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# Temporal expression of herpes simplex virus type 1 mRNA in murine retina

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## Abstract

*Purpose.* During maturation of herpes simplex virus type 1 (HSV) in infected murine retinal ganglion cells, new viral components are axonally transported in two phases. The viral envelope protein (gD) appears 48 hr before the capsid protein (VP5). Our hypothesis was that delayed appearance of VP5 mRNA in the infected eye causes the delayed expression of the VP5 protein in the axon.

*Methods.* HSV was injected into the ocular posterior chamber. Three to 24 hr later, the mice were euthanized, and the posterior eye was isolated. RNA was extracted, DNAase-treated, and used for amplification by reverse transcription-polymerase chain reaction (RT-PCR) using primers specific to gD, VP5 and ~~VP22, a tegument protein.~~

*Results.* VP22 and gD mRNAs are expressed 6 hr and VP5 mRNA is first detected 9 hr after infection.

*Conclusions.* The results establish that delayed transcription does not play a significant role in the 48-hr delay in VP5 appearance in the retinal axons.

**Keywords:** gD; herpes; retina; VP5, VP22

## Introduction

The neuropathogenesis of recurrent corneal keratitis from herpes simplex virus type 1 (HSV) involves several steps.[1] First, HSV enters mucous membrane epithelial cells and sensory nerve terminals of trigeminal ganglion cells. Second, the HSV is transported from the nerve endings to the host cell nucleus by retrograde axonal transport. Third, in the sensory ganglion cell bodies, the virus may either establish a latent infection or immediately begin to replicate new virions.[2] Fourth, the production of new viral DNA and mRNAs is carried out, and new HSV viral components are synthesized, mature, and are transported within trigeminal ganglion axons to reinfect the corneal epithelium.[3,4] The focus of this study is to examine the timing of appearance of specific viral mRNAs in the murine retina.

We have previously determined the arrival time of two viral proteins in the axonal compartment of infected retinal ganglion cells *in vivo*, using glycoprotein D (gD) as a marker of the envelope component of the virus and viral protein 5 (VP5) as a marker of the capsid component.[5] We found a 48-hr delay between the time of appearance of viral envelope protein and the time of appearance of capsid protein in the infected cell axon. The delayed, unsynchronized appearance of the capsid protein relative to the envelope protein in the axon could be the result of the delayed expression of the VP5 mRNA in the ganglion cell body.[6]

We have tested the hypothesis that the delayed appearance of VP5 protein in axons is due to retarded transcription of the VP5 mRNA in the infected eyes. We have used ~~reverse transcription-polymerase chain reaction (~~RT-PCR~~)~~ to determine the time at which the viral gene expression can be found for three mRNAs in retinal cells. These mRNAs were selected because of their expression of three proteins of the mature virus: the mRNA (1) for a major viral envelope protein (gD); (2) for a major capsid protein (VP5); and (3) for a major protein of viral tegument (VP22). We found that the mRNA for VP5 appeared at most 3 hr later than the first appearance of gD or VP22 mRNAs in the eye. Thus, the delay

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must be due to some subsequent step, such as protein processing, targeting or transport.

## Materials and methods

### Preparation of viral stock

African green monkey kidney (Vero) cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, and penicillin–streptomycin at 37°C ~~20~~ in P150 flasks. The Vero cells were infected at a multiplicity of infection (MOI) of 1.0 with F strain HSV (YBH J-3 substrain). After 48 hr, 100% of the cells were cytopathic. The medium and extracellular virions were collected and frozen and thawed three times. Cellular debris was removed by filtration through a 0.45-µm sterile filter (Millipore, Billerica, MA, USA). The filtrate was layered on a gradient of 10%, 30%, and 60% sucrose and centrifuged at 25 000 rpm for 2 hr at 4°C (SW28 rotor; Beckman, Fullerton, CA, USA). Virus was collected from the 30%–60% interface, resuspended in PBS, and recentrifuged at 25 000 rpm for 1 hr at 4°C. The pellet was isolated and resuspended in minimal essential medium (MEM) without serum and stored at −80°C. The concentration of virus was determined by standard plaque assay. This purified preparation of HSV was used for the remaining experiments.

### Intraocular injection

All procedures involving animals adhered to the Declaration of Helsinki, The Guiding Principles in the Care and Use of Animals, and to the guidelines of the UCSF Committee on Animal Research. Male BALB/c mice (5–6 weeks of age) were anesthetized by intraperitoneal injection of Avertin,[7] and the cornea was treated with a drop of 1% atropine: 0.05% proparacaine (1 : 1) to prevent any discomfort. All mice were infected with equivalent titers of plaque purified virus in sterile MEM without serum at concentrations of approximately $9 \times 10^4$ PFU/µl. The solution was drawn up into tubing connected to a 25-µl Hamilton syringe attached to a Hamilton repeating dispenser. After connecting the tubing to a 27-gauge needle, 2 µl of virus were injected into the vitreal chamber of each eye under a dissecting microscope. Approximately 30 s later, the needle was removed. Previous experiments have shown immunocytochemically that by 24 hr, the number of ganglion cells that are infected from an intravitreal injection varies among animals, due to differences in the placement of the needle in the vitreous chamber.[5] In the current experiments, we pooled tissues from 10 eyes to normalize the amount of viral mRNA recovered each treatment condition and time point. Each experiment ~~involving 10 eyes~~ was repeated three times.

### Tissue isolation

The mice were euthanized 3, 6, 9, 15, and 24 hr after infection. The eyes were removed, and the posterior half of the bulb, including the retina, lamina cribrosa, pigment epithelium, and choroid, but excluding the optic nerve, lens, ciliary body, and cornea, were analyzed. As negative controls, tissues from uninfected mice and from mice inoculated with MEM alone were similarly prepared. The infection, pooling, RNA isolation and PCR were done at least three times for each time point. ~~For each experiment, 10 eyes were homogenized and pooled for mRNA extraction.~~ Each sample was run for RT-PCR with gD, VP5, or VP22 mRNA at least three times.

Tissues were snap frozen in TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) and stored at −80°C. They were homogenized. The RNA was extracted, DNAse-treated, and purified using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNA was quantified using a spectrophotometer, and equal amounts of RNA were used for cDNA synthesis and amplification by RT-PCR. The minimum amount RNA used for RT-PCR was 50 ng. All of the primers were obtained from the Biomolecular Resource Center, UCSF. The RT-PCR was performed using viral gD primers: ATGGGAGGCAACTGTGCTAT and CTCGGTGCTCCAGGATAAAC; VP5 primers: GACGGGCTTTTCCACTTTATC and AAGCGTGCAGG AAGAAATTG; VP22 primers: CGCGATGAGTACGAGG ATCT and GAGGGCATAATCCGACTCGT; and 15S ribosomal control primers: TTCCGCAAGTTCACCTACC and CGGGCCGGCCATGCTTTACG. The steps were performed according to the Invitrogen Superscript One-Step RT-PCR with Platinum Taq kit (Invitrogen). According to the manufacturer, the sensitivity of this assay extends from 10 pg to 1 µg. The final concentration of our templates was within this range. The reverse transcription for cDNA synthesis was performed as one cycle: 50°C for 30 min followed by 94°C for 2 min. The PCR amplification step was performed for 30 cycles as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. A final extension was performed as one cycle at 72°C for 8 min. The PCR products were electrophoresed in a 2% agarose gel and visualized using a UV Trans-illuminator.

## Results

Viral RNA was isolated from infected eyes at 3, 6, 9, 15, and 24 hr postinfection. RT-PCR analysis was carried out on these samples to detect the presence of mRNAs for ~~the tegument protein~~ VP22, gD, and VP5. The mRNA for VP22 and gD were first recognized at 6 and for all time points up to 24 hr after infection (Fig. 1). The mRNA for capsid protein VP5 was first recognized at 9 hours (Fig. 1) and detected for the remaining times up to 24 hr postinfection. The internal positive control, 15S ribosomal RNA, was found consistently at all times of the assay. No viral mRNA was recovered from uninfected control tissues or from mice infected with only

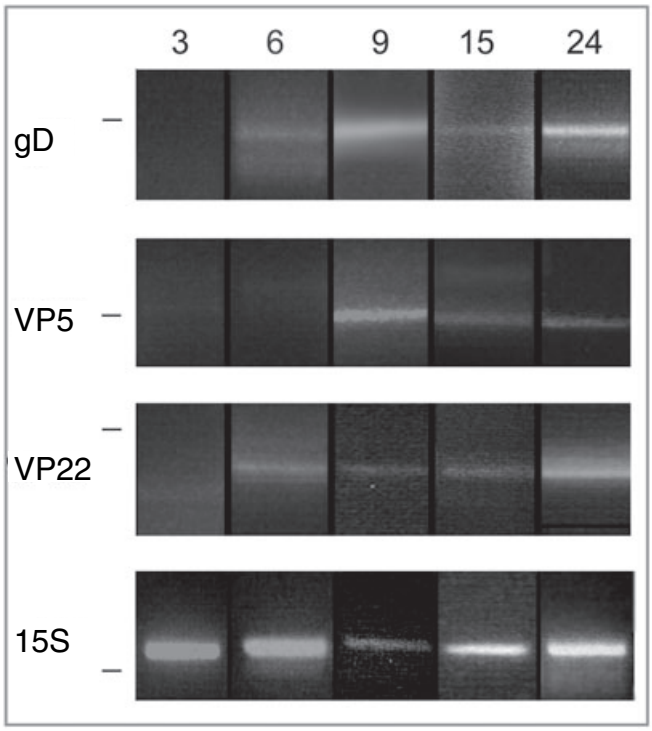


*Figure 1.* Time course of HSV transcription in infected eyes. The upper three panels show the expression profiles ~~of~~ gD, ~~of~~ VP5, and ~~of~~ VP22 mRNAs. The mRNAs for gD and VP22 first appear in the 6-hr sample, whereas the mRNA for VP5 is not detected until 9 hr postinfection. The lowest panel shows the expression profile of the internal control, 15S RNA. This mRNA was detected in all samples. On the left is the marker for 300 bp.

MEM. In sum, transcription of VP5 mRNA appeared to be delayed by 3 hr after gD and VP22 mRNA began to be synthesized.

## Discussion

We identified VP5 and gD proteins in retinal extracts from mice that were infected with HSV 48 hr ~~before euthanasia,~~[5] More recently, we found VP5, gD, and VP22 proteins in retinal tissues of mice that were infected for 24 hr (data not shown). Two days later, (or 3 days after infection), we identified only gD protein in the distal segments of the retinal ganglion cell axons.[5] In this paper, we have tested the hypothesis that the 4-day delay of transport of the capsid protein to the optic tract is due to a delayed initiation of mRNA expression in the eye.

Details of the precise timing of transcription of mRNAs and their half-lives have been established for HSV after infection of cultured Vero cells.[8] Weinheimer and McNight measured the transcription rates and mRNA accumulation of 11 HSV genes, including that of the major capsid protein (based on its molecular weight of 155 kDa,[9] presumably VP5) and several envelope proteins, including gB and gC in cultured cells.[8] They found these mRNAs were first recognized 3 hr after infection. In contrast, we found that the mRNA for gD and VP22 were first seen 6 hr after infection, and the mRNA for VP5 was first found 9 hr after infection.

One explanation for the 3-hr delay in appearance of HSV mRNA is that the access of HSV to cultured cells and to nervous tissue in the vitreal chamber is very different. Several barriers (that are absent in tissue culture) separate the vitreal chamber from the surface of retinal cells. Initially, the injected HSV reaches the basal lamina on the optic fiber layer. Heparan sulfate in the lamina will trap virus and impede its movement into retina proper. The Müller glial cell endfeet, which line the retinal surface and surround both retinal axons and retinal ganglion cell bodies, may also bind HSV and reduce the amount of virus available for further spread into the retina.[10]

A caveat of the current work is that we do not know which cells are making the early mRNAs. We have isolated the posterior half of the eye, removing the iris, ciliary body, lens, and cornea. Thus, the viral mRNA must be localized to retinal structures. The precise determination of which cells and when viral mRNAs are first transcribed in retinal ganglion cells awaits an alternative approach, such as laser capture of individual ganglion cell bodies for real time RT-PCR.

Our previous experiments were limited to one strain of HSV (F) and one strain of mouse (BALB/c). They allowed us to develop an efficient and reproducible assay of the axonal transport ~~of HSV~~ of viral subassemblies. We have extended these studies to viral mRNA transcription. Whether alternate viral and mouse strains would result in different patterns of infection, viral spread and efficiency of transport is beyond the scope of this study.

A naïve view of viral genesis in neurons might be that all viral proteins are expressed, transported, and assembled in concert at similar times. However, previous experiments conducted by this lab and other labs are inconsistent with this model of viral maturation.[5,11] During maturation of HSV in infected murine retinal ganglion cells, we found that new viral components are axonally transported in two phases.[5] The viral envelope protein (gD) appears 48 hr before the capsid protein (VP5). Understanding the mechanisms responsible for this delay is key to understanding the infection process in neurons. Among the many possibilities is that the mRNA transcription for capsid protein is significantly delayed in retinal cells. However, we have found only a 3-hr delay in appearance of the VP5 mRNA. This is clearly only a minor fraction of the 48-hr difference in delivery of VP5 protein to retinal ganglion cell axons. These results are significant in that they allow us to discount this possible cause of the delay and direct our future attention to regulation of viral protein expression in retinal ganglion cell bodies.

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