Detection of Herpes Simplex Virus Type 1 Gene Expression in Latently and Productively Infected Mouse Ganglia Using the Polymerase Chain Reaction

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SUMMARY

The polymerase chain reaction (PCR) was employed to detect herpes simplex virus (HSV) sequences in the DNA, and HSV gene expression in total cell RNA, extracted from cervical and trigeminal ganglia of mice during productive and latent infection with HSV-1, strain SC16. Such gene expression was detected in 1 μg or less of RNA, the quantity anticipated to be present in one or two cervical ganglia. Within the limits of the primers available, gene expression during latency appeared to be restricted to the latency-associated transcript (LAT). The 195 base portion of the LAT amplified by the PCR was sequenced and found to contain several base changes and deletions with respect to published sequences for different HSV strains. These mutations, within the putative open reading frame 2 of the LAT, formed stop or terminator signals, which suggests that the LAT does not act to establish or maintain latency through translation to a protein. The primers for the LAT also amplified a 300 bp fragment from any murine and some other mammalian RNAs. Apart from the oligonucleotide primers, this fragment did not show any homology with HSV.

INTRODUCTION

Infection with herpes simplex virus (HSV) is followed by the establishment of latency in the neurons of the sensory ganglia which innervate the site of primary infection (Goodpasture, 1929; Hill, 1985). The state of this latent virus is not clear: antigens characteristic of productive infection are not detectable (Itoyama et al., 1984), the virus genome may not be present as the linear molecule of the intact virion (Rock & Fraser, 1983, 1985) and in common with many, but not all (Croen et al., 1988), latent viral infections there appears to be very limited gene expression. Yet, with the right stimulus, the latently infected neurons can be induced to release infectious virus and recrudescence disease can sometimes be seen (Wildy et al., 1982; Harbour et al., 1983).

Although the major gene products of a productive HSV infection have not been detected in latency, a previously unknown viral transcript has recently been characterized in latently infected tissues (Stevens et al., 1987; Wagner et al., 1988a, b). This latency-associated transcript (LAT) is a 2.2 kb poly(A)-RNA, partly complementary to the 3' end of the ICP0 mRNA (Wagner et al., 1988a). There is evidence that the transcript can be spliced to give a smaller 1.5 kb species (Wechsler et al., 1988; Wagner et al., 1988b) but the extent of this splicing seems to vary with the animal host and the strain of virus (Wagner et al., 1988b). This viral transcript is present in productive infection, but at much lower levels (Wagner et al., 1988a). The LAT may have a role to play in the establishment and maintenance of latency, since it is more abundant in latent infection, and it is present in latently infected tissue from several species (rabbit, human, mouse). However, control of latency cannot be the exclusive function of the LAT since HSV
throughout because after inoculation with HSV they have a high incidence of latent infection. The majority were

...of any pair of primers, only one was added during eDNA synthesis; the second was added at the start

...of thermal cycling, eDNA synthesis, and ultimately amplification, would only occur if the initial sample contained

...killed (by sodium pentobarbitone overdose) the second and third pairs of cervical ganglia (neck-inoculated

...inoculated on the neck at 4 weeks of age using 10^5 p.f.u. of virus as described by Blyth

...mixed for storage at -70 °C.

...DNA and RNA from each sample (Maitland

...template for RNA transcription (see Lynas

...the 50 V.1 reaction mixture contained 16.6 raM-ammonium sulphate, 67 mM-Tris-HC1 pH 8.8, 6.7 raM-magnesium

...demonstrate that the reaction was working and not producing false positives.

...annealing at room temperature for 30 s and primer extension at 65 °C for 5 rain. The 30 cycles were performed by

...hand using Techne heating blocks and 1 unit of Taq polymerase (Anglian Biotech) was added every 10 cycles.

...extension time was reduced to 3 rain and Taq polymerase supplied by Cambio was used. A single addition of 2

...units of enzyme was made at the start of cycling and the reaction buffer contained 10 mM-Tris-HCl pH 8.3, 50 raM-

...preparation of nucleic acids. The batches of ganglia were homogenized in guanidinium thiocyanate-detergent

...mix (Chirgwin et al., 1979) and centrifuged on caesium trifluoroacetate gradients, which allows recovery of both

...samples were treated basically as described in Lynas et al., 1987). The nucleic acids recovered from the gradients were

...purified as described previously (Maitland et al., 1987).

...Samples of RNA and DNA for negative and positive PCR controls were prepared by the same method from

...samples of mouse liver and Vero cells productively infected with HSV-1 strain SC16. In all cases 1 µg of

...nucleic acid was routinely used as a template in the PCR.

...The DNA PCR. The method was based on that of Saiki et al., (1988), but with several differences. In most cases, the 50 µl reaction mixture contained 16-6 mm-ammonium sulphate, 67 mm-Tris–HCl pH 8-8, 6-7 mm-magnesium

...chloride, 1.5 µM-magnesium chloride with the same nucleotide and primer concentrations as above.

...application of this technique to the investigation of viral gene expression in latent infection will allow detection of very low levels of transcripts and should help to clarify the activity of the viral genome in latent infection of individual ganglia.

**METHODS**

**Infection of mice.** The virus used was HSV-1 strain SC16 (Hill et al., 1975). Female NIH inbred mice were used throughout because after inoculation with HSV they have a high incidence of latent infection. The majority were inoculated on the neck at 4 weeks of age using 10^5 p.f.u. of virus as described by Blyth et al. (1984). Some, however, were inoculated at 6 to 8 weeks of age with 10^6 p.f.u. per animal by scarification of the right eye (Tullo et al., 1982).

**Dissection of the ganglia.** During primary infection, the peak titre of infectious HSV in the ganglia occurs at 3 to 4 days post-inoculation. To provide acutely infected material, mice were killed at this time, but to obtain latently infected material the mice were not killed until at least 4 weeks post-inoculation. Immediately after the mice were killed (by sodium pentobarbitone overdose) the second and third pairs of cervical ganglia (neck-inoculated animals) or the right trigeminal ganglia (eye-inoculated animals) were removed with sterile instruments. The ganglia were immediately snap-frozen in liquid nitrogen and material from groups of 20 to 25 animals was combined for storage at -70 °C.

**Preparation of nucleic acids.** The batches of ganglia were homogenized in guanidinium thiocyanate-detergent mix (Chirgwin et al., 1979) and centrifuged on caesium trifluoroacetate gradients, which allows recovery of both DNA and RNA from each sample (Maitland et al., 1987). The nucleic acids recovered from the gradients were purified as described previously (Maitland et al., 1987).

Samples of RNA and DNA for negative and positive PCR controls were prepared by the same method from snap-frozen samples of mouse liver and Vero cells productively infected with HSV-1 strain SC16. In all cases 1 µg of nucleic acid was routinely used as a template in the PCR.

**The DNA PCR.** The method was based on that of Saiki et al., (1988), but with several differences. In most cases, the 50 µl reaction mixture contained 16-6 mm-ammonium sulphate, 67 mm-Tris–HCl pH 8-8, 6-7 mm-magnesium chloride, 10 µM-2-mercaptoethanol, 6-7 µM-EDTA, 33 µM each of dATP, dCTP, dGTP and dTTP, 10% DMSO and was 400 µM with respect to each 20 base primer. Each cycle consisted of denaturation at 91 °C for 30 s, annealing at room temperature for 30 s and primer extension at 65 °C for 5 min. The 30 cycles were performed by hand using Techne heating blocks and 1 unit of Taq polymerase (Anglian Biotech) was added every 10 cycles. However, some PCRs were also conducted using an automatic thermal cycler (Perkin Elmer), when primer extension time was reduced to 3 min and Taq polymerase supplied by Cambio was used. A single addition of 2 units of enzyme was made at the start of cycling and the reaction buffer contained 10 mm-Tris–HCl pH 8-3, 50 mm-potassium chloride, 1-5 mm-magnesium chloride with the same nucleotide and primer concentrations as above. Both methods gave comparable results. In all experiments positive and negative PCR controls were included to demonstrate that the reaction was working and not producing false positives.

The PCR products were analysed on a 12% polyacrylamide gel (prepared in Tris–borate buffer, electrophoresis at 20 W for 90 min). Molecular size standards (shown in Fig. 2) were the products of a HinfI digestion of ϕX174 replicative form DNA.

**RNA PCR.** The RNA samples were treated basically as described in Lynas et al., (1989), but with one major difference. Of any pair of primers, only one was added during cDNA synthesis; the second was added at the start of thermal cycling. cDNA synthesis, and ultimately amplification, would only occur if the initial sample contained RNA complementary to the primer added. Thus the PCR distinguished which DNA strand had served as the template for RNA transcription (see Lynas et al., 1989). Lyophilized samples of total cell RNA were redissolved in
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RESULTS

Detection of HSV DNA in latently infected ganglia

The TK, LAT and ICP0 DNA primers all readily amplified the corresponding HSV DNA in samples from productively infected mouse ganglia (Fig. 2, lanes 1 to 3).

The TK primers also demonstrated the presence of the HSV TK gene in DNA from latently infected ganglia (Fig. 2, lanes 4 to 6). However, repeated testing of these DNA samples did
Fig. 1. PCR primers for the detection of both ICP0 and LAT DNA and the expression of these genes. Exons are represented by open boxes and introns by lines. Primer 1 5' GACAGCAAAAATCCCCCTGAG 3', primer 2 5' ACGAGGGAAAACAATAAGGG 3', primer 3 5' TTCGGTCTCCGCTGAAGT 3', primer 4 5' AACTCGTGGGTGCTGATTGA 3', primer 5 5' GACCCTCCAGCCGTACGA 3'.

Fig. 2. Detection of HSV DNA in mouse ganglia by PCR. Lane 1, productively infected ganglia with TK primers; lane 2, productively infected ganglia with TK and ICP0 DNA primers 3 and 4; lane 3, productively infected ganglia with TK and LAT primers 1 and 2; lane 4, latently infected ganglia with TK primers; lane 5, latently infected ganglia with TK and ICP0 DNA primers 3 and 4; lane 6, latently infected ganglia with TK and LAT primers 1 and 2; lane 7, uninfected ganglia with TK primers. *HinfI*-digested φX174 replicative form DNA is shown on the left to provide molecular size standards (bp).
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produce some false negative results. These were ascribed to the minute amounts of HSV DNA present in latent infection (Puga et al., 1978), such that a single sample might rarely lack it altogether, and they underline the need for replicate tests on apparently negative material. No false positives were obtained, demonstrating that the primers are HSV-specific (Fig. 2, lane 7).

The LAT and ICP0 DNA primers have not yet reliably detected the presence of the HSV genome in latently infected ganglia. Even in productively infected samples, the ICP0 primers in particular were often less efficient than the TK primers (Fig. 2, lane 2). This failure may be related to the high G + C content (74%) of the ICP0 region of the genome (Perry et al., 1986), but a modification of the PCR method using 7-deaza-2'-deoxyguanosine, suggested to overcome the problem of stable hairpin loops forming in G + C-rich regions (McConlogue et al., 1988), was not beneficial in this instance.

Since the Taq polymerase is unlikely to be suffering inhibition (the TK primers work well with the same samples) we suggest that the failure to detect ICP0 DNA may be attributable to some unusual state of the virus in latency which is preventing the PCR from working on this small portion of the genome. Predigestion of the DNA with restriction enzymes might allow the primers to function normally, but since we can satisfactorily confirm HSV latency using the TK primers, we have not investigated this possibility further.

Viral gene expression in productively infected ganglia

The primers allow detection of RNA transcription from four regions of the viral genome (TK, LAT, ICP0-3' and ICP0-5'). Expression of all of these genes in productively infected ganglia was readily detectable by PCR (Fig. 3, lanes 1 to 5). Although the RNA PCR is not completely quantitative (Chelly et al., 1988; Lynas et al., 1989), the amplified LAT signal was consistently weak compared to that obtained using latently infected cell template RNA. This suggests that the LAT is present at much lower levels during productive infection in agreement with the findings of others (Spivak & Fraser, 1987; Wagner et al., 1988a).
(a)

Fig. 4. Analysis of the cross-reacting cellular sequence. Results of hybridizing (a) various mammalian total cell RNA (mouse liver, HEp-2 cells, human oral, rat liver, foetal kidney, monkey, mouse liver 2, lanes 1 to 7 respectively) and (b) murine DNA digested with restriction enzymes EcoRI, BamHI and HindIII (lanes 1 to 3, respectively) with the 'mouse-specific' fragment generated in an RNA PCR using the LAT primers. These results demonstrate that the template RNA is conserved in other species and is the product of a real gene rather than a DNA repeat sequence.

Viral gene expression in latently infected ganglia

Assay, on confluent Vero cells, of the supernatant medium from freshly excised homogenized cervical ganglia or portions of trigeminal ganglia that had been in organ culture for a week, produced characteristic HSV plaques, indicating that the ganglia were indeed latently infected with the virus. Using the HSV-specific primers available, the only viral transcript which could be detected in RNA from latently infected ganglia was the LAT (Fig. 3, lanes 7 to 10). PCRs were conducted using the TK, ICP0 RNA and 3' ICP0 primers but no amplified bands of the predicted sizes were ever detected. This confirms the findings of other workers using different methods (e.g. Croen et al., 1987; Rock et al., 1987a).

Amplification of the LAT fragment was also achieved using as little as 0.1 μg of total ganglion RNA as template. Although rare, false negatives were sometimes recorded in RNA PCRs. This again emphasizes the need for replicate analysis of apparently negative samples.

Cross-reaction of primers with cellular RNAs

The TK and ICP0 RNA primers never produced amplified bands of the predicted sizes when used on uninfected RNA (Fig. 3, lane 6), and furthermore they rarely produced bands of other sizes whether the origin of the target RNA was HSV-infected or not. Any such bands were always very faint. Similarly, the LAT primers never produced an amplified band of the predicted size (195 bp) with uninfected cell RNA, but in many PCRs using these primers and murine RNA from several tissues, an amplified band of approximately 300 bp was detected, irrespective of whether or not the RNA was from HSV-infected cells (Fig. 3, lanes 5, 10, 11). The band was often of comparable intensity to that amplified in HSV-infected samples from the LAT itself. It was also present, but at much lower levels, when human RNA was used. A corresponding band was not seen in DNA PCRs using the LAT primers on murine DNA, suggesting that the cellular transcript being amplified must be spliced in order to bring the
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primers into sufficient proximity for the PCR to work. In RNA, amplification occurred only when the LAT-specific primer was used to prime cDNA synthesis. Digestion of the amplified fragment with HaeIII, predicted to cleave the LAT into three fragments (87, 75 and 33 bp), had no effect, suggesting that this cellular RNA was not homologous to HSV RNA. When the cell-specific fragment was $^{32}$P-labelled and used as a probe on a Northern blot of RNA from several sources, it hybridized strongly to a 1.5 kb mouse RNA, and also weakly to a human and rat RNA of the same size (Fig. 4).

Fig. 5. Portions of the sequence for the 195 base region of the LAT amplified in this study illustrating some of the mutations found with respect to other published sequences. Asterisks indicate an identical base, - indicates a base deletion and any base changes are shown for the three strains. The reference strain (Perry et al., 1986) is 17 and shown below are KOS (M) (Wagner et al., 1988a, b) and the SC16 strain used in this study. The numbers at the beginning and end of the sequenced portion correspond to the nucleotide numbers in Perry & McGeoch (1988).
When the same probe was used on a Southern blot of murine DNA cut with different restriction enzymes, it hybridized to only one or two bands in each lane (Fig. 4), indicating that we were not merely detecting a repeat sequence in the mouse genome.

A portion of the DNA amplified from the mouse cellular RNA with the LAT primers was also sequenced. It was not found to have any homology with the HSV genome. An effort was made to identify the transcript by comparing the short sequence obtained with the GenBank database but no messenger RNA of significant homology could be found. Neither could it be identified by comparison of the translated polypeptide sequence with a protein database. However, it does appear from other evidence obtained here that the amplified fragment is the product of a cellular gene (see above).

Sequence data

The sequence of the amplified LAT fragment was established and compared with published data (from different strains of HSV-1) for the same region (Fig. 5) (Perry et al., 1986; Wagner et al., 1988a).

Two or three base changes and two single base deletions were recorded in the sequence of the product of our RNA PCR as compared with these reported sequences. These differences are unlikely to be artefacts of the PCR; direct sequencing of PCR products will average out any errors introduced by the polymerase in the way sequencing cloned PCR products cannot. A part of our sequence revealing these mutations is shown in Fig. 5. Since two of the mutations occurred in regions of particularly high G + C content, the sequencing was repeated using dITP as a nucleotide analogue for dGTP to eliminate any compression artefacts (Mills & Kramer, 1979). Identical results were obtained. Comparison of the sequences obtained for the amplified LAT fragment in latently and productively infected ganglia also revealed no differences.

Discussion

We have demonstrated that HSV gene expression can be detected in less than 1 μg of RNA extracted from the combined ganglia of several latently infected mice. A mouse cervical ganglion contains about 3000 neuronal cell bodies (Dr S. Nicolls, personal communication), but these represent only about 10% of all the cells present, the remainder being support cells of various kinds (Walz et al., 1976). An average mammalian cell is calculated to contain about 26 pg of RNA (Brandhorst & McConkey, 1974) although the RNA content of a neuron is likely to be somewhat less. Using 20 pg per individual cell as an estimate suggests that a murine cervical ganglion may contain about 0-6 μg RNA. A murine trigeminal ganglion is much larger and contains much more than 1 μg RNA (Puga & Notkins, 1987). So, even though we have pooled the ganglia from several mice to obtain the RNA used in this study, we have conclusively demonstrated that the quantity of RNA present in one or two mouse ganglia is sufficient to obtain a result in the PCR. It would therefore be possible to pool the ganglia from an individual animal or, with a suitable RNA extraction procedure, to subject individual ganglia to a PCR.

Within the limits of the primers available, the only HSV transcript that could be detected in latent infection was the LAT. It has been suggested that the role of the LAT in the establishment and maintenance of latency may be one of anti-sense regulation of the ICP0 gene (Stevens et al., 1987) which is important in activating the transcription of other immediate early genes and initiating the cascade of gene expression characteristic of productive HSV infection. This could be achieved by the LAT directly blocking the ICP0 mRNA preventing its translation and its trans-activating activity (Rock et al., 1987a). However, since we were unable to find any evidence of ICP0 transcription in latently infected ganglia, this possibility seems unlikely. Alternatively, the LAT could have a cis-acting effect in preventing ICP0 transcription. This hypothesis is not inconsistent with the results of the work reported here.

Although we found no evidence of viral transcripts other than LAT during latent infection, our results do not preclude the possibility that there are others. For example, ribonucleotide reductase is a good candidate for involvement in latency control; mRNA coding for one of its two subunits is produced very early in infection and has an unusual pattern of expression (Goldstein & Weller, 1988).
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Differences between published sequences and the sequence we obtained for the 195 base fragment of the LAT RNA amplified in the PCR were not unexpected. Strain variation has already been demonstrated in the sequence of the LAT (Perry & McGeoch, 1988; Wagner et al., 1988a). Both mutations and deletions are recorded in the sequence of KOS (M) when compared to strain 17. Many of these mutations, and all the deletions, occur between the suggested cap site and the start of the putative second open reading frame (ORF); the majority occur within the recently proposed intron (Wagner et al., 1988b). However, the 195 base fragment of LAT RNA sequenced here lies entirely within the putative ORF2. The base deletions and mutations found in SC16 introduce stop or terminator signals.

Although other workers postulate the existence of two ORFs in the LAT, this comes merely as computer predictions from the sequence and it is quite probable that they do not function as such (Perry & McGeoch, 1988). No translation product has yet been isolated; indeed the evidence for the existence of a poly(A)+ LAT is scant (Puga & Notkins, 1987; Spivak & Fraser, 1987) and such evidence has been suggested to be rather unreliable (Wagner et al., 1988a). In addition, the putative ORF1 disappears entirely in the spliced LAT species. Therefore it seems unlikely that the LAT acts in latency through its translation to a protein. However, it still remains possible that the LAT could code for an active peptide.

The amplification of a cellular RNA by the HSV LAT primers was somewhat surprising since the cDNA synthesis was conducted under high stringency conditions at 50 °C and the PCR itself at 65 °C. This necessitates a very high degree of primer–template homology for amplification to occur. Although the homology between the cellular RNA and the primers must have been good, there was no more general homology to HSV. The appearance of this cellular amplification product serves as a timely reminder that there may be considerable homology between cellular and HSV DNA and RNA sequences as suggested previously (Maitland et al., 1981; Puga & Notkins, 1987). Moreover, it emphasizes the need for careful interpretation of results, for example from in situ hybridization when using certain HSV probes.

By PCR, viral homology is seen with RNA only and could arise in two ways. Splicing of the RNA could result in a new sequence homologous to the primer, thus allowing amplification. Alternatively, splicing may bring two areas of existing homology sufficiently close so that the amplified product can be visualized on an acrylamide gel, the product from unspliced DNA being too large to be distinguished. In any event, we have been unable to identify the RNA which is unlikely to be a major species as it was difficult to detect by Northern blotting.

In conclusion, our results demonstrate that of those examined, the LAT is a major viral transcript in latent disease. We have detected this expression by the simple, highly sensitive PCR using 1 μg or less of RNA, equivalent to the amount present in one or two mouse cervical ganglia. The way is now open for very precise molecular study of gene expression in latent HSV infection.

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