The current study analyzed the herpes simplex virus type 1 (HSV-1) genome during suppressed and reactivated infection in vitro. Utilization of 3H-labeled HSV-1 provided a highly specific probe for intracellular localization, isolation, and characterization of the HSV genome after infection of rabbit trigeminal ganglion neurons. Restriction enzyme analysis of viral DNA extracted during both suppressed and reactivated infection matched that of the HSV1 control DNA. Viral DNAs extracted from both cellular and nuclear fractions of host cells exhibited identical patterns. No detectable alterations in terminal fragments were observed, which suggests that the HSV1 genome is both linear and nonintegrated during suppressed infection in this system. Invest Ophthalmol Vis Sci 28:391-394, 1987

Herpes simplex virus (HSV) is known for its ability to establish latent infection. Direct proof for the establishment of latency resides in the ability to recover virus, after resolution of acute disease, only via whole-cell culture methods. Supporting data are based on the observation by indirect methods of the presence of viral DNA sequences, virus-specific mRNA sequences, and virus-coded early nonstructural polypeptides in sensory ganglion neurons during latent infection.¹⁻⁵

One of the fundamental questions regarding the state of the viral genome during latent infection is whether viral DNA is incorporated into host cell DNA or remains an independent entity. The current study addresses this question by utilizing a system for acute and suppressed infections in rabbit trigeminal ganglion (TG) neuron explants.

It has been shown that radiolabeling of HSV-1 DNA with ³H-nucleosides provides a highly specific tracer for intracellular localization of the HSV-1 genome during acute and latent infections both in vivo and in vitro.⁶ In this study, ³H-labeled HSV-1 was utilized as a probe for isolation and characterization of the viral genome during latent and reactivated infections in rabbit TG neurons in vitro.

Materials and Methods. Preparation of ³H-labeled HSV-1 inoculum: Infection of confluent OMK monolayers was initiated by adsorption of 10⁷ plaque-forming units of HSV-1 for 1 hr at 37°C. Cultures were reconstituted with AP-MEM plus 10% fetal calf serum. Four hours after inoculation, 0.5 mCi of ³H-nucleosides in various combinations was added. When 3+ to 4+ cytopathogenic effect was evident (36–48 hr postinfection), the cells were harvested, dounce homogenized, and clarified by use of low-speed centrifugation at 4°C. Solid sucrose was added to the supernate to a final concentration of 0.2 M; the mixture was layered onto a 65% sucrose pad and centrifuged in an SW27 rotor at 25,000 rpm for 3 hr at 4°C. The virus was then aspirated from the sucrose-supernate interface and passed through a Sepharose CL2B column (Pharmacia Fine Chemicals; Uppsala, Sweden) at a flow rate of 50 ml per hour at 4°C. The peak fractions from column purification were assayed for infectivity and specific activity. Purity of the virus preparation was confirmed by electron microscopy.

Preparation and inoculation of TG cell cultures: Establishment of TG cultures, inoculation of neurons, and reactivation of infected cultures have been described previously by Dunkel et al.⁷

Extraction of viral DNA from infected TG neurons: In method A, neurons from 40 cultures were collected for each DNA extraction by gentle scraping followed by low-speed centrifugation. DNA was extracted from the resultant pellet by the Hirt procedure.⁸ The final DNA pellet was dissolved in 50 μl of water and analyzed.

In method B, DNA from the TG neuron pellet was extracted as described by Pignatti et al.⁹ After CsCl density gradient centrifugation, the peak fractions were combined and dialyzed in TE buffer. The DNA was precipitated by adding sodium acetate solution to a final concentration of 0.1 M plus 2 volumes of ethanol and storing at -20°C overnight. The DNA was collected by centrifugation, dissolved in a small volume of TE buffer, and analyzed.

Analyses of DNA: Portions of the DNA samples were cleaved with restriction endonucleases BamHI, EcoRI, and XhoI under conditions recommended by the supplier (Bethesda Research Laboratories; Bethesda, MD).
Table 1. Infectivity and specific activity of virus from Sepharose column fraction

<table>
<thead>
<tr>
<th>Tritium-labeled nucleosides</th>
<th>Titer (plaque-forming units/ml)</th>
<th>cpm/μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-thymidine</td>
<td>1.6 × 10⁷</td>
<td>5 × 10⁵</td>
</tr>
<tr>
<td>3H-thymidine</td>
<td>2.0 × 10⁷</td>
<td>1 × 10⁶</td>
</tr>
<tr>
<td>3H-deoxyguanosine</td>
<td>2.0 × 10⁷</td>
<td>6 × 10⁶</td>
</tr>
</tbody>
</table>

Restriction endonuclease fragments were separated by electrophoresis in 0.7% agarose gels, enhanced (EN3HANCE, New England Nuclear; Boston, MA), dried under vacuum, and exposed to X-ray film (XAR-5) for 10–15 days.

Results. Preparation and purification of the virus inoculum: The general scheme for labeling and purification of HSV-1 DNA is described in the Materials and Methods section. Increasing the number of labeled nucleosides from one to three resulted in a ten-fold increase in the specific activity of labeled virus (Table 1, Fig. 1). The introduction of sonication steps enhanced the virus titer and significantly decreased cell membrane fragment contaminants in the inoculum (Table 1).

Relationship of viral and host cell DNA during suppressed and reactivated infection: The restriction endonuclease patterns of viral DNA extracted during the suppressed and reactivated stages of infection were compatible with that of the control HSV-1 DNA. All of the DNA sequences appeared to be retained (Fig. 2). No alteration was observed in the terminal fragments with respect to molarity and size. These results were verified by XbaI and EcoRI digestion of the viral DNA extracted from TG neurons during latent (suppressed) infection (Fig. 3). No alteration in the HSV-1 genome was detectable when compared with purified control HSV-1 DNA. For comparison, DNAs extracted by method B from cellular and nuclear host cell fractions were analyzed. The restriction patterns were found to be identical to the control HSV-DNA (Fig. 3).

Discussion. One of the fundamental questions regarding the state of the viral genome in latency is whether viral DNA is incorporated into host cell DNA or remains an independent entity. In latently infected animals only small amounts of viral DNA are present. In the past, the difficulty in isolating such small quantities of viral DNA over a large background of host DNA has resulted in a poor understanding of the biochemical nature of the HSV-1 genome during latency.

To analyze the molecular aspects of establishment and maintenance of suppressed (latent) infection and its reversal (reactivation), it was essential to increase the cell population containing an HSV-1 genome capable of a productive infection. The in-vitro system described resulted in suppressed infection in 40–50% of the cultures, as compared with 15–20% in the original method. Radiolabeling of HSV-1 DNA with 3H-nucleosides (thymidine, deoxyadenosine, deoxyguanosine) provided a convenient probe for isolation and characterization of the viral genome after establishment of infection in TG neurons. This study reports optimal conditions for labeling and purification of HSV-1 DNA. The unique feature of this system is the isolation and characterization of a direct label from the latently infected TG neurons. The restriction endonuclease patterns of the DNAs extracted from infected neurons during suppressed and reactivated infection were consistent with those of the parental control HSV-1 DNA. The restriction enzyme patterns of DNAs from TG neuron cellular and nuclear fractions confirmed the above findings.
There was no detectable alteration in the sizes of terminal DNA fragments after digestion with three different restriction endonucleases (BamHI, Xbal, EcoRI). This finding suggests that the HSV-1 DNA is nonintegrated during the suppressed and reactivated stages in infection in this system. However, it does not rule out the possibility of minor HSV-1 DNA species being retained in an integrated form. This study represents the first isolation and characterization of a directly labeled HSV-1 genome in the rabbit in-vitro suppression model. Recently, using blot hybridization techniques, Wigdahl et al. have conducted similar studies in rat sensory neurons. Their results closely resemble our data, suggesting that the HSV-1 genome during suppressed infection in this model system is in a nonintegrated, linear form. In addition, the linear form of the HSV-1 genome detected in our current study resembles the form of the HSV-1 genome detected in several total human brain DNA specimens by Fraser et al. A perplexing point is that similar studies in murine models contradict these findings.

Although suppressed infection in the in-vitro system appears to correlate with latent infection in vivo (virus isolation, antigenic properties, ultrastructure), the molecular relationship between host cell and virus may differ. The answer to this question will await similar studies in vivo.

References


2. Green MT, Courtney RJ, and Dunkel EC: Detection of an immediate early herpes simplex virus type 1 polypeptide in trigem-
Localization of \(^{3}H\)-Thymidine-Labeled HSV-1 in Latently Infected Rabbit Trigeminal Ganglion Cells

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Although current data favor conservation of virus in a nonreplicating form during latency, the actual host cell-virus relationship during this quiescent period remains an enigma. The purpose of this study was to develop a highly specific probe for direct localization of the HSV-1 genome in an animal model that closely mimics human disease. Tritium-labeled HSV-1 was inoculated onto trigeminal ganglion (TG) neurons in vitro and onto New Zealand white rabbit corneas in vivo. During acute infection in vivo and after establishment of latency in vivo or suppressed infection in vitro the TG neurons were processed for autoradiography. Silver grains were localized over nuclei of 8–10% of TG neuron cell bodies during suppressed infection in vitro. Acute infection in vivo resulted in the localization of label over 5–10% of TG neuron cell bodies and satellite cells per section. During latency the label appeared over nuclei of 1–10% of TG bodies per section. This study shows that directly labeled HSV-1 can be found in TG neuron nuclei both in vivo and in vitro. It also suggests that HSV genetic material is lost from certain neurons when latency is established. Invest Ophthalmol Vis Sci 28:394–397, 1987

Although the fundamental relationship between the virus and the host cell during the latent stage of herpesvirus infection remains uncertain, most data appear to favor conservation of the virus in a nonreplicating form. Previous studies have utilized indirect techniques, such as DNA hybridization and antigenic expression, to localize the herpes simplex virus (HSV) genome during latency.\(^{1–5}\) A majority of these studies have been conducted in the murine system, which differs from human HSV infection in that spontaneous recurrence of disease does not occur.\(^{6,7}\) The purpose of this study was to develop a highly specific probe for direct localization of the HSV-1 genome in an animal model that closely mimics human HSV infection.

Materials and Methods. Preparation and isolation of \(^{3}H\)-thymidine-labeled HSV-1: Thymidine-starved, confluent primary rabbit kidney cell monolayers were infected with HSV-1 (McKrae strain), rehydrated, and incubated at 37°C until 3–4+ cytopathic effect was obtained. Cultures then were harvested by scraping, and the cells were disrupted by four strokes of a dounce homogenizer. Concentrated sucrose in phosphate buffer (0.125 M, pH 7.0) was added to the homogenate to a final concentration of 0.25 M followed by centrifugation at 1200 rpm for 10 min. Twenty-milliliter aliquots were centrifuged onto 65% sucrose pads in an SW27 rotor at 25,000 rpm for 6 hr at 4°C. The virus then was aspirated from the sucrose-supernate interface. Three-milliliter aliquots of the aspirate were passed through an 80-ml Sepharose CL2B column (Pharmacia Fine Chemicals; Uppsala, Sweden) at a flow rate of 50 ml per hour at 4°C. Fractions were collected and tested for infectivity and for incorporation of \(^{3}H\)-thymidine counts. Fractions containing infectious HSV-1 of high specific activity were pooled and brought to a final concentration of 0.25 M with sucrose. Four-milliliter samples were then layered on 5-ml 65% sucrose pads and were centrifuged at 40,000 rpm in an SW50.1 rotor for 1 hr at 4°C. After centrifugation, 0.5-ml supernate fractions were withdrawn, pooled, and assayed for infectivity and specific activity. To char-