Comparison of Properties of Mouse Cells Transformed Spontaneously by Ultraviolet Light-irradiated Herpes Simplex Virus or by Simian Virus 40


ABSTRACT

A cloned line of BALB/c-derived mouse cells (10E2) was used to compare the phenotypic properties of cells transformed either spontaneously, by UV-irradiated herpes simplex virus (UV-HSV), or by SV40. Cells transformed by SV40 (tumorigenic and non-tumorigenic) expressed several in vitro properties usually associated with transformed cells, including: (a) growth in serum-deficient medium; (b) high saturation density and loss of contact inhibition; and (c) anchorage independence in agarose and agar. Tumorigenic cells transformed spontaneously or by UV-HSV were indistinguishable from each other and did not express these properties. The SV40-transformed cells, but not the UV-HSV- or spontaneously transformed cells, also expressed transplantation rejection antigens. No correlation was found between any of the in vitro properties tested and tumor induction by 10E2 cells transformed either spontaneously, by UV-HSV, or by SV40.

No evidence of viral DNA, RNA, or proteins was found in cells transformed by UV-HSV within limitations of the applied techniques. The UV-HSV-transformed cells demonstrated no in vitro properties distinct from spontaneously transformed cells which could be ascribed to retained viral gene sequences. This differed from SV40-transformed cells, where retained viral gene sequences were apparently responsible for expression by the cells of in vitro properties usually associated with a transformed phenotype. The fact that 10E2 cells transformed by UV-HSV differed significantly from cells transformed by SV40 suggested that if a "transforming" segment of the herpes simplex virus genome was retained in the cells, it did not function to alter the in vitro growth properties of the cells, as occurred with SV40-transformed cells.

INTRODUCTION

Transformation of rodent cells in culture by HSV has been well documented since the original studies of Duff and Rapp (13), and at least some transformed cells have proven tumorigenic when inoculated into an appropriate host (4, 9, 13, 27–29). In addition to tumorigenic transformation, HSV can biochemically transform cells by introduction of the viral thymidine kinase gene into thymidine kinase-negative cells (35).

Although the mechanism of tumorigenic transformation by HSV has yet to be determined, 2 possibilities warrant consideration. First, HSV may function as a "typical" oncogenic DNA virus (e.g., SV40), whereby a segment of the viral genome with transforming properties is integrated into the cell DNA (41). Alternatively, HSV may function as a cocarcinogen (19–21), whereby transformation results from an HSV-induced alteration in one or more cell processes which may not require retention in the cells of any portion of the viral genome.

We have considered an alternative approach to gain some insight into the mechanism of HSV-induced cell transformation. The rationale for our studies is based on the observation that following prolonged subculturing of a BALB/c-derived clonal cell line (10E2), the cells could undergo spontaneous tumorigenic transformation without expressing several of the in vitro properties usually associated with a transformed phenotype. In contrast, when 10E2 cells were transformed by SV40, the cells did express several in vitro properties usually associated with a transformed phenotype, including: (a) high saturation density and loss of contact inhibition (46); (b) growth in serum-deficient medium (14, 26); and (c) anchorage independence (16, 30). By transforming the 10E2 cells with UV-HSV, we were able to compare the in vitro properties of the UV-HSV-transformed cells with those of cells transformed spontaneously or by SV40. On the basis of the phenotypic properties studied, we concluded that 10E2 cells transformed by UV-HSV were indistinguishable from cells transformed spontaneously but significantly different from cells transformed by SV40.

MATERIALS AND METHODS

Cells. The BALB/c-derived BLP cells (21) were cloned by seeding 25 to 100 cells in 100-mm Petri dishes and dispersing well-separated colonies with trypsin using cloning rings. Clone 10E2 cells derived from BLP cells and BALB/c 3T3 clone A31 cells (obtained from Dr. W. Brockman, University of Michigan) were grown in Eagle’s MEM supplemented with 10% heat-inactivated FCS, penicillin, and streptomycin. The cells were subcultured at 6- to 8-day intervals at a 1:8 split ratio.

Viruses. The type 2 HSV 333 strain was plaque-purified and propagated in Vero cells (19), and aliquots were stored at −70°. The virus was titrated in Vero cells using a methylocel-
lulose overlay and was used within 1 to 2 passages following plaque purification.

The small-plaque variant of SV40 (obtained from Dr. G. Khoury, National Cancer Institute) was propagated in Vero cells, harvested as above for HSV, and titrated in BSC-1 cells using an agar overlay.

Mycoplasma Testing. All control and transformed cells as well as virus pools were tested by R. Del Giudice (Frederick Cancer Research Center) and were found to be free of Mycoplasma or other contaminants.

UV Irradiation. Irradiation of HSV for periods of 2 to 12 min at 63 ergs/sq mm/sec was carried out as described (19).

HSV Neutralization. Hyperimmune rabbit anti-HSV serum (22) or preimmunization serum was added to virus at a 1:20 dilution. The mixtures were placed in a 34° water bath for 30 min, and residual virus was then assayed in Vero cells.

SV40 T-Antigen. Ethanol- or acetone-fixed cells on coverslips were stained by indirect immunofluorescence using hamster SV40 tumor-bearing serum (obtained from Office of Program Resources and Logistics, National Cancer Institute), or normal hamster serum and fluorescein isothiocyanate-conjugated goat anti-hamster serum (Meloy Laboratories, Springfield, Va.).

Tumor Induction. Sch: BALB/c/BOM Cr (nu/nu, nu/nu) DF athymic nude mice were obtained from ARS/Sprague-Dawley, Madison, Wis. Weanling conventional (immunocompetent) BALB/c mice were obtained from Microbiological Associates, Inc., Walkersville, Md.

Nude mice were inoculated s.c. in the anterolateral region of the dorsum (2) and were observed for an 8-week period. Conventional mice were similarly inoculated and were observed for a 16-week period. Five animals were inoculated for each test sample.

Transformation Assays. Cells (10E2) seeded in replicate at 4 to 5 ×10^4 in 75- to 250-cm^2 flasks were incubated for 3 days to obtain actively growing cultures. The medium was removed, 5 ml of UV-HSV or SV40 were added, and the cells were incubated for 2 hr, washed, and dispersed with trypsin. When IdUrd (Calbiochem-Behring Corp., San Diego, Calif.) was used, the cells were refed with fresh medium, drug (20 µg/ml) was added under subdued light, the cells were incubated for 2 to 8 hr, washed, and dispersed with trypsin.

UV-HSV-infected, IdUrd-treated, and control cells were seeded at 0.5 to 2 ×10^5 cells, and SV40-infected cells were seeded at 1 to 5 ×10^4 cells in 2 to 3 flasks (75 sq cm) for transformation with or without added PAA (Richmond Drug Co., Richmond, Va.) at 20 µg/ml. The cells were refed on Day 7 to remove residual PAA and were refed at 7- to 9-day intervals. The cells were subcultured at a 1:8 split ratio as required. Cells were also seeded in triplicate at 200 cells/25 sq cm flask with and without PAA for plating efficiency determination. These cells were incubated for 7 to 9 days, fixed with methanol, and stained with Giemsa stain for enumeration of colonies.

Multiplicities of infection with HSV were determined by counting the number of cells in one flask at the time of infection and titration of unirradiated virus in Vero cells. Similar procedures were used with SV40, except that the virus was titrated in BSC-1 cells.

Colony Formation in Semisolid Medium. A 4-ml base layer of either 0.5% agarose (SeaKem medium agarose; Marine Colloids Division, FMC Corp., Rockland, Maine) or 0.6% purified agar (Difco Laboratories, Detroit, Mich.) in MEM plus 15% FCS was placed in 60-mm Petri dishes and allowed to set at room temperature. Trypsin-dispersed single cells were added to agarose (0.25%) or agar (0.3%) in MEM plus 15% FCS, and 4 ml were poured on the base layer and allowed to set at room temperature. Incubation was at 37° in an atmosphere of 5% CO₂ and 95% relative humidity. Samples were tested in triplicate, and colony formation was determined after a 3-week incubation by counting the number of colonies per dish and determining colony diameters using an eyepiece reticle. Cells were tested at 10-fold dilutions ranging from 10^2 to 10^6 cells/dish.

In Situ Hybridization. HSV virions prepared as described (5) were used to obtain unit-length viral DNA which was labeled by nick-translation (39) with ^[3H]TTP (specific activity, 60 Ci/mmol) to give a final product with a specific activity of 1 to 2 ×10^7 cpm/µg DNA. In situ hybridization was done by the method of Pardee and Gall (36) for viral DNA and by the method of Harrison et al. (23) for viral RNA. Hybridization was carried out at 65° for 16 hr in Denhardt's buffer (11). Slides were dipped in Kodak NTB emulsion diluted 1:1 with water and exposed at 4° for 1 to 15 weeks.

Radioimmunoprecipitation. Mock-infected and HSV-2-infected cells were labeled between 3 and 24 hr postinfection either with 50 µCi of ^[35]S)methionine (Amersham/Searle Corp., Arlington Heights, III.) in 1 ml MEM containing 5% dialyzed FCS and 10% the usual concentration of methionine or with 20 µCi (14C)glucosamine (Amersham/Searle) in 1 ml MEM containing 5% FCS. The cells were washed 3 times with ice-cold Tris-buffered saline [0.01 M Tris (pH 7.4):0.001 M Na_2HPO_4:0.14 M NaCl], scraped, and suspended in Tris-buffered saline. The cells were sedimented at 1200 × g for 5 min at 4°C and extracts were prepared by resuspending the cells in 0.1 ml Tris (pH 8.0):0.5% Nonidet P-40:0.5% sodium deoxycholate:10% glycerol:2% ethanol:0.25 M phenylmethylsulfonyl fluoride and gently mixing for 1 hr at 4°C. Cell extracts (0.5-ml portions) were mixed with serum (20 µl) and subsequently mixed with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N. J.), and the immunoprecipitated proteins were analyzed by sodium dodecyl sulfate:polyacrylamide gel electrophoresis as described (47).

RESULTS

Properties of 10E2 Cells. The saturation density of the 10E2 cells in medium containing 10% serum remained constant through passages 30 to 35 following their clonal isolation and then progressively increased (Chart 1). Cell growth in medium containing 1% serum was not observed prior to passage 35, and cells tumorigenic in nude mice first appeared at passage 54 (Chart 1). Prior to passage 35, the cells could be maintained for at least 8 to 10 weeks without dislodging, despite weekly refeeding with growth medium. During this period, the cells displayed an epithelioid morphology (Fig. 1A). Subsequent to passage 35, the cells required more frequent subculturing, accompanied by a more heterogeneous cell population.

Prior to passage 44, the 10E2 cells showed no colonies in either agarose- or agar-containing medium (≤0.0003%). Colony formation in agarose (≤1%) was first observed at passage 48 and in agar (≤0.05%) at passage 54, but the diameter of the colonies remained small (≤0.05 mm), even when tumori-
Tumorigenic Transformation by HSV

Chart 1. Properties of 10E2 cells following clonal isolation (passage 0). The cells were passaged at 6- to 8-day intervals at a 1:8 split ratio. Cell growth in medium containing 1% (•) and 10% (A) serum was determined by seeding 5 x 10^5 cells in 75-sq cm flasks, refeeding on Day 5, and counting on Day 8. Tumorigenicity studies were carried out by inoculating 10^7 cells s.c. in the anterolateral region of the dorsum of 5 nude mice and maintaining the animals for at least 8 weeks.

The designation of “transformed” with respect to untreated or UV-HSV-infected cells was limited to those foci which proved tumorigenic in nude or conventional (immunocompetent) mice. Since SV40-transformed cells could be identified readily by their morphology (Fig. 1D) and by the presence of T-antigen, they were further classified as being tumorigenic or nontumorigenic in nude mice.

**Tumor Induction.** Tumors at the site of inoculation in nude mice were evident within 2 to 4 weeks and in conventional mice within 3 to 5 weeks, and their size increased progressively. Regressive tumors in nude mice were not observed with spontaneously, UV-HSV-, or SV40-transformed cells, while in conventional mice, tumors induced by SV40-transformed cells, but not by spontaneously or UV-HSV-transformed cells, regressed. Pathological examination indicated that all tumors were fibrosarcomas exhibiting varying degrees of undifferentiation.

All foci were tested in nude mice within 5 passages following their isolation. Spontaneously and UV-HSV-transformed cells showed a 100% tumor incidence when 10^7 and 10^6 cells were inoculated, while 20 to 100% of the animals developed tumors with 10^5 cells. Nontumorigenic morphological variants remained nontumorigenic for at least 25 subcultures. Three spontaneously transformed foci and 5 foci transformed by UV-HSV were cloned from Petri dishes. The clonal sublines showed the same tumor incidence in nude mice as did the parental cells. Several spontaneously and UV-HSV tumorigenically transformed foci, when tested in conventional mice, showed the same tumor incidence as in nude mice.

SV40-transformed foci were classified as tumorigenic even if only one of 5 nude mice developed tumors when 10^7 cells were inoculated. None of the SV40-transformed cells formed tumors when 10^5 cells were inoculated. All SV40-transformed cell-induced tumors were excised, propagated in vitro, and shown to contain T-antigen. Nontumorigenic SV40-transformed cells were present subsequent to passage 54. The plating efficiency of the cells on plastic remained constant at 50 to 60% from passage 5 through passage 61.

Cells with properties comparable to those described above were obtained readily by reconstituting low-passage 10E2 cells from the freezer or by recloning.

**Selection of Transformed Cells.** Infection of 10E2 cells with SV40 led to the appearance of well-demarcated foci of piled cells (Fig. 1D). Similar foci were never seen in untreated or in UV-HSV-infected cultures, although a variety of contact-inhibited morphological variants were observed in some of these cultures. When these morphological variants were isolated and propagated, cells tumorigenic in nude mice were readily identified as being spindle shaped and contact inhibited (Fig. 1, B and C). Spontaneously, UV-HSV-induced, and SV40-induced transformed cell foci were distinct (Fig. 2), and foci induced by UV-HSV (Fig. 1C) were indistinguishable from spontaneous foci (Fig. 1B), which appeared only after prolonged subculturing.

Fig. 1. Microscopic appearance of Giemsa-stained 10E2 cells. A, control cells at passage 8; tumorigenic cells transformed spontaneously (b), by HSV (c), or by SV40 (d). Scale: 100 = 1 mm.

Fig. 2. Appearance of Giemsa-stained UV-HSV- and SV40-transformed cell foci. A, control untransformed cells; B, UV-HSV-transformed foci; and C, SV40-transformed foci.
formed cells remained nontumorigenic for at least 25 subcultures. When SV40-transformed cells (tumorigenic and nontumorigenic) were cloned by isolating well-dispersed colonies either from plastic or from agarose or agar, the tumorigenic potential of the clonal sublines was similar to that of the parental cells.

Transformation by UV-HSV. Actively growing 10E2 cells were infected with UV-HSV (2 to 12 min irradiation) or were mock infected with medium alone or with medium containing extracts from uninfected Vero cells. The interval between subculturing (2 to 10 weeks) was determined by the passage level of the cells used. The remaining cultures at each passage were maintained for observation until the cells dislodged or the experiment was terminated.

Infection with 2-, 4-, and sometimes 6-min UV-HSV at a multiplicity of infection of 1 to 4 often resulted in breakout of infectious virus within 1 to 3 weeks. To minimize the breakout of HSV, PAA, an inhibitor of HSV DNA polymerase (31, 43), was added at a concentration (20 μg/ml) sufficient to inhibit >95% of HSV DNA synthesis and virus plaque formation (Chart 2). The PAA, which showed no adverse effect on cell growth or plating efficiency, was removed on Day 7.

Foci of spindle-shaped cells in UV-HSV-infected cultures (Fig. 1C) appeared within 1 to 3 months (1 to 4 subcultures), depending on the passage level of cells used for infection. These foci were isolated and propagated for testing, as were any morphologically altered areas lacking spindle-shaped cells which appeared either in mock-infected or virus-infected cultures. In several experiments, "normal" cell areas distant from foci were randomly isolated and propagated for testing. Prior to termination of an experiment, the total cell populations were tested in nude mice.

Most experiments were carried out with a virus multiplicity of 2 to 4. The results from a typical experiment are summarized in Table 1, where passage 17 cells were infected at a multiplicity of 4. Cultures infected with 4-min UV-HSV in the absence of PAA were destroyed due to breakout of HSV. Toxicity was observed with 4-min UV-HSV and to a lesser extent with 6-min UV-HSV. Foci of spindle-shaped cells appeared in cultures infected with 6-min UV-HSV, but not in the remaining infected or control cultures. The 11 foci isolated from 6-min UV-HSV-infected cultures (PAA treated and untreated) were tumorigenic in nude mice, while a variety of morphologically altered areas which were also isolated from the 6-min UV-HSV-infected cultures proved nontumorigenic (not shown). Only the 6-min UV-HSV-infected cell population was tumorigenic in nude mice.

A total of 14 experiments were carried out with 10E2 cells infected with UV-HSV with and without PAA treatment. In some experiments, cultures treated with IdUrd (20 μg/ml) were included, with the time of treatment being varied from 2 to 8 hr so that the cytotoxic effects of the drug were comparable to those observed with 6-min UV-HSV. The findings may be summarized as follows.

Tumorigenic cells in mock-infected cultures were initially observed when passage 40 cells were used. This differed from other findings where tumorigenic control cells did not appear until passage 54 (Chart 1) but is consistent with studies which suggest that cells maintained under experimental "stress" conditions of frequent refeeding and infrequent subculturing may transform sooner than cells subcultured at more frequent intervals (1).

Tumorigenic cells in UV-HSV-infected cultures were observed in 10 of 12 experiments carried out with passage 38 or earlier cells. In the 10 positive experiments, cultures treated with PAA showed tumorigenic cells, while in only 5 experiments were tumorigenic cells observed when PAA was omitted. This may be attributed either to breakout of infectious virus or to cell death without virus breakout in the absence of PAA.

Tumorigenic transformation was not observed in IdUrd-treated cultures.

Tumorigenic transformation by UV-HSV was usually limited to one UV dose (6 min) when virus multiplicities of 2 to 4 were used. With higher virus multiplicities (>4), tumorigenic cells...
also appeared in cultures infected with 8-min-irradiated virus and in one experiment with 10-min-irradiated virus. Conversely, with lower virus multiplicities (<2), tumorigenic cells appeared in cultures infected with virus irradiated for 2 and 4 min.

Tumorigenic transformation was not seen in cultures infected with neutralized virus. Neutralization was carried out prior to UV irradiation, and the virus titers were reduced by 2 to 3 log_{10}. In 3 experiments, tumorigenic cells appeared in cultures infected with virus treated with control serum but not in cultures infected with neutralized virus.

Although virus-induced cytotoxicity was often noted in cultures which ultimately showed tumorigenic foci, cytotoxicity was not required for transformation by UV-HSV, as evidenced when low virus multiplicities (<2) were used.

Cell counts over a 7-day period of control (mock infected) and UV-HSV-infected cultures indicated that virus-induced cytotoxic effects were expressed during the initial 48-hr postinfection. At 24 hr postinfection, the number of cells in control and UV-HSV-infected cultures was comparable and equal to the number of cells seeded. By 48 hr, the control cultures had undergone one cell division, while the relative number of UV-HSV-infected cells which had divided approximated the cytotoxic effects of the virus as determined by plating efficiency.

Subsequent to 48 hr, the doubling time of the cells in control and UV-HSV-infected cultures was similar (16 to 18 hr).

While transformation by UV-HSV was observed when 2 × 10^5 cells were seeded, it was observed rarely with 5 × 10^4 cells. We estimate a frequency of tumorigenic transformation by UV-HSV of 5 × 10^{-4} to 10^{-5} under the test conditions used. Attempts to test >2 × 10^6 cells proved frustrating due to the increased frequency of virus breakout, even when PAA was used.

Properties of Cells Surviving HSV Infection. Infection of 10E2 cells with UV-HSV often resulted in breakout of infectious HSV when low UV doses were used, and in most cases the cultures were completely destroyed. When these cultures were maintained for 2 to 4 months with weekly refeeding, approximately 26% (13 of 49) showed a few surviving cells which ultimately propagated to replenish the cultures. The properties of cells in 10 such cultures were studied with the following findings: (a) cells surviving HSV infection showed no morphological evidence of transformation and were routinely nontumorigenic in nude mice; (b) surviving cells showed no evidence of residual HSV infection by immunofluorescence or by testing for infectious virus by inoculation of extracts on permissive Vero cells; and (c) the sensitivity of surviving cells to HSV infection based on plaque formation was indistinguishable from control cells.

Transformation by SV40. Infection of 10E2 cells with SV40 was carried out in parallel with UV-HSV infection. The cells were infected with virus multiplicities ranging from 40 to 200, and morphologically transformed foci (Fig. 1D) appeared at a frequency of 0.1 to 1% within 3 to 4 weeks. Foci were isolated and shown to be positive for T-antigen in >95% of the cells, while normal areas isolated from outside the foci were negative for T-antigen. When tested in nude mice, >90% of SV40 morphologically transformed foci proved to be nontumorigenic (see Table 4).

Virus Sensitivity and Growth Properties of Transformed Cells. The sensitivity of UV-HSV-transformed cells to infection with HSV (based on plaque formation) was similar to that of control cells, while nontumorigenic SV40-transformed cells were slightly less sensitive (Table 2). The plating efficiencies of transformed cells (spontaneous, UV-HSV, and SV40) were comparable and were only slightly higher than those of control cells. Cells transformed spontaneously or by UV-HSV showed some growth in medium containing 1% serum but significantly less than that of cells transformed by SV40. A similar relationship was noted for cells grown in 10% serum, where spontaneously and UV-HSV-transformed cells were intermediate between control and SV40-transformed cells.

Anchorage Independence of Transformed Cells. Cells from transformed foci and negative control areas were initially tested within 5 passages from isolation for colony formation in agarose and agar in parallel with tumorigenicity testing in nude mice. Most cells were retested several times to determine changes in their growth potential in semisolid medium. Unless indicated otherwise, the results are based on the initial tests.

### Table 2

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sensitivity to HSV</th>
<th>Plating efficiency (%)</th>
<th>1% serum</th>
<th>10% serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransformed</td>
<td>0.57 ± 0.1 (8)</td>
<td>55 ± 4 (15)</td>
<td>2 ± 0.6 (24)</td>
<td>16 ± 3 (24)</td>
</tr>
<tr>
<td>Spontaneously transformed</td>
<td>NT</td>
<td>66 ± 7.2 (10)</td>
<td>8 ± 1.3 (12)</td>
<td>35 ± 2.3 (12)</td>
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<tr>
<td>HSV transformed</td>
<td>0.60 ± 0.12 (9)</td>
<td>62 ± 8.8 (13)</td>
<td>9 ± 1.1 (28)</td>
<td>33 ± 3.8 (28)</td>
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<tr>
<td>SV40 transformed</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontumorigenic</td>
<td>0.44 ± 0.13 (8)</td>
<td>65 ± 5.2 (14)</td>
<td>36 ± 5.9 (21)</td>
<td>73 ± 8.1 (21)</td>
</tr>
<tr>
<td>Tumorigenic</td>
<td>NT</td>
<td>68 ± 3.6 (8)</td>
<td>35 ± 3 (8)</td>
<td>69 ± 4.8 (8)</td>
</tr>
</tbody>
</table>

- a Cells infected with 100 to 200 plaque-forming units were overlayed with methylcellulose, and the number of plaques was determined on Day 3. Sensitivity = plaque-forming units in 10E2 cells/plaque-forming units in Vero cells.
- b Cells seeded at 5 × 10^3 in 75-cm^2 flasks were refed on Day 5 and counted on Day 8.
- c Cells transformed by SV40 were tested between passages 3 and 30.
- d Tumorigenic in nude or in conventional mice.
- e NT, not tested.
- f Numbers in parentheses, numbers of transformed foci tested.
- g Represents minimal values, since cells were being shed into medium.
- h Tumorigenic in nude but not in conventional mice.

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Tumorigenic cells isolated from passage 40 mock-infected cultures used in a transformation experiment showed no colony formation in agar and <1% in agarose, while tumorigenic cells isolated from passage 50 stock 10E2 cultures (see Chart 1) showed ≥44% colony formation in agarose and ≥2% in agar (Table 3). Tumorigenic cells transformed by UV-HSV showed a similar pattern, in that their ability to form colonies in semisolid medium was related to the passage level of cells used for infection. Cloned sublines from cells transformed spontaneously or by UV-HSV showed the same growth potential in semisolid medium as did the parent cell populations (not shown). Growth in semisolid medium of spontaneously and UV-HSV-transformed cells usually improved with increasing cell passage, but in several instances the colony-forming potential of the cells remained low even after 20 or more subcultures (see Table 5, Cells 3-5).

In contrast to spontaneously and UV-HSV tumorigenically transformed cells, SV40 morphologically transformed cells demonstrated good colony formation in agarose and agar, although >90% (93 of 102) of the cells failed to form tumors in nude mice (Table 4). Most SV40 morphologically transformed cells were classified as being nontumorigenic, with ≥15% colony formation in agarose and ≥1% in agar. Some cells in this category acquired tumorigenic potential after 30 or more subcultures, but this was interpreted as being due to spontaneous rather than to SV40-induced events.

To ensure that the observed colony formation in semisolid medium truly reflected the properties of tumorigenic cells in a population, tumor cell explants were also tested. In all cases, the colony-forming ability of explant cells approximated that of their respective parent cells when tested in agarose or agar.

Additional studies were carried out to confirm the differences noted in the ability of SV40 morphologically transformed cells (tumorigenic and nontumorigenic) and UV-HSV tumorigenically transformed cells to form colonies in semisolid medium (Tables 3 and 4). A UV-HSV tumorigenically transformed cell focus was cloned, and a clonal subline designated 3-5 was infected at passage 7 with SV40. Five transformed foci of piled cells were isolated at 3 weeks for testing (Table 5). The parent 3-5 cells showed poor colony formation in semisolid medium, while the SV40-transformed 3-5 cells (T-antigen positive) showed good colony formation in both agarose and agar. The 3-5 cells and SV40-transformed 3-5 cells both showed a 100% tumor incidence in nude mice when 105, 106, and 107 cells were tested, and the 3-5 cells showed the same tumor incidence in conventional mice. In contrast, the SV40-transformed 3-5 cells, when inoculated in conventional animals, showed initiation of tumor formation, but in every case the tumors regressed and the animals survived.

**SV40 Transformation of BALB/c 3T3 Cells.** Since >90% of SV40 morphologically transformed 10E2 cells failed to form tumors in nude mice (Table 4), studies were carried out to determine the properties of other mouse cells transformed by the same virus. BALB/c 3T3 cells were infected with SV40 using the same procedures as those used with 10E2 cells. Transformed foci of piled cells appeared within 3 to 4 weeks, and these were isolated and propagated for testing. The cells in all foci were shown to contain T-antigen.

Tumor formation in nude mice of SV40 morphologically transformed 3T3 cells was observed with 69% (22 of 32) of the foci tested, while control cells tested 6 times over a period encompassing 18 subcultures were consistently nontumorigenic. All SV40 morphologically transformed 3T3 cells showed efficient colony formation in agarose (13 to 90%) and agar (1 to 22%), regardless of the tumorigenic potential of the cells.

**Persistence of HSV in Transformed Cells.** Several procedures were used for detecting HSV DNA, RNA, or proteins in tumorigenic cells transformed by UV-HSV.

Cells grown on coverslips and fixed in ethanol or acetone were tested by indirect immunofluorescence using rabbit anti-HSV-1 and anti-HSV-2 sera (22), human anti-HSV-2 sera (25), and sera from conventional mice bearing UV-HSV-transformed

### Table 3

<table>
<thead>
<tr>
<th>Passage no. infected</th>
<th>No. of foci tested</th>
<th>Transformed cell</th>
<th>Collected in agarose</th>
<th>Collected in agarose</th>
<th>Diametere(mm)</th>
<th>Diametere(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>20</td>
<td>5-75</td>
<td>0.1-2.0</td>
<td>1-38</td>
<td>0.1-1.4</td>
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<tr>
<td>1</td>
<td>1</td>
<td>63</td>
<td>0.2-1.2</td>
<td>40</td>
<td>0.1-0.9</td>
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<tr>
<td>14</td>
<td>26</td>
<td>14-90</td>
<td>0.1-1.0</td>
<td>1-55</td>
<td>0.1-0.8</td>
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<tr>
<td>3</td>
<td>+</td>
<td>27-90</td>
<td>0.2-1.0</td>
<td>12-35</td>
<td>0.2-0.8</td>
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<tr>
<td>17</td>
<td>14</td>
<td>12-90</td>
<td>0.1-2.0</td>
<td>50</td>
<td>0.1-0.8</td>
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<tr>
<td>20</td>
<td>11</td>
<td>4-51</td>
<td>0.1-1.1</td>
<td>0.5-18</td>
<td>0.1-0.8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>36</td>
<td>0.2-1.1</td>
<td>0.001</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>17</td>
<td>5-90</td>
<td>0.1-1.4</td>
<td>5-31</td>
<td>0.1-0.7</td>
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<tr>
<td>3</td>
<td>+</td>
<td>10-50</td>
<td>0.2-1.0</td>
<td>0.1-0.8</td>
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</tr>
<tr>
<td>30</td>
<td>5</td>
<td>18-90</td>
<td>0.2-1.4</td>
<td>1-28</td>
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</tbody>
</table>

*All cells were tumorigenic in nude mice.

**Table 4**

| Passage number of 10E2 cells used for infection with SV40. Transformed foci were isolated after 3 to 4 weeks and were tested for tumorigenicity and colony formation in semisolid medium within 5 passages from isolation.

### Table 5

| Passage number of 10E2 cells from which spontaneously transformed foci were isolated. Passage number of 10E2 cells used for infection with UV-HSV. Transformed foci were isolated within 1 to 2 passages. Spontaneously and UV-HSV-transformed cells were tested for tumorigenicity and growth in semisolid medium within 5 passages from isolation.
cell lines were tested between passages 3 and 35. Positive with serum from tumor-bearing animals. When immunoprecipitated with rabbit or human anti-HSV sera, bands were observed in gels from productively infected cell extracts of control cells, and cells productively infected with HSV were absent of complement.

With 5 sera from tumor-bearing mice. These sera also failed to neutralize virus when tested in either the presence or the absence of complement.

Tumorigenic cells transformed spontaneously or by UV-HSV, control cells, and cells productively infected with HSV were labeled with [35S]methionine or [14C]glucosamine and immunoprecipitated with normal rabbit serum, rabbit or human anti-HSV-2 positive serum, or serum from conventional mice bearing HSV-transformed cell tumors. HSV-specific proteins were observed in gels from productively infected cell extracts when immunoprecipitated with rabbit or human anti-HSV serum, but not with control serum or with serum from tumor-bearing animals. No HSV-specific bands were seen in gels containing extracts of cells tumorigenically transformed by UV-HSV when reacted with rabbit or human anti-HSV serum or with serum from tumor-bearing animals.

HSV productively infected 10E2 cells and UV-HSV tumorigenically transformed cells (PAA treated and untreated) were tested for viral DNA and RNA by in situ hybridization. Six transformed cell lines were tested between passages 6 and 35. Autoradiographic grains were observed in productively infected cells tested for viral DNA and RNA following a 1-week exposure, but grains were not observed following a 15-week exposure in cells tumorigenically transformed by UV-HSV. Based on a genome size of 108 daltons for HSV, we estimate that at least 10 to 15% of the viral genome would have to be present at a level of one DNA copy/cell for detection by in situ hybridization, using our whole-genome probe with a specific activity of 1 to 2 x 107 cpm/μg DNA.

Several UV-HSV tumorigenically transformed 10E2 cell lines were also tested by McDougall, using in situ hybridization procedures which detected HSV RNA sequence in UV-HSV-transformed hamster cells (8). No evidence of expressed HSV RNA sequences was found in the transformed 10E2 cells.

**DISCUSSION**

We have compared the properties of cloned mouse cells (10E2) transformed spontaneously, by UV-HSV, or by SV40. Spontaneously transformed cells were not observed prior to the 40th passage of the cells. Cells transformed spontaneously or by UV-HSV were identified morphologically as being spindle shaped and contact inhibited (Fig. 1, B and C). The transformed nature of these cells was confirmed by demonstrating their ability to form tumors in nude or conventional (immunocompetent) mice. Cells transformed by SV40 (Fig. 1D) were identified by their morphology, by their loss of contact inhibition, and by the presence of T-antigen. Cells morphologically transformed by SV40 were further classified as being tumorigenic or non-tumorigenic in nude mice (Table 4).

Cells morphologically transformed by SV40 (tumorigenic and nontumorigenic) expressed several in vitro properties usually associated with a transformed phenotype (Tables 2 and 5; Fig. 1D), including: (a) high saturation density and loss of contact inhibition; (b) growth in serum-deficient medium; and (c) anchorage independence; spontaneously transformed cells did not express these properties (Tables 1 to 3; Fig. 1B). Based on their phenotypes, which included tumorigenic potential and in vitro properties, we found that cells transformed by UV-HSV were indistinguishable from those transformed spontaneously. The changes which were observed in the in vitro properties of spontaneously and UV-HSV-transformed cells were related more closely to their passage level than to their tumorigenic potential (Table 3).

Transformation by UV-HSV was hindered to some extent by the breakout of infectious virus. The use of PAA at a concentration which reduced viral DNA replication without adversely affecting cell growth or survival (Chart 2) helped alleviate this problem. We have no reason to suspect that PAA played any significant role in reducing viral DNA replication.

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* J. McDougall, personal communication.
role other than to reduce the breakout of virus and concomitant destruction of the cultures. Transformation by UV-HSV was observed in the absence of PAA, albeit at a lower frequency.

Tumorigenic cells which appeared in UV-HSV-infected 10E2 cultures expressed no detectable viral markers to prove that the cells had been transformed by virus. The fact that tumorigenic cells appeared in UV-HSV-infected cultures, but not in control or IdUrd-treated cultures, or in cultures infected with neutralized virus, suggested a role for UV-HSV in the transformation process. Two alternative possibilities were also considered: (a) infection with UV-HSV and the accompanying cytototoxicity may have selected preexisting tumorigenic cells in the population; and (b) infection with UV-HSV may have caused prolonged cytotoxic effects, which permitted individual cells in infected cultures to undergo more cell doublings and consequently to transform sooner than do cells in control cultures. Several findings argue against these alternative possibilities: (a) tumorigenic cells were observed in cultures infected with low virus multiplicities where cytotoxicity was not evident. When virus multiplicities of 2 to 4 were used, tumorigenic cells were seen in cultures infected with 6-min-irradiated virus but not in cultures infected with virus irradiated <6 min. Since the degree of cytotoxicity by UV-HSV was inversely related to the UV dose, tumorigenic cells should have appeared in cultures infected with virus irradiated <6 min if selection alone were responsible; (b) no evidence was found to suggest that UV-HSV-induced cytotoxicity persisted for a prolonged period, (c) cells surviving breakout of infectious virus following infection with UV-HSV did not demonstrate transformed properties or tumorigenic potential.

The phenotypic properties of tumorigenic 10E2 cells transformed by UV-HSV were significantly different from those of cells morphologically transformed by SV40 (tumorigenic and nontumorigenic) (Tables 2 to 5; Fig. 1). Consequently, while 10E2 cells transformed by SV40 acquired virus gene sequences which were apparently responsible for the expression of in vitro properties by the cells usually associated with transformation (Tables 2, 4, and 5; Fig. 1D), cells transformed by UV-HSV showed no in vitro properties distinct from spontaneously transformed cells which could be ascribed to retained virus gene sequences (Tables 2 and 3; Fig. 1, B and C). Similarly, while 10E2 cells transformed by SV40 demonstrated properties consistent with their expression of transplantation rejection antigens, in that tumors developed in nude but not in conventional mice, neither spontaneously nor UV-HSV-transformed cells demonstrated such properties, as evidenced by their ability to form tumors in both nude and conventional mice (Table 5). Similar conclusions with respect to the absence of transplantation rejection antigens have been reported for hamster cells transformed by UV-HSV (12, 24) where viral DNA is present (7, 17) and viral proteins are expressed (18, 37).

The possibility that 10E2 cells tumorigenically transformed by UV-HSV may have retained a relatively small segment of the viral genome was not excluded by our procedures. It is well established that transformation by other oncogenic DNA viruses, such as SV40, polyoma, and adenovirus, requires integration into the cell DNA of only a portion of the viral genome which is expressed in transformed cells (i.e., T-antigen). The absence of a relatively large amount of HSV DNA in transformed 10E2 cells, and its presence for at least a limited time in transformed hamster cells (7, 8, 17, 34), suggests a difference in the response of these cells to infection with UV-HSV. At present, no evidence is available concerning the state of the HSV genome in transformed hamster cells. One possibility, though admittedly speculative, is that retained HSV gene sequences may serve to "immortalize" the hamster cells, similar to what has been described for the Epstein-Barr virus in human cells (33). "Immortalization" by HSV may have particular relevance in primary hamster cells which normally undergo crisis after a limited number of cell doublings and either die or less frequently give rise to established cells lines (13, 27, 28, 44).

The majority (>90%) of 10E2 cells morphologically transformed by SV40 were nontumorigenic (Table 4), although the cells expressed several in vitro properties usually associated with a transformed phenotype (Tables 2 and 4; Fig. 1D). Testing for tumor formation was carried out in syngeneic nude mice to minimize immunological factors which could cause rejection of tumorigenic cells (16). Despite this precaution, we are aware that nude mice have been shown to be incapable of supporting tumor formation with certain tumor cells (45). The possibility that the majority of SV40-transformed 10E2 cells did not form tumors for reasons other than that they were nontumorigenic seems unlikely, based on 2 findings: (a) some SV40 morphologically transformed cells which initially were nontumorigenic did acquire tumorigenic potential after multiple subcultures. We attributed this to spontaneous rather than to SV40-induced events; (b) when tumorigenic cells transformed by UV-HSV were morphologically transformed by SV40, their tumor incidence in nude mice remained the same, but they no longer formed tumors in conventional mice (Table 5).

The inability of >90% of SV40 morphologically transformed 10E2 cells to form tumors in nude mice was apparently due to cellular rather than to viral properties (40), since 69% of 3T3 cells morphologically transformed by the same virus were tumorigenic. In the 10E2 system, therefore, the transforming and oncogenic potentials of SV40 were more closely related to the transforming activity of UV-HSV than to the abilities of the virus to induce tumorigenicity in all cell lines (reviewed in Ref. 45). Our findings with BALB/c-derived 10E2 cells indicated that none of the in vitro properties that we tested was necessarily correlated with the ability of transformed cells to form tumors. In the case of anchorage dependence, the findings with spontaneously and UV-HSV tumorigenically transformed 10E2 cells were less conclusive, since tumors were observed with ≥10⁵ cells, while only 10⁴ cells were tested in semisolid medium. The finding that colony formation in semisolid medium of tumor explants approximated the frequency observed with parental cells, however, is consistent with the conclusion that anchorage independence was not necessarily correlated with tumorigenicity in 10E2 cells. The situation with 3T3 cells was quite different, however.

³ B. Hampar, G. Khoury, and M. Zweig. Isolation of SV40-transformed mouse cells expressing a truncated T-antigen, manuscript in preparation.
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