Quantitation of Latent Varicella-Zoster Virus DNA in Human Trigeminal Ganglia by Polymerase Chain Reaction

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Competitive polymerase chain reaction was used to quantitate latent varicella-zoster virus (VZV) DNA in human trigeminal ganglia. Ganglionic DNA from five subjects was amplified with oligonucleotide primers specific for VZV gene 28. Two of the samples were also analyzed with primers specific for VZV gene 62. Our results indicated that there are 6 to 31 copies of the VZV genome in every 100,000 ganglionic cells.

After primary infection, varicella-zoster virus (VZV) becomes latent in dorsal root ganglia and reactivates decades later to produce zoster (6). Polymerase chain reaction (PCR) revealed latent VZV DNA in trigeminal ganglia from 13 of 15 (86%) humans and in thoracic ganglia from 9 of 17 (53%) humans; none of three thoracic ganglia from one seronegative individual contained VZV DNA (7). To develop an understanding of the establishment and maintenance of latency, it is necessary to know the cell type in which virus is latent, its abundance and configuration, and the extent of virus DNA expression. This study addressed the issue of virus DNA abundance during latency. We used a modified version of competitive PCR (5) to determine precisely the number of VZV DNA molecules in human ganglia during latency in comparison with the number in productively infected cells in tissue culture.

One or both trigeminal ganglia were obtained 2 to 24 h after death from five humans who died of illnesses unrelated to VZV infection (Table 1). Total DNA was extracted from human ganglia and VZV-infected BSC-1 cells as described previously (7). DNA sequences specific for VZV gene 28 (located in the unique long region of the virus genome) and for VZV gene 62 (located in the inverted repeat sequences bracketing the unique short region of the virus genome) were used for PCR amplifications. VZV genes 28 and 62 are separated by 58 kbp.

VZV gene 28. Figure 1 illustrates the construction of clones containing the wild-type and mutated VZV gene 28 sequences. DNAs extracted from a wild-type clone and a recombinant clone containing a mutated gene 28 were purified on a CsCl gradient. The recombinant DNAs were linearized with *EcoRI*, extracted with phenol (saturated with 1 M Tris-HCl [pH 8.0]), precipitated with ethanol, and redissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA concentration was determined from the average of five independent recordings of optical density at 260 nm. Solutions containing 25, 20, 10, 5, and 1 copy of mutant DNA per 10 μ l were prepared by serial dilution. Oligonucleotide primers (Operon Inc., Alameda, Calif.) specific for VZV gene 28 (4) were chosen from published sequences of VZV DNA (1).

DNA (1 μ g) extracted from one or both trigeminal ganglia was mixed in duplicate with 25, 20, 10, 5, 1, or 0 copies of the linearized recombinant clone containing the mutated gene 28 and used for PCR amplification in a total volume of 100 μ l. PCR and the analysis of products were performed as described before (7, 8). Briefly, duplicate samples containing 1 μ g of human ganglionic DNA were mixed with various amounts of the cloned mutated VZV gene 28 DNA and amplified for 40 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The elongation step (72°C) in the last cycle was for 7 min. An aliquot (50 μ l) of the PCR products was mixed with 5 μ l of 4 M NaCl, extracted with an equal volume of Tris-HCl (pH 8.0)-saturated phenol, precipitated with 2 volumes of ethanol, and redissolved in 50 μ l of TE buffer. A 12- μ l aliquot of the cleaned PCR products was then digested with *NcoI* for 16 h, separated on a 2% agarose gel, transferred onto a Zeta-probe (Bio-Rad, Richmond, Calif.) membrane, and hybridized with an end-labeled oligonucleotide probe located internal to the amplified DNA segment (7, 8). The filter was washed and exposed to Kodak X-OMAT film for 5 h at -70°C.

DNA from latently infected human ganglia was mixed with the recombinant clone containing the mutated gene 28 and amplified. *NcoI* digestion of the products yielded three fragments (250, 175, and 75 bp), two of which (250 and 175 bp) hybridized to the end-labeled oligonucleotide probe. Figure 2 shows the results of quantitative PCR on DNA from the trigeminal ganglia of subject 1. The Zeta-probe (Bio-Rad) membrane containing the radioactive bands was cut out, and the radioactivity was counted by liquid scintillation spectrophotometry. Background counts per minute (cpm) were determined from the no-DNA control (first lane in Fig. 2).

From the equation $Y = A(1 + R)^n$, where Y is the extent of amplification, A is the initial amount of DNA, R is the mean efficiency per cycle, and n is the number of cycles (9), the relationship between the number of copies of the mutated clone containing VZV gene 28 DNA added to the PCR mixture and the ratio of cpm in the mutant band to cpm in the wild-type band is linear. A first-order line of regression was fitted to the data points obtained by counting the cpm in the respective bands (Fig. 3). This line did not intersect the origin (as would be expected), indicating a margin of error of ± 2 copies of VZV DNA.

The number of copies of VZV DNA in 1 μ g of ganglionic

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TABLE 1. Amount of VZV DNA in human trigeminal ganglia

Subject no.	Age (yr)/sex ^a	VZV antibody present ^b	Cause of death	No. of copies of VZV gene 28 DNA/ μ g of total DNA ^c	No. of copies of VZV gene 62 DNA/ μ g of total DNA ^c
1	54/F	+	Peritonitis	11.0 (2)	ND ^d
2	44/M	+	Pulmonary embolism	17.0 (1)	ND
3	62/M	+	Cardiac arrest	15.0 (1)	53 (1)
4	49/F	+	Myocardial infarction	10.0 (1)	9 (1)
5	67/M	+	Sepsis and pneumonia	30.0 (1)	ND

^a F, female; M, male.^b Determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radioimmunoprecipitation (3).^c Numbers in parentheses indicate how many trigeminal ganglia were tested.^d ND, not determined.

DNA was determined from the point where the cpm in the mutant band and in the wild-type band were equivalent. Trigeminal ganglia from subject 1 contained 11 copies of latent VZV gene 28 DNA per μ g of cell DNA. Identical results were obtained in duplicate experiments (not shown). The same protocol was used to analyze DNA extracted from the trigeminal ganglia of five humans and revealed the presence of 10 to 30 copies of VZV gene 28 DNA in each microgram of total ganglionic DNA (Table 1).

We are aware of the caveat of Gilliland et al. (5) that in the use of mutant templates as competitors in assays in which the primer is rate limiting, annealing may occur between heterologous strands of mutant and wild-type templates. However, heteroduplexes would not be cleaved by *Nco*I. To standardize conditions, heteroduplex formation needs to be

maximized by heating the PCR products at 94°C for 4 min and then cooling them. If the mutant and wild-type templates are present in a 1:1 molar ratio prior to PCR, the ratio of products from the mutated template to those from the wild-type template will be 1:3 because of heteroduplex formation. Under conditions of heteroduplex formation, we observed that the point of equivalence was not altered, indicating that our primers were indeed in excess in the amplification reaction.

VZV gene 62. Trigeminal ganglia from two subjects were also studied by competitive PCR for VZV gene 62. For the primer pair used for VZV gene 62 amplification, the rightward ends are located between nucleotides 107835 and 107859 and the leftward ends are located between nucleotides 107915 and 107935 on the VZV genome (1). This

FIG. 1. Construction of recombinant clones for quantitative PCR with VZV gene 28. The VZV *Eco*RI-B fragment (10.6 kbp) (a gift from Stephen Straus) was digested with *Apa*I, and the 1.5-kb *Eco*RI-*Apa*I (E-A) fragment containing the 5' terminus of VZV gene 28 was purified from an agarose gel and cloned into a transcription vector [pGEM7Zf(+); Promega] which had been digested with *Eco*RI and *Apa*I. The recombinant clone (wild type) has an *Nco*I site between the two primer sequences (1 and 2) used to amplify a segment of VZV gene 28. The wild-type recombinant clone was linearized at the *Nco*I site, the restriction site was destroyed by being filled in with the Klenow fragment of *Escherichia coli* DNA polymerase, and the DNA was religated and used to transform *E. coli* DH5 α . The mutant recombinant clone was used to amplify a 250-bp segment of VZV gene 28 which lacked the *Nco*I site. After amplification, the wild-type recombinant clone and the VZV DNA from human ganglia produce a 250-bp segment of VZV gene 28, which was digested with *Nco*I to obtain two fragments (175 and 75 bp). *Nco*I digestion of the amplification products from a mixture of the wild-type and the mutant recombinant clones yielded three fragments of 250, 175, and 75 bp.

FIG. 2. Quantitative PCR. Total DNA (1 μ g) isolated from the pooled trigeminal ganglia of subject 1 was mixed in duplicate with 25, 20, 10, 5, 1, or 0 copies of the linearized mutant gene 28 clone in a total volume of 100 μ l and used for PCR amplification. As a control, no DNA was included in one of the reaction tubes. DNA from VZV-infected BSC-1 cells was included as a positive control. The sizes of the PCR products detected are indicated.

should generate a 100-bp PCR product. The internal oligonucleotide probe used for detection of the PCR product of VZV gene 62 is located between nucleotides 107861 and 107885 on the virus genome (1). A mutated single-stranded DNA was then used to determine the copy number of latent VZV DNA in the trigeminal ganglia of subjects 3 and 4 as described for VZV gene 28.

To construct the mutant VZV gene 62, a synthetic 100-nucleotide single-stranded DNA containing an A to T substitution at positions 52 and 53 (corresponding to positions 107887 and 107888 in the VZV genome) was obtained (Operon, Inc.). The substitution in the synthetic 100-mer generated a unique *Eco*RI site. Whereas the 100-bp PCR product generated from ganglionic DNA (wild type) does not contain an internal *Eco*RI site, the 100-bp amplification product from the synthetic (mutant) single-stranded DNA produces 54-bp and 46-bp *Eco*RI fragments. The products were detected and quantitated by using a labeled internal oligonucleotide as described above.

At the end of the first cycle of the competitive PCR, a single copy of a 100-bp double-stranded segment is produced from the mutant single-stranded DNA, whereas four copies of 100-bp double-stranded DNA are produced from wild-

FIG. 3. Quantitation of latent VZV DNA in human trigeminal ganglia by competitive PCR. Amplification of VZV gene 28-specific sequences from DNA extracted from the trigeminal ganglia of subject 1 and analysis of the products were performed as described in the text. The cpm for each sample was determined by averaging samples from duplicate lanes (see Fig. 2). The point of equivalence (cpm in the mutant [Mut] band/cpm in the wild-type [WT] band = 1.0) was obtained at 11 copies of input mutant DNA.

type VZV gene 62, since there are two copies of gene 62 in the VZV genome. Therefore, at the point of equivalence, the number of cpm in the mutant band is one-fourth of that in the wild-type band.

The number of copies of VZV in DNA from the trigeminal ganglia of subjects 3 and 4 (Table 1) for gene 62 was determined by this analysis. Between 9 and 53 copies of VZV gene 62 DNA were present in each microgram of total ganglionic DNA (Table 1). Given the ability of PCR to detect small numbers of copies of DNA molecules, these numbers compare favorably with the 10 to 15 copies of VZV gene 28 detected in DNA from the trigeminal ganglia of subjects 3 and 4.

DNA from purified VZV virions was also quantitated by competitive PCR. With either the gene 28 or gene 62 primers, 7.309×10^9 copies of VZV DNA were detected in 1 μ g of virion DNA (data not shown), in contrast to 9 to 53 copies of VZV DNA per μ g in normal human trigeminal ganglia. Furthermore, 1 μ g of VZV DNA (125 kbp) contains 7×10^9 molecules, which is in agreement with the quantity determined by competitive PCR. Each eukaryotic cell contains 6×10^{-6} μ g of DNA (10). We detected 9 to 53 copies of VZV DNA per μ g of ganglionic DNA, or 6×10^{-5} to 31×10^{-5} VZV DNA molecules per cell. This corresponds to approximately 6 to 31 copies of VZV DNA in every 100,000 ganglionic cells. In normal human ganglia latently infected with herpes simplex virus type 1, approximately 1,000 to 10,000 copies of herpes simplex virus DNA have been detected in every 100,000 cells (2). The relationship between the greater (166- to 322-fold) abundance of herpes simplex virus DNA than of VZV DNA in latently infected human ganglia and the frequent reactivation of herpes simplex virus in the young versus VZV reactivation in the elderly remains to be determined.

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