Focus Formation and Neoplastic Transformation by Herpes Simplex Virus Type 2 Inactivated Intracellularly by 5-Bromo-2'-Deoxyuridine and Near UV Light

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The induction of focus formation in low serum and of neoplastic transformation of Syrian hamster embryo cells was examined after the expression of herpes simplex virus type 2 functions. Syrian hamster embryo cells infected at a high multiplicity (5 PFU/cell) with 5-bromo-2'-deoxyuridine-labeled herpes simplex virus type 2 (11% substitution of thymidine residues) were exposed to near UV light irradiation at various times postinfection. This procedure specifically inactivated the viral genome, while having little, if any, effect on the unlabeled cellular DNA. Focus formation in 1% serum and neoplastic transformation were observed in cells exposed to virus inactivated before infection, but the frequency was enhanced (15- to 27-fold) in cells in which the virus was inactivated at 4 to 8 h postinfection. Only 2 of 45 independently isolated foci were capable of establishing tumorigenic lines. The established lines exhibited phenotypic alterations characteristic of a transformed state, including reduced serum requirement, anchorage-independent growth, and tumorigenicity. They retained viral DNA sequences and, even at relatively late passage, expressed viral antigens, including ICP 10.

By analogy to the initiation/promotion phenomena described in chemical carcinogenesis (14), transforming functions of DNA tumor viruses have been shown to reside in two distinct complementation groups, one responsible for the "initiation," and the other for the "maintenance," of a transformed phenotype. Viruses having a defect in the maintenance-related complementation group induce morphologically altered foci. However, most of these fail to establish tumorigenic lines (13, 15).

Recent data also suggest that more than one viral function may be involved in transformation by herpes simplex virus type 2 (HSV-2). Thus (i) a viral gene mapping outside the thymidine kinase region on the HSV-1 genome greatly enhances the frequency of thymidine kinase transformation (31), and (ii) HSV-2 DNA sequences responsible for focus formation (32) map at a different site on the HSV-2 genome than those involved in the establishment of immortalized lines capable of inducing tumors in immunocompetent hosts (neoplastic transformation) (20). If different viral functions are involved in focus formation and neoplastic transformation, the virus involvement in each process could conceivably be assessed by inactivating the viral genome at specific intervals during a synchronous replicative cycle, thereby interrupting its sequential

expression. The studies described in this report demonstrate that viral functions expressed before 4 h postinfection (p.i.) enhance (15- to 27-fold) the frequency of both focus formation and neoplastic transformation. Although most (43 out of 45) foci failed to establish immortalized lines, all established lines capable of inducing tumors in newborn animals retained and expressed viral DNA, suggesting that viral functions other than those involved in frequency enhancement might be required for the acquisition of tumorigenic potential.

MATERIALS AND METHODS

Cells. Syrian hamster embryo (SHE) cells, prepared from 11-day-old embryos (E1a:Eng strain; Engle Laboratories, Farmburg, Ind.), were grown in 100-mm plates in IBR modified Dulbecco Eagle reinforced medium (ERM) (Biolabs, Northbrook, Ill.) supplemented with 10% fetal bovine serum (FBS) (Reheis Chemical Co., Kankakee, Ill.).

Virus. HSV-2 strain ANG [HSV-2 (ANG)], originally obtained from L. Kucera (Bowman-Gray School of Medicine, Wake Forest University, Winston-Salem, N.C.), was plaque purified in SHE cells before use in these studies. Infectivity assays were done on SHE cells under liquid overlay containing 0.2% pooled human IgG as described previously (1).

BuDR labeling of viral DNA. SHE cells (4×10^6) were infected with HSV-2 (ANG) (5 PFU/cell), and at

2 h p.i., they were overlaid with ERM medium with 2% FBS (ERM-2% FBS) and increasing concentrations (3 to 30 μ M) of 5-bromo-2'-deoxyuridine (BUdR). To prevent BUdR-induced cytotoxicity due to starvation for deoxycytidine nucleosides (30), the medium was supplemented with 0.2 mM deoxycytidine. Virus was harvested at 24 h p.i. by seven cycles of freezing and thawing. All of the procedures involving BUdR were carried out under red safety light.

To estimate the amount of BUdR incorporated into viral DNA, 4×10^6 SHE cells, infected with 5 PFU of HSV-2 (ANG) per cell were exposed to 10 or 30 μ M BUdR and 5 μ Ci of [3 H]thymidine (New England Nuclear Corp., Boston, Mass.; specific activity, 57 Ci/mmol) or [3 H]BUdR (Amersham Corp., Arlington Heights, Ill.; specific activity, 5 Ci/mmol) per ml from 4 to 24 h p.i. At this time, the infected cells were scraped, pelleted by centrifugation at $3,000 \times g$ for 10 min, lysed in 1 ml of 0.1% Sarkosyl in $1 \times$ SSC (150 mM NaCl-15 mM sodium citrate), and digested with 0.1% pronase for 5 h at 37°C (10). The extract (0.2 ml) was mixed with an equal volume of similarly prepared extract from HSV-2 (ANG)-infected cells grown in the presence of 0.13 μ Ci of [14 C]thymidine (New England Nuclear Corp.; specific activity, 21 mCi/mmol) but in the absence of BUdR. The mixture was layered on CsCl gradients ($\rho = 1.74$ g/cm 3 in $1 \times$ SSC) and centrifuged at $83,000 \times g$ for 70 h in a Beckman 50 Ti rotor. Samples of fractions collected by bottom puncture were precipitated with 5% cold trichloroacetic acid onto membrane filters (Millipore Corp., Bedford, Mass.) and counted. Densities were determined by refractive index. The percentage of thymidine residues substituted by BUdR was calculated from the increase in DNA density by the formula of Luk and Bick (26):

% substitution

$$= \frac{\rho_{\text{substituted}} - \rho_{\text{unsubstituted}}}{\% T/50 \times 0.1730} \times 100$$

where % *T* is the percentage of thymidine residues in the DNA (15.5% based on a 69% guanine plus cytosine content for HSV-2 [22]) and 0.1730 represents the maximum increase in density for a totally substituted polydeoxyadenylic acid-polydeoxythymidylic acid polymer.

Inactivation of BUdR-HSV-2 (ANG) during the replicative cycle. Early passage SHE cells ($\leq$ passage 4) were trypsinized and exposed in suspension (10^5 cells) to 5 PFU of BUdR-HSV-2 (ANG) per cell. After adsorption for 2 h at 37°C, the cells were pelleted by centrifugation at $3,000 \times g$ and diluted to final concentrations of 10^4 , 10^3 , and 10^2 cells/ml. Mixtures containing 1 ml of these cells and 1×10^5 freshly trypsinized, uninfected SHE cells were plated onto 60-mm plates, overlaid with ERM-2% FBS containing 0.2% pooled human IgG (ERM-2% FBS-IgG), and incubated at 37°C. At various times after seeding (see Fig. 4), the medium was aspirated and replaced with 1 ml of phosphate-buffered saline containing 0.2% bovine serum albumin. The cells were irradiated with near UV light (nUV; wavelength = 300 to 400 nm) for 30 min with two 15-W black light fluorescent bulbs (General Electric F15T8/BLB) in a Black-Ray reflector (model XX-15; Ultraviolet Products, Inc., San Ga-

briel, Calif.) at 60 μ W/mm 2 (60,000 ergs/cm 2 per s) as measured by a Black-Ray meter. Irradiated cultures were overlaid with 5 ml of ERM-2% FBS-IgG and reincubated at 37°C for 48 h. Those displaying virus plaques were fixed and stained with Giemsa (1). The others were reincubated at 37°C for 5 days, at which time those cultures that were still free of viral cytopathic effect (CPE) were assayed for transformation.

Focus formation was assessed in 1% serum (9). Briefly, cultures were washed with phosphate-buffered saline and incubated in ERM-1% FBS with twice-weekly medium changes. After 5 weeks, the cells were examined for the presence of morphologically altered foci containing ≥ 40 cells. Neoplastic transformation was assayed by the continuous-passage procedure (19). In this assay, confluent cultures were repeatedly passaged in ERM-10% FBS at 1:10 or 1:20 split ratios. Under these conditions, normal SHE cells enter crisis and senescence within 10 to 15 passages (19). Lines that escaped senescence were assayed for phenotypic properties characteristic of transformation.

Phenotypic properties characteristic of transformation. The ability of transformed cells to grow in 10% or 1% serum (12) was determined by plating 100 trypsinized cells on 35-mm dishes in ERM-10% FBS. After 18 h at 37°C to allow attachment, the medium was replaced with ERM-1% FBS (low-serum assay), and the cells were reincubated at 37°C. At 7 days after the initial seeding, the plates were washed with phosphate-buffered saline, fixed in absolute methanol and stained with Giemsa, and colonies containing ≥ 40 cells were counted. Colony-forming efficiency was expressed as the number of colonies $\times 100$ /number of cells seeded. Saturation density was determined by plating 2×10^5 transformed cells in ERM-10% serum in 60-mm dishes. Replicate dishes were trypsinized at 2, 4, 6, and 8 days after seeding, and the number of cells was counted with a Coulter Counter. The maximum number of cells per plate at the time of maximal growth was considered the saturation density.

For the assay of anchorage-independent growth (28), cells were diluted to concentrations of 10^3 or 10^4 cells per 4 ml of ERM-10% FBS medium with 0.3% agarose and 0.1% peptone (Difco Laboratories, Detroit, Mich.) at 45°C and layered on top of a solidified basal layer (4 ml) of ERM-10% FBS containing 0.6% agarose and 0.2% peptone. After 14 to 21 days of incubation at 37°C, clones larger than 20 to 30 cells were scored. Cloning efficiency was expressed as the number of clones $\times 100$ /number of cells seeded. Tumorigenic potential was determined by the subcutaneous inoculation of 2×10^5 transformed cells (in 0.1 ml of ERM without serum) into newborn (1-day-old) hamsters. Animals were examined weekly for tumor appearance.

Viral antigens and antisera. Antisera used in these studies were prepared against: (i) total soluble antigenic mixtures (SAM) of HEp-2 cells infected with HSV-2 (G) for 24 h (36); and (ii) ICP 10, a high-molecular-weight (160,000) viral protein (36) made in cells infected in the presence of cycloheximide (8 h) and actinomycin D (4 h) (37), purified to radiochemical homogeneity (38), and recently shown to be phosphorylated and DNA binding (C. C. Smith and L.

Aurelian, unpublished data). Rabbits or mice were inoculated subcutaneously at biweekly intervals for 4 weeks with antigens homogenized in complete Freund adjuvant as described elsewhere (38). They were bled by cardiac puncture 1 day before each antigen injection and 10 days after the final inoculation. Anti-SAM sera were repeatedly adsorbed with uninfected HEP-2 cells, and all sera were fractionated on linear 10 to 40% (wt/vol) sucrose gradients. Immunoglobulins were quantitated and shown to be free of cross-contamination by radial immunodiffusion (3, 38). Virus specificity has previously been established (36–38) and was confirmed in these series (see Table 4). Visna antigen, consisting of supernatants from infected cells in minimal essential medium-1% heat-inactivated lamb serum, and the sheep anti-Visna serum were received from O. Narayan, The Johns Hopkins Medical Institutions.

Complement fixation and immunofluorescent assays. The high sensitivity and reproducibility of the microquantitative complement fixation assay were described previously (3, 4, 37–39, 42). The assay was performed in triplicate with optimal concentrations of antigen and immunoglobulin G (IgG), and reaction was considered positive if more than 10% of the complement was fixed (3, 4, 38). Indirect immunofluorescence was performed as previously described (3, 38), using the IgG fraction of the anti-SAM and anti-ICP 10 sera and fluorescein-conjugated goat anti-rabbit or anti-mouse IgG (γ -chain specific; Cappel Laboratories, Downingtown, Pa.).

DNA hybridization. Viral DNA was extracted from cytoplasmic HSV-2 (ANG) virions (30) and centrifuged on NaI density gradients containing 0.5 μ g of ethidium bromide per ml as previously described (41). DNA obtained from normal and transformed cells was extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with ether and centrifuged on CsCl density gradients containing 0.5 μ g of ethidium bromide per ml (10). High-density DNA ($\rho = 1.71$ to 1.74 g/cm³) was prepared from samples sheared by repeated forcing through a bent 25-gauge needle before the CsCl centrifugation (25). Ethidium bromide and NaI or CsCl were removed by extraction with isoamyl alcohol followed by extensive dialysis against 50 mM Tris-hydrochloride, pH 7.6.

Viral DNA was labeled in vitro with 1 mCi each of [³²P]dGTP and [³²P]dCTP (Amersham Corp.; specific activity, >400 Ci/mmol) by a modification of the nick translation system (43). Unlabeled cellular or viral DNA in 50 mM Tris-hydrochloride (pH 7.6) was denatured with 1 M KOH for 1 h at room temperature and neutralized with 5 M HCl in 1 M Tris-hydrochloride, pH 7.6. Denatured DNA (10 μ g) was spotted on nitrocellulose membrane filters (Schleicher & Schuell Co., Keene, N.H.; BA 85) which had previously been soaked in 2 $\times$ SSC and dried. The spotted filters were incubated at 60°C overnight in preincubation buffer consisting of 50% formamide, 5 $\times$ SSC, 200 μ g of heat-denatured salmon sperm DNA per ml, and 5 $\times$ Denhardt reagent (1 $\times$ = 0.2 mg each of bovine serum albumin, Ficoll-400, and polyvinylpyrrolidone per ml), and exposed to a ³²P-labeled heat-denatured viral DNA probe (1 $\times$ 10⁷ cpm) in hybridization buffer (50% formamide, 1 $\times$ Denhardt reagent, 100 μ g of heat-de-

natured salmon sperm DNA per ml, 20 mM sodium phosphate [pH 6.5], and 10% sodium dextran sulfate) for 24 h at 60°C (35). After hybridization, the filters were washed three times each with 0.1% sodium dodecyl sulfate in SSC buffer of decreasing concentrations (2 $\times$, 0.3 $\times$, 0.1 $\times$) for 30 min at 60°C. They were dried at 80°C for 2 h and subjected to autoradiography for 6 days at -70°C. The extent of hybridization was quantitated by scanning the autoradiograms with a Joyce-Loebel Chromoscan equipped with an integrator that records counts proportional to the product of the horizontal and vertical displacements of the pen (36–38).

RESULTS

BUDR incorporation into viral DNA. DNA extracted from SHE cells infected with 5 PFU of HSV-2 (ANG) per cell and exposed to [³H]BUDR (5 μ Ci/ml) in the presence of 30 μ M BUDR from 4 to 24 h p.i. was cocentrifuged on CsCl density gradients with DNA extracted from cells infected in the absence of BUDR and similarly labeled with [¹⁴C]thymidine (0.13 μ Ci/ml). The tritium label (Fig. 1A) localized in one major band with an apparent density of 1.745 g/cm³, whereas the peak of the ¹⁴C label migrated at a density of 1.727 g/cm³. A similar increase in the density of viral DNA was observed when virus grown in the presence of 30 μ M BUDR was labeled with [³H]thymidine (Fig. 1B). This density shift represents a 33.6% BUDR substitution of the thymidine residues as determined by the formula of Luk and Bick (26). Based on similar calculations, it can be estimated that in the presence of 10 μ M BUDR (Fig. 1C) viral DNA ($\rho = 1.733$) exhibits 11% substitution of its thymidine residues. In similar double-labeling experiments with mock-infected SHE cells, the tritium-labeled DNA (cells grown in the absence of BUDR) localized in one band with an apparent density of 1.703 g/cm³, whereas the ¹⁴C-labeled DNA (cells grown in the presence of 30 μ M BUDR) migrated at a density of 1.721 g/cm³ (Fig. 1D).

Infectivity of BUDR-labeled HSV-2. To establish optimal conditions for the fragmentation of BUDR-labeled viral DNA by exposure to nUV light (18, 33), virus grown in the presence of increasing (3 to 100 μ M) concentrations of BUDR was exposed to nUV light (wavelength, 300 to 400 nm) for 0 to 30 min and assayed for residual infectivity (Fig. 2). Virus grown in medium containing 3 to 10 μ M BUDR retained most (85 and 60%, respectively) of its infectivity. However, it was rapidly inactivated by exposure to nUV light, with maximal inactivation specific to nUV (3 $\times$ 10⁵ to 8 $\times$ 10² PFU/ml) occurring when 11% of the thymidine residues had been substituted by BUDR (10 μ M) (Fig. 2). Unsub-

FIG. 1. *CsCl* density gradients. (A) DNA extracted from SHE cells infected with 5 PFU of HSV-2 (ANG) per cell and labeled from 4 to 24 h p.i. with [^3H]BUDr (5 $\mu\text{Ci}/\text{ml}$) and 30 μM BUDr. (B) DNA extracted from cells infected as in (A), labeled with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$) and 30 μM BUDr. (C) DNA extracted from cells infected as in (A), labeled with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$) and 10 μM BUDr. In (A) through (C) ^3H -labeled DNA (●) was cocentrifuged with DNA from similarly infected cells grown in the absence of BUDr and labeled with [^{14}C]thymidine (0.13 $\mu\text{Ci}/\text{ml}$) (○). (D) DNA extracted from mock-infected SHE cells labeled from 4 to 24 h p.i. with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$) and 30 μM BUDr (●) was cocentrifuged with DNA extracted from similarly mock-infected SHE cells labeled with [^{14}C]thymidine in the absence of BUDr (○).

stituted virus was virtually unaffected (20% reduction). Accordingly, virus grown in the presence of 10 μM BUDr was used in all subsequent experiments, and inactivation was performed by exposure to nUV for 30 min. The effect of nUV irradiation on the growth characteristics of normal uninfected SHE cells was studied in parallel experiments. Mixtures containing 10^2 mock-infected and 10^5 freshly trypsinized SHE cells were seeded in 35-mm dishes, exposed to nUV light for 30 min, and reincubated at 37°C. At daily intervals (7 days total) cells in replicate dishes

were trypsinized, resuspended in 1 ml of ERM, and counted (Fig. 3A). Cloning efficiency after nUV irradiation was determined by seeding 10^4 cells per 35-mm dish, irradiating for 0 to 30 min (at 2, 4, 6, 8, and 10 h after seeding), and reincubating the cells at 37°C in ERM-10% FBS. Eight days later the cells were fixed and stained, and colonies larger than 40 cells were scored (Fig. 3B). Consistent with previous observations (6), we found that exposure of uninfected SHE cells to nUV for 30 min under conditions identical to those used to inactivate BUDr-labeled virus (BUDr-HSV-2) did not affect their growth (Fig. 3A) or their cloning efficiency (Fig. 3B).

Inactivation of BUDr-HSV-2 during the virus replicative cycle. SHE cells infected with BUDr-HSV-2 in suspension (multiplicity of infection = 5 PFU/cell) and diluted to final

FIG. 2. Kinetics of inactivation by nUV light of virus grown in the presence (3 to 100 μM) or the absence of BUDr.

FIG. 3. (A) Growth curve of mock-infected SHE cells before (○) and after (●) exposure to 30 min of nUV irradiation. (B) Cloning efficiency of SHE cells (10^4 per 35-mm dish) exposed to nUV irradiation at 2 and 4 h after seeding. Similar results were obtained at all other time intervals (data not shown).

concentrations of 10^4 , 10^3 , and 10^2 cells/ml were mixed (1:1) with 10^5 uninfected SHE cells/ml and plated (2 ml/plate) in ERM-2% FBS-0.2% IgG. At various times after plating, one set of cultures was exposed to nUV for 30 min, and the other served as a nonirradiated control. After reincubation at 37°C for 48 h, cultures displaying characteristic plaque formation were fixed, stained, and scored for infectious virus. In parallel experiments, infected cells were pulsed with [3 H]thymidine (5 μ Ci/ml) for 30 min at 0, 1, 3, 5, 7, and 9 h p.i. Similarly pulsed uninfected cells served as controls. The DNA extracted with sodium dodecyl sulfate-pronase (10) was centrifuged on CsCl density gradients. Tritium incorporation into cellular DNA ($\rho = 1.701$ g/cm 3) decreased rapidly after infection and was essentially abolished by 7 h p.i. Synthesis of viral DNA ($\rho = 1.727$ g/cm 3) was first detected at 5 h and reached high levels by 9 h p.i. The extent of virus production in cells infected with BUDR-labeled virus and exposed to nUV at various times after infection is summarized in Fig. 4, along with the rate of viral DNA synthesis. Although all nonirradiated cultures displayed characteristic plaque formation, virus infectivity was reduced by nUV exposure up to 8 h p.i. By 10 h p.i., in the presence of progeny unsubsti-

tuted viral DNA (Fig. 4), exposure to nUV was no longer effective. Cultures (containing 10^2 infected cells) in which the infectivity of the BUDR-labeled virus was inactivated by nUV exposure (0 to 8 h p.i.) and which did not display characteristic plaque formation (Table 1) were reincubated at 37°C for 5 days. At this time, cultures still free of virus CPE were assayed for transformation.

Transformation assays. Uninfected and BUDR-HSV-2-infected cultures that did not display virus characteristic plaque formation in the experiment described in Fig. 4 were assayed for transformation according to their ability to (i) form morphologically altered foci in 1% serum (9), and (ii) escape senescence and establish tumorigenic lines (19). Foci of densely growing cells (≥ 40 cells), easily distinguishable from the surrounding monolayer, were observed only in the HSV-2-treated cultures. They were not seen in "mock"-infected (12 plates) controls (Table 1). The frequency of focus formation in low serum was expressed as the number of foci $\times$ 100/the total number of cells seeded. Virus, inactivated before infection, induced focus formation in 1% serum; however, a 15- to 23-fold increase in frequency was observed when virus was inactivated at 4 to 8 h p.i. (Table 1). A total

FIG. 4. Replicate plates containing mixtures of SHE cells (10^4 /ml) previously exposed to 5 PFU of BUDR-HSV-2 (ANG) per cell for 2 h and 10^5 freshly trypsinized uninfected SHE cells were irradiated with nUV light for 30 min at the times p.i. indicated on the abscissa and incubated at 37°C for 48 h. Virus plaques were scored at that time, and the results of two separate experiments are expressed as virus titers (●). Similar results were obtained in plates containing 10^3 infected cells/ml (data not shown). Virus titers in plates containing similar mixtures of infected (10^4 or 10^3 /ml) and uninfected (10^5) cells that were not exposed to nUV light and were scored for plaque formation in parallel were 2×10^4 PFU/ml (□). The rate of viral DNA synthesis (○) was measured in SHE cells infected with 5 PFU of HSV-2 (ANG) per cell pulsed (30 min) with [3 H]thymidine at the times p.i. listed on the abscissa. The results are expressed as total counts per minute migrating on CsCl density gradients at the apparent density (1.727 g/cm 3) of viral DNA.

TABLE 1. Frequency of focus formation in 1% serum

Time of nUV ^a exposure	No. of foci ^b			Frequency ^c (%)
	Expt 1	Expt 2	Total	
Mock infected ^d	0	0	0	0
Pre-inactivated ^e	1	1	2	0.5
0 h	1	1	2	0.5
2 h	2	1	3	0.7
4 h	25	20	45	11.3
6 h	16	13	29	7.3
8 h	9 ^f	8 ^f	17	8.5
10 h	CPE ^g	CPE	CPE	— ^h
12 h	CPE	CPE	CPE	—

^a A total of 100 SHE cells infected with 5 PFU of BUDR-HSV-2 (ANG) per cell were seeded with 10⁵ uninfected cells and irradiated with nUV at the indicated times.

^b Each time point represents total foci on two plates.

^c Frequency is expressed as the total number of foci (experiments 1 and 2)/the number of seeded plates × cells seeded per plate.

^d Twelve plates, containing mixtures of 10² mock-infected SHE cells and 10⁵ freshly trypsinized uninfected SHE cells each, were irradiated with nUV at the various time points.

^e In a separate experiment, four plates were seeded with mixtures containing 10⁵ SHE cells infected with 5 PFU of BUDR-HSV-2 (ANG) per cell exposed to nUV before infection and 10⁵ freshly trypsinized uninfected cells. A total of 25 foci were observed on these four plates (frequency, 0.63%). They were isolated and subcultured, and one of them grew into an established line.

^f One of two plates in each experiment showed signs of viral CPE and was discarded. Frequency was calculated on the basis of surviving cultures only.

^g All plates exhibited viral CPE and were discarded.

^h —, Not applicable.

of 45 foci were picked and independently subcultured in ERM-10% FBS. A total of 25 were picked from cells infected with inactivated virus, and 10, 7, and 3 foci were picked from infected cells irradiated at 4, 6, and 8 h (Table 1), respectively. Only two (one from the pre-inactivated plate and one from the 4-h-p.i. plate) survived passage and grew into established lines. The others senesced within 2 to 5 subculture passages. Similar results were reported by Kimura et al. (23). The data suggest that focus formation even in low serum is not equivalent to the establishment of transformed lines with tumorigenic potential.

In another series of experiments, immortalized lines were established by the continuous passage assay (19) and were assayed for tumorigenicity (Table 2). Cells mock infected with phosphate-buffered saline instead of virus and exposed to nUV irradiation for 30 min senesced at passage 10 to 15, consistent with previous observations (6, 19). On the other hand, some of the virus-

infected cultures developed colonies of rapidly growing morphologically altered cells that overgrew the cultures and established immortal lines capable of inducing tumors in newborn animals (Table 3). The frequency of transformation, estimated as the number of established lines × 100/(the number of plates × the number infected cells seeded per plate), was maximal when cultures were exposed to nUV light at 4 to 8 h p.i. (Table 2). It was significantly lower in cultures exposed to virus inactivated before infection or at 0 to 2 h p.i.

Phenotypic properties of transformed cells. Six lines (Table 3) established from independent cultures displayed phenotypic alterations associated with transformation, including (i) loss of contact inhibition of growth (12), (ii) reduced serum requirement (12), (iii) anchorage independence (28), and (iv) tumorigenicity in syngeneic animals. Cells grew more rapidly and achieved much higher saturation densities than normal SHE cells. They manifested high cloning efficiencies in 1% serum even when seeded at low density (100 cells per 35-mm dish) and displayed anchorage-independent growth in 0.3% agarose. When inoculated subcutaneously (2 × 10⁶ cells) into newborn (1-day-old) hamsters at posttreatment passages (PTP) 22 to 54, they caused tumors in 20 to 100% of inoculated animals, with latent periods ranging from 9 to 22 weeks. Histopathologically, the tumors were invasive anaplastic fibrosarcomas.

Expression of viral antigens in transformed cells. Extracts from the six transformed lines studied in these series, but not from normal SHE cells, fixed complement with IgG from anti-SAM and anti-ICP 10 sera (Table 4). The reactivity was virus specific, as evidenced by the observations that (i) anti-SAM and anti-ICP 10 IgG fixed complement with extracts of HSV-2-infected, but not uninfected, HEp-2 cells; (ii) preimmune serum IgG did not fix complement with transformed cell extracts; (iii) the reactivity of the anti-SAM and anti-ICP 10 IgG was adsorbed with extracts of HSV-2-infected, but not uninfected, HEp-2 cells; and (iv) sheep anti-Visna serum did not fix complement with the transformed cell extracts nor with extracts from HSV-2-infected cells. Extracts from SHE cells or mock-infected HEp-2 cells were negative throughout (Table 4).

Lines HBU-1, HT6A, and HT8A were also assayed with mouse anti-ICP 10 and preimmune serum IgG in indirect immunofluorescence. All were positive with the anti-ICP 10 but not with the preimmune IgG (Fig. 5). The staining was cytoplasmic and perinuclear. It was similar to that detected in HEp-2 cells infected with HSV-

TABLE 2. Frequency of neoplastic transformation

Time of nUV ^a exposure	No. of lines ^b			% Frequency (lines/cells seeded)
	Expt 1	Expt 2	Total	
Mock infected ^c	0	0	0	0
Pre-inactivated ^d	0	0	0	0
0 h	0	0	0	0
2 h	0	0	0	0
4 h	2	1	3	0.38
6 h	1	1	2	0.25
8 h	2 ^e	0	2	0.33
10 h	CPE/	CPE	CPE	— ^g
12 h	CPE	CPE	CPE	—

^a A total of 100 SHE cells infected with 5 PFU of BUdR-HSV-2 (ANG) per cell were seeded with 10⁵ uninfected cells and irradiated with nUV at the indicated times. They were passed at 1:10 or 1:20 split ratios as they became confluent.

^b Four plates were seeded in each experiment with 100 virus-infected cells. Frequency calculations are based on the assumption that each line represents at least one transforming event.

^c Twelve plates, containing mixtures of 10² mock-infected SHE cells and 10⁵ freshly trypsinized uninfected SHE cells each, were irradiated with nUV at the various time points.

^d In a separate experiment, 14 cultures were seeded with mixtures containing 10³ SHE cells infected with 5 PFU of BUdR-HSV-2 (ANG) per cell exposed to nUV (30 min) before infection and 10⁵ freshly trypsinized uninfected cells. Two established lines were obtained (frequency = 0.014%).

^e Two of a total of eight plates (experiments 1 and 2) developed CPE and were discarded. Frequency was calculated based on surviving cells.

^f All plates exhibited viral CPE and were discarded.

^g —, Not applicable.

2 in the presence of cycloheximide followed by actinomycin D (38), conditions permitting only the synthesis of "immediate early" viral proteins (17), a major component of which is ICP 10 (37). Staining was adsorbed with extracts of 4-h-infected, but not uninfected, cells; SHE cells or uninfected HEp-2 cells exposed to cycloheximide and actinomycin D did not stain (38). Finally, sera from hamsters bearing tumors induced by HBU-1, HT6A, and HT8A cells stained cycloheximide-actinomycin D-treated HSV-2-infected cells (17, 38), but did not stain similarly treated uninfected cells. Sera from normal hamsters were nonreactive (data not shown). This finding is consistent with the interpretation that viral proteins are expressed in the tumors induced by these transformed lines.

DNA hybridization. Two series of experiments were done to test for the presence of viral DNA in the transformed cells. In the first series, total DNA (10 µg) from SHE (passage 5), HT8A

(PTP 63), and HBU-1 (PTP 64) cells denatured with 1 M KOH was spotted on nitrocellulose membrane filters and hybridized with ³²P-labeled virion DNA at 60°C for 24 h (Fig. 6A). In the second series, spots were prepared with 10 µg of high-density DNA (1.71 to 1.74 g/cm³) from SHE (passage 5), HBU-1 (PTP 64), HT4A (PTP 34), and HT6A (PTP 61) cells and hybridized as above (Fig. 6B). Hybridization was observed with total DNA or high-density DNA from all the transformed lines examined in these series. The reaction was specific. Serial dilutions of unlabeled HSV-2 (ANG) DNA from two different preparations were mixed with 10 µg of salmon sperm DNA and spotted on membrane filters (Millipore Corp.). Hybridization was observed with as little as 10⁻⁴ µg of viral DNA. Preincubation of the viral DNA (10⁻³ µg) (Fig. 6C) or of the total DNA from the transformed cells (data not shown) with 20 µg of DNase per ml (in 10 mM MgCl₂-200 mM NaCl-4 mM KCl-3 mM sodium phosphate, pH 7.0) for 5 h at 37°C before denaturation and spotting abolished the hybridization. Furthermore, hybridization was not observed with salmon sperm or normal SHE cell DNA. The extent of hybridization was quantitated by densitometric scanning of the autoradiograms. Comparison between the two series of experiments is impossible, since (as evidenced by HBU-1) it appears that the extent of the hybridization was significantly greater with high-density DNA than with an equal amount of total cell DNA. Similar conclusions were independently reached by Leiden et al. (25). However, comparison between the extent of hybridization in the lines studied within each series indicates that different amounts of viral DNA were retained by the various transformed lines (Fig. 6A and B).

DISCUSSION

The salient feature of these studies is the finding that HSV-2, inactivated by a novel procedure consisting of BUdR and nUV light, retained the capacity to induce morphologically transformed foci in low serum and establish tumorigenic lines. Similar results were obtained with BUdR-labeled virus inactivated by nUV irradiation (λ = 300 to 400 nm) before infection or at very early times (0 to 2 h) p.i. However, the frequency both of focus formation and of neoplastic transformation (establishment of tumorigenic lines) was increased (15- to 27-fold) when BUdR-labeled virus was inactivated at 4 h p.i. (Tables 1 and 2). The frequency of neoplastic transformation (0 to 0.38%) was significantly lower than that of focus formation (0.5 to 11.3%) and, consistent with the observation of

TABLE 3. Phenotypic characteristics of SHE cells transformed by HSV-2 inactivated at various times p.i.

Cell line ^a	Phenotypic characteristic					
	Saturation density ($\times 10^6$ cells/cm ²)	Cloning efficiency at low cell density ^b in:		Colony-forming efficiency ^c in 0.3% agarose	Tumorigenicity ^d	Avg latent period for tumors (wk)
		1% serum	10% serum			
SHE (passage 4; no virus, BUdR + nUV treated)	1.2	0	0	0	0/12	— ^e
HBV-1 (PTP 35; pre-inactivated virus)	3.9	12.5	25	2.9	2/6	13
HT4A (PTP 22; 4 h p.i.)	4.6	25	31	1.8	3/6	22
HT4B (PTP 44; 4 h p.i.)	3.4	20	30	10.0	12/12	9
HT4C (PTP 54; 4 h p.i.)	3.0	70	100	3.6	1/5	18
HT6A (PTP 54; 6 h p.i.)	4.2	10	35	6.2	2/6	12
HT8A (PTP 36; 8 h p.i.)	4.7	37	50	23.6	4/7	9

^a Cell lines were independently derived from SHE cell cultures exposed to BUdR-labeled virus and irradiated with nUV light at the times p.i. indicated.

^b A total of 100 cells were seeded per 35-mm dish. Colonies of ≥ 40 cells were counted at day 10. Cloning efficiency = the number of colonies/the number of cells seeded. Normal SHE cells do not clone under these conditions.

^c Cells (10^4) were seeded per 35-mm dish. Colonies of >20 to 30 cells were counted at 2 to 3 weeks. Cloning efficiency = the number of colonies $\times 100/10^4$.

^d Newborn hamsters were injected subcutaneously with 2×10^6 transformed or mock-infected cells in 0.1 ml of ERM without serum. Values represent the number of hamsters developing tumors/the number of hamsters injected.

^e —, None.

Kimura et al. (23), only 2 of 45 subcultured foci gave rise to continuous tumorigenic lines. However, all six lines examined in these series retained viral DNA sequences, expressed viral antigens, and were tumorigenic in newborn immunocompetent animals.

To assess the involvement of HSV-2 gene expression in the induction of focus formation and neoplastic transformation, the viral genome labeled with BUdR was inactivated intracellularly by exposure to nUV light (18, 33) after sequential expression of viral functions. We found that virus grown in the presence of $10 \mu\text{M}$ of BUdR (11% substitution of thymidine residues) retained most of its infectivity before irradiation, but was specifically inactivated by exposure to nUV (Fig. 2). The residual infectivity (1 in 10^3 PFU) after nUV treatment, probably due to the presence of unsubstituted or minimally substituted DNA molecules (viz. ^3H -labeled shoulder at $\rho = 1.727 \text{ g/cm}^3$ in Fig. 1B), was excluded from transformation assays by plating 100 infected cells per dish (Tables 1 and 2). Virus grown in the presence of 30 to $100 \mu\text{M}$ BUdR was not used, as it displayed reduced (>1 log) infectivity before nUV irradiation, presumably due to nonspecific BUdR toxicity (29).

Significantly, irradiation with nUV had virtually no effect on the growth and cloning properties of normal uninfected SHE cells (Fig. 3). The fate of normal SHE cells exposed to nUV has also been extensively investigated by other

colleagues in our laboratory with similar results. SHE cells exposed to nUV for 30 min do not show any detectable increase in mutation frequency (40) nor in the frequency of focus formation or neoplastic transformation (6; Tables 1 and 2). If transformation is a nonspecific event due to the induction of DNA repair enzymes (11) by the inactivation of a replicating viral DNA and does not require the expression of a viral gene(s), it would be expected to reach maximal levels at the time of maximal viral DNA synthesis (≥ 9 h p.i.). However, we found that the frequency of transformation was maximal (Tables 1 and 2) when virus was inactivated virtually before the onset of viral DNA synthesis (4 h p.i.; Fig. 4).

The replication of a viral DNA strand lasts 20 min (34). Newly synthesized viral DNA requires more than 1 and up to 2 h to mature before participating in another round of replication (7, 16, 21). Since two complete rounds of replication are required to generate viral DNA that is insensitive to nUV irradiation, these time constraints suggest that, in a synchronized infection, both viral DNA strands should be unsubstituted and, therefore, resistant to nUV fragmentation at 4 h after the onset of viral DNA synthesis. Indeed, we found that in our system viral DNA synthesis began at 5 h p.i. It reached highest levels at 9 h, at which time the virus infectivity was largely (95%) resistant to nUV inactivation, presumably due to the appearance of infectious, totally un-

TABLE 4. Complement-fixing potential for HSV-2-transformed cells of antisera to HSV-2 antigens

Antigen ^a	% Complement fixed						Visna ^c
	SAM	Pre-immune	Ad-sorbed ^b SAM	ICP 10	Pre-immune	Ad-sorbed ^b ICP 10	
HBU-1 (PTP 35)	90.3	0	0	20.3	0	0	0
HT4A (PTP 19)	90.2	0	7.2	23.1	9.6	2.2	0
HT4B (PTP 21)	42.9	0	ND ^d	21.6	0.8	ND	0
HT4C (PTP 17)	76.9	0	ND	16.3	5.9	ND	0
HT6A (PTP 53)	88.9	6.4	ND	43.0	3.6	ND	0
HT8A (PTP 35)	91.1	7.3	3.8	77.2	0	5.0	0
SHE	0	0	0	0	0	4.7	ND
HSV-2-infected HEp-2 (4 h)	ND	ND	ND	28.1	3.0	0	ND
HSV-2-infected HEp-2 (24 h)	41.4	2.7	0	0	0	8.6	0
Mock-infected HEp-2	0	0	2.2	7.8	0	0	1.2
Visna	3	7	ND	9	0	ND	77.8

^a Extracts were prepared as described (36-38). ICP 10 was made in HEp-2 cells infected with HSV-2 for 4 h (37, 38). Extracts of 24-h-infected cells were used in preparing SAM (36). Control antigen consisted of HEp-2 cells mock infected with phosphate-buffered saline for 4 or 24 h. Antigens were assayed at 20 μ g of protein with IgG at a 1/4 dilution. Reaction was considered positive if more than 10% of the complement was fixed.

^b Anti-SAM and anti-ICP 10 were adsorbed with extracts of 24- and 4-h-infected cells, respectively, and with extracts of mock-infected cells. Reactivity was not abrogated by adsorption with extracts of mock-infected cells (data not shown).

^c Optimal concentrations of sheep anti-Visna serum (1/32) and of Visna antigen (1/4) were used.

^d ND, Not done.

FIG. 5. Immunofluorescent staining. (a) HBU-1 cells stained with mouse anti-ICP 10 IgG. (b) HT8A cells stained with mouse anti-ICP 10 IgG. Notice perinuclear location of the staining. (c) HT8A cells stained with mouse preimmune IgG. (d) HT8A cells stained with mouse anti-ICP 10 IgG adsorbed with extracts of 4-h-HSV-2-infected cells as described else-

substituted DNA.

Cultures surviving the BUdR-nUV treatment were assayed for focus formation in low serum and neoplastic transformation, since it appears that focus formation does not correlate with tumorigenicity (5, 13, 15, 23) and may be induced by HSV-2 DNA sequences different from those that induce neoplastic transformation (20, 32). The frequency of both focus formation and neoplastic transformation was enhanced 15- to 27-fold in cells in which virus replication was allowed to proceed for 4 (but not for 2 h) p.i. It did not increase at 6 and 8 p.i. (Tables 1 and 2). Given that fragmentation by nUV irradiation was a random event (33), similar fragmentation of the viral genes occurred at 2 and 4 h p.i. Therefore, the finding that the frequency of transformation was not enhanced in cells exposed to nUV at 2 h p.i. suggests that a viral gene(s) that codes for the enhancing functions is inactivated after their expression (in the 4-h cells). It follows that viral antigens (such as ICP 10) expressed in lines established with virus irradiated at 4 to 8 h p.i. are probably encoded by gene(s) that have escaped inactivation. As such, they are distinct from the gene(s) that codes for the transformation-enhancing functions. The identity of the transformation-enhancing functions is unknown, and their role in the establishment of tumorigenic lines remains a matter of

where (38). Staining was not abrogated by adsorption with extracts of uninfected cells.

FIG. 6. Spot hybridization with HSV-2 (ANG) [³²P]DNA. (A) Total DNA from HBU-1, SHE, and HT8A cells. (B) High-density DNA from HBU-1, HT4A, HT6A, and SHE cells. (C) Serial dilutions of unlabeled HSV-2 (ANG) DNA, salmon sperm DNA, and DNase-treated HSV-2 (ANG) DNA from two different preparations (a, b). Numbers under peaks refer to areas as determined by integrator counts.

conjecture. However, the finding that the transformation frequency was virtually identical in cells exposed to nUV irradiation at 4, 6, and 8 h p.i. suggests that enhancing functions are expressed by 4 h p.i.

It is particularly significant that most (43 of 45) transformed foci failed to establish immortal lines, suggesting that viral functions other than those involved in focus formation are required for the establishment of tumorigenic lines. However, all lines studied in these series were positive for viral information. Using the spot hybridization assay, a sensitive screening procedure successfully employed for the detection of viral DNA in Epstein-Barr virus-transformed cells (8), with DNA samples prepared as described by Leiden et al. (25), we identified viral DNA sequences in all of the established tumorigenic lines studied in these series (Fig. 6). The hybridization appears to be specific for viral DNA sequences since (i) the viral DNA used as a

probe was extracted from purified cytoplasmic virions and was free of contaminating cellular DNA sequences (30), (ii) hybridization was not observed with salmon sperm DNA nor with total or high-density DNA from normal SHE cells (Fig. 6A and B), and (iii) pretreatment of the DNA with DNase abolished the hybridization. Accordingly, it is unlikely that the hybridization represents reactivity with viral RNA or small amounts of contaminating cellular DNA sequences. However, the data do not rule out the possibility that the lines contain an amplified cellular sequence with homology to HSV sequences.

Transformed lines (PTPs 17 to 53) were positive for viral antigens, including ICP 10, a viral protein expressed in cells neoplastically transformed by specific viral DNA sequences (20). The specificity of the antisera and of their reactivity in complement fixation or immunofluorescence or both is established (36-38) and was

confirmed in these series (Table 4, Fig. 5). The finding that sera from hamsters bearing tumors induced by HBU-1, HT6A, and HT8A cells specifically stained cycloheximide-actinomycin D-treated HSV-2-infected cells in immunofluorescence suggests that at least some immediate early proteins (a major component of which is ICP 10 [37]) can be expressed *in vivo*. Similar conclusions were reached by MacNab et al. (27) in an identical assay with sera from rats bearing tumors induced by HSV-2-transformed rat cells. Finally, HBU-1 (PTP 53), HT4A (PTP 68), HT6A (PTP 80), and HT8A (PTP 61) were also found to be positive for virus-specific mRNA as determined by *in situ* hybridization with ³²P-labeled HSV-2 DNA (M. Manak et al., manuscript in preparation).

It should be pointed out that the DNA sequences that are maintained and expressed in the HSV-2-transformed cells do not necessarily play a role in the maintenance of the transformed phenotype. Indeed, it has been shown that cells can maintain and express any extraneous DNA (44) even when growth conditions do not select in its favor. However, it may be particularly significant that, although fragmentation of the viral genome was a random event, all of the established lines studied in these series expressed at least one common viral antigen (ICP 10) that had also been identified in cells neoplastically transformed with a specific viral DNA fragment (20). The expression of ICP 10 appears to correlate well with anchorage-independent growth and tumorigenicity (2; M. Manak et al., in preparation).

Taken in toto, the data suggest that viral functions(s) expressed from 2 to 4 h p.i. enhance the frequency of both focus formation and neoplastic transformation, possibly by creating conditions, such as increased cellular DNA synthesis (24), that are conducive to the establishment of a transformed state. However, most foci fail to grow into established lines, suggesting that distinct viral functions are involved in focus formation and neoplastic transformation. Viral functions, for which ICP 10 appears to be a reasonable marker, seem to be selectively expressed in neoplastically transformed lines. However, their involvement, if any, in the maintenance of a transformed state, is unclear. Final conclusions pertaining to the identity of viral transforming functions and their role in the transformation event must await the results of studies, in progress in our laboratory, designed to (i) develop a better understanding of HSV-2 immediate early functions and (ii) identify the HSV-2 DNA sequences expressed or retained and expressed in transformed foci and tumorigenic lines.

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