-free water were added to 500 ng of total RNA extracted from control African green monkey dorsal root ganglia as described above. Each dilution of ganglionic RNA containing known numbers of VZV gene 21, 29, and 63 and HSV-1 LAT transcripts was reverse transcribed, and the cDNA was quantitated by real-time PCR. For each sample, a reaction lacking reverse transcriptase was prepared and similarly quantitated.

RESULTS

HSV-1 and VZV DNA in human TG. Samples from individuals described in Table 1 were analyzed using real-time PCR quantitation. Tables 3 and 4 present the amounts of HSV-1 and VZV DNA in 100 ng of DNA extracted from individual human TG. Each independent analysis contained no template DNA controls and, in all cases, no PCR product was detected in samples lacking template. The real-time PCR amplification of virus DNA standards was linear over a range from 10⁶ to 10¹⁰ copies ($r^2 > 0.95$).

The mean (average) and the standard error of the mean (SEM) for replicate assays were compared by signed rank tests. Spearman rank sum test comparing the results from each set of primers showed significant correlation between all HSV-1 primers and between all VZV primers: HSV-1 ICP27 versus HSV-1 LAT, $P < 0.1$ ($n = 15$); HSV-1 UL44 versus HSV-1 UL54, $P < 0.05$ ($n = 8$); VZV 21 versus VZV 63, $P < 0.0005$ ($n = 34$); VZV 21 versus VZV 29, $P < 0.1$ ($n = 12$); and VZV

29 versus VZV 63, $P < 0.05$ ($n = 12$). Similar pairwise analysis of the mean virus DNA copies showed no significant differences (Wilcoxon rank sum, $P > 0.2$). Thus, the results for HSV-1 UL44 and UL54 or LAT and ICP27 primer sets were combined to yield the mean HSV-1 DNA copy number per 100 ng of DNA for each individual ganglion. Similarly, the results for VZV gene 21 and gene 63 or for gene 29, gene 21, and gene 63 were combined to yield the mean VZV DNA copy number per 100 ng of DNA for each individual ganglion.

Of the 17 individuals analyzed, HSV-1 DNA was detected in both TG from 11 subjects and not detected in either ganglion from 5 subjects (Table 3). HSV-1 DNA was present in the left TG of subject 2 but was absent in the right TG. VZV DNA was found in both TG from all 17 individuals (Table 4). Comparison of the mean HSV-1 DNA copy number detected in the left TG to that detected in the right TG showed significant correlation (Spearman rank sum, $P < 0.025$, $n = 12$) and no significant difference (Wilcoxon rank sum, $P > 0.2$). Similar analysis showed the left and right mean VZV DNA copy numbers to be highly correlated (Spearman rank sum, $P < 0.0005$, $n = 17$), with no significant differences (Wilcoxon rank sum, $P > 0.2$). Thus, for each subject, the virus DNA copy numbers obtained from individual left and right TG were combined to yield the average HSV-1 and VZV DNA burdens in 100 ng of DNA per subject.

As quantitated in Table 3 and shown in Fig. 1, the average HSV-1 DNA copy number in the 12 subjects which contained detectable levels of the virus DNA ranged from 42.9 to 677.9 (mean, 195.0; SEM, 168). The average VZV DNA copy number per 100 ng of TG DNA of the 17 subjects (Table 4, Fig. 1) ranged from 37.0 to 3560.5 (mean, 579.9; SEM, 847.8). In the instances where both virus DNA were present, comparison of the mean HSV-1 DNA copy number to the mean VZV DNA copy number within the same subject showed no significant correlation (Spearman rank sum, $P > 0.2$, $n = 12$) and a modest difference in the means (Wilcoxon rank sum, $0.2 > P > 0.1$, $n = 12$).

Quantitative RT PCR. Polyadenylated VZV gene 21, 29, and 63 transcripts, along with nonpolyadenylated HSV-1 LAT transcripts, were in vitro synthesized. After DNase treatment, each transcript produced a discrete band at the appropriate size on denaturing agarose gels. After removal of unincorporated ribonucleotides and quantitation by determining the optical density at 260 nm, the number of transcripts synthesized was calculated. Figure 2 shows the real-time PCR quantitation of cDNA synthesized from dilutions of 10^6 to 10^{-1} copies of each transcript. Using VZV gene 29-specific primers and probes to generate standard curves, DNA quantitation was linear over a 7-log range of standard, purified VZV DNA ($r^2 > 0.99$) and sensitive to a limit of 10 DNA molecules (Fig. 2A). Similar results were obtained using primers and probes specific for VZV genes 21 and 63 using VZV DNA, as well as HSV-1 LAT using HSV DNA (not shown). Figure 2B shows the RT-dependent cDNA yield as determined by real-time PCR from various amounts of synthetic virus transcripts (input RNA as determined by measuring the optical density). A linear relation is evident between the number of template RNA molecules and the yield of cDNA for each transcript assayed. Overall, the assay was approximately 10-fold more sensitive for the polyadenylated VZV transcripts than for the nonpolyadenylated HSV-1 LAT transcripts. VZV gene 21, 29, and 63 cDNAs were detected when as few as one copy of each transcript was reverse transcribed. HSV-1 LAT cDNA was detected when as few as 10 copies of the synthetic LAT transcript was reverse transcribed. Interestingly, when one transcript from each VZV gene was reverse transcribed, approximately 100 copies of

FIG. 1. HSV-1 and VZV DNA burden in human TG. Total DNA was extracted from left and right human TG and the virus DNA copy number per 100 ng was determined by real-time PCR. Statistical analysis allowed condensation of the results from individual ganglia to yield virus DNA copy numbers in 100 ng per subject. Subject numbers refer to the individuals described in Table 1; data quantitation is shown in Tables 3 and 4. VZV DNA (○) was found in the TG from all 17 subjects, while HSV-1 DNA (●) was found in 12 of the 17 subjects.

cDNA were detected. An amplification of cDNA was also detected for the synthetic HSV-1 LAT transcripts in that when 10 copies of HSV-1 LAT RNA were reverse transcribed, 100 copies of LAT-specific cDNA was detected. The same proportional increase in cDNA yield was maintained with all four transcripts as the amount of template RNA molecules increased.

HSV-1 and VZV latent transcripts. Table 5 shows the real-time PCR quantitation of cDNA synthesized from RNA extracted from individual ganglia obtained from 11 individuals (subjects 1 to 11, Table 1). Columns 3 and 4 represent copies of GAPDH molecules quantitated in the cDNA prepared from 80 ng of total RNA (2% of the cDNA synthesized from 4 μ g of RNA) in replicate samples. The average number of GAPDH molecules (column 5) ranges from 117 to 4,478.5. The standard error of replicate GAPDH quantitations for each TG (SEM, column 6) shows that within an individual cDNA synthesis reaction, quantitation of the number of molecules is accurately obtained using real-time PCR. Columns 7 to 10 list the average quantities of HSV-1 LAT and VZV gene 21, 29, and 63 transcripts normalized to the average number of cellular GAPDH transcripts in two replicate samples obtained from the same cDNA synthesis reaction. HSV-1 LAT transcripts were found in all TG containing HSV-1 DNA but not in TG that did not contain HSV-1 DNA (column 7). The average number of HSV-1 LAT transcripts per 400 ng of total TG RNA (10% of 4 μ g of total RNA) ranged from 19.9 to 645 (not shown). Normalizing the number of HSV-1 LAT transcripts to that of GAPDH showed that the amount of LAT transcripts ranged over 2 orders of magnitude. In latently infected ganglia, there was no statistical correlation between the number of HSV-1 DNA copies and LAT expression (linear regression, $r^2 = 0.40$).

Table 5 also shows the real-time PCR quantitation of VZV gene 21, 29, and 63 transcripts normalized to cellular GAPDH transcripts in the same cDNA preparations from which HSV-1 LAT transcripts were quantitated. Gene 21 transcripts were detected in five individual TG (column 8) and gene 29 transcripts were detected in 3 individual TG (column 9). Gene 63

FIG. 2. Sensitivity of real-time PCR. Dilutions from 10^6 to 10^{-1} copies of polyadenylated VZV gene 21, 29, and 63 transcripts and nonpolyadenylated HSV-1 LAT transcript per 10 μ l of nuclease-free water were added to 500 ng of total RNA extracted from control African green monkey dorsal root ganglia. Each dilution was reverse transcribed, and the cDNA was subjected to real-time PCR using gene 29 reagents. (A) Standard curve generated using VZV genomic DNA as described in Materials and Methods. ●, standards; ○, unknowns (cDNA samples shown in panel B). (B) Calculated gene 21, gene 29, gene 63, and LAT copy numbers in cDNA generated from synthetic RNA, relative to the standard curve shown in panel A (gene 29) and the standard curves for gene 21, gene 63, and LAT (not shown).

transcripts were the most prevalent, being present in 19 of the 22 individual TG (column 10). Normalizing the number of VZV transcripts to that of GAPDH showed that the abundance of gene 21 transcripts spanned 1,430-fold, gene 29 transcripts ranged over 20-fold, and gene 63 transcripts ranged over 2,000-fold. At a confidence level of $>85\%$, there was a statistical correlation between the number of VZV DNA copies and the amount of gene 63 expression (linear regression, $r^2 = 0.13$).

DISCUSSION

Latent infection and virus recrudescence are central themes among the neurotropic alphaherpesviruses. This study represents the first in which both HSV-1 and VZV DNA copy numbers and the numbers of latently associated HSV-1 and VZV transcripts were quantitated in the same individual human ganglia removed at autopsy.

Latent herpesvirus DNA burdens. In this study of 17 individuals, HSV-1 DNA was detected in both TG of 11 individuals, in only a single TG from one individual, and in no TG samples from 5 individuals (overall, 70.6% positive). In contrast, VZV DNA was found in both TG from all individuals. These findings are in accordance with the previous detection of HSV-1 DNA in TG from 72.7% and VZV DNA in TG from 90.9% of 11 individuals using conventional PCR (21). Liedtke

et al. (18) found HSV-1 DNA in 100% and VZV DNA in 67% of TG from 39 individuals 50 to 80 years old using nested PCR analysis. Recently, Pevenstein et al. (27) reported a higher number of individuals latently infected with VZV (79 to 87%) than those with HSV-1 (53%) following analysis of a single TG obtained from 15 subjects. Using real-time PCR, they detected a mean of $2,902 \pm 1,082$ copies of HSV-1 DNA and 258 ± 38 copies of VZV DNA per 10^5 ganglionic cells. In the present study we used real-time PCR to detect HSV-1 and VZV DNA in both TG from 17 individuals. Statistical analysis allowed the condensations of data obtained from individual primer pairs and individual TG to yield an average virus DNA burden per 100 ng of DNA per subject. Assuming 15.6 pg of DNA per TG cell (27) to linearly transform our data, the number of HSV-1 DNA copies per 10^5 ganglionic cells ranged from 669 to 10,575 (mean, 3,042; SEM, 3,274; $n = 12$). Similarly, the number of VZV DNA copies per 10^5 cells ranged from 577 to 55,543 (mean, 9,046; SEM, 13,225; $n = 17$).

The numbers of HSV-1 copies detected in this study compare well with the previous results of Pevenstein et al. (27). However, we detected on average 35-fold more copies of VZV DNA per 10^5 ganglionic cells. The large range of VZV DNA burden within the individual subjects may in part explain the higher mean VZV DNA detected in our study. The median VZV DNA copy number per 10^5 ganglionic cells is 357. The mean VZV DNA copy number per 10^5 ganglionic cells of the

TABLE 5. Quantitation of latent HSV-1 and VZV gene transcripts in individual human TG

Subject	TG location	GAP#1	GAP#2	Avg GAP	SEM	LAT/GAP ^a	21/GAP ^a	29/GAP ^a	63/GAP ^a
1	Left	2,985.7	2,486.0	2,735.9	249.9	444.1	6.2	0.0	168.1
	Right	578.6	723.8	651.2	72.6	1,673.8	0.0	0.0	0.0
2	Left	125.4	108.7	117.0	8.4	23,070.0	0.0	1,153.5	2,785.5
	Right	475.2	273.5	374.3	100.9	0.0	85.5	427.4	2,254.6
3	Left	1,046.9	931.2	989.1	57.8	2,578.2	0.0	55.6	307.4
	Right	727.1	684.1	705.6	21.5	3,755.8	0.0	0.0	9.9
4	Left	2,297.0	1,882.9	2,090.0	207.1	1,100.5	8.6	0.0	150.2
	Right	1,489.2	1,364.9	1,427.1	62.2	1,226.3	0.0	0.0	121.2
5	Left	1,855.1	1,077.8	1,466.5	388.7	0.0	0.0	0.0	0.0
	Right	1,055.3	1,293.2	1,174.3	119.0	0.0	0.0	0.0	38.3
6	Left	2,037.1	2,328.6	2,182.9	145.8	0.0	0.0	0.0	318.9
	Right	2,228.3	2,004.6	2,116.5	111.9	0.0	0.0	0.0	2.8
7	Left	4,564.6	3,908.8	4,236.7	327.9	743.5	0.0	0.0	789.5
	Right	2,963.9	2,991.5	2,977.7	13.8	2,166.1	0.0	0.0	1,105.2
8	Left	4,444.6	4,512.3	4,478.5	33.9	44.3	0.0	0.0	118.1
	Right	4,912.0	4,630.3	4,771.2	140.9	880.3	0.0	0.0	162.9
9	Left	1,696.2	1,643.9	1,670.1	26.2	0.0	0.0	0.0	1.2
	Right	703.3	874.7	789.0	85.7	0.0	0.0	0.0	0.0
10	Left	704.9	729.4	717.2	12.3	1,150.4	0.0	0.0	7.0
	Right	939.9	656.0	798.0	141.9	4,636.8	0.0	0.0	38.9
11	Left	1,659.6	1,652.0	1,655.8	3.8	111.7	1.2	0.0	16.9
	Right	3,329.8	3,278.7	3,304.3	25.6	690.0	0.6	0.0	93.5

^a Calculated as follows: (number of virus gene transcripts/number of GAPDH transcripts) $\times 10^4$. GAP, GAPDH.

eight individuals below the median is 112 (SEM, 57) and for the eight individuals above the median is 1,075 (SEM, 965). The 10-fold difference in the mean VZV DNA copy number for individual samples above and below the median could be due to limited virus reactivation. However, the time separating death and autopsy was not correlated with the VZV DNA copy number in the TG. The average time between death and autopsy (tissue removal and flash freezing in liquid nitrogen) for the eight subjects whose mean VZV DNA copy number was below the median was 18.3 h (SEM, 6.7 h). Similarly, 17.8 h (SEM, 5.0 h) is the average time between death and autopsy for the eight subjects whose mean VZV DNA copy number was above the population median. While the immediate cause of death, underlying illness, or resuscitative procedures may contribute to VZV reactivation, the sample size precludes statistical association. Further, the narrow range of HSV-1 DNA copies per subject compared to that of VZV suggests that if VZV reactivation had occurred, the initiation event did not induce HSV-1 reactivation. An alternative explanation for the wide range in the VZV DNA copy number per subject stems from the biology of the virus. While HSV-1 latency is established by retrograde transport of the virus from the periphery, both retrograde transport and hematogenous infection of the ganglion during primary viremia may be involved in the establishment of VZV latency. Primary VZV infection is generally spread over more dermatomes than is primary HSV-1 infection and, therefore, one would expect more ganglionic cells latently infected with VZV than HSV-1. Also, the severity of primary VZV varies within the population; thus, one may also expect a variation in latent VZV burden.

Latent HSV-1 and VZV gene transcription. The function of the HSV-1 LAT transcript is being debated (24, 28); however, the basic consensus is that TG latently infected with wild-type virus express LAT. Consistent with this assertion, we detected LAT transcripts in all TG samples shown to contain HSV-1 DNA. For comparison between individual TG and among subjects, the LAT copy number was normalized to the number of GAPDH transcripts within the same RT reaction. The relative expression of HSV-1 LAT varied by >520-fold, with no asso-

ciation between the number of latent HSV-1 DNA copies and LAT expression.

Polyadenylated transcripts mapping to VZV genes 21, 29, and 63 have been detected in RNA extracted from latently infected human TG. VZV gene 62 transcripts have been detected by Northern blot analysis of poly(A) selected human TG RNA (22). We have also detected VZV gene 62 transcripts in a cDNA library constructed from poly(A) selected human TG RNA (2). However when the 3' terminus of the gene 62 transcripts were cloned and sequenced, no polyadenosine tract was detected. Thus, although circumstantial evidence exists that VZV gene 62 is a polyadenylated transcript in latently infected human ganglia, no sequence evidence has been obtained. Therefore, in this initial study, we concentrated our quantitation efforts on the three VZV transcripts that have been shown to be polyadenylated in latently infected human TG.

With the exception of gene 21 transcripts, VZV transcripts have been detected in samples obtained from pooled ganglia (3). The present study is the first to quantitate multiple VZV genes in individual TG. The expression of VZV gene 21, 29, and 63 transcripts relative to cellular GAPDH transcripts allows the comparison of the abundance of these transcripts between left and right TG of the same individual along with the relative abundance between the TG of different subjects. Our results indicate that, of the three transcripts analyzed, gene 63 is closely associated with latent VZV infection, as all but two individual TG expressed detectable levels of gene 63 transcripts. Further, at a confidence level of 85%, gene 63 expression is related to the amount of VZV DNA detected within the individual TG (linear regression, $r^2 = 0.13$). From 11 subjects we tested, gene 21 and 29 transcripts were detected in 5 and 3 of the 22 latently infected TG, respectively. In only one ganglion (i.e., the right ganglion, subject 2) were all three VZV transcripts detected.

Despite extensive investigation by numerous laboratories, the function of latently expressed herpesvirus transcripts has not been conclusively determined. Indeed, the lack of an animal model for VZV latency and hence the requirement to analyze human autopsy tissue has always raised concerns in

interpreting the data. Further confounding the analysis has been the pooling of tissue from numerous individuals. Here we have shown that VZV gene 63 is consistently detected in individual latently infected human TG. VZV gene 63 encodes a 30.5-kDa nuclear-located phosphoprotein detected in latently infected TG neuronal cells and in dermal nerves of herpes zoster biopsies (20, 26, 34). Although the protein encoded by gene 63 is classified as immediate early, its lack of transcription transactivation suggests that this protein plays a subordinate role in modulating VZV gene expression (13). Whether gene 63 is critically involved in latency or whether gene 63 transcription is initiated in response to neuronal death awaits further experimentation.

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