Oncogenic Transformation by Equine Herpesviruses

II. Coestabulation of Persistent Infection and Oncogenic Transformation of Hamster Embryo Cells by Equine Herpesvirus Type 1 Preparations Enriched for Defective Interfering Particles

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Infection of permissive hamster embryo cells with virus preparations enriched for defective interfering (DI) particles of equine herpesvirus type 1 (EHV-1) resulted in persistent infection and oncogenic transformation. Six cell lines, designated DI-5 to -10, exhibited biological properties (immortality, increased saturation density, growth in soft agar, etc.) inherent to transformed cells, but 2 to 18% of the total cells in these cell lines were shown to release virus as judged by electron microscope studies and infectious center assays. The released virus was shown to be standard EHV-1 and not to contain DI particles as determined by density measurements of the viral DNA in the analytical ultracentrifuge and by interference assays using the released virus. Tumorigenicity studies revealed that inoculation of these persistently infected cells into newborn LSH inbred hamsters resulted in a lethal, fulminating hepatitis, whereas inoculation into older immunocompetent hamsters (+4 weeks) led to the development of metastatic fibrous sarcomas. Tumor cell lines (DI-5T to -10T) established from these sarcomas were shown to be transplantable and virus nonproducers. Hybridization analyses of cellular DNAs from DI transformed and tumor cell lines using 32P-labeled genomic EHV-1 DNA as probes indicated that the whole virus genome was detectable in multiple copies (23 to 45) in the transformed cells and that DNA sequences representing only 43.5 to 56.6% of the virus genome were present in amounts of 2 to 4 copies per cell in the DI tumor cells. Expression of these viral DNA sequences was demonstrated by the detection of virus-neutralizing antibodies, 50% neutralizing dose titer ranging from 1:50 to 1:1,000, in the sera of animals inoculated with either the virus-producing transformed cells or the virus-nonproducing tumor cells. Further, EHV-1-specific proteins were detected in the membrane and the perinuclear region of both DI transformed and tumor cells by indirect immunofluorescent assays using antisera against EHV-1 structural antigens, EHV-1 nonstructural antigens, or preparations of EHV-1 DI particles. The roles of DI particles in mediating persistent infection and cellular transformation are discussed.

The interaction of oncogenic DNA viruses with animal cells may precipitate the establishment of one or more cellular states that include cytocidal infection, latency, persistent infection, and cellular transformation. Although the coexistence of these latter two types of virus-cell interactions has been noted for many years, the underlying mechanisms accounting for their mutualistic relationship have yet to be elucidated. The accompaniment of cellular transformation by persistent infection was observed initially with papovavirus systems, in which a small percentage of semipermissive cells that survived primary infection with simian virus 40 or polyoma virus underwent transformation and commenced virus production after extended periods in culture (6, 11, 17, 34). The viral DNA in these cells has been found to persist in an integrated state or as free viral DNA arising from excision of genomes integrated into host cell chromosomes (4, 36). Recently, Takemoto and co-workers (35) have reported the establishment of persistent infection in human fetal brain cells transformed by BK virus; however, these cells differed from polyoma and simian virus 40 persistently infected and transformed cells in that only a small fraction of the BK virus-infected human fetal brain cells expressed T antigens. These BK cells lacked integrated BK virus genomes but contained nonintegrated episomal viral DNA, which may explain the different expression of viral genes in these cells. The lymphotropic her-
pesivirus such as Epstein-Barr virus have been shown to be able to mediate persistent infection and malignant transformation concurrently in the cultures of B lymphocytes (8). The viral genome in these cells has been shown to exist in a nonintegrated episomal (8, 18) and/or an integrated (1, 2) state.

Viral entities that may be responsible for initiating and maintaining persistent infection and oncogenic transformation, separately or together, may be defective interfering (DI) particles. Holland and others (14, 15) have suggested that DI particles of vesicular stomatitis virus (VSV) and other viruses play a prominent role in the initiation and perpetuation of persistent infection, which may be the end result of the capacity of DI particles to inhibit replication of the standard virus. In addition, the defective viral genotype of herpesvirus has been associated by Schroder et al. (33) with herpesvirus transformation, since the presence of defective virus in stocks of herpesvirus type 1 strain Angelotti was shown to increase the survival rates of HSV-1-infected cells during abortive infection at 42°C. Dunn and colleagues (7) reported that HSV-1 persistently infected BHK/3T3 cells were able to grow in agarose, but these workers did not ascertain whether these cells were stably transformed or oncogenic.

It has been our goal to understand the roles of DI particles in different types of virus-cell interactions by investigating the responses elicited by infection of primary mammalian cultures or established cell lines with DI particles of equine herpesviruses (EHV). These viruses provide suitable models for such studies because (i) EHV type 1 (EHV-1) is able to oncogenically transform permissive hamster embryo cells as a result of abortive infection with UV-irradiated EHV-1 (30), (ii) DI particles of EHV-1 may be generated readily in vivo (3) and in vitro (12, 13), and (iii) EHV form a triad of distinct but related viruses that exhibit diversity in their biological potentials (26). In this report, we describe the establishment of persistently infected and oncogenically transformed hamster embryo cells by infection with virus preparations enriched for EHV-1 DI particles. In addition, evidence for the presence, persistence, and expression of viral genomes in these cells is presented. The role of DI particles of this herpesvirus in initiating and maintaining persistent infection of permissive cells is discussed in light of recent findings concerning the mechanisms of persistent infection with other viruses.

**MATERIALS AND METHODS**

Cells. The L-M strain of mouse fibroblasts used for in vitro propagation of EHV-1 was grown in YLPP suspension medium (yeast extract, lactalbumin hydrolysate, and peptone) containing 3% fetal bovine serum (FBS; Flow Laboratories, Rockville, Md.) as described previously (25). Primary hamster embryo fibroblast (HEF) cultures were established from hamster embryos (LSH inbred strain; Charles River Lakeview, Wilmington, Mass.) and were grown in Eagle minimal essential medium (EMEM; Microbiological Associates, Walkerville, Md.) as described previously (30). Secondary HEF cultures were employed to propagate EHV-1 used to immunize animals for antibody production. EHV-1 DI transformed cells, designated DI-5 to DI-10, and DI tumor cell lines, designated DI-5T to DI-10T, were grown in monolayer cultures in EMEM supplemented with 5% FBS. Tumor cell lines were established from sarcoma tissues as described previously (30).

Viruses. EHV-1, Kentucky A strain, was propagated and quantitated by methods described previously (25). To ensure that these virus preparations contained only a single species of viral DNA with a buoyant density of 1.716 g/cm³, viral DNA released from purified virus was analyzed in the model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, Calif.).

EHV-1 DI particles were generated in L-M cells by repeated high-multiplicity, serial, undiluted passage as described previously (12, 13). Portions of the supernatant virus of selected DI passages were used as virus inoculum for transformation experiments and as antigen for production of antiserum. The amount of DI particles in each passage was quantitated by measuring the amount of high-density (1.724 g/cm³) viral DNA present in purified supernatant particles by CsCl isopycnic banding in the analytical ultracentrifuge as described previously (12).

VSV, Indiana strain, was a gift from M. Hunt and was used in superinfection experiments. Virus was grown in L-M cells and quantitated by plaque assay (15).

**Assays for transformed cells properties.** Various passages (15 to 75) of DI transformed cell lines were assayed for several biological transformation properties, including colony formation in soft agar, growth in low concentrations of serum, resistance to superinfection with the homologous virus, altered growth properties, and altered cellular morphology, by methods described by Robinson et al. (30).

**Tumorigenicity tests.** Control cells (HEF cells) or test cells (DI transformed and DI tumor cells) were harvested at various passages by trypsinization, rinsed twice with phosphate-buffered saline (PBS) and 10% FBS, resuspended in EMEM at cell concentrations ranging from 1 x 10⁵ to 5 x 10⁶ cells/ml, and inoculated subcutaneously (0.2 ml/animal) into newborn or immunocompetent (+4 weeks old) LSH syngeneic hamsters. Animals were monitored daily for symptoms of herpesvirus infection and for development of palpable tumors. The methods have been described previously (30) for the electron microscopy (EM) and histopathological examination of sections of vital organs and tumor tissues removed from these animals at various intervals postinoculation (p.i.).

**Tests for production and induction of virus from cells.** Supernatant medium and disrupted cell
lysates of DI transformed and tumor cells were assayed for the presence of EHV-1 viral particles by plaque method on permissive L-M cells and by EM as previously described (27). In addition, infectious center assays were performed to determine the number of virus-producing cells within a population of persistently infected DI transformed cultures as described previously (12). Briefly, 10^6 L-M cells were either mock infected with 50 ml of medium or infected with 20 or 50 ml of the supernatant from DI transformed cells (3.0 x 10^2 to 5.0 x 10^3 cells); this was designated as 0 h p.i. After incubation at 37°C for 1.5 h, the L-M cells were collected and rinsed (three times) with 50 ml of growth medium, challenged with standard EHV-1 at several multiplicity of infection (MOI) (0.1 to 5.0 PFU/cell), and incubated for 1.5 h at 37°C. After absorption of the standard virus, the cells were collected, rinsed (three times) with 50 ml of medium, and resuspended in 50 ml of fresh medium. At various times p.i., portions of the virus-cell suspension were removed, stored at -70°C, and assayed later for infectious virus by the plaque method (28). In some experiments, supernatant virus from a passage of EHV-1 DI particles was used as a positive control (12).

Analytical density gradient centrifugation. The methods for CsCl equilibrium density analysis were those described by Henry et al. (12). Viral DNA (2 μg/ml) that had been isolated from purified virus released by DI transformed cells or from purified standard EHV-1 virus was mixed with CsCl solution to a density of 1.7 g/ml and was sedimented by centrifugation in the model E ultracentrifuge at 44,770 rpm for 24 h at 22°C in an AnF rotor. Densityrometer tracings of DNA were made with a Joyce-Loebl chromoscan as previously described (12).

Isolation and radiolabeling of viral DNA. EHV-1 virions released from infected L-M cells were purified by rate velocity centrifugation in dextran-10 gradients of polyethylene glycol 6000 precipitates of extracellular virus as previously described (28). EHV-1 DNA was isolated from purified virions by treatment with sodium dodeyl sulfate (final concentration, 0.5%) and autodigested pronase (1 mg/ml) for 3 h at 37°C. After two cycles of extraction with TE buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA) saturated-pentan at room temperature and one cycle of extraction with chloroform-n-butanol (24:1), the viral DNA was precipitated with 95% ethanol, redissolved in TE buffer, and banded in CsCl equilibrium gradients (12). Purified EHV-1 DNA was radiolabeled in vitro with [α-32P]dTTP and [α-32P]dATP (New England Nuclear, Boston, Mass.) by nick translation with Escherichia coli DNA polymerase I (Boehringer Mannheim, Indianapolis, Ind.) as described by Maniatis et al. (19). The specific activity of the [32P]-labeled EHV-1 DNA ranged from 0.9 x 10^6 to 2.5 x 10^6 cpm per μg of DNA.

Isolation of cellular DNA for hybridization. DNA from normal HEF cells, DI transformed cells, and DI tumor cells was prepared for hybridization essentially as described by Robinson et al. (30). It should be noted that monolayers of DI transformed cells, which were virus producers, were washed with several changes of a 0.01 M EDTA-0.25% trypsin solution before harvesting to remove any residual virus absorbed to the cell surface.

Hybridization of viral DNA with DI transformed and tumor cell DNAs. The detection and quantitation of EHV-1 DNA sequences in DI transformed cells (virus-producing cells) were determined by DNA-DNA reassociation kinetic studies as described previously (9). EHV-1 [32P]DNA labeled by nick translation (2.0 x 10^6 cpm/μg of DNA) and cold EHV-1 DNA were sonicated to a fragment size of 300 to 600 nucleotides. Then 2.7 x 10^6 cpm (0.135 μg) of EHV-1 [32P]DNA was allowed to reanneal at 63°C in the presence of DI transformed cell DNAs (1 mg) in 1 ml of 1X hybridization buffer (0.1 M Tris-hydrochloride, pH 8.1, 0.025 M EDTA, and 1 M NaCl). The ratio of EHV-1 DNA to cellular DNA was 6.25 genome equivalents per cell. Control DNAs included mixtures of calf thymus DNA (1 mg) either with 2.7 x 10^6 cpm of EHV-1 [32P]DNA (6.25 genomes/cell) or with 2.7 x 10^6 cpm of EHV-1 [32P]DNA and 0.311 μg of unlabeled EHV-1 DNA (20.6 genomes/cell). Hybrid DNA mixtures were dispersed into 100-μl portions, sealed in capillary pipettes, heat denatured at 110°C for 15 min, and allowed to reassocitate for time intervals indicated in Fig. 9. At given times, samples were removed and frozen at -70°C until all reactions were completed. Single-stranded and double-stranded DNAs were separated by hydroxyapatite chromatography (12), and trichloroacetic acid-precipitable counts were measured by liquid scintillation spectroscopy. Reassociation kinetics of randomly sheared viral DNA were determined by the equation, C/Co = 1/1 + KCot, where C0 and C are acid precipitable counts of unla
diated DNA at time t = 0 and t, respectively, and K is the second-order reaction rate constant (23). The methods and rationale for analysis of viral DNA sequences in DI tumor cells were essentially those described previously (30). Briefly, 2 x 10^6 cpm of EHV-1 [32P]DNA (0.001 μg) was mixed with DI tumor DNAs (5 mg) in 1 ml of 1X hybridization buffer and 20% (vol/vol) formamide (Matheson, Coleman, and Bell, Elk Grove, Ill.). The molar ratio of cellular DNA to viral probe DNA was 50:1, which allows for the detection of approximately a single copy of a 2.8-kilobase segment of the EHV-1 genome. Control reaction mixtures consisted of 5 mg of calf thymus DNA with either 2 x 10^6 cpm of EHV-1 [32P]DNA (0.001 μg) or 2 x 10^6 cpm of EHV-1 [32P]DNA and 0.05 μg of cold EHV-1 DNA. DNA reaction mixtures were prepared for reasscociation as outlined above and were allowed to reassocitate at 63°C for time intervals indicated in Fig. 9. Unreassocitated DNA and reassocitated DNA were fractionated by hydroxyapatite chromatography as above. The percentage of viral genome represented in DI tumor cells and the number of copies...
per cell were quantitated by formulas of Fujinaga et al. (10).

**Viruses neutralization assays.** Indirect detection of viral antigens in DI transformed or tumor cells was made by assaying sera (complement inactivated at 56°C for 30 min) from tumor-bearing hamsters for anti-EHV-1 antibody by virus neutralization tests (5). Blood was collected (2 weeks after palpable tumor developed) by cardiac puncture of hamsters inoculated with DI transformed or tumor cells. Serial dilutions of control (animals inoculated with HEF cells) and tumor-bearing sera were made and reacted with 5,000 PFU of EHV-1 at 4°C for 30 min. Portions (0.1 ml) of serum-virus mixtures were assayed for residual virus by the plaque method of Perdue et al. (28).

**Antisera and immunofluorescence.** Detection of viral antigens on the surface membrane and in the cytoplasm of DI transformed and tumor cells was made by indirect immunofluorescence. Antisera were prepared in LSH inbred hamsters and New Zealand rabbits against the structural proteins of purified EHV-1 virions (30), purified virus from EHV-1 DI passages, or structural and nonstructural viral poly-peptides present in EHV-1-infected HEF cells. The latter antisera was prepared by inoculating animals with nuclear and cytoplasmic fractions of 24-h EHV-1-infected (MOI = 10 PFU/cell) HEF cells clarified after sonication by centrifugation at 33,000 x g for 90 min; protein in the clarified extracts was diluted to the appropriate protein concentration in PBS. In some experiments, antibodies to only EHV-1 nonstructural components were selected for and prepared by adsorption of antisera to EHV-1-infected HEF cells with 1.5 x 10^11 virus particles/ml under conditions described previously (30). Preparations of envelope proteins and immunization procedures were described previously (30).

Indirect immunofluorescence using the above antisera and fluorescein-conjugated anti-immunoglobulins was performed on methanol- or acetone-fixed DI transformed and tumor cells, as well as uninfected and EHV-1-infected HEF cells, as described previously (30). All antisera were absorbed routinely with intact and lysed hamster liver and spleen cells before use to remove Forbsman antigens and were complement inactivated by heating at 56°C for 30 min. Dilutions of all antisera were assayed for optimal immunofluorescence to EHV-1 antigens and for neutralizing activity.

**RESULTS**

Establishment of transformed and persistently infected cultures by EHV-1 DI particles. Primary cultures of permissive HEF cells (2 x 10^6 cells/25-cm² plastic flask) were infected with virus preparations enriched for DI particles of EHV-1 at an MOI equal to 50 PFU/cell or 650 total viral particles/cell, of which 65% of the viral particles were DI virus as judged by the content of defective DNA species (1.724 g/cm³). Further features of the in vitro generation, interference capacity, genome size and composition, and polypeptide composition of these EHV-1 DI virus preparations have been described (12, 13). After a 2-h period of virus adsorption, cultures were rinsed with several changes of medium, maintained at 37°C, and fed with fresh medium every 3rd day. Within 14 days of infection, the majority of the cells exhibited marked viral cytopathology, characterized by margination of the chromatin and intranuclear inclusions, and by 21 days p.i. greater than 90% of the cells were destroyed by viral cytolysis.

After 4 to 5 weeks of infection, numerous scattered areas of surviving cells in each flask were observed to develop into discrete foci or colonies. Individual foci were isolated, dispersed into a single-cell suspension by trypsinization, and transferred into new flasks. From these cultures six independent cell lines, designated DI-5 through DI-10, were established and subcultured thereafter at a 1:10 dilution every 3rd day. All of these cell lines have been passaged continuously in monolayer culture for over 2 years (>800 cell divisions) in our laboratory. In contrast, HEF cell cultures infected with standard EHV-1 (no DI particles) succumbed to complete cytolysis, and uninfected HEF cell cultures failed to form foci and survived only 10 to 12 passages in vitro (<27 cell divisions).

The morphological appearances of control HEF cells and DI cells were examined by light microscopy after differential staining of cultures (Fig. 1). Microscopic studies revealed that cultures of all DI cell lines exhibited two distinct cell types which were a predominant class of spindle-shaped, fibroblastic cells and a second, but minor, class of cells that had a rounded, cytopathic appearance similar to that exhibited by EHV-1-infected HEF cells early in productive infection. The morphology of the latter class of cells was accentuated by enlarged, darkly staining nuclei and suggested the possibility of virus production in these cells. Together these two cell types formed an altered-network pattern as compared with the uniform fibroblastic monolayers of HEF cell cultures (Fig. 1). The ratio of the fibroblastic to rounded cell types varied from 5:1 to 9:1 for all six DI cell lines, but the ratio of these cell types remained constant throughout the passage of each cell line. The DI cells grew to several layers of thickness in culture and formed colonies on solid substrates, thus demonstrating a loss of contact inhibition.

**Transformation properties of EHV-1 DI cell lines.** Experiments examining the growth and biological properties of EHV-1 DI cells were carried out to determine whether these cells had undergone the transformation event. Those properties chosen to discern morphological transformation of these cells included indefinite passage in culture (immortality), saturation den-
sity, generation time, serum dependency, growth in soft agar, and resistance to superinfection with EHV-1 (Table 1).

The ability of the DI cells to be passaged for greater than 800 cell divisions under the subculturing conditions described above offered evidence that these cells had undergone the immortality event to become permanent cell lines. Under optimal culturing conditions, the DI cells grew to saturation densities of 1.3- to 1.6-fold greater than control cells, and their doubling time was 23 to 34% less than that of uninfected HEF cells. The DI cell lines, unlike uninfected HEF cells, were able to grow to maximal saturation densities at equal rates in growth medium supplemented with either 0.5 or 5% serum. All six DI cell lines, in contrast to HEF cells, formed colonies in soft agar at very high efficiencies (60 to 75%) and thus exhibited loss of both contact inhibition and anchorage dependency. The above growth properties of the DI cell lines provided a firm basis for classifying these cells as morphological transformants.

As a preliminary means of determining whether EHV-1 mediated this cellular event, superinfection experiments with EHV-1, the homologous virus, or VSV, a heterologous virus, were conducted on the DI cells at several MOI. In the case of the virus-nonproducing HEF cells transformed by UV-irradiated EHV-1 (30), resistance to superinfection was monitored readily by EM and by the demonstration that these cells failed to support EHV-1 replication. However, in the DI cell system, determination of resistance to superinfection with exogenous EHV-1 was complicated by the spontaneous release of virus from these cells. Therefore, it was necessary to titer virus in the supernatants from DI cells before challenge with superinfecting virus and to provide a baseline of virus in this system. DI cells were resistant to superinfection with EHV-1 (Table 1), since no significant increase in virus titers was detected at 24 or 48 h p.i. and infection with exogenous EHV-1 at high MOI (50 PFU/cell) did not cause increased cytopathology or altered cell growth. In contrast, infection with VSV resulted not only in marked cytopathology, but also in production
of high titer of this heterologous virus in all the DI cell lines. Additional experiments revealed that these cells supported the replication of Mengo virus (data not shown). These data considered with the above growth properties of DI cells suggested that these cells had been transformed by EHV-1.

**Characterization of viral particles released from DI cells.** To determine whether the DI transformed cells contained and released viral particles, several approaches were undertaken to detect, quantitate, and characterize released virus (standard or defective genotypes) and to enumerate the percentage of virus-producing cells, if any, in these transformed cell lines. First, to detect viral particles within the ultrastructural framework of the two cell types, detailed EM analyses of the DI cells and their extracellular fluids were made (Table 2 and Fig. 2). EHV-1 virions were discerned in all six DI transformed cell lines, and all three nucleocapsid species (H, I, and L; 27) were found in the nucleoplasm of these cells (Fig. 2). From these EM studies, it was demonstrated that 2 to 7% of the total cell population in the DI transformed cultures contained viral particles (Table 2) and that viral particles were limited to the rounded, cytopathic cell type.

Dilutions of all DI cells were cultivated with permissive L-M cells in infectious center assays to determine the number of cells that produced infectious virus. Results from these assays indicated that all six transformed cell lines contained a subset of cells ranging from 3 to 20% of the total cell population that produced infectious virus (Table 2). Therefore, these data substantiated the EM results showing that only a small percentage of the total cell population in these cultures were active virus producers. The finding that higher values were obtained by the infectious center assays than in EM studies was expected due to the release of small amounts of virus after the washing cycle.

Titters of infectious virus released from the DI transformed cells ranged from $1 \times 10^3$ to $2 \times 10^6$ PFU/ml. The ratio of virus-producing to virus-nonproducing cells and the titers of released virus from DI transformed cells did not vary significantly during passage of DI cells. Virus production was not stimulated by various means of induction, such as UrdR treatment, growth at various temperatures, or cocultivation with permissive cells. From these data, it was concluded that the morphological transformation of the DI cells was accompanied by persistent infection.

To characterize the type (standard or defective) of virus released from the DI transformed cultures, it was necessary to conduct interference assays of released virus and density analyses of DNA in these virus particles. In the interference assays, L-M cells were mock infected or infected with portions of supernatant from confluent monolayers of DI cells. After an absorption period of 1.5 h, the L-M cells were harvested, rinsed with several changes of growth medium, and infected with standard EHV-1. The infection proceeded for an additional 24 h, and the capacity of the released virus to interfere with the expression of monolayers of DI cells.
Table 2. Virus production and induction in DI transformed and DI tumor cells

<table>
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<tr>
<th>Cell line</th>
<th>Supernatant virus yield (PFU/ml)</th>
<th>% of cells Persistently Infected</th>
<th>Infectious center assay</th>
<th>EM</th>
<th>Cocultivation with LM cells (PFU/ml)</th>
<th>Temp induction (PFU/ml)</th>
<th>IUdR (PFU/ml)</th>
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* Supernatant media from cells were assayed 24 h after subculturing for infectious EHV-1 by plaque assay as described in Materials and Methods.
* Conducted as described in Materials and Methods.
* Cells were harvested at various times, fixed with 2% glutaraldehyde, postfixed with 1% osmium tetroxide, and embedded in DER 222-732 resin. Thin sections were prepared and stained with uranyl acetate followed by lead citrate as described previously (27). Numerous sections representing hundreds of cells were examined in an RCA-EMU 3G electron microscope for subviral particles, virions, and morphological features of virus replication.
* Normal, transformed, or tumor cell cultures (10^6 cells) were washed (three times) in culture medium and seeded into 25-cm² flasks together with LM cells (2.0 × 10^6 cells) permissive for EHV-1. Cultures were incubated at 37°C and monitored frequently for EHV-1-specific cytopathic effect. Supernatants were removed at 48 h intervals and assayed for infectious virus.
* DI and DI tumor cell cultures were incubated at 31, 39, and 41°C for 24 h, and the amount of infectious virus was assayed.
* Cells (2.5 × 10^6) were treated for various time periods (ranging from 2 to 48 h) with either 25 or 50 µg of IUdR per ml followed by overlay with fresh medium. Supernatants and disrupted cells were assayed separately 24 h posttreatment for infectious EHV-1 by plaque assay and by EM as described in footnote c.
* Refers to all six DI tumor cell lines (DI-5T to DI-10T).
* Cells (10^6 in 1 ml) were disrupted by freeze-thawing and sonication and were assayed for infectious virus.

Results of these interference assays indicated that the released viral particles from all DI cell lines tested did not possess interfering activity (Fig. 3).

The DNA of the virus released from the DI cells was shown to be comprised of only standard EHV-1 DNA ($\rho = 1.716$ g/cm³) as determined by density analyses in an analytical ultracentrifuge. Scan tracings of DNA from standard EHV-1 virions and from purified preparations of virus released from DI cells are depicted in Fig. 4. The standard EHV-1 DNA banded as a single peak with a density of 1.716 g/cm³. The DNA of the virus released from various passages of DI-5 and DI-7 cells cosedimented with standard virus DNA. The findings that the released viral particles were infectious for permissive cells, lacked interference activity, and possessed standard EHV-1 DNA supported the conclusion that the DI transformed cultures did not produce DI particles. Thus, production of DI virus particles does not appear to be required for maintenance of the persistently infected state displayed by these cultures.

Oncogenicity tests of DI transformed cells. To determine whether the virus-producing DI transformed cells possessed oncogenic potential, DI cells at various passage levels were inoculated subcutaneously into newborn and immunocompetent (+4 weeks old) LSH syngeneic hamsters. The results of these experiments indicated a differential host response that was dependent upon the immunological maturity of the animal (Table 3). Newborn hamsters succumbed within 36 h to a fulminating viral hepatitis which is a pathognomonic feature of the EHV-1 in vivo model (24). However, older animals with mature immune systems developed palpable tumors within 6 to 8 weeks p.i., and all six DI transformed cell lines were oncogenic when inoculated in amounts of $1 \times 10^5$ to $5 \times 10^6$ cells in a single injection. All tumors were invasive and metastasized to major organ systems such as lung, kidney, and central nervous system.

Sections of all tumors and vital organs from animals inoculated with DI transformed cells were fixed and stained by a variety of standard procedures for histopathological examination (Fig. 1C). These studies revealed that all tumors were malignant sarcomas (subclass fibrous histiocytomas) and that the histopathology of these tumors was the same as that obtained in ham-
Fig. 2. Electron micrographs of DI transformed and persistently infected cells. Cell line and passage number are indicated. Extracellular virion, intranuclear capsid species, and virus particles at nuclear membrane are displayed. Bar = 200 nm.

Tumor cell lines were established from tumor tissues of animals inoculated with DI transformed cell lines and were designated DI-5T through DI-10T, according to the parent cell...
Fig. 3. Assay for interference capacity of virus released from DI transformed and persistently infected cell cultures. Assays were carried out as described in Materials and Methods. Mock infected at 0 h; infected with standard EHV-1 (MOI = 5) at 1.5 h p.i. (●). Infected with 20 ml of D-15 supernatant (0.38 PFU/cell; 164 particles/cell) at 0 h; infected with standard EHV-1 (MOI = 5) at 1.5 h (x). Infected with 20 ml of DI-5 cell supernatant at 0 h; infected with standard EHV-1 (MOI = 0.1) at 1.5 h (○). Infected with 20 ml of DI-7 cell supernatant at 0 h; infected with standard EHV-1 (MOI = 0.1) at 1.5 h (△). Infected with 50 ml of DI-8 cell supernatant at 0 h; infected with standard EHV-1 (MOI = 0.1) at 1.5 h (■). Infected with 50 ml of DI-10 cell supernatant at 0 h; infected with standard EHV-1 (MOI = 0.1) at 1.5 h (▲).

Fig. 4. Density analyses of viral DNA present in virus released from DI transformed and persistently infected cultures. Virus present in the supernatants of DI-5 and DI-7 cells was purified as described in Materials and Methods. Conditions for lysis of viral particles and analytical ultracentrifugation of DNA in CsCl were as described in the text. Scan tracings of the viral DNA bands at 260 nm are shown with the inner and outer references designated IR and OR, respectively. Densities were calculated with reference to Micrococcus luteus DNA (ρ = 1.731 g/cm³) as marker in all centrifugations. (A) Standard EHV-1 DNA (----) and viral DNA from EHV-1 DI passages used in transformation assays (---). (B) Viral DNA from released particles of low (△) and high (▲) passages of DI-5 cells. (C) Viral DNA from released particles of low (○) and high (●) passages of DI-7 cells.
and by failure to detect infectious virus by infections center assay, plaque assay, and prolonged cocultivation of tumor cells with cells permissive for EHV-1. Also, additional experiments revealed that the production of infectious virus or physical particles could not be induced by chemical (UdR) or physical treatment of DI tumor cell lines (Table 2). Lastly, it should be noted that, in contrast to the virus-producing DI transformed cell cultures, the virus-nonproducing DI tumor cell lines were comprised of only the fibroblastic cell type.

**Viral DNA sequences in productive DI transformed cells and in DI tumor cells.** To determine whether the persistently infected and transformed cultures of DI cells contained complete copies of the EHV-1 genome, total DI cell DNA was analyzed by reassociation kinetics with $^{32}$P-labeled EHV-1 DNA. Reassociation kinetics followed the second-order reaction rate relationship described previously by Nonoyama and Pagano (23), and the rate of reassociation was directly proportional to the amount of viral DNA (unlabeled) added to the reaction. Hence, a linear relationship existed between the rate of reassociation and the number of copies of viral DNA per cell. Reconstruction experiments using different concentrations of unlabeled standard EHV-1 DNA (1, 10, 50, 100, 250, 500, and 1,000 µg/ml) were conducted to validate this hybridization approach (data not shown). The positive control reassociation reaction contained 14.35 genome equivalents of unlabeled EHV-1 DNA per cell mixed with 6.25 genome equivalents of $^{32}$P-labeled viral probe DNA per cell and provided a reference for calculating the copy number of viral DNA in the cell DNAs.

Total cell DNA from the productive DI transformed cells accelerated the rate of reassociation of the viral probe (Fig. 5). By comparing the half $C_{50}$ values of the rates of reassociation of the control reaction with those of the DI cell DNA reactions and by using the relationship between the number of reassociation and the number of genomes per cell, the number of genome equivalents per diploid cell contained in DI-5, DI-8, and DI-10 cells was found to be 23.5, 26.6, and 43.7, respectively (Table 4). The kinetics of completed reassociation reactions of the viral probe DNA in the presence of total DI cell DNA were quite similar to those of the control reactions and indicated that the viral DNA in the productive DI transformed cells possessed the full complement of standard type EHV-1 DNA sequences.

Since virus could be neither detected nor induced from any of the DI tumor cells, DNA-DNA reassociation studies were designed on the assumption that viral DNA sequences in these cells represented only a fraction of the EHV-1 genome (9). EHV-1 $^{32}$P]DNA (0.001 µg/ml) was allowed to reanneal in the presence of DI tumor cell DNAs (5 mg/ml), hamster cell DNA (5 mg/ml), or hamster cell DNA (5 mg/ml) mixed with randomly sheared, unlabeled EHV-1 DNA at 63°C for at least 360 h. The molar ratio of cellular DNA to viral DNA was 50:1, which was sufficient to detect a single copy of a viral DNA sequence 2.8 kilobases in size under the reannealing conditions prescribed in Materials and Methods. Viral DNA sequences were detected in total cell DNAs from the four DI tumor cell lines tested, as the radioactively labeled viral probe exhibited an accelerated rate of reannealing in the presence of tumor cell DNA indicating multiple copies (Fig. 6). In addition, reassociation curves for tumor cell DNAs, unlike that of control EHV-1 DNA, were biphasic, indicating partial homology. The biphasic nature of these curves was observed at low $C_{50}$ values, but is not apparent in the curves as plotted in Fig. 6. The fraction of viral DNA sequences represented in these cells and the number of copies per tumor cell were estimated by reassociation equations previously described (10) and are summarized in Table 4. The DI tumor cell lines were shown to contain viral DNA sequences representing from 42.2 to 56.7% of the EHV-1 genome in amounts of 2.2 to 3.8 copies per cell. Thus, low passages of DI tumor cells contained approximately one-half of the viral genome, similar to UV-irradiated EHV-1 transformed and tumor cell lines (30).

**Viral antigens in productive DI transformed cells and tumor cell lines.** The
expression of EHV-1 antigens in DI transformed and tumor cells was investigated by neutralizing antibody assays and indirect immunofluorescence. Sera from hamsters challenged with DI transformed and tumor cell inocula were complement inactivated, diluted, and assayed for neutralizing antibodies to EHV-1 by plaque assay as described previously (30). As expected, high titers of neutralizing antibodies to EHV-1 were found in animals inoculated with any of the virus-producing DI transformed cell lines (Table 5). In addition, sera from animals inoculated with virus-nonproducing DI tumor cell lines, exemplified by DI-5T in Table 5, contained neutralizing antibodies to EHV-1. These data suggested that both the DI transformed and tumor cell lines expressed viral antigens which elicit antibodies whose activity neutralizes viral infectivity.

To discern the number of cells expressing viral antigens and to determine whether these viral antigens were expressed differently in the two cell types of DI transformed cultures, immunofluorescent studies using antisera to EHV-1 structural or nonstructural antigens, or both, were performed (Fig. 7). EHV-1 structural antigens were detected in both the nonproductive fibroblastic cell type and the productive rounded cell type when treated with rabbit or hamster antisera to purified EHV-1 virions (Fig. 7, center panel). This fluorescence was localized to the cytoplasmic membrane and the cytoplasmic regions of the DI transformed cells but was more intense in the rounded cell type than in the
fibroblastic cell type. Quantitation of positively staining cells revealed that greater than 75% of the cells in the DI transformed cultures expressed detectable EHV-1 structural polypeptides. Nonstructural antigens were detected readily in the nuclei of both cell types of the DI transformed cultures, when the cells were treated with virion-absorbed antisera to EHV-1-infected cell lysates (data not shown). The percentage of cells within the total cell population of the DI transformed cultures expressing nonstructural viral polypeptides was similar to that which expressed structural viral polypeptides. Therefore, the viral genetic information in both productive and nonproductive cell types within DI transformed cultures was expressed as viral antigens. Nonstructural and structural EHV-1 antigens were found in all DI tumor cell lines when the above antisera were used (Fig. 7, right panel); the percentage of fluorescent cells was greater than 60% with either antisera. Therefore, the viral DNA sequences harbored in DI transformed and tumor cells were expressed as viral polypeptides.

**DISCUSSION**

The data presented in this report demonstrate that EHV-1 preparations enriched for DI particles can mediate persistent infection and oncogenic transformation of hamster embryo cells. The DI cell cultures were shown to contain cells that had undergone morphological transformation and were tumorigenic in syngeneic hamsters. However, all six DI cell lines characterized to date were shown to be comprised of two different cell types: (i) a virus-nonproducing cell type constituting the majority of the total cell population and exhibiting a fibroblastic appearance, and (ii) a minor population consisting of rounded, cytopathic cells that produced infectious EHV-1 particles. The virus released by the persistently infected DI transformed cell cultures was judged to be standard EHV-1 and not a temperature-sensitive variant. In addition, DI particles could no longer be detected in these cultures as demonstrated by the failure of released virus to inhibit replication of standard virus in interference assays and by the absence of the heavy-density viral DNA species (1.724 g/cm³; the defective EHV-1 genotype) in the released virus as determined by analytical ultracentrifugation. Further evidence that the virus released from these cultures was standard EHV-1 was the demonstration that newborn hamsters challenged with the virus-producing DI transformed cell cultures rapidly succumbed to a fulminating viral hepatitis, a hallmark of EHV-1 replication in the in vivo hamster model (24).

The presence of oncogenically transformed cells within these mixed cell cultures was indicated by the development of fibrous sarcomas in older, immunocompetent hamsters, which were able to survive the virus infection. Tumor cells and the subsequently derived tumor cell lines were shown to be virus nonproducers by several methods and to be transplantable in both newborn and adult hamsters.

As expected, the DI transformed cells contained multiple copies of the EHV-1 genome and expressed both EHV-1 structural and nonstructural polypeptides, though a greater amount of viral antigens was expressed in the productive cell type. The DI tumor cell lines, which were virus nonproducers, harbored only a few copies of portions of the viral genome and expressed both structural and nonstructural viral antigens. Recently, we have cloned restriction fragments encompassing the whole EHV-1 genome into the plasmid pBR322 and phage vector lambda Charon 4A (R. A. Robinson, P. W. Tucker, and D. J. O’Callaghan, in Proceedings of the Fifth Herpesvirus Workshop, Cold Spring Harbor Symposium, in press) and have used these as probes in blot hybridization analyses. These studies have confirmed that only selected portions of the EHV-1 genome are integrated in the DI tumor cell DNA (26).

It is likely that the establishment of this persistent herpesvirus infection in permissive hamster embryo cells requires an intricate balance between cell survival and sufficient viral replication to maintain virus production. One of the
Fig. 6. Hybridization of EHV-1 [\(^{32}\)P]DNA with total cell DNAs of DI tumor cells. EHV-1 [\(^{32}\)P]DNA (0.001 
\(\mu\)g) labeled by nick translation (specific activity, \(2.0 \times 10^8\) cpm/\(\mu\)g) was allowed to reanneal in the presence of 5 mg of DI tumor cell DNA or calf thymus DNA. The molar ratio of cellular DNA to viral DNA probe, designated as \(N\) value, was 50:1. A reaction mixture of 50 ng of cold EHV-1 DNA and 5 mg of calf thymus DNA with 1 ng of EHV-1 [\(^{32}\)P]DNA served as a positive control. Reaction portions (100 
\(\mu\)l) were sealed in capillary pipettes, denatured at 1100 C for 15 min, and allowed to reanneal at 63°C. Samples were removed at various times up to 360 h and stored at \(-70°C. Unreassociated and reassociated DNAs were fractionated by hydroxyapatite chromatography as described in Materials and Methods.

Table 5. Neutralizing antibody titers to EHV-1 of sera from hamsters inoculated with DI transformed and DI tumor cells

<table>
<thead>
<tr>
<th>Cell type in inoculum</th>
<th>Passage no.</th>
<th>% of EHV-1 PFU neutralized by serum dilution of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
<td>1:4</td>
</tr>
<tr>
<td>HEF</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>EHV-1-infected HEF</td>
<td></td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-5</td>
<td>20-25</td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-6</td>
<td>20-25</td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-7</td>
<td>20-25</td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-8</td>
<td>20-25</td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-9</td>
<td>20-25</td>
<td>+99.9</td>
</tr>
<tr>
<td>DI-10</td>
<td>20-25</td>
<td>88.7</td>
</tr>
<tr>
<td>DI-5T(^a)</td>
<td>1-5</td>
<td>+99.9</td>
</tr>
</tbody>
</table>

\(^a\) Immunocompetent LSH inbred hamsters were inoculated subcutaneously with \(3.0 \times 10^6\) cells in a volume of 0.2 ml. Sera were collected after development of palpable tumors (median time, 5 to 6 weeks), heated at 56°C for 30 min to inactivate complement, diluted with virus diluent, and incubated with \(5 \times 10^7\) total PFU of EHV-1 at 4°C for 30 min. Virus-serum reactions were diluted, and the amount of neutralized virus was determined by plaque assay in L-M cells.

\(^b\) All DI tumors cells were shown to elicit anti-EHV-1 antibody formation, but for the sake of clarity only the data from DI-5T experiments are shown.
predominant factors that may be responsible for initiating persistence at the population level is DI particles (14, 15, 32), since they have the ability to inhibit standard virus replication and to restrict viral cytolytic activities. Several lines of evidence from this study support the conclusion that EHV-1 DI particles initiated the persistent state observed in these DI transformed cultures. First, the presence of DI particles in the virus preparations was essential for establishment of a persistent infection, since HEF cells are permissive for EHV-1 replication. Indeed, HEF cells fail to survive infection with standard EHV-1 (30), whereas DI cell lines established with virus containing DI particles have never undergone a period of crisis during the more than 75 passages in vitro. Second, all EHV-1 cell cultures were resistant to superinfection with the homologous virus but not with a heterologous virus, since VSV infection of these cultures resulted in widespread and rapid cytolysis. Resistance to superinfection with the homologous virus has been associated with DI particle-mediated persistent infection in several other virus systems (15, 16, 20, 32). Third, preparations of EHV-1 DI particles used in these studies have been shown to have high levels of interference activity and to inhibit production of EHV-1 by greater than 1,000 fold (12, 13).

Although DI particles can mediate the establishment of EHV-1 persistent infection, our data are not consistent with the hypothesis that EHV-1 DI particles are necessary to maintain the persistent infection in these cultures, since the presence of DI particles in the released virus could not be shown by either interference assays or density analyses of the viral DNA. It is possible that low levels of EHV-1 DI particles may have escaped detection by our methods and that DI particles may persist at very low levels. However, in light of our recent findings that the genome of EHV-1 DI particles originates from the S region (26) and that the presence of reiterated sequences is manifested by biphasic reassociation kinetics (12), the results of these hybridization studies (Fig. 5) do not support the possibility that significant amounts of defective DNA are conserved within these cells. The persistent infection exhibited by the EHV-1 DI transformed cell cultures resembles Semliki Forest virus persistence established by DI particles (20). In this system, sustained production of DI particles was not required, since interferon was shown to be the primary factor in maintaining this persistence. Successful superinfection of our EHV-1 persistently infected cells was obtained with heterologous viruses (VSV or Mengo virus). Although this finding usually rules out the possibility of interferon-mediated persistence (16, 32), assays to detect small amounts of interferon in the DI cell lines are being conducted to resolve this question.

The initial goal of our studies with EHV-1 DI particles in permissive cell cultures was to investigate the effects of DI virus on the transforming potential of this herpesvirus. EHV-1 preparations enriched with DI particles were more efficient than UV-irradiated standard virus in trans-
forming primary HEF cells (30), and this observation is similar to that of Schroder et al. (33) in experiments on the nonproductive transformation of rat embryo fibroblasts by HSV-1 stocks containing a defective genotype. Recently, we have obtained evidence that viral DNA sequences mapping at approximately 0.3 to 0.4 in the L region of the EHV-1 genome are associated with oncogenic transformation (26, 31) and that the DI genome originates from EHV-1 S-region sequences (26; S. A. Dauenhauser, R. A. Robinson, B. E. Henry, and D. J. O'Callaghan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, S99, p. 224). Therefore, at the present time we propose a model in which these EHV-1 DI particles per se do not initiate oncogenic transformation, but temper the cytocidal infection caused by standard infectious virus and thereby allow L-region-transforming sequences to be expressed. DI particle interference activities may provide conditions that are required for or increase the likelihood of stable integration or expression of EHV-1 gene sequences coding for morphological transformation.

Although the intracellular states of herpesvirus DNA in host chromosomes were not addressed directly in these studies, integration of subgenomic fragments of the EHV-1 genome in the DI transformed and DI tumor cells as well as nonintegrated forms of covalently closed circular EHV-1 genomes in the productive DI transformed cultures are possible states for maintaining EHV-1 DNA in these cells as judged from results in the Epstein-Barr virus lymphoblastoid cell systems (2, 8). Indeed, the nature of the viral DNA (integrated or episomal forms) may explain the regulation of the two herpesvirus-permissive host cell states involved in the EHV-1 DI transformed cultures. A prerequisite for studying such relationships in the persistently infected and transformed cells of this herpesvirus system would be the isolation of productive and nonproductive cell clones from the mixed DI cultures. Recently, we have been successful in establishing several nonproductive cell clones from three of the DI transformed cell lines, and studies to elucidate the physical state of the intracellular viral DNA in these cells are in progress.

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LITERATURE CITED