DETERMINATION OF THE DNA TARGET SEQUENCE OF POORLY REACTIVABLE STRAIN HSZP OF HERPES SIMPLEX VIRUS TYPE 1 BY POLYMERASE CHAIN REACTION

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Summary. – HSZP strain of herpes simplex virus type 1 (HSV-1) – unlike strains KOS, SC16 and ANGpath – established latency in the homolateral trigeminal ganglion of mice at a limited rate (21%) when tested by reactivation of latent virus in culture. If a nested polymerase chain reaction (PCR) for virus DNA detection was used, the positivity rate was 63 – 100%. The detection rate of HSZP DNA in acutely infected gangla did not differ from that of SC16 DNA provided that the sensitivity of PCR was below 20 pg of HSZP DNA per 0.5 μg of total ganglionic DNA. The nested PCR assessed at least 200 fg of HSZP DNA per 0.5 μg of ganglionic DNA.

Key words: herpes simplex virus type 1; latent infection; polymerase chain reaction; reactivation

Introduction

A latent virus infection established in the regional sensory ganglion after peripheral HSV administration is usually detected by explantation of neural tissue or its co-cultivation with indicator cells. Extensive studies on molecular level (Ho et al., 1992) have showed that latent HSV DNA in the nucleus is possibly circular and episomal (rewieved by Fraser *et al.*, 1986), probably associated with nucleosomes forming a chromatin-like structure (Deshmane and Fraser, 1989), and its expression is extremely limited. The only transcripts detectable during latency are latency-associated transcripts (LAT) and their precise role has not been elucidated yet. Nevertheless, they may operate at maintenance and/or reactivation of latency rather than at its establishment (Fraser *et al.*, 1992). The expression of LAT is not essential for latent infection (Steiner *et al.*, 1989).

PCR was succesfully applied to assess the presence of HSV-1 DNA in cerebrospinal fluid during HSV encephalitis (Aurelius *et al.*, 1991; Guffond *et al.*, 1994; Kúdelová *et al.*, 1995), and to demonstrate the presence of latent HSV DNA in extracts of neural tissues (Lynas *et al.*, 1989; Cantin *et al.*, 1991). Gressens and Martin (1994) confirmed the presence of HSV-2 LAT in ganglionic pseudounipolar neurons, but not in the neurons of CNS, though the HSV-2 thymidine kinase gene sequences were detected in both neurons.

The peculiar biological properties of HSV-1 strain HSZP were described previously (Szántó *et al.*, 1972). This strain is not pathogenic for mice after peripheral inoculation and is defective in the early shut-off function (Matis and Szántó, 1985). Here we report that the HSZP strain establishes well a latent infection in homolateral trigeminal ganglion of mice. The latent virus is poorly reactivable in culture, but its DNA sequences are well detectable by PCR.

Abbreviations: EDTA = ethylenediamine tetraacetate; gB = glycoprotein B; HSV = herpes simplex virus; LAT = latency-associated transcript; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate; Tris = tris-(hydroxymethyl)-aminomethane

Materials and Methods

Virus. KOS strain originated from Dr. J.L. Melnick's collection (WHO Collaborating Center for Virus Reference

and Research, Houston, TX, USA), ANGpath strain from the Institute of Virus Research, German Cancer Research Center, Heidelberg, F.R.G. and SC16 strain was kindly provided by Dr. T. Hill, University of Bristol, Bristol, U.K. The domestic HSZP strain, originally isolated from a patient's lip, was passaged in rabbit lung cells and Vero cells, and was adapted to chick embryo cells.

Animals. DBA/2 mice from the breed Velaz (Czech Republic) were kept under standard conditions. The virus dose was inoculated intracutaneously into the right lip. In acute and latent infection the both trigeminal ganglia and the brain stem at the entrance zone of the right trigeminal nerve root were removed under sterile conditions on days 3-6 and 38-41 p.i., respectively, and either cultured or used for DNA extraction.

Tissue explantation was described previously (Rajčáni *et al.*, 1990). Briefly, the minced tissue fragments were kept in plastic Petri dishes for 10 days in culture. The RPMI medium supplemented with 10% foetal calf serum, antibiotics and 8 μmol/l 5-azacytidine as inducer (Whitby *et al.*, 1987) was changed for a fresh one on days 4 and 7 in culture. The explanted tissue fragments were examined by day 10, and the medium on days 4, 7 and 10 in culture.

Virus detection was done in Vero cell monolayers grown on 24-well microplates. Samples of media and of 10% suspensions prepared from explants after termination of explantation (pooled fragments from each sample were collected) were inoculated into Vero cell cultures and the latter were incubated for 3-5 days at 37°C in 4% CO_2 atmosphere.

DNA extraction from ganglionic samples was carried out according to Sambrook *et al.* (1989). Ganglionic tissue (5 – 10 mg) was digested for 1 hr at 37°C in 10 mmol/l Tris-HCl pH 8.0 with 0.1 mol/l EDTA, 0.5% SDS and 20 μg/ml pancreatic RNase (DNase-free, Boehringer), and then for 3 hrs at 50°C in the same buffer complemented with 100 μg/ml proteinase K. The suspension was gently mixed and extracted with phenol for 10 mins. The water phase was repeatedly extracted twice as before. DNA was ethanol-precipitated, washed, air dried, and dissolved in 50 μl of TE buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA pH 8.0). The DNA yield ranged from 7 to 30 μg per ganglion.

PCR. A modification of the standard procedure using Taq polymerase (Saiki *et al.*, 1988) was employed. The downstream primer P11 (5'-CCA AAA GAT GCA CAT GCG GTT-3', nt 53,023 – 53,043 of UL27 (McGeoch *et al.*, 1988)), and the reversed upstream primer P12 (5'-AAG GTG GTG ATG GGA CTC GTG-3', nt 53 544 – nt 53 524 of UL27) were designed to flank the 522 bp fragment coding for the cytoplasmic portion of glycoprotein B (gB) (the syn[1] locus). The primers were synthetized using the Pharmacia Gene Assembler according to the manufacturer's procedure. Usually 0.25 – 1.0 μg of ganglionic DNA or 100 pg of purified HSZP DNA (used as positive control) were amplified in 50 or 100 μl reaction volume with 0.2 mmol/l dNTP mixture (Perkin Elmer Cetus), 0.5 μmol/l primers and 2.5 U of Taq polymerase (Boehringer) in the corresponding buffer. The reaction mixture was denatured for 7 mins at 94°C and cycled 35 times (2 mins at 96°C, 2 mins either at 42°C (low stringent conditions) or 52°C (stringent conditions) and 3 mins (prolonged by 3 secs at each cycle) at 72°C). The products of the PCR (first PCR) were used as template for the second, nested PCR.

The nested PCR was performed with the downstream primer 11N (5'-AAC AAA CCC CCC ATC ACA GGT-3', nt 53,067 – 53,087 of UL27) and the reversed primer 12N (5'-GGC CTT CTT CGC CTT TCG CTA-3', nt 53,428 – 53,408 of UL27), flanking a 362 bp nested fragment. The nested PCR had 25 cycles (96°C for 2 mins, 56°C for 1.5 min, and 72°C for 2 mins) or 35 cycles, when the PCR products were not sufficiently distinct in the gel. PCR products were visualized by electrophoresis in 1.5% agarose gel containing 0.5 μg/μl ethidium bromide. DNA size markers VI (154 – 2176 bp) and VIII (37 – 1114 bp) (Boehringer) were used. The positive and negative controls were included in each amplification, as well as control with distilled water instead of template. We followed the guidelines suggested by Kwok and Higuchi (1989) to avoid contamination.

SmaI digestion. A 20 μl-aliquote of PCR reaction mixture was incubated with 2 U of *Sma*I (Boehringer) for 4 hrs at 25°C.

Results

Reactivation in culture of latent infection with HSZP, ANGpath, KOS and SC16 strains

The pathogenic strains ANGpath and SC16 and the non-pathogenic strains KOS and HSZP were used to establish a latent infection in the homolateral trigeminal ganglia of DBA/2 mice after inoculation into right lip. Non-pathogen-

Table 1. Latent infection of trigeminal ganglia and brain stem of DBA/2 mice inoculated with HSV-1 strains ANGpath, SC16, KOS and HSZP

Virus strain	Inoculation dose	Frequency of reactivated virus RTG	LTG
ANGpath	1×10^5 PFU	23/29 (79%)	4/29 (14%)
SC16	1×10^4 PFU	14/20 (70%)	ND
KOS	5×10^5 PFU	14/17 (82%)	3/17 (17.6%)
HSZP	2×10^6 PFU	6/28 (21%)	0/28 (0.%)

RTG – right trigeminal ganglion; LTG – left trigeminal ganglion; ND = not done.

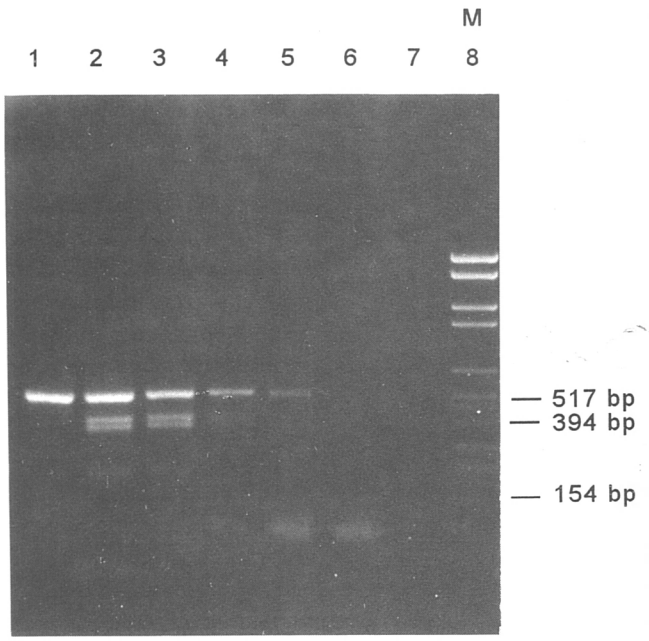

Fig. 1
Sensitivity of the first PCR with purified HSZP DNA under low stringency
Agarose gel electrophoresis of PCR products. Various amounts of DNA used in PCR: 2 ng (lane 1), 200 pg (lane 2), 20 pg (lane 3), 2 pg (lane 4), 200 fg (lane 5), 20 fg (lane 6). No DNA (lane 7). Size marker DNAs (lane 8).

ic strains HSZP and KOS did not kill mice when given in a dose of 5 x 10^5 – 2 x 10^6 PFU per animal, while SC16 strain was lethal for the half of mice already in a dose as low as 5 x 10^3 PFU. Some samples yielded virus when collected by day 10 in culture, many samples shed the reactivated virus into medium since day 7 in culture. As shown in Table 1, the reactivation rate of latent HSZP strain in culture was 21% only, while that of other virus strains was higher (79% – 82%). In addition, strains ANGpath and KOS also spread to the co[illegible]lateral ganglion, a property known for sev[illegible]trains (Rajčáni *et al.*, 1977).

The sensivity of PCR [illegible] primers P11 and P12 was 0.2 – 2 pg of purified HSZP DNA. Multiple DNA products were amplified at the annealing temperature of 42°C (Fig. 1, lanes 2-4), but only a single virus-specific 522 bp band was produced at the annealing temperature of 52°C (Fig. 2). The sensitivity of HSZP DNA detection decreased to 2 – 20 pg in the presence of 0.25 – 0.5 µg of control mouse ganglionic DNA (Fig. 2, lanes 12-19). In the second, nested PCR, the sensitivity increased 10-100 times and as few as 20 fg of purified HSZP DNA might have been detected (data not shown). Thus, samples which showed no visible products in the first PCR, became positive in the second, nested PCR. Fig. 3 (lane 19) shows that 20 pg of purified HSZP DNA in the absence of heterologous DNA could

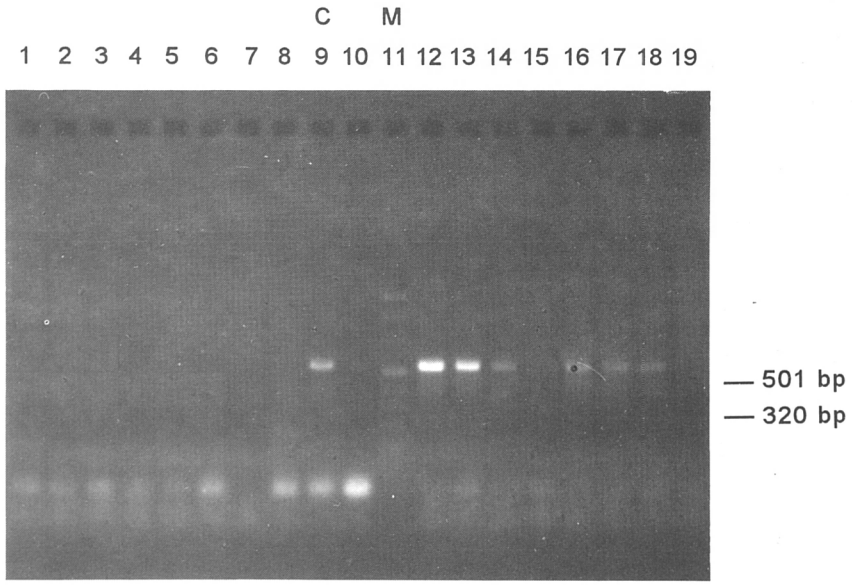

Fig. 2
Products of the first PCR with ganglionic DNA from mice latently infected with HSZP strain
Agarose gel electrophoresis of PCR products. Ganglionic DNA was isolated from mice at day 38 p.i. PCR products of ganglionic DNA samples # 27(lane 1), # 28 (lane 2), # 29 (lane 3), # 30 (lane 4), # 31 (lane 5), # 32 (lane 6), # 33 (lane 7), # 34 (lane 8). HSZP DNA, 100 pg (lane 9). No DNA (lane 10). Size marker DNAs (lane 11). PCR products of HSZP DNA 2 ng (lanes 12, 16), 200 pg (lanes 13, 17), 20 pg (lanes 14,18), 2 pg (lanes 15, 19) in the presence of 0.25 µg of control ganglionic DNA (lanes 12-15) and of 0.5 µg of control ganglionic DNA (lanes 16-19).

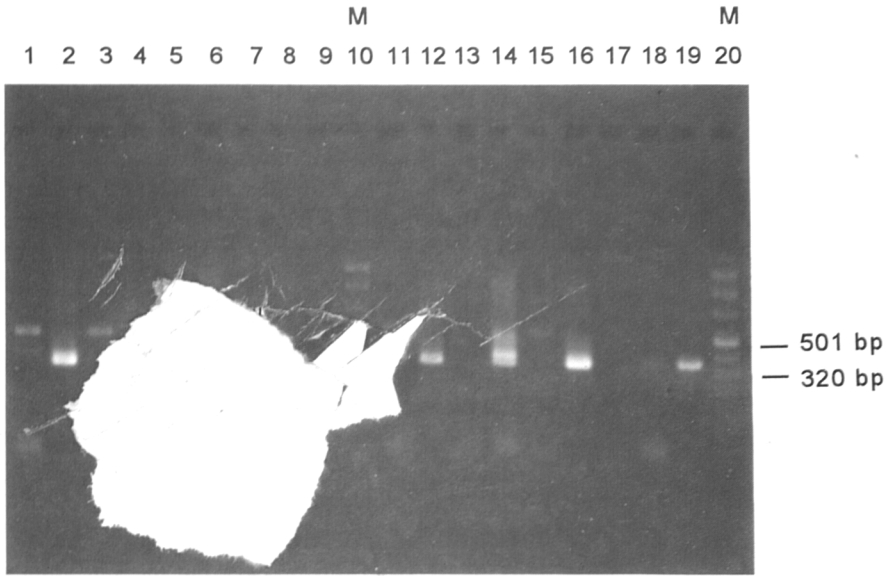

Fig. 3

Products of the first and second (nested) PCR with various amounts of purified HSZP DNA

Agarose gel electrophoresis of PCR products. The first PCR with 20 pg (lanes 1, 11, 13), 2 pg (lane 3), 200 fg (lanes 5, 15, 17) and 20 fg (lane 7) of HSZP DNA in the absence of control ganglionic DNA (lanes 1, 3, 5, 7), and in the presence of 0.25 μg (lanes 11, 15) and of 1 μg (lanes 13, 17) of control ganglionic DNA. No DNA (lane 9). The second (nested) PCR with 0.1 μl from the first PCR of samples 1, 3, 5, 11, 15 (lanes 2, 4, 6, 12, 16), and with 10 μl from the first PCR of samples 7, 13, 17 (lanes 8, 14, 18). The first PCR performed with the nested primers and 20 pg of purified HSZP DNA (lane 19). Size marker DNAs (lanes 10, 20).

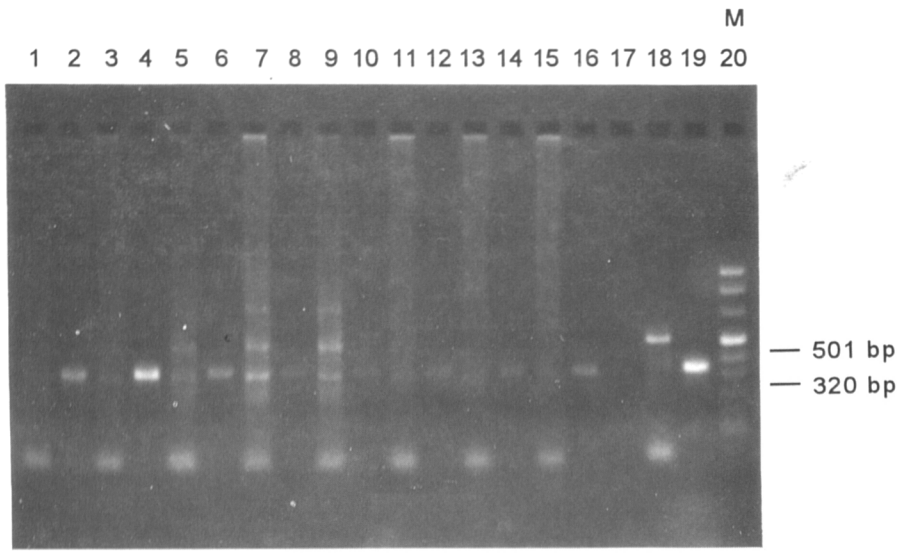

Fig. 4

Products of the first and second (nested) PCR with ganglionic DNA from mice latently infected wit'. .ISZP strain

Agarose gel electrophoresis of PCR products. Ganglionic DNA was isolated from mice at day 40 p.i. Ganglionic DNA samples # 72 (lanes 1, 2), # 73 (lanes 3, 4), # 74 (lanes 5, 6), # 76 (lanes 7, 8), # 77 (lanes 9, 10), # 80 (lanes 11, 12), # 81 (lanes 13, 14), # 82 (lanes 15, 16). No DNA (lane 17). The first PCR with 0.5 μg of DNA (lanes 1, 3, 5, 7, 9, 11, 13, 15). The second (nested) PCR with 10 μl from the first PCR of samples 1, 3, 13 (lanes 2, 4, 14) and with 0.1 μl from the first PCR of samples 5, 7, 9, 11, 15 (lanes 6, 8, 10, 12, 16). The nested PCR with 1 μl from the first PCR of sample 18 (lane 19). Size marker DNAs (lane 20).

be easily detected but yielded only a faint band in the presence of 1 μg of heterologous DNA (lane 13).

Detection of HSZP DNA target sequence in the trigeminal ganglion of latently infected mice by PCR

In the first trial, 8 ganglia from DBA/2 mice with established HSZP latent infection (38 days p.i.) were negative in the first PCR under stringent conditions (Fig. 2, lanes 1-8); it revealed a sensitivity of 2 – 20 pg of HSZP DNA per 0.5 μg of ganglionic DNA (Fig. 2, lanes 16-19). In PCR performed under low stringent conditions, 7 of them showed faint PCR products (data not shown). The nested PCR confirmed the presence of HSZP DNA in the range of 200 fg to 2 pg per 0.5 μg of ganglionic DNA. In the second trial, out of 12 ganglia from HSZP latently infected mice (40 days p.i.). 9 were positive under stringent conditions in the first PCR and each ganglion was positive in the nested PCR (Fig. 4). Summing up, the first PCR was positive in 16 of 20 homolateral ganglionic samples (80%), while 19 of 20 samples were found positive by the nested PCR (Table 2).

The specificity of HSV DNA detection was confirmed by digestion of the PCR products with *Sma*I (Fig. 5). The 522 bp product was cleaved into the 280 and 242 bp subfragments, and the 362 bp product was cleaved into the 198 and 164 bp subfragments in agreement with the theoretical assumptions.

Detection of SC16 and ANGpath DNA target sequences in the trigeminal ganglion of acutely and latently infected-mice

In the first PCR with nested primers, 7 out of 8 ganglia from mice acutely infected with SC16 strain (3 days p.i.) were positive (Fig. 6). One sample showed a borderline positivity, probably due to low DNA content. At day 6 p.i., 9 of 11 examined ganglia were positive in the first PCR, while all samples were positive in the second, nested PCR.

Five of 6 ganglia from mice latently infected with strain SC16 (40 days p.i.) were positive in the first PCR and all samples were positive in the second, nested PCR (Table 2). The ANGpath DNA target sequence was assessed in mice with established latency by day 41 p.i., but not during acute infection. Out of 16 samples examined 10 were positive and the rest remained negative even in the second, nested PCR (Table 2).

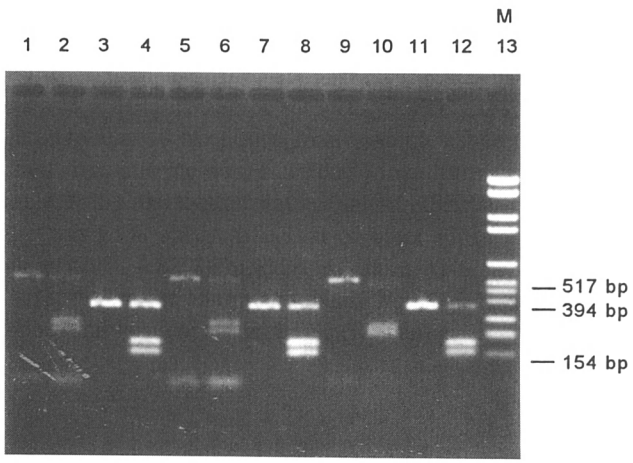

Fig. 5
***Sma*I-digestion of PCR products**
Agarose gel electrophoresis. *Sma*I-digested (lanes 2, 6) and undigested (lanes 1, 5) products of the first PCR of purified HSZP DNA. *Sma*I-digested (lanes 4, 8) and undigested (lanes 3, 7) products the second (nested) PCR of the samples shown on lanes 1, 5. *Sma*I-digested (lane 10) and undigested (lane 9) products of the first PCR with ganglionic DNA isolated from a mouse latently infected with HSZP strain. *Sma*I-digested (lane 12) and undigested (lane 11) products of the second (nested) PCR of the sample shown on lane 11. Size marker DNAs (lane 13).

Table 2. Detection of HSV-1 DNA by PCR in trigeminal ganglia of DBA/2 mice during acute and latent infection with strains SC16, ANGpath and HSZP

Virus strain	Day p.i.	Positivity rate		Positivity %	
		1.PCR	2.PCR (nested)	1.PCR	2.PCR (nested)
SC16	3	7/8	ND	88%	ND
	6	9/11	10/11	82%	91%
	40	5/6	6/6	83%	100%
ANGpath	41	10/16	10/16	63%	63%
HSZP	38 and 40	16/20	19/20[a]	80%	95%

[a]Assayed at the sensitivity of 200 fg of HSZP DNA in the presence of 0.5 μg of total ganglionic DNA.
ND = not done.

Discussion

The maximal sensitivity of the nested PCR in our experiments was in the range of 20 – 200 fg HSZP DNA per 0.5 μg of total ganglionic DNA. Assuming that the mouse chromosomal DNA consists of 3.3×10^9 bp, 0.5 μg of cell DNA corresponds to 1.5×10^5 cells (Brown,1991). The threshold sensitivity of the PCR used in our experiments represents a detection of 20 fg HSZP DNA (130 HSV DNA molecules), corresponding to a single HSZP DNA molecule present in 1000 or lower ganglionic cells. Simmons *et al.* (1992) have found that the frequency of occurence of SC16 strain genome in mouse dorsal root ganglia was in the range from 0.001 to 0.056 per cell. Using ^{3}H-thymidine-labelled HSV-1 for establishment of latency in rabbit trigeminal ganglia, the label appeared in 1 – 10% of neu-

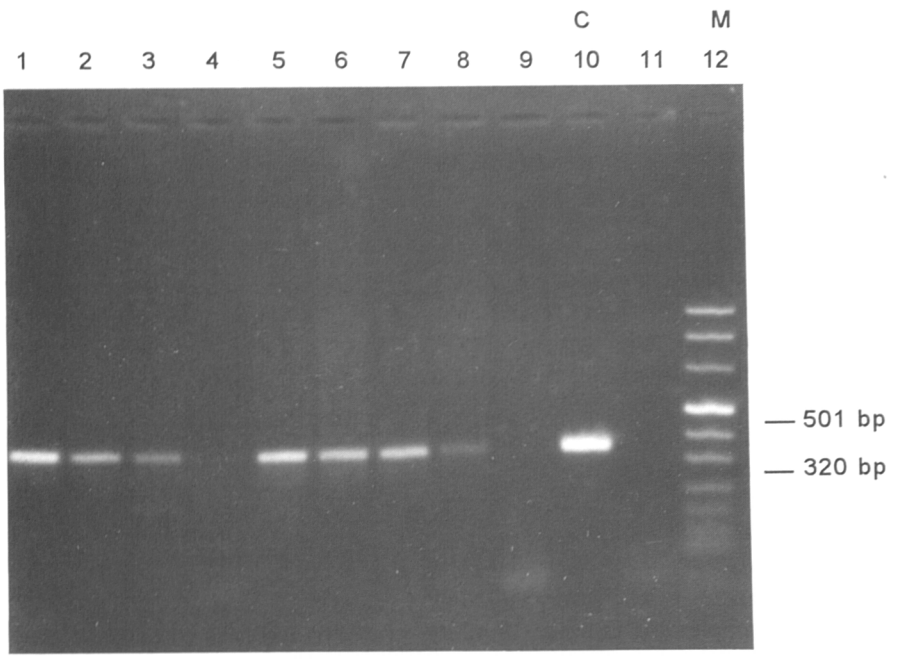

Fig. 6

Products of the first PCR using nested primers with ganglionic DNA from mice acutely infected with SC16 strain

Agarose gel electrophoresis of PCR products. Ganglionic DNA was isolated from mice at day 3 p.i. Ganglionic DBA samples # 91 (lane 1), # 92 (lane 2), # 93 (lane 3), # 94 (lane 4), # 95 (lane 5), # 96 (lane 6, # 97 (lane 7), # 98 (lane 8). No DNA (lane 11). Control ganglionic DNA (lane 9). HSZP DNA (lane 10). Size marker DNAs (lane 12).

rons (Green *et al.*, 1987). The amount of LAT in expressing neurons ranged from 0.08 to 0.3 per cell suggesting that LAT-positive neurons contained many (approximatelly 8-280) copies of HSV-specific RNA molecules in accord with earlier estimates of 0.3 – 3% of latency-related RNA-bearing neurons (Rock *et al.*, 1987). We found that the HSZP strain could establish a latent infection similarly to other strains even when it was poorly reactivable in culture. This is consistent with results of studies of latency established with defective HSV strains. These results could be helpful in elucidating the role of viral genes in the establishment and maintance of latency. It was shown that thymidine kinase-negative mutants, which poorly replicate during the acute post-inoculation phase, could establish latency in the sensory ganglia but could not be reactivated (Kosz-Vnenchak *et al.*, 1990). When latent in ganglia of mice, these mutants were demonstrated by PCR (Friedrich *et al.*, 1990). Steiner *et al.* (1990) have studied a HSV mutant defective in the virion transactivation factor Vmw65 (α-trans-inducing factor, VP16). This mutant was not detectable in mouse ganglion, but was reactivable from latency with fair efficiency. Further studies on latency with transcription- and replication-defective HSV mutants (ICP 4, ICP 27, DNA polymerase, ribonucleotide reductase) have suggested that latent infection was established and maintained even when transcription and replication were restricted (Sedarati *et al.*, 1993). Quantitative PCR of these HSV-1 mutants renders possible to detect one copy of HSV DNA in 10^3 – 10^4 ganglionic cells (Katz *et al.*, 1990). This is in a quite good agreement with our present results of PCR analysis of ganglia from mice latently infected with strain HSZP characteristics with limited explantation reactivation rate. The fact that HSZP virus, which poorly replicates at periphery during acute infection, is able to establish latency but is poorly reactivable, needs elucidation.

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