Phenotypic Transformation of the Host Cell Enhances Polyoma Pseudovirion Formation

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Phenotypic transformation of the host cell affected the formation of polyoma pseudovirions. Polyoma virus infection of various transformed derivatives of mouse 3T3 cells resulted in the formation of predominantly pseudovirions, whereas infection of mouse 3T3 cells produced mainly polyoma virus. The effect that transformation of the host cell had on polyoma pseudovirus formation was further demonstrated by using phenotypic revertants isolated from some of the transformed cell lines. The revertants were characterized by their morphology, saturation densities, and colony-forming ability in methylcellulose suspension. By these criteria they were distinct from their transformed parents and similar to 3T3 cells. After infection, the revertants produced predominantly polyoma virus and few pseudovirus. Thus, for the cell lines used in this study, phenotypic transformation enhanced the formation of polyoma pseudovirions.

A polyoma pseudovirion is a virus-like particle which contains a double-stranded, linear fragment of host cell DNA encapsidated in a polyoma protein coat. Polyoma pseudovirions were first reported by Michel et al. (12) and, independently, by Winocour (19) in virus preparations from baby mouse kidney (BMK) cells infected with polyoma virus. Since this discovery, pseudovirions have also been reported in preparations of simian virus 40 (SV40) (11). Their biological significance and the mechanism which results in their formation are unknown.

The relative amounts of polyoma virions and pseudovirions that result from a lytic polyoma virus infection appear to be dependent upon the host cell. Yelton and Aposhian (20) demonstrated that polyoma virus infection of primary mouse embryo (PME) cells produced predominantly pseudovirus, whereas infection of BMK and mouse 3T3 cells yielded mainly polyoma virus. The quantity of pseudovirus formed in these infections correlated with the degree of chromosome fragmentation that occurred in the different cell types. When numerous fragments were produced, as in the case of PME cells infected with polyoma virus, a large proportion of the progeny were pseudovirions. When little fragmentation occurred, as with BMK or 3T3 cells, most of the progeny were infectious polyoma virus. The results of other investigators have also demonstrated this host cell effect on pseudovirus formation in both polyoma virus and SV40 infections. Basilico and Burstin (1)

reported that polyoma virus infection of a mouse-hamster hybrid cell line produced about 90% pseudovirus and only 10% polyoma virus. In this case, polyoma virus replicated poorly in the hybrid cell and little viral DNA was available for encapsidation. Levine and Teresky (11) found that SV40 pseudovirions were produced in primary African green monkey kidney cells but were not produced in the established cell lines, CV-1 and BSC-1. Ritzi and Levine (14, 15) found that the pseudovirions produced in African green monkey kidney cells were formed late during infection and were related to the fragmentation of the host cell chromatin that occurred in these cells. Little or no fragmentation of host cell DNA occurred in the CV-1 and BSC-1 cells. In his studies of the intracellular events involved in polyoma pseudovirus formation, Türler found that pseudovirions were formed preferentially late in infection (18). He further demonstrated a close linkage between cellular and polyoma DNA synthesis and suggested that the proportion of pseudovirus in a viral preparation was inversely related to the amount of polyoma DNA synthesized during the infection.

One explanation for this host cell effect is that embryo cells express some function which influences the outcome of polyoma infection and that this function is turned off in "adult" cells. Evidence exists to show that 3T3 cells are similar to adult cells and distinct from embryo cells (16). Since transformation of cells often leads to reexpression of embryonic functions, we undertook the present study to determine whether transformation of a cell would enhance the formation of polyoma pseudovirus. If it did, it

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would be possible to investigate which of the changes brought on by transformation are important in the formation of pseudovirus. Such knowledge will aid in our understanding of the mechanisms involved in pseudovirus formation and will give us further insights into the lytic interaction of a potentially oncogenic virus with a host cell.

In this paper, we have extended the present knowledge available concerning the host cell effect on the formation of polyoma pseudovirions. Specifically, we investigated the effect the phenotypic transformation of the host cell had on polyoma pseudovirus formation and found that such transformation enhanced the formation of pseudovirus.

MATERIALS AND METHODS

Medium and reagents. The cell culture medium used in these experiments was Dulbecco modified Eagle medium, containing 10% calf serum (GIBCO Laboratories or KC Biologicals). Phosphate-buffered saline (PBS) was prepared according to Dulbecco and Vogt (8). Adsorption buffer for virus infections was Hanks salts solutions buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) adjusted to pH 6.2 with 1 N HCl as described by Benjamin and Goldman (2). [*methyl*-³H]thymidine (20 Ci/mmol) was purchased from New England Nuclear Corp. Receptor-destroying enzyme was purchased from Microbiological Associates, Inc. Methylcellulose (4,000 centipoise) was obtained from Fisher Scientific Co. DNase I and salt-free, highly purified concanavalin A (ConA) were purchased from Sigma Chemical Co.

Virus and cells. The small-plaque variant of the Toronto strain of polyoma virus was used in these experiments and was obtained from H. V. Aposhian. Virus stocks were prepared by infecting Swiss PME cells at a multiplicity of infection of 1 PFU/cell with virus that had been plaque purified on secondary mouse embryo cells.

Cell lines 3T3, 3T6, and 3T12, derived from Swiss White Webster mice, were obtained from Howard Green. The following cell lines, derived from BALB/c mice, were obtained from George Todaro: 3T3-A31 (BALB/c 3T3 clone A31); SVT2 (3T3-A31 transformed by SV40); and B77-A31 (3T3-A31 transformed by avian sarcoma virus, strain B77). PME cells were prepared from whole embryos taken from 15- to 17-day pregnant mice. Cells were grown in 60- or 150-mm plastic petri dishes (Falcon) and were incubated at 37°C in a humidified incubator containing 10% CO₂ and 90% air.

Virus infections. To establish a polyoma virus infection in various cell lines, 1.8×10^6 cells were seeded in 150-mm-diameter plastic petri plates. After the cells had attached (within 4 h after seeding), they were infected at a multiplicity of infection of 1 PFU/cell. The virus was allowed to adsorb to the cells for 1.5 h in adsorption buffer. The adsorption buffer was removed, the infected cells were washed once with medium, and then fresh medium containing 1% calf serum and 1 μ Ci of [³H]thymidine per ml was added

to the infected cultures. This infection procedure is similar to one previously described by Benjamin and Goldman (2).

Virus extraction and purification. Virus was harvested from infected cultures 96 h postinfection by a modification of a procedure described by Crawford (5). Infected cultures were frozen at -20°C and thawed with agitation and scraping with a Teflon scraper to remove the cells from the surface of the culture dish. The cells and medium from the infected cultures were combined and centrifuged for 20 min at $2,000 \times g$ to pellet the cell debris. The supernatant medium was decanted and saved. The pelleted cell debris was suspended in PBS, frozen, thawed, and treated with receptor-destroying enzyme at a final concentration of 40 U/ml for 3 h at 37°C. The suspension was then adjusted to pH 8.5 by adding 1 M NaHCO₃ and incubated for an additional 30 min at 37°C. The cell debris was again pelleted, and the virus-containing, supernatant fluid was decanted and saved. Virus was extracted from the cell debris in this manner at least three times. Hemagglutination assays were performed, as described by Eddy et al. (9), on the supernatant medium and each receptor-destroying enzyme extract to follow the progress of virus extraction. The viral extracts and supernatant culture medium were combined and then centrifuged at 25,000 rpm for 3 h at 30°C, using the Beckman type 30 rotor to pellet the virus. Each virus pellet was suspended in 2 ml of PBS and treated with DNase I at a final concentration of 1 mg/ml for 1 h at 37°C.

The virus was then purified by first centrifuging for 3 h at $60,000 \times g$ through a layer of 10% sucrose into a CsCl cushion. The virus-containing fractions were combined, adjusted to 1.326 g/ml with CsCl, and centrifuged to equilibrium at $100,000 \times g$ for 18 h. The CsCl gradient was fractionated, and the virus-containing fractions (as determined by hemagglutination assays) were combined and then dialyzed against PBS.

Analysis of DNA. Sedimentation analysis of viral and pseudoviral DNA was done by using preformed 5 to 20% alkaline sucrose gradients (pH 12.5). Fractions were collected (0.10 ml/fraction) directly into scintillation vials. The alkalinity of the samples was neutralized with 1 drop of glacial acetic acid, and the radioactivity of each sample was determined in a Packard Tri-Carb (model 3320) scintillation counter. The scintillation fluid consisted of 3.6 g of 2,5-diphenyloxazole (PPO) and 0.045 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene (POPOP) dissolved in 1 liter of a mixture of toluene-Triton X-100-water (6:3:1).

Analysis of polyoma viral and pseudoviral DNA by DNA-DNA hybridizations was done by the Denhardt membrane filter technique as modified by Qasba and Aposhian (13). Hybridizations were carried out at 65°C in 3 $\times$ SSC (SSC = 0.15 NaCl plus 0.015 sodium citrate).

Isolation of revertants. The ConA selection procedure described by Culp and Black (6) was used to isolate phenotype revertants from transformed cell lines. When the cells were about 95 to 100% confluent, the medium was changed and ConA was added to the fresh medium at a final concentration of 50 μ g/ml. These treated cultures were incubated at 37°C for 48 h. The medium and dead cells were then removed

from the dish, and the remaining cells were washed repeatedly with PBS. Fresh medium containing 10% fetal calf serum was added to the plates, and the plates were incubated until colonies appeared (1 to 2 weeks). Colonies were observed microscopically, and those colonies made up of cells having a flat morphology similar to that of 3T3 cells were cloned.

Characterization of revertants. Growth curves were done on each of the revertant clones, using the procedure previously described by Culp et al. (7). The colony-forming ability of the revertant clones and parent transformed cells in methylcellulose was determined by the method of Stoker et al. (17).

RESULTS

Production of pseudovirus in cell lines derived from mouse embryo cells. Several laboratories have shown that the host cell used for polyoma infection can influence the proportion of pseudovirus formed during the infection (20). Somewhat surprisingly, Yelton and Aposhian (20) found that 3T3 cells (a cell line derived from mouse embryos) produced relatively few pseudovirions, whereas mouse embryo cells produced a large proportion of pseudovirions. To further investigate the influence of the host cell on polyoma pseudovirus production, a number of different mouse cell lines were infected with polyoma virus, and the progeny were analyzed for their relative content of pseudovirus and polyoma virus. Two Swiss mouse cell lines, 3T6 and 3T12 (also derived from mouse embryos), as well as Swiss 3T3 and secondary mouse embryo cells were tested for their ability to produce polyoma pseudovirus. Both the polyoma virions and the pseudovirions were radioactively labeled by adding medium containing [^3H]thymidine after the virus adsorption period. The progeny viruses produced by 96 h postinfection were purified, and their content of 53S (polyoma) and 16S (pseudoviral) DNA was determined by sedimentation through alkaline sucrose gradients (Fig. 1). Calculation of percentage of polyoma virus was determined by dividing the counts per minute in the 53S region by the total counts in the 16S and 53S regions. The percentage of pseudovirus was determined by dividing the counts per minute in the 16S region by the same sum. That the amounts of radioactive 53S and 16S DNA indicate the amounts of polyoma virus and pseudovirus, respectively, in the purified virus preparation had been shown previously by DNA-DNA hybridization (20). This was also supported by DNA-DNA hybridization data that we obtained on these preparations (data not shown). The hybridization data consistently showed 3 to 7% more polyoma virus than did the alkaline sucrose gradient analysis. This was due to a small amount of nicking of the 53S DNA which occurred and resulted in polyoma DNA

FIG. 1. 53S and 16S DNA in virus particles purified from different Swiss mouse cells after infection with polyoma virus. The DNA was labeled by the addition of [^3H]thymidine to the medium after the virus adsorption period. Virus was purified by a centrifugation in sucrose and CsCl gradients as described in Materials and Methods. The purified virus was layered onto 5 to 20% alkaline sucrose gradients for analysis of its DNA content. ^{14}C -labeled marker DNA was used to determine the position of the 53S and 16S regions of the gradients.

which also sedimented at 16S. In no instance did more than 7% of the 16S DNA hybridize to polyoma DNA. The 3T6 and 3T12 cells produced pseudovirus in about the same proportion as SME cells (Fig. 1). In these three cell types, pseudovirus represented from 85 to 90% of the progeny produced during polyoma virus infection; only 10 to 15% of the virions contained polyoma DNA. Infection of 3T3 cells, however, produced about 38% pseudovirus, with the remaining 62% of the particles containing polyoma DNA.

Influence of transformation of the host cell on pseudovirus formation. The data in Fig. 1 also suggest that there is some function associated with embryonic cells which leads to the formation of a large proportion of pseudovirus (we presume that 3T3 cells lost this property during the process of selection which led to their isolation). To test this possibility, transformed derivatives of 3T3 were infected with polyoma, and the progeny were analyzed for pseudovirus. The data presented in Fig. 2 suggest that phenotypic transformation of host cells may enhance polyoma pseudovirion formation. Previous studies (12, 19, 20) on polyoma pseudovirus formation had been conducted with

Swiss mouse cells; however, the data in Fig. 2 were obtained after polyoma virus infection of BALB/c mouse cells. The relative amounts of pseudovirus and polyoma virus produced by infection of BALB/c 3T3 and SME cells were similar to those obtained after infection of Swiss 3T3 or SME cells with polyoma virus. Infection of two transformed BALB/c mouse cell lines, SVT2 and B77, yielded 78 and 74% pseudovirus, respectively.

Isolation and characterization of revertants of transformed cells. To further test the hypothesis that transformation of the host cell may enhance polyoma pseudovirion production, a variety of phenotypic revertants of transformed mouse cells were isolated and then tested for their ability to produce pseudovirus. Using a ConA selection technique (6), revertants of the transformed cell lines, SVT2 and B77, were isolated and cloned. The morphology, saturation density, and colony-forming ability in semisolid medium of these revertants and their parents were determined.

Since selection of the revertants had involved microscopic scanning of cells and isolation of those cells resembling the morphology of 3T3 cells, the revertant clones were characterized by a flat, polygonal morphology. When confluent, the revertant cells formed monolayers with no overlapping cells. Cultures of revertant clones showed very few rounded or spindle-shaped cells, which were abundant in cultures of the parent transformed cells. The photographs

shown in Fig. 3 depict the flat morphology of the revertants and demonstrate their similarity to 3T3 cells.

Growth curves were performed for all of the phenotypic revertants and their parental transformed cells to determine saturation densities. For each cell type, the maximum number of cells obtainable on a 60-mm-diameter dish was defined as its saturation density. The saturation densities of the transformed and revertant cells are presented in Table 1. The saturation densities of the B77 revertant clones were similar to those of the BALB/c 3T3 cells and were less than one-half the value determined for the parent B77 cells. Although the saturation densities of the SVT2 revertant clones were significantly higher than those of the BALB/c 3T3 cells, they were still about one-half those of the parent SVT2 cells. The revertants also showed density inhibition of growth unlike their parent (SVT2 or B77) cells, which continued to divide as long as fresh medium was supplied.

Data obtained on the ability of revertant and transformed cell lines to form colonies in medium containing methylcellulose are also shown in Table 1. The phenotypic revertant cells and 3T3 cells were unable to form colonies in methylcellulose, whereas the SVT2 and B77 cells formed macroscopic colonies within 15 days after seeding. Similar results were also obtained when soft agar was used instead of methylcellulose as a semisolid medium.

Polyoma pseudovirion production in revertant cells. Previous descriptions of phenotypic revertants of transformed cells have shown that selection for reversion in one property, such as flat morphology, is often accompanied by reversion of many other transformed properties such as saturation density and anchorage dependence (7). The data presented here on the characteristics of the revertants that have been isolated are consistent with these previous observations. Since reversion typically affects a variety of phenotypic characteristics, it was possible that the factor(s) responsible for the enhanced pseudovirion formation in transformed cells might also revert. To test this hypothesis, some of the revertants were infected with polyoma virus and the progeny were analyzed for pseudovirions. These data are presented in Fig. 4 and summarized in Table 2, along with the results obtained with the various other cell types used in this study for comparison. The revertants of both SVT2 and B77-A31 cells demonstrated a reduction in their ability to produce polyoma pseudovirions to levels that were roughly equivalent to those found with BALB/c 3T3 cells. Polyoma virus replicated well in all of the cell lines (Table 2). About the same

FIG. 2. 53S and 16S DNA in virus particles purified from different BALB/c mouse cells after infection with polyoma virus. Conditions were as described in Fig. 1.

FIG. 3. Morphology of BALB/c 3T3 cells (a), SV40-transformed 3T3 cells (b), avian sarcoma virus (B77)-transformed 3T3 cells (c), ConA-selected revertant cells of SVT2 (d), and ConA-selected revertants of B77 (e). All cells were seeded at approximately one-fourth confluence in medium containing 10% calf serum. Three days later the cells were fixed with methanol, stained with Giemsa, and photographed. Bar equals 25 μ m.

TABLE 1. Characteristics of revertant cells

Cell line	Satura- tion density ($\times 10^{-6}$) ^a	Col- ony forma- tion (%) ^b	Morphology
BALB/c 3T3	1.8	0	Flat, polygonal
SVT2	20	5.4	Rounded, spindle shaped
SVT2 revertant clone A4	15	0 ^c	Flat, polygonal
SVT2 revertant clone A7	11	0 ^c	Flat, polygonal
B77-A31	6	3.8	Rounded, spindle shaped
B77 revertant clone 1	1.7	0	Flat, polygonal
B77 revertant clone 3	2.8	0	Flat, polygonal

^a Cells per 60-mm plastic petri dish.

^b Determined by dividing the number of macroscopic colonies that formed in methylcellulose medium after a 15-day incubation by the number of cells seeded per 60-mm dish (5×10^3 cells/dish).

^c Although these cells did not form colonies within the 15-day incubation period, extended incubation for 1 month allowed about 1% of the cells to form colonies.

FIG. 4. 53S and 16S DNA in virus particles purified from revertant cell lines after infection with polyoma virus. Conditions were as described in Fig. 1.

TABLE 2. *Relative amounts of pseudovirus and polyoma virus produced in a variety of cell lines*

Cell line	HAU/10 ⁷ cells at 96 h postin- fection ^a	Relative amt of polyoma DNA at 36 h postin- fection ^b	% Poly- oma virus ^c	% Pseu- dovi- rus ^c
3T3 ^d	4,150	1.35	61	39
SME ^d	11,200	1.00	10	90
SVT2	17,200	1.15	22	78
SVT2 revertant clone A4	1,800	1.06	64	36
SVT2 revertant clone A7	8,600	ND ^e	77	23
B77-A31	16,700	0.91	26	74
B77 revertant clone 1	3,300	1.06	75	25
B77 revertant clone 3	2,200	ND	68	32
3T6	32,300	ND	9	91
3T12	9,450	ND	15	85

^a Average of two or more experiments. HAU, Hemagglutination units.

^b Cells were infected and labeled with [³H]thymidine, and Hirt extracts were prepared at 36 h postinfection. A portion of the extract was then centrifuged to equilibrium in CsCl-ethidium bromide and the number of counts found in the closed circular DNA region of the gradient was determined. After correction for mock-infected cells, this number was divided by the number obtained with infected SME cells.

^c Determined by alkaline sucrose gradients.

^d Derived from both BALB/c and Swiss mice.

^e ND, Not determined.

amount of polyoma DNA was produced in each of the cell lines. Thus, the large amount of pseudovirus formed in the embryonic and transformed cell lines is not due to poor replication of polyoma virus as has been reported in some systems (1).

DISCUSSION

The data presented in this paper indicate that phenotypic transformation of the host cell affects the relative amounts of pseudovirions and polyoma virions produced during a polyoma virus infection. Progeny resulting from a polyoma virus infection of a variety of transformed mouse cell types consisted of predominantly pseudovirus, whereas mouse 3T3 cells produced mainly polyoma virus. The influence that transformation of the host cell had on polyoma pseudovirion formation was further exemplified by isolation of phenotypic revertants of some of the transformed cell lines used in this study. The progeny resulting from a polyoma virus infection in these revertant cells consisted of mainly polyoma virus, with relatively small amounts of

pseudovirus produced. Thus, there was an association between a cell line being phenotypically transformed and that cell line producing high proportions of pseudovirus relative to polyoma virus when infected with polyoma virus.

Yelton and Aposhian (20) demonstrated that the host cell influences the relative amounts of pseudovirus and polyoma virus produced during polyoma infection. The data presented here confirmed these results by showing that polyoma virus infection of 3T3 cells produced low proportions of pseudovirions, and infection of mouse embryo cells produced high proportions of pseudovirions. The effects of the host cell on polyoma pseudovirion production may now be extended to include the influence that transformation of the host cell has on polyoma pseudovirion formation.

Both embryonic and transformed mouse cells formed predominantly pseudovirus relative to polyoma virus upon infection with polyoma virus. Embryonic and transformed cells, of course, display many common features (4). Antigenic, hormonal, and enzymatic similarities have been demonstrated between embryonic and transformed cells (3, 4). Shier and Trotter (16) have reported that 3T3 cells have an adult esterase isozyme pattern similar to that found in mouse kidney fibroblasts. However, transformed derivatives of 3T3 cells have an esterase isozyme pattern characteristic of mouse embryo cells. They concluded that 3T3 cells, despite their embryonic origin, are adult in nature and that transformation of such cells leads to retrogenesis and the reexpression of oncofetal genes. The results reported here indicate that the ability of a host cell to produce a high proportion of polyoma pseudovirus may represent another similarity between embryonic and transformed cells.

The cells used in this study offered an opportunity to explore the possibility that an embryonic gene function may be important in polyoma pseudovirion formation. All cell types used in these experiments were originally derived from mouse embryos. Thus, it is possible that during establishment of the 3T3 cell line, the process of selection resulted in a reduced expression of the embryonic gene(s) which is important in pseudovirion production. Transformation of the 3T3 cells by viruses or selection of spontaneous transformants allowed for reexpression of the putative embryonic gene(s). Isolation of phenotypic revertants of the transformed cells resulted in cell lines in which the expression of the embryonic gene(s) was again shut down.

A function that might be important to pseudovirus formation could be one that promotes fragmentation of the host cell genome during polyoma virus infection. Extensive fragmenta-

tion of the host genome has been correlated with the formation of high proportions of pseudovirions in both polyoma virus (26) and SV40 (15) infections. Yelton and Aposhian (20) studied the relative amounts of polyoma DNA and fragmented host cell DNA found intracellularly in PME, BMK, and 3T3 cells at different times postinfection. In their experiments, the low-molecular-weight DNA was extracted from cells by the method of Hirt (10) and was analyzed in alkaline sucrose gradients. Their results showed that the relative amounts of intracellular fragmented host cell DNA correlated with the amount of pseudovirus produced. Our preliminary experiments with polyoma virus-infected, transformed mouse cells indicate that fragmentation of the host genome also correlates with their ability to produce high proportions of pseudovirus.

A more thorough elucidation of the factor(s) common to embryonic and transformed cells which are involved in pseudovirus formation should provide a better understanding of the mechanisms involved in polyoma pseudovirion formation.

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