

## ORIGINAL ARTICLES

## Latency-associated transcripts in corneas and ganglia of HSV-1 infected rabbits

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### Abstract

**Herpes simplex virus (HSV) establishes latent infection in the sensory neuron and possibly in non-neuronal tissue, particularly the cornea. During latency only one region of the HSV genome is transcribed, producing RNAs known as latency associated transcripts (LAT). The gene for LAT overlaps with the HSV gene for the protein ICPO in the downstream regions of both genes. Latency can occur in the absence of LAT. This study reports the detection of ICPO/LAT and thymidine kinase (TK) gene fragments by the polymerase chain reaction in DNA extracted from the corneas and trigeminal ganglia of latently infected rabbits. Both genes were detected in four of four trigeminal ganglia tested and in three of five corneas tested. More importantly, this study reports the first detection of LAT in RNA extracted from 9% of corneas from latently infected rabbits (n=22) by the polymerase chain reaction. LAT was detected in RNA from 100% of the corresponding trigeminal ganglia (n=22). Although LAT is not essential for latency, it remains the only known molecular marker for latent HSV infections. Detection of LAT in these rabbit corneas suggests that HSV latency may occur in this non-neuronal tissue and that reactivation from non-neuronal tissue may occur at a low frequency in animals in which HSV latency has been established.**

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The sensory neuron is acknowledged as the principal site for HSV latency,<sup>1-4</sup> but other neural tissue, specifically the autonomic ganglia, also harbour latent HSV.<sup>5</sup> Before a herpetic infection can be described as latent in a particular target tissue by the operational definition<sup>6</sup> two conditions must be met: (1) at the time the host animal is killed, cell-free infectious virus must not be detectable (the detection of cell-free infectious virus implies acute infection or reactivation); (2) virus must be detectable after a period of organ culture of the target tissue.

A latency definition based on events occurring at a molecular level does not exist. Latency-associated transcripts (LAT) were first reported in 1987.<sup>7</sup> This limited transcription of the HSV viral genome has been demonstrated in mice,<sup>7,8</sup> rabbits,<sup>9,10</sup> and man.<sup>11-15</sup> Since then LAT has been shown to be non-essential for the establishment of latency in studies using HSV-1×HSV-2 deletion mutants<sup>16-18</sup> in mice. Other reports suggest that LAT may facilitate reactivation from organ culture<sup>17,18</sup> and reactivation induced *in vivo* in rabbits by iontophoresis of epinephrine.<sup>19</sup>

With the operational definition of HSV latency, non-neuronal sites, including human,<sup>20-22</sup> rabbit,<sup>23,24</sup> and mouse<sup>25</sup> corneas, have been shown to support latent HSV infections. The frequency of HSV isolation from human and animal corneas is low, ranging from 11 to 30%.<sup>20-25</sup> These frequencies are comparable to those obtained in similar studies of mouse skin and footpads.<sup>26,27</sup> However, *in situ* hybridisation applied to human corneas by means of 'sense' and 'antisense' single-stranded RNA probes from the ICPO gene, which overlaps with LAT, failed to detect HSV transcription in human corneas.<sup>28</sup> Other studies using HSV probes which encompass the whole genome have detected HSV DNA by *in situ* hybridisation in rabbit corneas up to day 60 post injection,<sup>29</sup> and HSV DNA by dot blot hybridisation in herpes infected human corneas removed at keratoplasty.<sup>30</sup>

Investigation of gene expression during latency is difficult, as so few cells appear to harbour latent HSV.<sup>31</sup> The polymerase chain reaction (PCR) is an extremely sensitive tool for the analysis of viral gene expression.<sup>32,33</sup> This technique has been used to detect HSV gene expression in mouse ganglia.<sup>34,35</sup> We now report on the use of this technique to detect HSV gene expression in the corneas and trigeminal ganglia of latently infected rabbits.

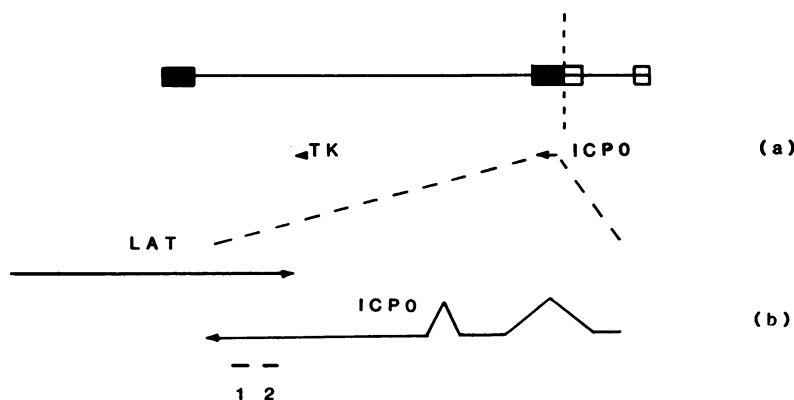


Figure 1 (a) Prototype orientation of HSV-1 genome, showing location of TK and ICPO genes. (b) Expanded ICPO gene showing area of overlap with LAT and site of intragenic LAT/ICPO primers.

Table 1 Clinical history of corneas subjected to DNA extraction

Rabbit number	Virus strain	Antiviral treatment	Reactivation by iontophoresis		Corneal scarring		Killed (day PI)
			OU	OD	OS	OD	
MI1	MI	ND	ND	ND	-	-	5
HSV1	17 Syn+	ND	ND	ND	DU	DU	5
J67	17 Syn+	ND	No	*	+	*	85
J56	McKrae	ND	Yes	Yes	-	-	81
1421	McKrae	P, A	ND	ND	-	-	41

Iontophoresis: Yes, virus reactivated; No, virus not reactivated. Corneal scarring: +, present; -, absent. Antiviral treatment: P, =PMEA [9-(2-phosphonomethoxyethyl) adenine]; A, =acyclovir. ND, not done. MI, mock-infected. DU, dendritic ulcer. \*J67: The cornea OS and both trigeminal ganglia were mishandled during processing and therefore unavailable for other analysis.

## Materials and methods

### RABBITS AND VIRAL INOCULATION

The unscarified eyes of New Zealand white rabbits (1.5–2.5 kg) were infected with a suspension of either HSV-1 strain 17 Syn<sup>+</sup> or McKrae at 10<sup>7</sup> plaque forming units (PFU) in 20 µl. Primary corneal infection was verified by slit-lamp biomicroscopic examination (SLE) between days 4 and 8 post inoculation.

When killed (days 41–147 post inoculation), the rabbits had been used in either antiviral or reactivation experiments. Rabbits in the antiviral experiments had been treated with either acyclovir or PMEA [9-(2-phosphonyl-methoxyethyl)-adenine]<sup>36</sup> during the primary infection between days 3 and 8 post inoculation. One rabbit (no 954) was an untreated control in antiviral experiments. Additional rabbits were used as mock-infected controls and acutely infected controls (sacrificed on day 5 post inoculation). The corneas were assessed clinically by slit-lamp biomicroscopic examination and preclear tear film was collected and monitored as previously described<sup>37</sup> to detect the presence of infectious HSV.

### PREPARATION OF NUCLEIC ACIDS FROM GANGLIA AND CORNEAS

Immediately after the rabbits were killed the corneas and trigeminal ganglia were removed aseptically and frozen in liquid nitrogen before storage at -70°C. DNA was extracted from corneas and trigeminal ganglia by standard methods.<sup>38</sup> For RNA extraction individual

corneas were ground under liquid nitrogen, and the tissue was homogenised briefly with a probe (TEKMAR, Cincinnati, OH) in guanidinium thiocyanate, then centrifuged on caesium chloride.<sup>39</sup> Homogenisation was unnecessary in the preparation of trigeminal ganglia. Samples of RNA for positive and negative PCR controls were prepared by the same method using acutely infected (sacrificed on day 5 PI) and mock-infected rabbits, respectively.

### DNA POLYMERASE CHAIN REACTION (PCR)

The method of Saiki *et al* was used.<sup>35</sup> Briefly, the polymerase chain reaction has three steps which are repeated in sequence for many cycles: denaturation, in which double-stranded DNA is melted to single strands; annealing, in which primers locate and adhere to the complementary single-stranded DNA template; and extension, in which dNTPs are polymerised, complementing the bases on the single-stranded DNA template. This primer extension phase is mediated by the heat stable Taq DNA polymerase. Each cycle theoretically doubles the number of copies of template DNA.

Molecular weight standards were the Hae III digest of øX174 RF DNA. The gels were prepared and used as previously described.<sup>34,35</sup> They were stained with ethidium bromide and observed under UV light. Figures 2 and 3 represent prints of the negative of the gel.

### RNA PCR

RNA was converted to single stranded DNA (cDNA) by means of reverse transcriptase and the addition of an appropriate primer.<sup>35</sup> cDNA synthesis occurs only if the initial RNA sample is complementary to the primer. The polymerase chain reaction thus identifies which RNA strand serves as the template for RNA transcription. This reaction product was used as a cDNA template in the PCR.

### DESIGN OF PRIMERS

Oligonucleotide primers (20 bases) have been synthesised and previously described<sup>34,35</sup> for three HSV genes: the thymidine kinase (TK), LAT, and ICPO genes (Fig 1). The TK and ICPO/LAT fragments amplified are 110 and 195 base pairs, respectively. The LAT and ICPO genes are partly complementary at the three prime ends. Selective addition of the downstream primer ensures cDNA synthesis when the initial RNA sample is complementary to the DNA primer added. Because the LAT and ICPO genes are complementary at the three prime ends, a positive result may be obtained if contaminating ICPO DNA is present. To eliminate the possibility of a false positive arising from contaminating ICPO DNA in this manner, polymerase chain reactions were also performed on RNA which had not been processed to cDNA.

### EXPERIMENTAL DESIGN

Five corneas from three rabbits with latent HSV-1 and four trigeminal ganglia from two of these

Table 2 Clinical history of corneas subjected to RNA extraction

Rabbit number	Virus strain	Antiviral treatment	Reactivation by iontophoresis		Corneal scarring		Killed (day PI)
			OU	OD	OS	OD	
MI2*	MI	ND	ND	ND	-	-	5
HSV2*	17 Syn+	ND	ND	ND	DU	DU	5
H67	17 Syn+	ND	Yes	Yes	-	-	145
H59	17 Syn+	ND	No	No	-	-	143
1414	McKrae	P	ND	ND	-	-	41
1419	McKrae	P	ND	ND	-	-	41
954	McKrae	ND	ND	ND	+	+	41
1409	McKrae	A	ND	ND	+	-	41
J52	McKrae	ND	No	Yes	-	-	94
J53	McKrae	ND	Yes	No	+	+	94
J41	McKrae	ND	Yes	Yes	-	-	147
J45	McKrae	ND	Yes	Yes	-	-	147
J47	McKrae	ND	Yes	Yes	-	-	147

Iontophoresis: Yes, virus reactivated; No, virus not reactivated. Corneal scarring: +, present; -, absent. Antiviral treatment: P, =PMEA [9-(2-phosphonomethoxyethyl) adenine]; A, =acyclovir. ND, not done. MI, mock-infected. DU, dendritic ulcer. \*Controls.

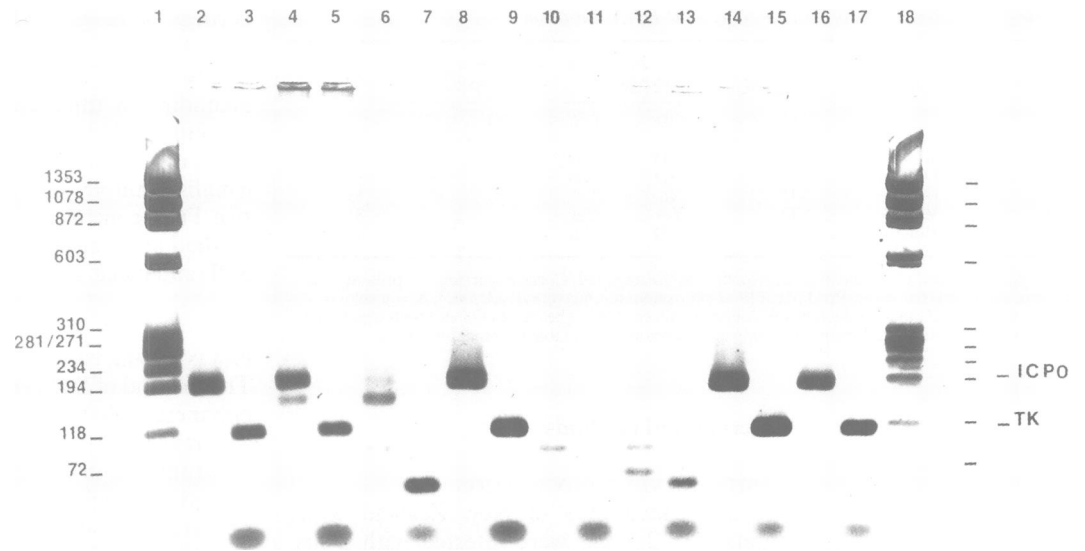


Figure 2 DNA PCR. Lanes 1, 18 marker  $\phi$ X174 RF DNA; lanes 2, 3 right cornea 1421; lanes 4, 5 right trigeminal ganglion 1421; lanes 6, 7 right cornea J56; lanes 8, 9 right trigeminal ganglion J56; lanes 10, 11 mock-infected cornea; lanes 12, 13 mock-infected trigeminal ganglion; lanes 14, 15 acutely infected cornea; lanes 16, 17 acutely infected trigeminal ganglion. Even numbered lanes (except 18) had ICPO/LAT primers added during the PCR reaction; product 195 bp. Odd numbered lanes (except 1) had TK primers added during the PCR reaction; product 110 bp.

rabbits were used for DNA analysis (Table 1). Twenty-two corneas and 22 trigeminal ganglia from 11 rabbits with latent HSV-1 were subjected to RNA extraction (Table 2). DNA and RNA were extracted from the corneas and trigeminal ganglia of mock-infected and HSV-1 acutely infected rabbits and used as controls (Table 1, 2).

HSV-1 latent rabbits, which were killed between days 41 and 147 post inoculation. One of the five corneas in the group of DNA extractions had scarring in more than two quadrants. Five corneas (three rabbits) of the 22 eyes in the group of RNA extractions had dense scarring in two or more quadrants.

## Results

### OCULAR SWABBING AND SLIT-LAMP EXAMINATION AT DEATH

With the exception of the acutely infected rabbits, no rabbit had an active herpetic infection when killed as shown by epithelial ulceration. HSV was detected only in the tear film of the acutely infected control rabbits immediately before death and not in the tear film of any of the

### DNA POLYMERASE CHAIN REACTION

With previously described intragenic primers with characterised products for the TK and ICPO genes<sup>34,35</sup> and polymerase chain reaction detected HSV-1 DNA sequences for the thymidine kinase (110 bp) and ICPO genes (195 bp) in all four ganglia from latent rabbits analysed (Fig 2, lanes 4, 5, 8, 9). The corresponding trigeminal ganglion from one rabbit (J67) was not available for use in this study. With the same

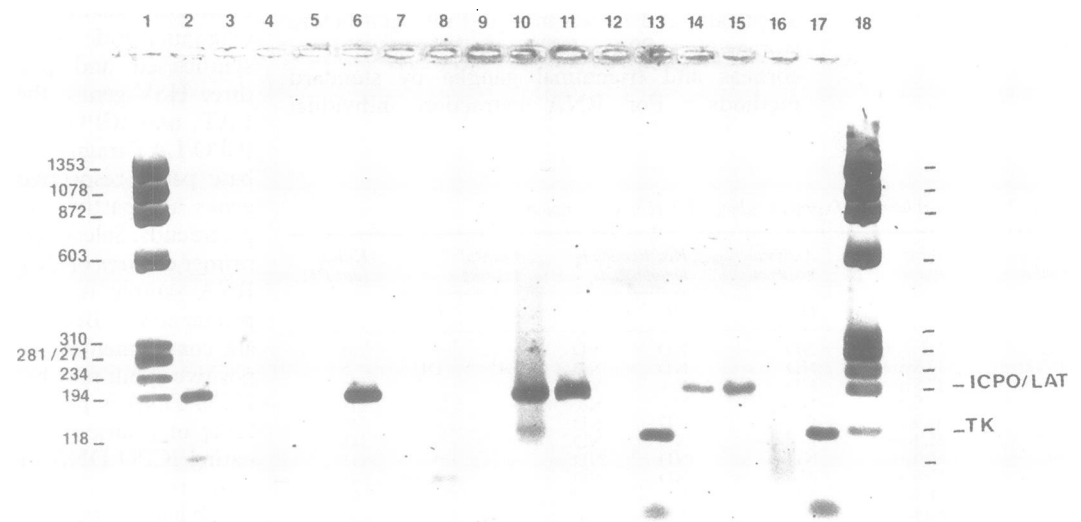


Figure 3 Lanes 1, 18 marker  $\phi$ X174 RF DNA; lanes 2-5 right cornea 954; lanes 6-9 right trigeminal ganglion 954; lanes 10-13 acutely infected cornea; lanes 14-17 acutely infected trigeminal ganglion. Lanes 2, 6, 10, 14 primer 2 added during reverse transcription. Lanes 3, 7, 11, 15 primer 1 added during reverse transcription. Lanes 4, 8, 12, 16 no reverse transcription, PCR on RNA. Lanes 5, 9, 13, 17; downstream TK primer added during reverse transcription. Upstream and downstream primers were added to samples 2-17 during the PCR.

intragenic primers the TK and ICPO genes were detected by polymerase chain reaction in three of the five corneas studied: J67 right (not shown in Fig 2); 1421 right and left. No TK or ICPO genes were detected in either cornea of rabbit J56 despite positive results from the trigeminal ganglia (Fig 2, lanes 2 and 3, 6 and 7). The PCR failed to detect HSV sequences in two mock-infected corneas and the two corresponding trigeminal ganglia (Fig 2, lanes 10–13). TK and ICPO genes were detectable by PCR in DNA extracted from both corneas and both trigeminal ganglia of an acutely infected rabbit sacrificed on day 5 post inoculation (Fig 2, lanes 14–17).

Additional bands smaller than the predicted PCR products are evident (Fig 2, lanes 4, 6, 7, 10, 12, and 13). These may represent incomplete primer extension products or an area of partial homology between the primers and the host genome. Previous sequence analysis of a 300 bp PCR product obtained with the same ICPO/LAT primers revealed homology with only the oligonucleotide primers.<sup>35</sup>

#### RNA POLYMERASE CHAIN REACTION

LAT was detected in all 22 trigeminal ganglia from HSV-1 latent rabbits by PCR on cDNA after addition of the downstream LAT primer (Fig 3, lane 6) during the reverse transcriptase reaction. In contrast, detection of LAT in rabbit corneas after the above procedure was limited, with only two positives from 22 corneas analysed (9%) (Fig 3, lane 2). The two positive results were obtained from the same rabbit (no 954), which was killed on day 41 post inoculation. Interestingly, both corneas were scarred. The PCR performed on cDNA which had the upstream LAT primer was negative (Fig 3, lane 3). The PCR performed on the RNA sample without a reverse transcription reaction was also negative (Fig 3, lane 4). These two negative results controlled for the presence of contaminating cDNA. Thus the detection of LAT is attributable solely to the presence of antisense RNA in the extracted tissue. Similar control results were also obtained for the latently infected trigeminal ganglia (Fig 2, lanes 7, 8). In each PCR run, appropriate positive (HSV-infected) and negative (mock-infected) controls were included.

The two corneal specimens positive for LAT (no 954) were also assessed for the presence of TK transcripts using a reverse transcriptase procedure followed by PCR. TK transcripts were not detectable (Fig 3, lane 5) on two PCR assays. Four of the 22 trigeminal ganglia positive for LAT were also tested for TK transcripts with similar results – that is, no TK transcripts were detected (Fig 3, lane 9).

In contrast, in acutely infected corneas and trigeminal ganglia LAT, ICPO, and TK transcripts were detectable when the appropriate downstream primer was added during the reverse transcriptase reaction, followed by PCR (Fig 3, lanes 10, 11, 13–15, 17). The results of the polymerase chain reaction performed on RNA using upstream and downstream primer without a reverse transcriptase reaction were negative (Fig 3, lanes 12, 16), implying that contaminating

DNA was absent. Reverse transcription and the PCR were also performed on DNA and RNA from mock-infected animals with negative results (data not shown).

#### Discussion

The reduced frequency of viral detection in non-neuronal tissue,<sup>20–27</sup> compared with neuronal tissue, after organ culture raises the question, 'Is this apparent latency genuine?' Other possible explanations exist, namely, that a persistent low-grade infection is occurring or that a reactivation event has just occurred and requires virus amplification before it can be detected. Under these conditions the possibility also exists that a relatively insensitive assay system for infectious virus may yield a false negative result.

An additional problem with the concept of non-neuronal latency is reconciling the detection of infectious HSV with the detection of genomic HSV in nucleic acid studies. The nucleic acid studies detect HSV DNA at a frequency greater than the detection of infectious HSV from non-neuronal tissue during organ culture. Specifically, in-situ hybridisation studies using HSV probes which encompassed the whole genome detected HSV DNA in 100% (26 eyes) of rabbit corneas up to 60 days post inoculation, yet no infectious HSV was detected from a separate sample of the same corneas after organ culture.<sup>29</sup> A further study using HSV probes which encompass the whole genome detected HSV DNA by dot blot hybridisation in 50% (seven of 14 eyes) of human corneas.<sup>30</sup> The use of HSV DNA probes which encompass the whole genome does not distinguish between a fragment of HSV DNA and the intact whole genome being retained in the cornea. It is therefore conceivable that the whole genome was not present.

In our experiments TK and ICPO genes were detected by the polymerase chain reaction from the DNA of all ganglia analysed (4/4). The same genes were detected by PCR in three of five corneas. When the animals were killed, there was no gross evidence of an active corneal herpetic infection, and culture of ocular swabs did not yield infectious HSV. Although the absence of gross evidence and negative HSV culture do not absolutely exclude the possibility of an asymptomatic herpetic infection, the presumption was made that no acute HSV infection was present in the trigeminal ganglia and corneas.

The detection of HSV DNA in the cornea confirms previous findings with in-situ hybridisation,<sup>29</sup> dot blot hybridisation,<sup>30</sup> and PCR (E Dunkel; D Willey; personal communication). However, the presence of HSV DNA in the cornea does not imply latency. Our results of three out of five specimens (60%) with HSV DNA detected by PCR is less than the 100% reported by the in-situ hybridisation technique in rabbits<sup>29</sup> and similar to the 50% reported for the dot blot hybridisation technique in human corneas.<sup>30</sup>

Is the DNA detected by the molecular biological techniques functional, or merely a fragmented form of DNA, either incorporated or unincorporated in the host cell DNA? Current

evidence suggests that viral DNA in latency may be extrachromosomal in a circular episomal form<sup>40</sup> and associated with nucleosomes.<sup>41</sup>

In our RNA studies LAT was detectable in all trigeminal ganglia by PCR following a reverse transcription reaction. This confirms reports by workers using different methods.<sup>9,11</sup> By contrast, LAT was detectable in only two of 22 corneas (9%). Both corneas were scarred and obtained from the same rabbit (no 954), killed at day 41 post inoculation. This date represents the earliest time after inoculation when latently infected rabbits were killed. The result is similar to the reported range (11–30%) of infectious HSV detection from animal and human corneas after organ culture.<sup>20–25</sup> After a reverse transcription reaction, TK was not detectable by PCR in the two corneas positive for LAT (no 954) or in any of four LAT-positive trigeminal ganglia tested. This result, combined with the failure to detect ICPO after a reverse transcription reaction, suggests that small scale HSV replication (reactivation) was not occurring.

LAT has been shown to be non-essential in the establishment and maintenance of latent infection in murine sensory neurons<sup>42</sup> and also has been postulated to have a role in the facilitation of reactivation.<sup>17–19</sup> Since reactivation can arise only from a preceding latent infection, we suggest that, by inference, LAT remains a useful, if imperfect, molecular marker for latent HSV infection. The limited detection of LAT from the cornea of latently infected rabbits implies that the cornea may be a non-neuronal site where latency can occur.

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