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Analysis of the Herpes Simplex Virus Genome During In Vitro Latency in Human Diploid Fibroblasts and Rat Sensory Neurons

BRIAN WIGDAHL,[1] ADRIENNE C. SCHECK,[1] RICHARD J. ZIEGLER,[2] ERIK DE CLERCQ,[3] AND FRED RAPP[1]*

Department of Microbiology and Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033[1]; Department of Medical Microbiology and Immunology, University of Minnesota—Duluth School of Medicine, Duluth, Minnesota 55812[2]; and Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium[3]

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We have previously designed in vitro model systems to characterize the herpes simplex virus type 1 (HSV-1) genome during in vitro virus latency. Latency was established by treatment of infected human embryo lung fibroblast (HEL-F) cells or rat fetal neurons with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and human leukocyte interferon and was maintained by increasing the incubation temperature after inhibitor removal. Virus was reactivated by reducing the incubation temperature. We have now examined the HSV-1-specific DNA content of latently infected HEL-F cells and rat fetal neurons treated with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and human leukocyte interferon and increased temperature. The HEL-F cell population contained, on an average, between 0.25 and 0.5 copies of most, if not all, HSV-1 *Hin*dIII and *Xba*I DNA fragments per haploid cell genome equivalent. In contrast, the latently infected neurons contained, on an average, 8 to 10 copies per haploid cell genome equivalent of most HSV-1 *Bam*HI DNA fragments. There was no detectable alteration in size or molarity of the HSV-1 terminal or junction DNA fragments obtained by *Hin*dIII, *Xba*I, or *Bam*HI digestion of the latently infected neuron or HEL-F cell DNA, as compared with digestion of a reconstruction mixture of purified HSV-1 virion and HEL-F cell DNAs. These data suggest that the predominant form of the HSV-1 genome in either latently infected cell population is nonintegrated, linear, and nonconcatameric.

The interaction of herpes simplex virus (HSV) with its human host can result in one or more cell-virus interactions that include cytocidal infection (40), latent infection (4, 41), and cellular transformation (16, 32). Because the latent HSV genome can be harbored in humans and can be reactivated to produce recurrent clinical disease, establishment and maintenance of virus latency and reactivation are current foci of research. To date, these studies have employed human tissue (3, 11, 12, 42) and both in vitro (13, 19, 29, 49–51) and in vivo (30, 41) model systems. In addition, as HSV has been implicated in the etiology of certain human cancers (33), the potential role of the latent HSV genome in the oncogenic process is currently being assessed.

We have previously described infections of human embryo lung fibroblast (HEL-F) cells with HSV type 1 (HSV-1) or HSV type 2 (HSV-2) in which the virus genome is maintained in a repressed form by increased temperature after virus replication is partially blocked by treatment with 1-β-D-arabinofuranosylcytosine (ara-C; 6, 7, 24, 43, 44). Infectious center assays indicate that less than 0.0002 to 0.02% of the cell population contains a virus genome that can be reactivated (7, 24, 44). To analyze the molecular aspects of establishment and maintenance of the virus latency and reactivation processes, it was essential for us to increase the fraction of the cell population containing an HSV genome that could be activated into a productive replication cycle.

Recent studies have used human leukocyte interferon (IFN-α) in combination with the relatively noncytotoxic (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU; 8–10) and increased temperature (40.5°C) to establish and maintain the HSV-1 genome in a repressed form after high-multiplicity infection of HEL-F cells with HSV-1 (45). BVDU is known to inhibit viral DNA polymerase (2), although it can also be incorporated into virus DNA (1, 21), presumably by interfering with expression of HSV late or γ genes (14, 17) and possibly a subset of HSV early or β genes (14, 17), while allowing complete expression of the immediate early α genes (14, 17). In addition, human interferon blocks the deleterious effects of HSV on human chromosomes (25, 26). As described with repressed HSV-1 or HSV-2 infections established by ara-C treatment (6, 7, 43, 44), HSV-1 can be activated either by reducing the temperature or superinfecting with human cytomegalovirus. However, combined IFN-α and BVDU treatment resulted in an increase in the minimum number of cells (from 0.02 to 3%) that can be activated to produce infectious HSV-1 (45).

Because HEL-F cells were used as the host cell, the validity of this in vitro latency model was further assessed in a cell of neuronal origin (46). BVDU or combination BVDU and IFN-α treatment of HSV-1-infected rat fetal neurons isolated from the dorsal root ganglia repressed HSV-1 replication. Analogous to repressed HSV-1 infections in HEL-F cells (45), the HSV-1 genome was maintained in a repressed form in rat fetal neurons by increasing the incubation temperature to 40.5°C after inhibitors were removed. In addition, virus was predictably reactivated from neurons by reducing the incubation temperature to 37°C. In this study, we examined the HSV-1 genome (i) during establishment and maintenance of the repressed infection in HEL-F cells, (ii) during maintenance of the repressed infection in rat fetal neurons, and (iii) during reactivation.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of HEL-F, primary rabbit kidney (PRK), and baby hamster kidney-21 (BHK-21) cells were grown as previously described (6, 7, 43, 44, 50, 51). Neuron cell cultures were prepared as previously de-

* Corresponding author.

scribed (5, 48, 50, 51). Briefly, dorsal root ganglion cells were obtained from 17-day-old Sprague-Dawley rat embryos. The dorsal root ganglion cells were dissociated with 0.25% trypsin in Hanks balanced salt solution free of magnesium and calcium for 45 min at 37°C. Washed cells were suspended in Eagle minimum essential medium (E-MEM) containing 10% fetal calf serum, 10% heat-inactivated horse serum, and 200 U of nerve growth factor (Collaborative Research Inc., Lexington, Mass.) per ml, 0.5% glucose, and 0.5% chicken embryo extract (GIBCO Laboratories, Grand Island, N.Y.) [E-MEM (10/10)]. Resuspended cells were seeded on collagen-coated, 35-mm plastic plates (approximately 3×10^4 neurons per plate) and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. After 24 h, medium was removed and the cultures were treated for 24 h with E-MEM (10/10) containing 10 μM uridine and the mitotic inhibitors ara-C (10 μM) and fluorodeoxyuridine (10 μM). The neuron cell cultures were subsequently alternated at 24-h intervals between E-MEM (10/10) and E-MEM (10/10) containing uridine and mitotic inhibitors for 7 days. Before use, neuron cell cultures were maintained for 2 to 4 days in E-MEM (10/10). After treatment of neuron cell cultures with uridine and mitotic inhibitors, cultures consisted of greater than 99.9% neurons with only an occasional contaminating fibroblast or Schwann cell. Such contaminating cells had aberrant nuclear and cytoplasmic morphology as a result of treatment with mitotic inhibitors. Only neuron cell cultures of 99.9% or greater purity were used for experimentation.

HSV-1 (strain Patton) stocks were obtained by replication in HEL-F cells as described previously (6, 7, 43, 44), and HEL-F mock-infecting fluid was prepared from uninfected HEL-F cells in a manner analogous to the preparation of the virus stock (6, 7, 43, 44). Infectious HSV-1 was quantitated by plaque assay on HEL-F, PRK, or BHK-21 cells (6, 7).

Establishment and maintenance of HSV-1 genome in repressed form and virus reactivation. The procedure for the establishment and maintenance of the HSV-1 genome in repressed form was performed essentially as previously described for HEL-F cells (45) or rat fetal neurons (46). Briefly, HEL-F cells (10^6 cells per culture) or essentially pure rat fetal neurons (3×10^4 neurons per culture) were pretreated with medium containing BVDU (30 μM) and 125 IU of IFN-α (6.5×10^5 IU/mg of protein; kindly provided by Life Sciences Research Laboratories, St. Petersburg, Fla.) per ml at 37°C, infected with 2.5 PFU of HSV-1 per cell, and treated for 7 days at 37°C with the same inhibitor combination. Due to the inherent difficulty in quantitating the number of neurons per culture, the multiplicity of infection may vary to a greater extent when neuron cell cultures are infected than when HEL-F cell cultures are infected. To maintain the HSV-1 genome in a repressed form, the infected HEL-F or rat fetal neuron cells were incubated at 40.5°C after removal of inhibitors on day 7. HSV-1 replication was activated by reducing the incubation temperature from 40.5 to 37°C.

Preparation of plasmid and virus DNAs. HSV-1 recombinant clones were obtained from R. W. Hyman, The Pennsylvania State University College of Medicine, Hershey, Pa. (18, 22, 36). The HSV-1 *Eco*RI DNA fragments were cloned into λ and pACYC184 vectors, whereas the HSV-1 *Hin*dIII and *Bam*HI DNA fragments were cloned into pBR322. HSV-1 recombinant plasmids were purified as described previously (8). HSV-1 DNA was purified from virions isolated from infected cells as described previously (7, 43, 44).

Isolation of cell DNA. DNA was extracted from neurons or HEL-F cells as previously described (47) with several modifications. The cells (10^6) were harvested during the inhibitor treatment or after maintenance at 40.5°C by scraping them into phosphate-buffered saline and centrifuging at $1,000 \times g$ for 10 min. The pellet was washed twice with phosphate-buffered saline and suspended in 1 ml of TNE (10 mM Tris-hydrochloride [pH 8.0], 150 mM NaCl, 10 mM EDTA), and sodium dodecyl sulfate and proteinase K were added to 0.5% and 200 μg/ml, respectively. The lysate was incubated at 37°C for 12 h, extracted in succession with TNE-saturated phenol and chloroform, and precipitated for 12 h at −20°C with 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of isopropanol. The precipitated DNA was washed twice with 70% ethanol and absolute ethanol and dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1.0 mM EDTA. The DNA was then treated with 50 μg of RNase A per ml at 37°C for 2 h.

Analysis of HSV-1-specific DNA by blot hybridization. The DNA extracted from cells was digested with restriction endonuclease *Hin*dIII, *Xba*I, or *Bam*HI according to the manufacturer's conditions (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Digestions (20 μg of DNA per digestion) were performed at an enzyme-to-DNA ratio of 4 U/μg of DNA for 3 h at 37°C. After 3 h, another 4 U of restriction endonuclease per μg of DNA was added, and the digestion was continued for an additional 3 h. Reactions were terminated by the addition of a 0.25 volume of 0.25 M Tris-hydrochloride (pH 8.0) containing 30% (wt/vol) Ficoll, 0.5% (wt/vol) sodium dodecyl sulfate, 60 mM EDTA, and 0.2% (wt/vol) bromophenol blue, heated for 3 min at 70°C, and immediately placed on ice. The terminated digestion was subjected to electrophoresis through a submerged 0.5% agarose (Bio-Rad Laboratories, Richmond, Calif.) horizontal slab gel at 1.5 V/cm for 16 to 18 h in a buffer containing 40 mM Tris-hydrochloride (pH 8.0), 5 mM sodium acetate, and 1 mM EDTA. DNA fragments were visualized under short-wave UV light after staining for 5 min in a solution of ethidium bromide (2 μg/ml). The digested, electrophoretically separated DNA was blot transferred (39) to diazotized aminophenylthioether paper (37) and hybridized to selected probe DNA radiolabeled in vitro by nick translation, and autoradiography was performed with Kodak XAR-5 film and DuPont Cronex Lightning Plus screens at −70°C for 24 to 72 h. Hybridizations were performed at 48°C in 50% formamide and 5× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M sodium chloride). Nick-translated probe DNAs were prepared essentially by a micromodification (20) of the method described by Rigby et al. (34). The reaction mixture consisted of 50 mM Tris-hydrochloride (pH 7.5), 50 mM $MgSO_4$, 50 μg of bovine serum albumin per ml, 10 mM dithiothreitol, 10 μM dATP, 10 μM dTTP, 125 μCi (1 Ci is 3.7×10^{10} Bq) of [α-^{32}P]dCTP (3,200 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 125 μCi of [α-^{32}P]dGTP (3,200 Ci/mmol), 0.1 ng of DNase I (Worthington Biochemicals, Freehold, N.J.), 2 U of DNA polymerase I (Boehringer Mannheim Biochemicals), and 100 ng of DNA in a reaction volume of 0.04 ml. The reaction was carried out for 3.5 h at 14°C and then terminated, and radiolabeled DNA was purified as described previously (20, 34). Specific activities of 5×10^8 to 2×10^9 cpm/μg of DNA were achieved. The radiolabeled probe DNA used was a genomic proportion mixture of HSV-1 recombinant plasmids consisting of HSV-1 *Eco*RI D, G, N, F, M, O, L, I, and H DNA fragments inserted in pACYC184 and HSV-1 *Hin*dIII K and L DNA fragments inserted in pBR322 (18, 22, 36) representing the entire HSV-1 long unique and short unique DNA sequences (Fig. 1).

The HSV-1 joint region *Bam*HI S-P (K) DNA fragment was partially purified from the recombinant plasmid, radiola-

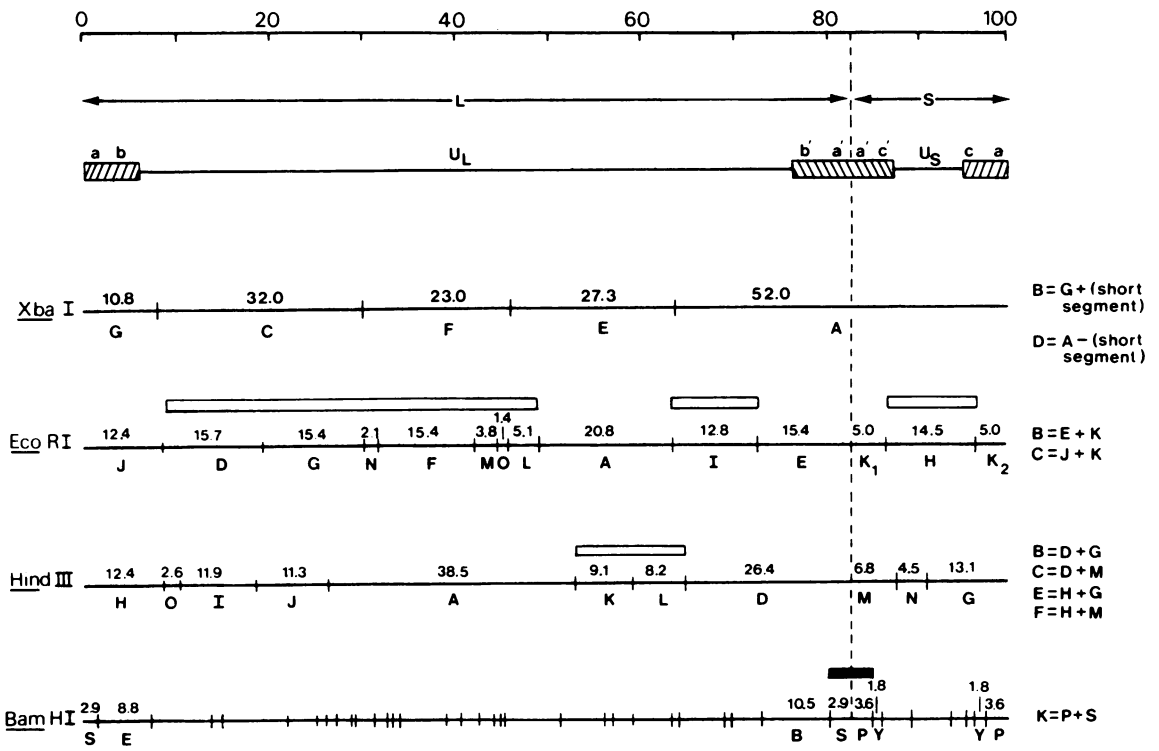

FIG. 1. Location of HSV-1 recombinant DNA hybridization probes on physical map of the prototype HSV-1 genome. The HSV-1 genome contains a long component (L) consisting of a long unique segment (U_L) bounded by inverted repeat regions and a short component (S) consisting of a short unique segment (U_S) bounded by inverted repeat regions. The long and short components of the genome invert relative to each other producing four isomeric forms (15). The genome is divided into 100 map units, each representing approximately 1.63 kilobase pairs (kb). The HSV-1 restriction endonuclease DNA fragments that have been included as recombinant DNA molecules in the unique sequence probe DNA are indicated by the open rectangles above the physical map. The physical map location of the *Bam*HI S-P (K) hybridization probe DNA is indicated by the closed rectangle. The *Bam*HI S-P (K) DNA fragment encompasses the junction of the long and short components, resulting in a single *Bam*HI junction DNA fragment in each isomeric form. The physical map of the HSV-1 genome and the physical location of the recombinant DNA fragments have been described previously (15, 18, 22, 23, 28, 36, 38).

beled in vitro, and also used as probe. Purification of the *Bam*HI S-P (K) DNA fragment insert was performed to increase the sensitivity of the probe DNA to increase the probability of detecting minor species of altered HSV-1 terminal DNA fragments. pBR322 containing the HSV-1 *Bam*HI S-P (K) DNA fragment (100 μg) was digested with *Bam*HI under the conditions specified by the manufacturer (Boehringer Mannheim Biochemicals). After complete digestion by agarose gel electrophoresis was confirmed, the digested DNA was centrifuged to equilibrium in an ethidium bromide-CsCl buoyant density gradient at 87,000 × *g* for 72 h at 20°C. After removal of the lower buoyant density pBR322 DNA from the top of the gradient, the higher buoyant density HSV-1 *Bam*HI S-P (K) DNA fragment was recovered by side puncture of the gradient. The ethidium bromide was removed from the DNA by extraction four times with *n*-butanol in TE buffer (0.01 M Tris-hydrochloride [pH 8.0] and 0.001 M EDTA), and the DNA was dialyzed twice for 24 h against 2,000 volumes of TE buffer. As estimated by agarose gel electrophoresis and subsequent ethidium bromide staining, the HSV-1 *Bam*HI S-P (K) DNA fragment obtained in this manner was at least 80% pure.

RESULTS

Repression and activation of HSV-1 replication in HEL-F cells and isolated rat fetal neurons. Previous studies have demonstrated that HEL-F cells or isolated rat fetal neurons pretreated with combined BVDU (10 μg/ml) and IFN-α (100 IU/ml) and infected with 2.5 PFU/ml of HSV-1 per cell retain the virus in a noninfectious or latent form after a 7-day treatment interval. Combined BVDU and IFN-α treatment resulted in elimination of detectable infectious HSV-1 within 3 days in both infected cell types (Fig. 2). Infectious HSV-1 remained undetectable during the remainder of the treatment interval. After removal of the inhibitor on day 7, HSV-1 was detectable within 3 days at 37°C with progressive virus-specific cytopathology and eventual total destruction of HEL-F cell or neuron cell cultures (Fig. 2A). However, as reported previously (45, 46), if the incubation temperature of HSV-1-infected, inhibitor-treated HEL-F cells or neurons was increased from 37 to 40.5°C, HSV-1 could be maintained in a noninfectious form for at least 60 or 15 days, respectively (data not shown). Replication of HSV-1 was activated in either latently infected cell type by reducing the incubation temperature from 40.5 to 37°C. Infectious virus was usually detectable within 1 to 3 days after temperature reduction, and maximum virus synthesis was attained after 4 to 7 days at 37°C (Fig. 2B), with extensive virus-specific cytopathology.

The infectious virus synthesized after reduction of the incubation temperature of latently infected HEL-F cells from 40.5 to 37°C was identified by DNA blot hybridization to be parental-like input virus (Fig. 3). HSV-infected HEL-F cells were treated with combined BVDU and IFN-α for 7 days and maintained at 40.5°C for an additional 7 days. Total cell DNA was partially purified from HSV-1-infected, inhibi-

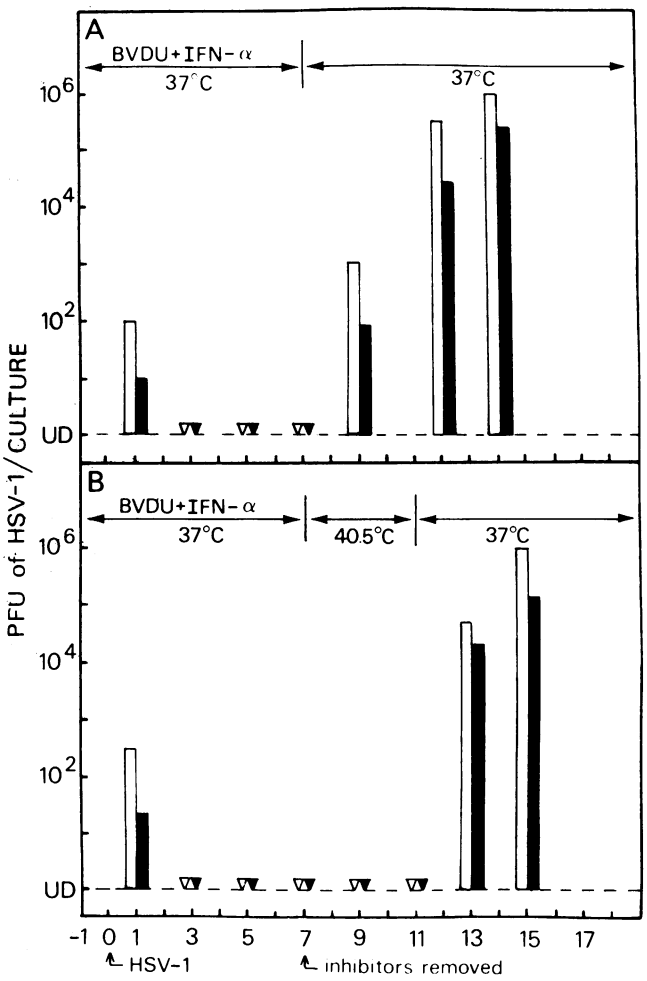

FIG. 2. Repression and activation of HSV-1 replication in HEL-F cells and rat fetal neurons. HEL-F cells (open symbols) or rat fetal neurons (closed symbols) were pretreated with combined BVDU (30 μM) and IFN-α (125 IU/ml) for 24 h, infected with 2.5 PFU of HSV-1 per cell, and treated every 24 h for 7 days with the same inhibitor combination. After 7 days, the inhibitors were removed, and the cultures were washed with Tris-buffered saline and incubated at either 37 or 40.5°C in maintenance medium without inhibitors. Maintenance medium was changed every third day after inhibitor removal. Virus replication was activated from either latently infected HEL-F cells or rat fetal neurons by incubating at 37°C after inhibitor removal (A) or reducing the incubation temperature from 40.5 to 37°C (B). All cultures were harvested and assayed for infectious virus by plaque assay in BHK-21 or PRK cells (6, 7, 43, 44). Undetectable (UD) quantities of virus (<5 PFU per culture) are indicated by the inverted triangles.

tor-treated HEL-F cells 4 days after reduction of the temperature from 40.5 to 37°C. The DNA was digested with *Xba*I (Fig. 3A), *Bam*HI (Fig. 3B), or *Hin*dIII (Fig. 3C), blot transferred, and hybridized with a ^{32}P-radiolabeled probe consisting of a genomic proportion mixture of recombinant plasmids containing the unique HSV-1 DNA sequences, and autoradiography was performed. No alterations in the reactivated virus genome (Fig. 3A, B, and C, lanes 2) were detectable by this method as compared with purified, parental input virion HSV-1 DNA (Fig. 3A, B, and C, lanes 1). This result was also confirmed by restriction endonuclease (*Xba*I and *Eco*RI) digestion of HSV-1 DNA purified from virus reactivated from latently infected HEL-F cells (data not shown).

Detection of HSV-1 unique DNA sequences during virus latency in HEL-F cells. Infectious center analyses have demonstrated that a minimum of 1 to 3% of the HSV-1-infected HEL-F cells treated with combined BVDU and IFN-α and subsequently maintained at 40.5°C contain a virus genome that can be activated into a productive replicative cycle (45). To examine the latently infected HEL-F cell population for HSV-1 DNA sequences, blot hybridization was performed. Total cellular DNA was purified from HSV-1-infected cells after a 1-, 3-, or 7-day inhibitor treatment and 4 or 8 days subsequent to inhibitor removal and increase in incubation temperature from 37 to 40.5°C. The DNAs were digested with *Xba*I (Fig. 4A) or *Hin*dIII (Fig. 4B), subjected to electrophoresis, blot transferred, and hybridized with a ^{32}P-radiolabeled genomic proportion mixture of recombinant plasmids containing greater than 95% of the unique HSV-1 DNA sequences (Fig. 1), and autoradiography was performed. Most, if not all, HSV-1 unique DNA sequences from the long and short genome components were retained within the latently infected HEL-F cell population 8 days after inhibitor removal and maintenance at 40.5°C (Fig. 4). The relative molar ratios of most HSV-1 *Xba*I and *Hin*dIII DNA fragments obtained by digestion of DNA isolated from latently infected HEL-F cells were comparable to those obtained after digestion of HSV-1 virion DNA in the reconstruction experiment. However, some small relative molarity increases in the larger HSV-1 restriction endonuclease DNA fragments obtained by digestion of DNA isolated from latently infected HEL-F cells were noted. This result is

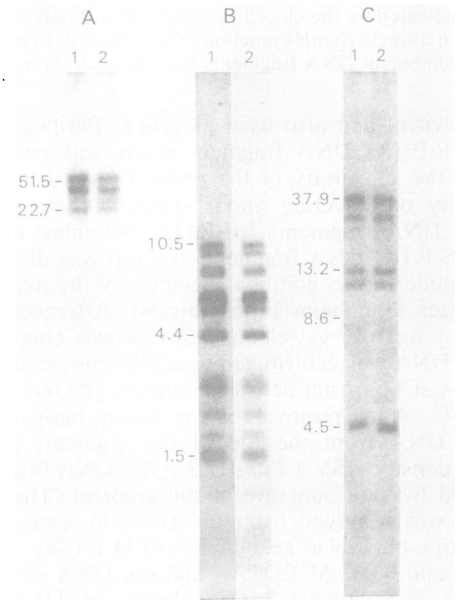

FIG. 3. DNA blot hybridization analysis of virus reactivated from latently infected HEL-F cells. Purified HSV-1 virion DNA (lanes 1) or total cellular DNA, isolated from HSV-1-infected, inhibitor-treated HEL-F cells 4 days after reduction of the temperature from 40.5 to 37°C (lanes 2), was digested with *Xba*I (A), *Bam*HI (B), or *Hin*dIII (C), subjected to electrophoresis, blot transferred, and hybridized with a genomic proportion mixture of recombinant plasmids (Fig. 1); autoradiography was then performed. Sizes (kb) of the DNA fragments are indicated at the left of each panel.

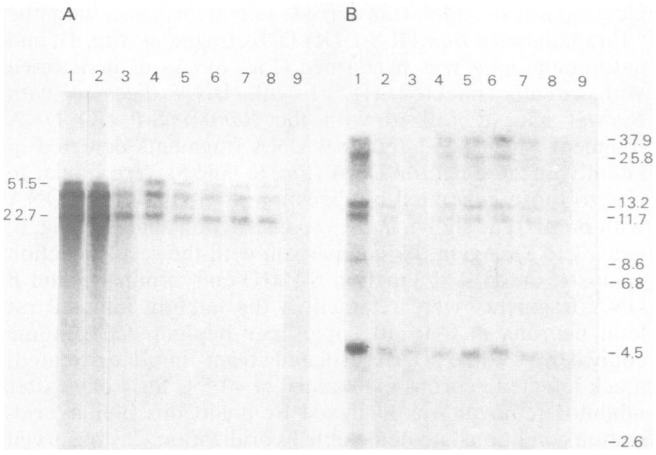

FIG. 4. Detection of HSV-1 unique DNA sequences in latently infected HEL-F cells by blot hybridization. DNA was isolated from HSV-1-infected HEL-F cells after a 1-, 3-, or 7-day treatment with combined BVDU and IFN-α (A and B, lanes 4, 5, and 6, respectively), 4 or 8 days subsequent to inhibitor removal and increase in incubation temperature from 37 to 40.5°C (A and B, lanes 7 and 8, respectively), or from HEL-F cells that were mock infected, inhibitor treated, and incubated at 40.5°C for 8 days after inhibitor removal (A and B, lane 9). The DNAs were digested with *Xba*I (A) or *Hin*dIII (B), subjected to electrophoresis, blot transferred, and hybridized with a ^{32}P-radiolabeled genomic proportion mixture of recombinant plasmids containing HSV-1 unique DNA sequence inserts; autoradiography was performed (3-day autoradiograph exposure). Sizes (kb) of the DNA fragments are indicated at the left of panel A and at the right of panel B. The reconstruction experiment was performed with 2, 1, or 0.5 copies of HSV-1 virion DNA (purified by two successive CsCl equilibrium buoyant density centrifugations) per haploid cell genome equivalent (A and B, lanes 1 to 3, respectively). A total of 20 μg of an HSV-1 and HEL-F cell DNA reconstruction was then digested with *Xba*I (A) or *Hin*dIII (B) and analyzed as described for panels A and B, lanes 4 to 9.

difficult to interpret because the HSV-1 virion DNA used in the reconstruction analyses was purified by two successive CsCl buoyant density equilibrium centrifugation steps and may, therefore, have reduced apparent relative molarities of larger restriction endonuclease DNA fragments.

Compared with the reconstruction analyses with purified HSV-1 extracellular virion and HEL-F cell DNAs, HSV-1 DNA fragments obtained by *Xba*I (Fig. 4A) or *Hin*dIII (Fig. 4B) digestion were retained at 0.25 to 0.5 copies per haploid cell genome equivalent. However, some reduction in retained HSV-1 DNA sequences was apparent between day 3 of the treatment interval and day 8 after inhibitor removal and increase in temperature. No further reduction in HSV-1 DNA sequences was noted after extended incubation of HSV-1-infected, inhibitor-treated cultures at 40.5°C (data not shown). Under analogous experimental conditions, no hybridization to DNA isolated from latently infected HEL-F cells was detected with ^{32}P-radiolabeled pBR322 or pACYC plasmid DNAs as probe (data not shown).

Detection of HSV-1 junction and terminal DNA sequences during virus latency in HEL-F cells. After demonstrating the maintenance of most, probably all, unique HSV-1 DNA sequences in latently infected HEL-F cells, it was of interest to examine the form of these HSV-1 sequences. As one approach to determine whether the HSV-1 genome was present in an integrated or nonintegrated form, we used the HSV-1 *Bam*HI S-P (K) DNA fragment as a probe (Fig. 1) to detect the authentic HSV-1 junction and terminal DNA fragments in latently infected HEL-F cells (Fig. 5). Again, total cellular DNA was isolated from HSV-1-infected, inhibitor-treated HEL-F cells 1, 3, and 7 days after infection and after incubation at 40.5°C for 4 and 8 days after inhibitor removal. The DNAs were digested with *Xba*I (Fig. 5A) or *Bam*HI (Fig. 5B), subjected to electrophoresis, blot transferred, and hybridized with the ^{32}P-radiolabeled *Bam*HI S-P (K) DNA fragment, and autoradiography was performed. There was no detectable alteration in the size of the HSV-1 terminal DNA fragments obtained by digestion of latently infected HEL-F cell DNA with *Xba*I or *Bam*HI as compared with the authentic terminal DNA fragments generated by digestion of the HSV-1 virion DNA in the reconstruction experiment (Fig. 5). There was also no detectable alteration in the size or molarity of HSV-1 terminal DNA fragments generated by *Hin*dIII digestion (data not shown). The fact that there were no detectable alterations in the molecular weights of HSV-1 terminal DNA fragments obtained by digestion of DNA from latently infected HEL-F cells with three different restriction endonucleases suggests that the predominant form of HSV-1 DNA retained is nonintegrated. In addition, densitometer scans of autoradiographs of DNA isolated from latently infected HEL-F cells, digested with *Xba*I or *Bam*HI, and hybridized with the *Bam*HI S-P (K) DNA fragment demonstrated that the molar ratios of the junction S-P (K) to the terminal S or P DNA fragment resembled those obtained by digestion of HSV-1 virion DNA in the reconstruction experiment (data not shown). These

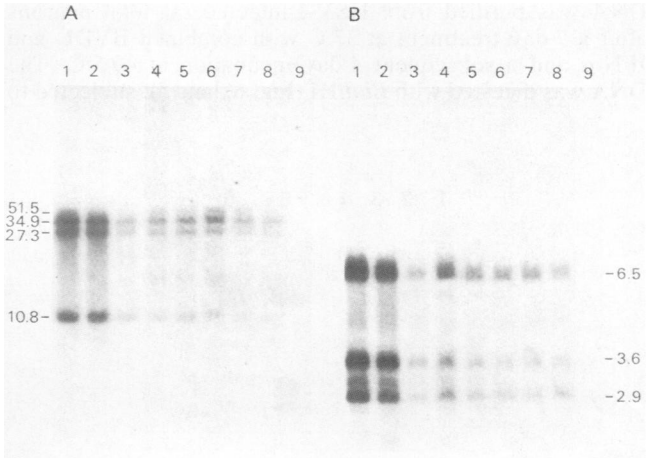

FIG. 5. Detection of HSV-1 junction and terminal DNA fragments in latently infected HEL-F cells by blot hybridization. DNA was isolated from HSV-1-infected HEL-F cells after a 1-, 3-, or 7-day treatment with combined BVDU and IFN-α (A and B, lanes 4, 5, and 6, respectively), 4 or 8 days subsequent to inhibitor removal and increase in incubation temperature from 37 to 40.5°C (A and B, lanes 7 and 8, respectively), or from HEL-F cells that were mock infected, inhibitor treated, and incubated at 40.5°C for 8 days after inhibitor removal (A and B, lane 9). The DNAs were digested with *Xba*I (A) or *Bam*HI (B), subjected to electrophoresis, blot transferred, and hybridized with a ^{32}P-radiolabeled *Bam*HI S-P (K) DNA fragment partially purified from the vector DNA; and autoradiography was performed (3-day autoradiograph exposure). The reconstruction experiment was performed with 2, 1, or 0.5 copies of purified HSV-1 virion DNA per haploid cell genome equivalent (A and B, lanes 1 to 3, respectively). A total of 20 μg of an HSV-1 and HEL-F cell DNA reconstruction was then digested with *Xba*I (A) or *Bam*HI (B) and analyzed as described for panels A and B, lanes 4 to 9. Sizes (kb) of the DNA fragments are indicated at the left of panel A and at the right of panel B.

data suggest that the nonintegrated HSV-1 genome was predominantly a nonconcatameric, linear form rather than a circular or concatameric form. As determined by comparison with the reconstruction analysis, the HSV-1 junction and terminal DNA fragments were retained in the latently infected HEL-F cell population at approximately 0.5 copies per haploid cell genome equivalent (Fig. 5): a result consistent with the retention of HSV-1 DNA fragments from the unique segments of the genome. Densitometric analyses of autoradiographs of DNA isolated from latently infected HEL-F cells, digested with *Hin*dIII, and hybridized with the *Bam*HI S-P (K) DNA fragment demonstrated the approximate equal molar presence of all four HSV-1 *Hin*dIII DNA fragments encompassing the junction between the HSV-1 long and short segments of the genome (data not shown). These results suggest the retention of each of the four isomers of the HSV-1 genome in latently infected HEL-F cells.

Detection of HSV-1 DNA sequences during virus latency in isolated rat fetal neurons. Although infectious center analysis of latently infected rat fetal neurons maintained at 40.5°C after removal of BVDU and IFN-α has not been performed, HSV-1-specific immunofluorescence was apparent in approximately 50% of the neurons 3 days after inhibitor removal and incubation at 37°C (46). Analogous experimentation with latently infected HEL-F cells has demonstrated that approximately 10% of the cell population expresses HSV-1 antigens early after reactivation (data not shown). To examine the latently infected rat fetal neurons for HSV-1 DNA sequences at a time when no infectious virus was detectable, blot hybridization was performed. Total cell DNA was purified from HSV-1-infected rat fetal neurons after a 7-day treatment at 37°C with combined BVDU and IFN-α and a subsequent 4-day incubation at 40.5°C. The DNA was digested with *Bam*HI (Fig. 6, lane 5), subjected to electrophoresis, blot transferred, and hybridized with the ^{32}P-radiolabeled *Bam*HI S-P (K) DNA fragment (Fig. 1), and autoradiography was performed (Fig. 6). As demonstrated with latently infected HEL-F cell DNA digested with *Bam*HI and hybridized with the *Bam*HI S-P (K) DNA fragment, the HSV-1 terminal DNA fragments detected in latently infected neuron DNA (Fig. 6, lane 5) were similar in size to those generated by digestion of HSV-1 virion DNA with *Bam*HI, as shown in the reconstruction analysis (Fig. 6, lanes 1 to 3). Again, by comparison with the reconstruction analysis, the HSV-1 junction S-P (K) and terminal S and P DNA fragments were retained in the latently infected rat fetal neurons at 8 to 10 copies per haploid cell genome equivalent. When DNA isolated from inhibitor-treated, mock-infected neurons maintained at 40.5°C for 4 days after inhibitor removal was analyzed by analogous blot hybridization conditions, no detectable hybridization was observed (data not shown).

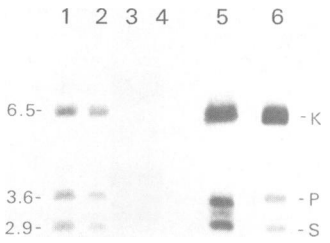

FIG. 6. Detection of HSV-1 junction and terminal DNA fragments in latently infected rat fetal neurons by blot hybridization. DNA was isolated from HSV-1-infected rat fetal neurons after a 7-day treatment with combined BVDU and IFN-α and a subsequent 4-day incubation after inhibitor removal and increase in temperature from 37 to 40.5°C, digested with *Bam*HI, subjected to electrophoresis, blot transferred, and hybridized with a ^{32}P-radiolabeled *Bam*HI S-P (K) DNA fragment partially purified from the vector DNA, and autoradiography was performed (lane 5). The reconstruction experiment was performed with 2, 1, 0.5, or 0 copies of purified HSV-1 virion DNA per haploid cell genome equivalent (lanes 1 to 4, respectively). A total of 20 μg of an HSV-1 and HEL-F cell DNA reconstruction was then digested with *Bam*HI and analyzed as described for lane 5. DNA (1.0 μg) was also isolated from HSV-1-infected rat fetal neurons 24 h postinfection (lane 6), digested with *Bam*HI, and analyzed as described for lane 5. Lanes 1 to 5, 3-day autoradiograph exposure; lane 6, 3-h autoradiograph exposure. Sizes (kb) of the DNA fragments are indicated at the left of panel A and at the right of panel B.

To compare molar ratios of the HSV-1 junction with terminal DNA fragments, densitometric analysis was performed. A densitometric scan of an autoradiographic exposure of DNA from latently infected rat fetal neuron DNA digested with *Bam*HI and hybridized with the *Bam*HI S-P (K) DNA fragment (Fig. 7B) demonstrated that the molar ratios of the junction S-P (K) to the terminal S or P DNA fragment resembled those obtained by digestion of HSV-1 virion DNA in the reconstruction experiment (Fig. 7A). Digestion of HSV-1 DNA obtained from virions isolated from productively infected rat fetal neurons rather than HEL-F cells resulted in a similar junction-to-terminal DNA fragment molarity ratio (data not shown). This ratio contrasts with that obtained with DNA isolated from productively infected rat fetal neurons 24 h after infection, digested with *Bam*HI, and hybridized with the *Bam*HI S-P (K) DNA fragment (Fig. 7C); the junction DNA fragment was markedly increased in molarity compared with either of the two terminal S or P DNA fragments, presumably due to the presence of replicating concatameric DNA. Autoradiographic exposures for relative quantitation of the junction and terminal DNA fragments were chosen for densitometric analyses so as to equalize the exposure of the terminal DNA fragments and with consideration given to the linearity of film response. These results, like those obtained by analysis of the HSV-1 genome in latently infected HEL-F cells, suggest that the predominant form of the virus genome in latently infected rat fetal neurons was also a nonintegrated, nonconcatameric, linear form. However, a quantitative comparison suggests that there was a greater retention of the HSV-1 DNA sequences in the latently infected rat fetal neurons as opposed to the latently infected HEL-F cells.

To analyze whether HSV-1 unique DNA sequences were retained to the same extent in latently infected rat fetal neurons as the junction and terminal DNA fragments, DNA was isolated from latently infected neurons after a 4-day incubation at 40.5°C, digested with *Bam*HI, subjected to electrophoresis, and blot transferred. The DNA blots were hybridized with the ^{32}P-radiolabeled genomic proportion mixture of recombinant plasmids (Fig. 1) containing the unique HSV DNA sequences (Fig. 8A), the *Eco*RI L DNA fragment (Fig. 1) from the middle of the long unique segment (Fig. 8B), or the *Eco*RI H DNA fragment (Fig. 1) encompassing the entire short unique segment (Fig. 8C). As demonstrated in each hybridization, most unique HSV-1 DNA sequences were maintained in the latently infected rat fetal neuron population at approximately four copies per haploid genome equivalent. This result was quantitatively

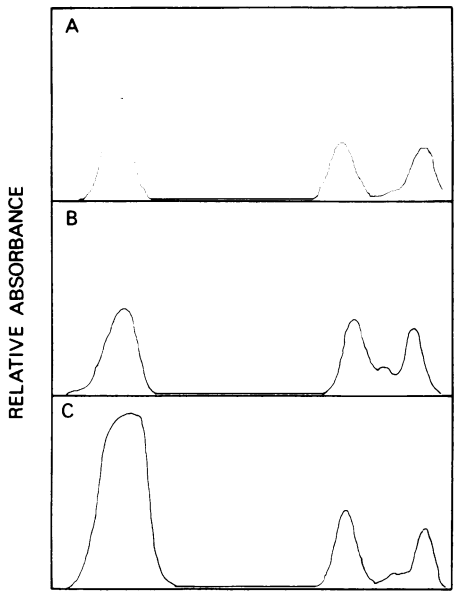

FIG. 7. Comparison of HSV-1 junction-to-terminal DNA fragment molarity ratios by densitometric analysis. (A) HSV-1 virion and HEL-F cell reconstruction DNA (2.0 copies of HSV-1 genome per haploid cell genome equivalent) was digested with *Bam*HI, subjected to electrophoresis, blot transferred, and hybridized with a partially purified ^{32}P-radiolabeled *Bam*HI S-P (K) DNA fragment; autoradiography was performed, and a 3-day exposure was analyzed by densitometry. (B) DNA was isolated from HSV-1-infected rat fetal neurons after a 7-day treatment with BVDU and IFN-α and a subsequent 4-day incubation after removal of inhibitor and increase in temperature from 37 to 40.5°C and was analyzed as described for (A) (2-day exposure of autoradiograph). (C) DNA (1.0 μg) was isolated from HSV-1-infected rat fetal neurons 24 h postinfection and analyzed as described for (A) (3-h exposure of autoradiograph). From left to right in each panel, the peaks represent the HSV-1 *Bam*HI K, P, and S DNA fragments, respectively. Autoradiographic exposures used for densitometric analysis were linear with respect to the film response to radioactivity.

consistent with the retention of the HSV-1 junction and terminal DNA fragments but not unexpected since synthesis of HSV-1 antigens has been detected in a large proportion of the infected neuron population early after inhibitor removal and maintenance at 37°C.

DISCUSSION

We have previously described the design and partial biological characterization of in vitro HSV latency models with HEL-F cells and rat fetal neurons as the host cell types (45, 46). The in vitro latent infections have been established and maintained by combined BVDU and IFN-α treatment and subsequent increased incubation temperature. Predictable reactivation of virus synthesis has been attained by reducing incubation temperature.

We now report for the first time the molecular characterization of the HSV genome during in vitro virus latency in a model system in which virus reactivation can be attained. The results suggest that the predominant form of the HSV-1 genome during in vitro virus latency in HEL-F cells or rat fetal neurons was nonintegrated, unit-length, linear DNA. However, this does not preclude the possibility of minor undetectable, yet important, HSV DNA species being retained in an integrated or nonintegrated plasmid or concatameric form.

Although substantial altered HSV-1 terminal DNA fragment species were not detected in latently infected HEL-F cell DNA, the possibility of integration of the HSV genome during in vitro virus latency cannot be completely eliminated. However, it is likely that the use of three different restriction endonucleases (*Xba*I, *Bam*HI, and *Hin*dIII) would result in detection of a substantially altered HSV-1 terminal DNA fragment species, suggesting HEL-F cell DNA sequences covalently joined to HSV-1 terminal DNA fragments. Any small alteration in terminal DNA fragment size may also be attributed to reiteration of the "a" sequence in the terminal repeat region since this phenomenon has been documented in lytic in vitro HSV infection (28). In addition, because homology has been reported between segments of HSV inverted repeat DNA sequences and repetitive sequences in mammalian cell DNA (27, 31), the presence of minor species of apparent terminal HSV DNA fragments would be difficult to interpret.

Quantitative estimation by comparison to reconstruction experiments suggested retention of approximately 0.5 and 2 copies of the HSV genome per haploid HEL-F cell and rat fetal neuron genome equivalent, respectively. However, it is not possible by this technique to ascertain the distribution of the HSV-1 genome in either latently infected cell population. Preliminary DNA-RNA in situ hybridization studies suggest that perhaps 30% of latently infected rat fetal neurons maintained at 40.5°C contained virus-specific RNA. These data suggest that the HSV-1 genome was present in more than just a few select rat fetal neurons. Since, on an average,

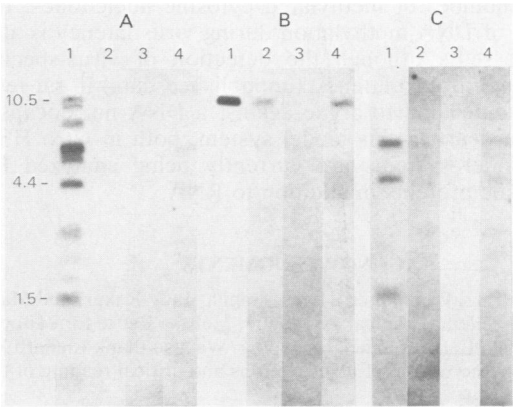

FIG. 8. Detection of HSV-1 unique DNA sequences in latently infected rat fetal neurons by blot hybridization. DNA was isolated from HSV-1-infected rat fetal neurons after a 7-day treatment with combined BVDU and IFN-α and a subsequent 4-day incubation after inhibitor removal and increase in temperature from 37 to 40.5°C, digested with *Bam*HI, subjected to electrophoresis, and blot transferred (A, B, and C, lanes 4). The reconstruction experiment was performed with 2 and 0 copies of purified HSV-1 virion DNA per haploid cell genome equivalent (A, B, and C, lanes 2 and 3, respectively). A total of 20 μg of an HSV-1 and HEL-F cell DNA reconstruction was then digested with *Bam*HI and analyzed as described for lane 4 in each panel. DNA (1.0 μg) was also isolated from HSV-1-infected rat fetal neurons 24 h postinfection, digested with *Bam*HI, and analyzed as described for lane 1 in A, B, and C. Blot-transferred DNAs were hybridized with a ^{32}P-radiolabeled genomic proportion mixture of recombinant plasmids containing HSV-1 unique DNA sequence inserts (A), *Eco*RI L DNA fragment (B), or *Eco*RI H DNA fragment (C) (3-day exposure of autoradiograph). Sizes (kb) of the DNA fragments are indicated at the left.

greater than two copies of the HSV-1 genome per haploid cell genome equivalent were retained in latently infected rat fetal neurons, it is also likely that many cells contained more than one HSV-1 genome equivalent. The fraction of the HSV genomic content that may be defective during in vitro virus latency in either cell type is not yet clear.

Recent DNA blot hybridization studies by Rock and Fraser have demonstrated that most, if not all, of the HSV-1 genome is present in central nervous tissue of latently infected mice (35). In addition, although the HSV-1 junction DNA fragment could be detected, authentic HSV-1 terminal DNA fragments could not be detected in total mouse brain or pooled trigeminal ganglia DNA. However, in contrast to this result, Fraser and co-workers previously detected HSV-1 terminal as well as junction DNA fragments of authentic size in human brain DNA (11), suggesting that the HSV-1 genome is in a nonintegrated, unit-length, linear form in at least a portion of the specimens examined. The predominant nonintegrated, unit-length, linear form of the HSV-1 genome retained during virus latency in the HEL-F cell or rat fetal neuron in vitro model system resembled the form of the HSV-1 genome detected in several total brain DNA specimens by Fraser and co-workers (11).

Present studies are directed at a delineation of the intracellular location of the HSV-1 genome during in vitro virus latency. Since the HSV-1 genome appears to be in a nonintegrated, linear form, as opposed to a plasmid or a replicating concatameric form (15, 23), the possibility of the genome being maintained in a nucleosomal or nucleocapsid structure is also being examined. Furthermore, since Youssoufian and co-workers (49) have demonstrated that virus DNA in HSV-1 latently infected human lymphoblastoid cells contained an increased number of methylated cytosine nucleotides, the possibility of DNA methylation during virus latency is also being assessed. Although the detection of virus-specific RNA during in vitro latency (unpublished data) in rat fetal neurons would tend to argue against a DNA nucleocapsid structure at least in this model system, both in vitro HSV latency model systems are currently being analyzed for virus-specific proteins in addition to RNA.

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