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Quantitative Assay for Transformation of 3T3 Cells by Herpes Simplex Virus Type 2

RONALD DUFF[1] AND FRED RAPP*

Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033

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The interaction of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) with Swiss/3T3 cells was investigated. Virus-induced cytopathic effects developed in the absence of production of infectious virus. HSV-2 inactivated with UV light (2, 4, 6, and 8 min) also induced cell death in the absence of virus replication. Cell death was not detectable after infection by HSV-2 that had been inactivated by UV irradiation for 10, 12, and 14 min. 3T3 cells infected with UV-inactivated virus (10 and 12 min) continued to replicate past the contact-inhibited monolayer normally associated with these cells. Infection of 3T3 cells with UV-irradiated HSV-2 also induced the development of transformed foci. Transformed cells with an epithelioid or fibroblastoid morphology were identified and isolated. All HSV-2-transformed cell lines contained HSV-2-specific antigens detectable by immunofluorescence techniques. The maximum frequency of HSV-2-induced transformation was 3×10^5 PFU per transformed focus, and the observed transformation could be inhibited by pretreatment of the virus with specific antiserum. No type C particles were detected within five cell culture passages after transformation by HSV-2. Type C virus particles were detected after 10 cell culture passages of the HSV-2-transformed cell lines.

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) induce the transformation of normal cells into cells with altered properties (2-5, 9, 10). Cells from a variety of animal species have been used in these experiments, including hamster, rat, mouse, and human cells. However, the frequency of transformation observed in these reports has generally been too low to allow a quantitative analysis of the transforming event.

Experiments described in this communication illustrate the use of 3T3 cells as the host cell for development of a quantitative assay designed to evaluate the transforming potential of HSV-2. The system uses HSV-2 inactivated by UV irradiation and yields a frequency of transformation sufficiently large to permit semiquantitation of the transforming frequency induced by the virus. The system should be useful for determination of the events regulating transformation by HSV, as well as the mechanism of virus latency within the transformed cell.

MATERIALS AND METHODS

Cells. Swiss/3T3 cells were obtained from A. Lipton (Pennsylvania State University College of Medicine, Hershey, Pa.) and grown as previously described by other investigators (13). They were grown in 100-mm plastic petri dishes in Dulbecco's modification of Eagle medium supplemented with 10% calf serum and 0.225% $NaHCO_3$.

Rabbit kidney cells for use in HSV-2 assays were prepared from weanling animals (21 to 28 days old) by dispersion with 0.25% trypsin (3). These cells were grown in 60-mm plastic petri dishes containing Eagle medium with 10% fetal calf serum, 10% tryptose phosphate broth, and 0.225% $NaHCO_3$.

Virus. HSV-2 (strain 333) and HSV-1 (strain Patton) were obtained from W. Rawls (Baylor College of Medicine, Houston, Tex.). Virus stocks used for the experiments in this paper were prepared as previously described (3). HSV-1 and HSV-2 stocks were passed a total of three times in human embryonic kidney cells (at low multiplicity of infection of 0.01 PFU/cell) before use in the 3T3 cells. Virus stocks for infectivity and for transformation studies were inactivated by UV irradiation at a dose of 42 ergs/s/mm^2. The appropriate virus concentration (1.5 ml) was placed in a 60-mm plastic petri dish and exposed to the UV source accompanied by continuous, gentle shaking.

Virus infectivity assay. The assay method for HSV-1 and HSV-2 in rabbit kidney cells has been previously described (3, 11). Briefly, monolayers of rabbit kidney cells were inoculated with dilutions of the herpesvirus that were to be assayed for infectivity. After virus adsorption for 1 h, the cell cultures were overlayed with Eagle medium containing 10% fetal calf serum, 10% tryptose phosphate broth, 0.225%

[1] Present address: Department of Virology, Abbott Laboratories, North Chicago, Ill. 60064.

$NaHCO_3$, and 0.5% methylcellulose. A solution of 0.001% neutral red was added to each petri dish 4 days after infection, and the plaques were counted 2 to 3 h later.

Virus transformation assay. Swiss/3T3 cells (10^6 cells) were infected with HSV-2, strain 333 (5×10^6 PFU) while in suspension with gentle shaking. One hour after virus infection, each virus-cell mixture was placed in four 60-mm plastic petri dishes containing 5 ml of Dulbecco's modification of Eagle medium supplemented with 10% calf serum. Medium was changed on each culture every 3 days. Culture dishes were stained with Wright Stain 28 days after initial virus infection, and transformed foci were identified and counted microscopically.

IF techniques. The indirect method of immunofluorescence (IF) was used to detect HSV-specific antigens in transformed Swiss/3T3 cells. This technique has been described in detail elsewhere (3, 5).

The presence of RNA tumor virus-specific gs antigens was detected by direct IF tests (12). Cells were grown on round cover slips (18 mm in diameter) for 24 h. The cultures were washed three times in Tris-buffered saline (pH 7.4), air dried, and fixed in acetone for 3 min. The presence of gs antigens was determined by using specific antimurine gs serum obtained from R. E. Wilsnack (Huntingdon Research Center, Baltimore, Md.). Serum (0.01 ml) was placed on each culture and adsorbed for 30 min, and the cultures were washed three additional times with Tris-buffered saline. The J20-NRK cell line was used as the positive control in these experiments (7). Specific fluorescence was detected with a Zeiss microscope with a UV light source.

Neutralization of HSV. HSV-2 (0.5 ml containing 10^7 PFU) was mixed with 0.5 ml of undiluted serum obtained from a convalescent patient after a primary infection with HSV-2 (strain 333). Human serum containing no detectable neutralizing antibodies against HSV-1 or HSV-2 was used as a negative control. Virus-serum mixtures were incubated in a 37 C water bath for 1 h. After incubation, the virus-serum mixtures were mixed with the 3T3 cells and infected in suspension as described above. Twenty-eight days after infection, the cultures were stained with Wright Stain and the transformed foci were counted.

Detection of C-type particles. HSV-2-transformed 3T3 cells were examined for the presence of RNA-containing virus particles with a density of 1.15 to 1.18 g/cm³ in sucrose gradients (12). Cell lines were grown in Eagle medium supplemented with 10% dialyzed fetal calf serum, 0.225% $NaHCO_3$, and 100 μCi of [^{3}H]uridine/ml. After 18 h the medium was examined for ^{3}H-labeled type C particles. The supernatant fraction was centrifuged at 27,000 $\times$ g for 10 min and the pellet was discarded. The supernatant fraction was again centrifuged through 20 ml of a 10% sucrose gradient (Schwarz-Mann; ribonuclease free) at 30,000 $\times$ g for 2 h. The pellet was suspended in 1 ml of ethylenediaminetetraacetic acid phosphate buffer (0.0015 M EDTA, 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.0015 M NaH_2PO_4, 0.02 M KCl), and a 0.2-ml amount of the pellet was centrifuged through 15 to 60% sucrose at 19,000 $\times$ g. The radioactivity was determined after precipitation with tricarboxylic acid and a scintillation cocktail of toluene and omnifluor.

RESULTS

Replication of HSV in Swiss/3T3 cells. Swiss/3T3 cells were tested for their ability to support the normal replicative cycle of either HSV-1 or HSV-2 before the initiation of transformation studies. Confluent monolayers of cells infected at a multiplicity of infection of 1 PFU/cell developed extensive virus-specific cytopathic effects (CPE). Apparently, progeny HSV was not produced (Fig. 1). Neither HSV-1 nor HSV-2 appeared to replicate. The step in the virus replicative cycle that is blocked and is responsible for the inhibition of virus replication has not been determined at the present time; however, the development of virus-induced CPE in the absence of normal virus replication indicates that some early steps in the replicative cycle of HSV are not inhibited.

The extensive CPE, which developed between 6 and 24 h after virus infection, was followed by death of the host cell and made it necessary to partially inactivate the HSV-2 before use in cellular transformation studies. An initial step in the development of a transformation system

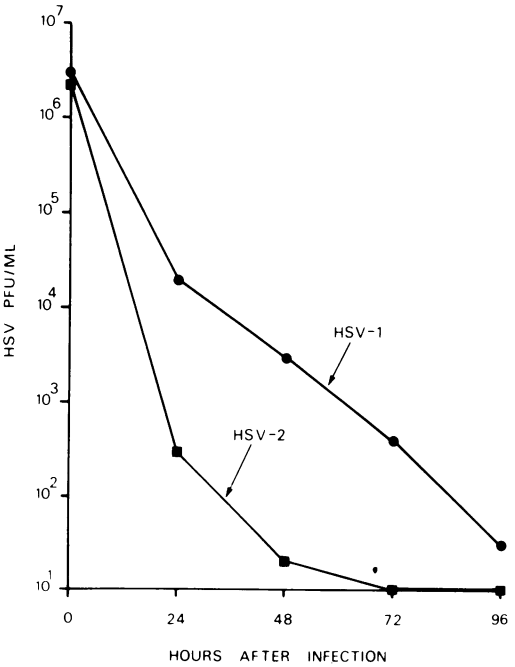

FIG. 1. *Replication of HSV-1 (strain Patton) and HSN-2 (strain 333) in monolayer cultures of Swiss/3T3 cells. Infected cultures were harvested 0, 24, 48, 72, and 96 h after initial virus infection. The harvested samples were frozen and thawed twice, sonically treated, and assayed for infectious virus (PFU) in rabbit kidney cell monolayers by the plaque method.*

utilizing HSV-2 and Swiss/3T3 cells was designed to establish the effects of UV-irradiated virus on the growth of the cells. Swiss/3T3 cells were infected at a multiplicity of infection of five while in suspension with UV-irradiated HSV-2, as described. After virus adsorption, the infected cells were placed in 60-mm plastic petri dishes at a cell density of 2.5×10^5 cells per dish. One day after virus infection, the early CPE induced by HSV-2 was related to the length of UV inactivation of the virus. Longer UV treatment of the virus lessened the CPE induced by the inactivated virus. Five days after initial virus adsorption, cells from five petri dishes for each UV irradiation time of virus were harvested by trypsinization. The HSV-2 had been irradiated for 0, 2, 4, 6, 8, 10, 12, or 14 min. Cells in each petri dish were counted in a Coulter counter and compared to uninfected control cultures (Fig. 2). Cultures containing cells that had been infected with HSV-2 after irradiation for 2, 4, 6, or 8 min were partially inhibited, and the number of cells in each plate was less than the number of cells in control plates. However, cultures containing cells that had been infected by HSV-2 inactivated for 10 or 12 min contained more cells than did the uninfected control cultures. The number of cells in cultures containing virus that was inactivated for 14 min was identical when compared to the uninfected control cultures. The variation in counts did not generally exceed 10%. The increase in the total number of cells per culture treated with virus irradiated for 10 or 12 min was evidence that HSV-2 has the potential to stimulate 3T3 cell growth past normal cell density usually found in contact-inhibited cultures of noninfected cells.

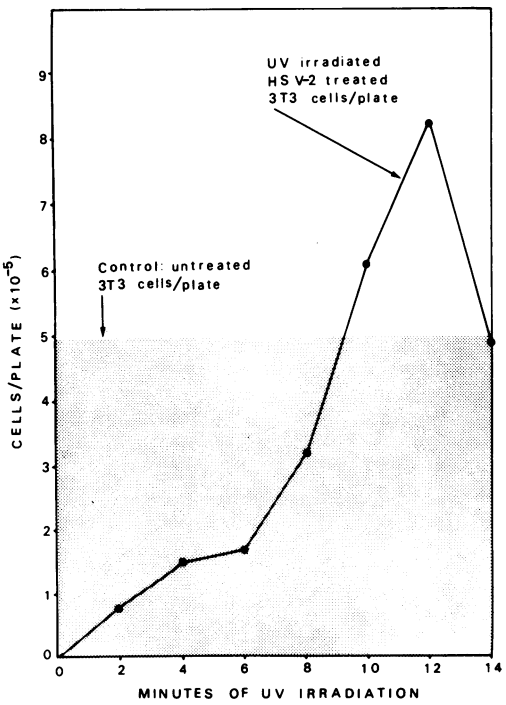

FIG. 2. *Induction of virus-specific cell death and cell stimulation by UV-irradiated HSV-2 in Swiss/3T3 cultures. Cells were infected in suspension by inactivated HSV-2 and grown in 60-mm plastic petri dishes. Five days after initial virus infection, the cells in each plate were harvested by trypsinization, and the number of cells were counted in a Coulter counter. The results are presented as the number of cells per culture dish and are the average of 12 cultures per time of UV irradiation.*

Transformation of Swiss/3T3 cells by UV-irradiated HSV-2. Swiss/3T3 cells were infected with HSV-2 as described above. The infecting virus had been previously irradiated for 2, 4, 6, 8, or 10 min. The infected cultures were grown for 28 days and stained with Wright Stain before the counting of transformed foci. Two morphological types of foci were observed (Fig. 3 and 4). The first type was of fibroblastoid morphology, and the second was somewhat epithelioid or cuboidal in morphology. Cells from foci of both morphological types were isolated from unstained foci in duplicate culture dishes and grown into established cell lines. Cells from the isolated lines could be morphologically differentiated into fibroblastoid and epithelioid cell types. Both contained specific HSV-2 antigens as determined by indirect IF techniques using hamster antiserum or serum from hamsters bearing tumors induced by cells transformed by HSV-2. A granular, cytoplasmic fluorescence was observed in about 10 to 30% of the transformed cells. The antigens were detected after transformation and through 12 passages (when this report was written).

After exposure to Wright Stain, the transformed foci on five replicate plates (for each inactivation time of HSV-2) were counted and compared to the initial number of virus PFU in the inoculum before irradiation (Fig. 5). No transformed foci were observed in uninfected control cultures. The number of transformed foci increased in relation to the length of virus inactivation and reached a maximum between 6 and 8 min of UV irradiation. After 8 min of irradiation, the number of transformed foci decreased.

Neutralization of transforming activity by specific antiserum. A direct method to demonstrate the involvement of HSV-2 in the in vitro transformation of Swiss/3T3 cells is the neutralization of UV-inactivated virus by specific antiserum directed against HSV-2. Previous studies

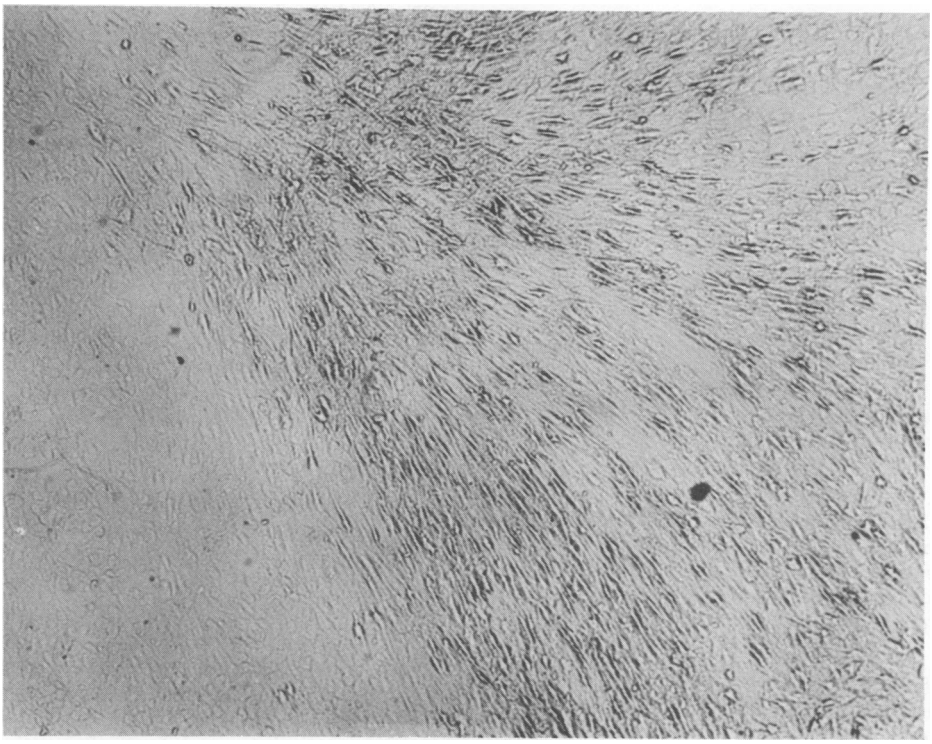

FIG. 3. *Photomicrograph of a transformed focus of Swiss/3T3 cells after infection by UV-irradiated HSV-2 (strain 333). Most cells in the culture have a predominantly fibroblastoid morphology. ×100.*

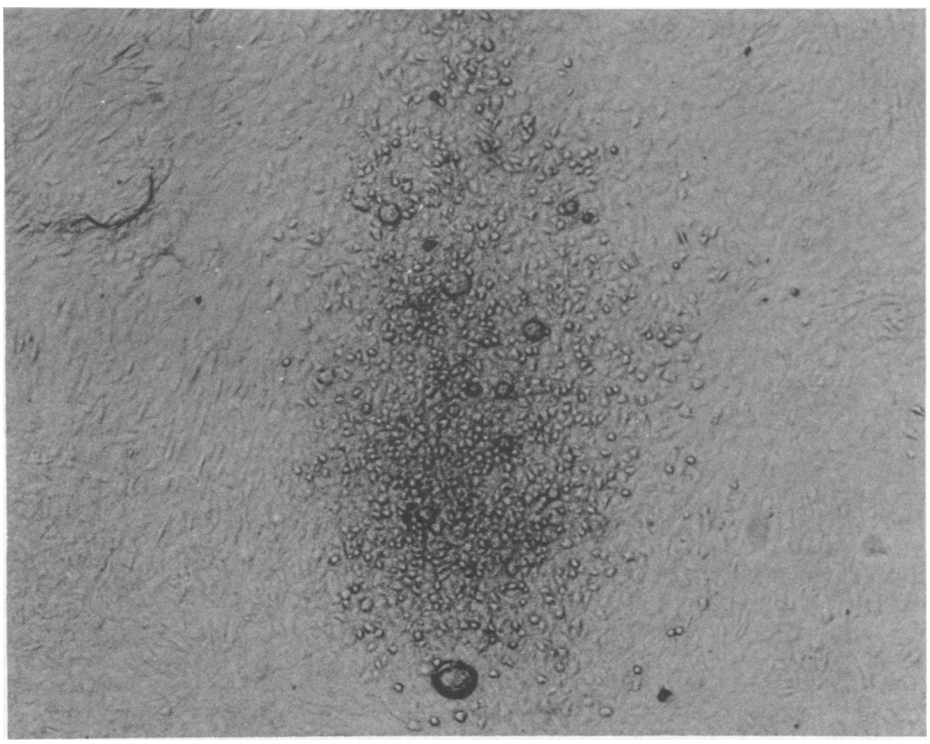

FIG. 4. *Photomicrograph of a transformed focus of Swiss/3T3 cells after infection by UV-irradiated HSV-2 (strain 333). Most cells in the focus have a predominantly epithelioid morphology. ×100.*

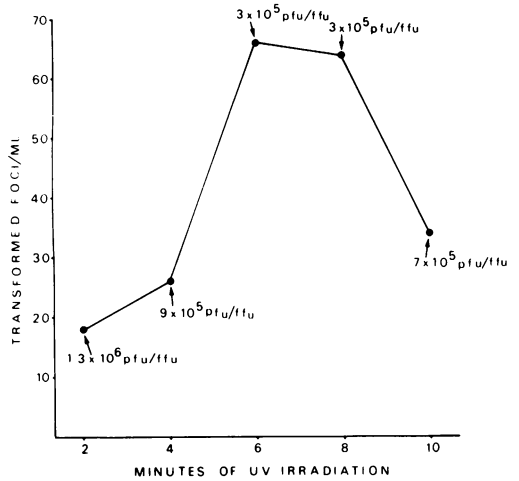

FIG. 5. *Transforming frequency of Swiss/3T3 cells after infection with UV-irradiated HSV-2. Transformed foci were counted 28 days after virus infection of the cells. The number of foci per milliliter of infecting virus is an average of 12 replicate plates for each time of virus inactivation.*

in this laboratory did not permit such tests, due to the extremely low frequency of HSV-2-induced transformation of hamster embryo cells (3). However, the relatively high transforming frequency by HSV-2 in the 3T3 cells permitted meaningful neutralization studies.

HSV-2, after UV irradiation for 4, 6, 8, or 10 min, was incubated with either anti-HSV-2 serum or control serum for 1 h at 37 C. The virus-serum mixtures were then adsorbed onto the 3T3 cells for 1 h while in suspension. The infected cells were placed in 60-mm plastic petri dishes and grown for 28 days. The cultures were then stained with Wright Stain and the number of transformed foci was counted. The results are shown in Fig. 6 and are expressed as the percentage of transformed foci compared to control cultures not treated with serum in which the virus was inactivated for 6 min by UV irradiation. Cultures infected with neutralized virus contained fewer foci than did cultures infected with non-neutralized HSV-2. Focus reduction was not complete; however, when reduction in the number of foci was compared to the reduction of PFU in rabbit kidney cells, the reduction in virus activity was comparable in both cases. The potency of the antiserum used for these experiments was therefore not sufficient to neutralize all virus in the inoculum due to the large amount of virus required to induce statistically significant numbers of transformed foci.

Induction of type C particles in HSV-2-transformed Swiss/3T3 cells. Oncornavirus particles have been induced in 3T3 cells after spontaneous transformation, chemically induced transformation, and virus-induced transformation (1, 8, 13). The activation of these particles was probably the result of an endogenous virus genome within the 3T3 cells. However, the exact role of these viruses in the initial transformation event is not yet clear.

To determine the presence of type C particles in HSV-2-transformed cells, cultures were grown in the presence of [^{3}H]uridine for 18 h. Type C particles were detected by determination of the uptake of radioactive uridine into particles with a density of 1.15 to 1.18 g/cm^3 after density centrifugation in sucrose gradients. By this procedure, no type C particles could be detected in HSV-2-transformed 3T3 cells following five cell culture passages after the initial transformation (Fig. 7A). However, type C particles were detected following 10 cell culture passages after the induction of transformation by HSV-2 (Fig. 7B). Four cell lines were tested with identical results.

The four cell lines were also tested by direct IF techniques at passages 5 and 10 for the presence of oncornavirus gs antigens in the transformed cells. Specific fluorescence could

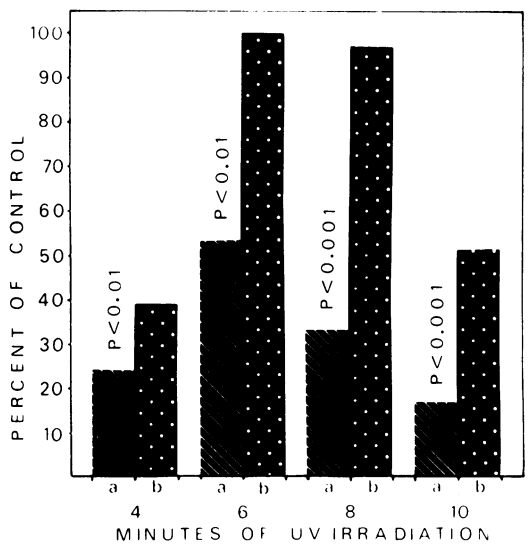

FIG. 6. *Neutralization of the transforming potential of HSV-2 (strain 333) by specific anti-HSV-2 serum (a) and normal serum (b). Specific HSV-2 antiserum was obtained from a patient with a primary herpetic infection induced by HSV-2 (strain 333). The results are presented as the percentage of transformed foci compared to the number of transformed foci that developed in control cultures not treated with serum. The statistical significance of the reduction after virus neutralization was determined by using the Student's t test.*

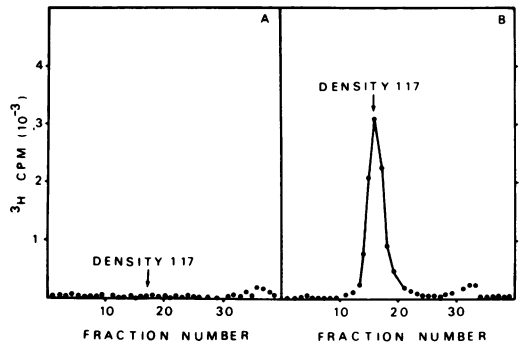

FIG. 7. *Sucrose density gradient centrifugation of virions with labeled RNA from HSV-2 (strain 333)-transformed Swiss/3T3 cells. Transformed cells were examined at cell passage five (Fig. 7A) and at cell passage 10 (Fig. 7B) after initial transformation by HSV-2. Cells were labeled with 100 μCi of [^{3}H]uridine/ml for 18 h and processed as described in Materials and Methods.*

not be detected at passage 5, but was detected in a majority of the cells at passage 10.

Both results can be interpreted as evidence that the initial transformation did not immediately induce the production of type C particles or expression of the type C genome. Continued cell passage then resulted in activation of type C particles and virus release into the culture medium.

DISCUSSION

The technique of quantitative transformation studies is one method for evaluating the transforming potential of HSV, and indirectly, the potential role of HSV isolates in human cancer. Although Swiss/3T3 cells are relatively resistant to the replication of both HSV-1 and HSV-2, they are not resistant to some early events after virus infection which result in the death of the infected cell. This early initiation of cell death makes it necessary to partially inactivate the virus genome by UV irradiation before cellular transformation can be measured. Data presented in this communication confirm that the genome site for cellular cytopathology is more susceptible to UV inactivation than is the site for cellular transformation. As a result, cellular transformation can be detected after a UV irradiation dose capable of removing all detectable CPE induced by the virus. The CPE and transforming potential must be separable if, in fact, transformation is to occur.

Results presented in this paper also show that, under selected conditions, HSV-2 can stimulate 3T3 cells to replicate beyond the normal contact-inhibited monolayer usually reached in cultures of these cells. Similar stimulation of cell replication has also been described using other systems of transforming viruses (13–15). The number of transformed foci induced by inactivated HSV-2 is dependent upon the dose of UV employed. The number of transformed foci increases up to a maximum and then decreases after higher doses. This pattern of transforming frequency is probably the result of two factors: (i) the removal of virus-induced cell death by UV inactivation, allowing infected cells to survive and to express the transforming potential of the virus; (ii) the inactivation of the transforming potential of the virus by UV irradiation resulting in a decrease in the number of transformed foci after longer periods of inactivation. It is impossible to separate the two events in the 3T3 system at this time and, as a result, the maximum transforming potential of HSV-2 cannot be determined. However, the transforming potential of HSV-2 after UV irradiation is relatively high in the Swiss/3T3 system.

Previous studies in this laboratory of the transforming potential of HSV-1 and HSV-2 used hamster embryo cells (3–5). During these studies the transforming frequency was extremely low and, as a result, quantitation was not achieved. Therefore, experiments to determine whether the transforming effect of HSV-2 could be neutralized by specific antiserum were not attempted. The transforming frequency of HSV-2 in 3T3 cells was sufficiently high to allow neutralization experiments, and the role of HSV-2 in cellular transformation was confirmed by neutralization of HSV-2-induced transformation. Reduction in the number of transformed foci after treatment of the virus with specific antiserum is direct evidence of involvement of the virus in the observed transforming event and strengthens the classification of HSV-2 as a tumor virus.

A primary role of oncornaviruses in all tumors has been postulated and investigated in detail (6, 16). However, type C particles have not been consistently detected (12) in hamster cells transformed by HSV-2 (Spiegelman, personal communication; Girardi, personal communication). It was therefore of interest to determine the activation of type C particles in the 3T3 cells which are known to carry a latent oncornavirus genome. Type C particles were detected after 10 cell culture passages of the transformed cells. However, no particles were detected after five cell culture passages. One explanation for this result is that insufficient numbers of particles were produced during the initial transformation event and, as a result, this low level of virus could not be detected by the uridine

labeling technique that was employed.

IF studies did not support this concept and group-specific antigens were also not detectable after five cell passages, but these antigens were detectable after cell passage 10. A second explanation for the absence of type C particles during the early cell passages is that type C particle synthesis was not involved in the primary transformation event but was activated after continued cell passage by nonspecific genome alterations. Such activation of a latent virus genome after transformation by a second, distinct virus has also been found using hamster cells latently infected with measles virus, followed by transformation with the PARA (defective SV40)-adenovirus 7 hybrid (Duff, unpublished data). The release of measles virions is induced in this system after transformation. However, measles virus is not involved in the oncogenic conversion of the cells. It is possible that such a relationship exists between the endogenous type C particles found in the 3T3 cells and the inducer of transformation, HSV-2. The quantitative transformation system described in this paper therefore represents an excellent tool with which to further study the interaction of RNA and DNA-containing viruses within transformed cells.

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