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Reversion in Polyoma-transformed Cells: Retransformation, Induced Antigens and Tumorigenicity

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We studied the properties of two morphologically reverted cell clones isolated as chromosomal segregants from a "hybrid" clone of BHK 21/13 hamster fibroblasts, transformed with polyoma virus. Both clones were less tumorigenic than control transformed cells. They contained no detectable polyoma-specific complement-fixing antigen. Induced transplantation antigen also appeared to be lost or reduced. Both clones could be retransformed with polyoma virus, suggesting that their reversion is due to the loss of viral genes from the transformed cell.

Clones of cells which had regained certain of the characters of their untransformed precursors were isolated among 6-thioguanine (TG)-resistant variants of polyoma virus-transformed BHK21/13 hamster fibroblasts. The resistant variants were selected from a "hybrid" subline obtained by fusion of a TG-resistant cell and a 5-bromodeoxyuridine (BUDR)-resistant cell. The reverted clones had lost the random growth pattern of transformed cells and grew in orderly parallel array. They also had a greatly diminished colony-forming efficiency when cultured in agar suspension (5). Since resistance to TG was found to be correlated with a loss of chromosomes, it is likely that reversion is due to a loss of factors that control the transformed phenotype and which are associated with one or more chromosomes. These factors may be viral genes, since Westphal and Dulbecco (8) have shown that cells transformed by polyoma virus and simian virus 40 (SV40) contain viral polynucleotide sequences in their nuclear deoxyribonucleic acid. Reversion, however, may result either from a loss of viral genes associated with chromosomes or from a loss of cell genes required for the expression of the transformed phenotype. To study these alternatives, revertant cells were tested for their susceptibility to retransformation and for the presence of polyoma-specific transplantation and complement-fixing antigens. The specificity of these antigens suggests that they are primary viral products. Their presence in transformed cells is often regarded as evidence that persistent viral gene activity is necessary for the maintenance of the transformed state. The retransformation test was performed on the assumption that reinfection would not cause revertant cells to recover the transformed phenotype if loss of cellular, rather than viral, genes was responsible for reversion.

In the present paper, we show that the two revertant clones tested were susceptible to retransformation, that they contained no detectable polyoma-specific complement-fixing antigens, and that they had considerably reduced tumorigenicity. Polyoma-specific transplantation antigen also appeared to be lost or reduced in the revertant clones. An attempt to isolate revertant cells among BUDR-resistant clones obtained from the same hybrid subline was unsuccessful.

MATERIALS AND METHODS

Culture methods and transformation assays were as described previously (5).

Cell lines: Hy3-5. This polyoma-transformed hybrid cell clone was derived from the hamster fibroblast line BHK21/13 (3). An untransformed hybrid clone was obtained by culturing the two BHK21/13 variant sublines, B1 (resistant to BUDR) and T6 (resistant to TG), together in medium containing aminopterin, hypoxanthine, thymidine, and glycine (5). By infecting cells of the hybrid clone with polyoma virus, a transformed subline was obtained and clonally isolated in agar suspension culture. Both normal and transformed hybrid lines were shown to be resistant to aminopterin and sensitive to TG and BUDR. Survival of the Hy3-5 cells after exposure to TG was about 5×10^{-5} and after exposure to BUDR was about 5×10^{-6}. The Hy3-5 cells displayed typical transformed morphology. Their colony-forming efficiency in agar suspension and in fluid medium was

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approximately 40%. After about 200 generations in culture, the modal chromosome number was 75.

ST13. This is a typical TG-resistant subline derived from a randomly selected Hy3-5 colony surviving in 2×10^{-5} M TG. It was morphologically transformed and had a high colony-forming efficiency in agar suspension culture. Its modal chromosome number was 55.

ST17-5 and ST14-1. These clones were derived from two TG-resistant sublines (ST17 and ST14) originally selected for their apparently normal morphology among Hy3-5 survivors to 2×10^{-5} M TG. Their plating efficiencies in agar medium were very low. The modal chromosome numbers of ST17-5 and ST14-1 were 57 and 64, respectively.

Transplantation tests. Cells were inoculated subcutaneously in the backs of 6- to 10-week-old, male Syrian hamsters. Cultures were trypsinized and suspended in tris(hydroxymethyl)aminomethane-saline plus 0.5% calf serum (TC). The resulting tumors were scored after 8 weeks. A lump of 1 cm in diameter was considered to be a tumor. No regression was found in any tumors.

Hamsters were immunized by injecting approximately 5×10^7 plaque-forming units (PFU) of the small plaque mutant of polyoma virus intraperitoneally. Virus was diluted in TC. Two injections were given at an interval of 1 or 2 weeks, and the cell challenge was made 1 week after the second dose of virus. Control animals received two injections of TC.

Hamsters were also immunized with X-irradiated cells (3,500 r). Each hamster was injected subcutaneously in the left hind leg with two doses of 10^6 irradiated cells. The second injection was 10 days after the first. The irradiated cells did not induce tumors.

Complement fixation tests. The microtechnique of Sever was used (6). Complement was fresh guinea-pig serum. Antigens were prepared from cell cultures by washing confluent cell sheets with Veronal buffer (VB) and then by scraping the cells into VB. The cell suspension was washed twice in VB, and a 20 to 50% suspension was frozen and thawed three times. The antigen was clarified by low-speed centrifugation. Polyoma-induced hamster tumor antigen and polyoma "T" hamster antiserum were obtained from Flow Laboratories, Inc., Rockville, Md. Serum from adult hamsters injected with polyoma virus was also used to test for "T" antigen. This serum possesses complement-fixing antibodies against polyoma "T" antigen and has the advantage that it does not contain any activity against normal isoantigens. In antigen titrations, graded dilutions of antigen were tested against graded dilution of antibody (chess-board titration).

RESULTS

Retransformation with polyoma virus. Table 1 shows the results of a typical transformation test performed with ST17-5 and ST14-1 cells and, for comparison, a transformation test with the normal parental lines B1 and T6. At an input multiplicity of 400 PFU/cell, the transformation frequency of ST17-5 cells was similar to that of the TG-resistant parental line T6, whereas that of ST14-1 was lower. Colonies from agar suspension cultures seeded with infected ST17-5 or ST14-1 cells gave rise to morphologically transformed cultures when transferred to fluid medium, had high colony-forming efficiencies when cultured in agar, and were shown to contain polyoma-specific complement-fixing antigen (*see below*).

TABLE 1. *Transformation frequency of revertants and of parental lines in agar suspension culture*[a]

Subline	Plating efficiency in fluid medium (a)	Plating efficiency in agar medium (b)	Plating efficiency in agar medium of viable cells (b/a × 100)
	%	%	%
ST17-5	42.0	0.44	1.0
ST14-1	11.6	0.07	0.6
B1	30.2	0.93	3.1
T6	26.0	0.39	1.5

[a] Input multiplicity of infection = 400 PFU/cell; 10^5 infected cells plated per culture (five dishes).

Complement fixation tests. Tissue culture antigens prepared from ST17-5 and ST14-1 cells did not fix complement with three batches of hamster polyoma T antiserum from Flow Laboratories and with two batches of pooled serum from hamsters injected with polyoma virus. These sera all fixed complement with polyoma hamster tumor antigens and with antigens prepared from four clones of BHK21/13 cells transformed by polyoma virus but not with BHK21/13 antigen. Dilutions of antigens equivalent to 0.5 to 1.0% (v/v) extracts of Hy3-5 cells, ST13 cells, or retransformed ST17-5 cells (*see above*) all fixed two units of complement when in combination with the optimal dilutions (usually about 1:40) of the various sera described above. In parallel titrations, these antigens were at least as active as those prepared from polyoma-transformed BHK21/13 lines.

Transplantation tests. The most striking feature of these tests was the much lower tumor-inducing capacity of ST17-5 and ST14-1 cells compared with their precursors Hy3-5 and the ST13 cells (Table 2). The ST14-1 cells induced small tumors (after 14 weeks) in about 50% of hamsters inoculated with 10^6 cells. This degree of tumor induction is comparable to that found in low-culture passages of the parent clone BHK21/13. The transplantability of these cells can be attributed to a small proportion (about 0.001%) of highly tumorigenic variant cells (O. Jarrett and I. Macpherson, Intern. J. Cancer, *in press*). In

TABLE 2. *Tumor production by hybrid polyoma-transformed cells and revertants in normal and polyoma-treated hamsters*

Subline	No. of cells inoculated	No. of hamsters with tumors/no. of hamsters inoculated			
		Control animals		Polyoma-treated animals	
		Expt 1	Expt 2	Expt 1	Expt 2
Hy3-5	10^2	4/4		0/3	
	10^3	4/4		2/4	
	10^4	4/4		2/2	
ST13	10^2	4/4	7/8	1/4	3/8
	10^3	4/4		4/4	
ST17-5	10^3	0/4	2/4	1/4	2/4
	10^4	2/4	7/8	4/4	8/8
	10^5	4/4		4/4	
ST14-1	10^5	0/4		1/4	
	10^6	0/4		0/4	
	10^7		5/8		8/8

contrast, 10^2 Hy3-5 cells produced rapidly growing tumors in less than 4 weeks. None of 10 animals with tumors induced by ST17-5 cells produced antibodies against T antigen (Flow Laboratories). Of three animals with tumors induced by ST14-1 cells, two produced sera that fixed complement with antigens from a variety of cells, including human HEp-2, rat embryo, and BHK-21. The other six sera were negative. (Sera were considered positive when they fixed two units of complement with two units of T antigen at a dilution of 1:8 or higher.)

The results of implanting the transformed cells (Hy3-5 and ST13) and the revertant cells (ST17-5 and ST14-1) in polyoma-immunized hamsters indicated that polyoma-specific transplantation antigen is present in the hybrid and in the transformed segregant, whereas it is depleted or lost in the revertants (Table 2). The same indication was given by an experiment in which transformed cells were implanted in animals immunized with X-irradiated cells from revertant and transformed lines (Table 3).

Attempts to isolate BUDR-resistant revertants from HY3-5 cells. When Hy3-5 cultures were first tested for the presence of parental types (i.e., cells resistant to BUDR or TG), it appeared that only TG-resistant clones could readily be isolated (5). Subsequent tests showed that failure to observe BUDR-resistant colonies was due to their inhibition by persisting sensitive cells when high cell concentrations were used. With appropriate cell concentrations, BUDR-resistant colonies also developed from Hy3-5 cultures treated with 10^{-4} M BUDR. Their frequency, however, was lower than that of TG-resistant colonies ($\leq 10^{-5}$), and the karyotype of BUDR-resistant clones was not consistently reduced. No clear indication of reversion was apparent when a sample of BUDR-resistant clones was examined and tested in agar suspension culture (Table 4). An equivalent sample of TG-resistant colonies would yield a number of clones displaying different degrees of reversion to the normal morphology and a reduced ability to grow in agar (5).

Failure to observe revertants among BUDR-resistant clones could be related to the fact that fewer chromosomes appear to be lost by these variants. It should also be mentioned that selection for resistance to BUDR may not favor revertants, since transformation confers on BHK-21 cells a higher degree of resistance to this analogue (I. Macpherson, *unpublished data*).

TABLE 3. *Capacity of different cell lines to induce immunity in hamsters inoculated with polyoma-transformed cells*

Immunizing cells (X-irradiated)	No. of hamsters with tumors/no. of hamsters inoculated with 5×10^3 Hy3-5 cells	Per cent
Hy3-5	1/9	11
ST13	0/6	0
ST14-1	3/8	37
ST17-5	5/12	42
BHK21/13	6/9	67

TABLE 4. *Plating efficiency in agar suspension culture of clones derived from Hy3-5 survivors to 5-bromodeoxyuridine*

Clone no.	Morphology	Modal chromosome no.	Per cent plating efficiency in fluid medium (a)	Per cent plating efficiency in agar medium (b)	Per cent plating efficiency in agar-medium of viable cells (b/a × 100)
1	Transformed		16.1	14.9	92
2	Transformed	65	54.4	48.1	88
3	Transformed	71	22.9	20.1	88
4	Transformed		37.2	29.3	79
5	Transformed		8.7	9.9	114
6	Transformed	63	12.7	13.1	103
7	Transformed		44.1	41.1	93
8	Transformed		5.3	4.6	87
9	Intermediate	66	27.6	24.5	89
10	Intermediate	69	27.2	19.6	72

DISCUSSION

Our results tend to support the hypothesis that the reversion of polyoma-induced transformation observed in chromosomal segregants obtained from a hybrid subline of BHK21/13 hamster fibroblasts is due to a loss of viral genes from the transformed cell. The disappearance of detectable polyoma-induced antigens from the revertant clones suggests that reversion is not simply a modulation of the cell's response to viral gene products, but rather that viral genes are absent or are not functioning as they do in the transformed cell. Similarly, Weiss et al. (7) reported the loss of T antigen from hybrids between normal mouse and SV40-transformed human cells, and the loss of T antigen appeared to be correlated with the loss of human chromosomes. In the cases described here, the possibility that the revertant cells had lost a cellular function conditioning the expression of viral genes, rather than a viral function, was tested by reinfecting the revertant cells. If reversion was phenotypic, the addition of structural (viral) genes would not be expected to restore the transformed phenotype. Such a test presupposes that the revertant cell still contains loci available for interaction with the transforming virus. This requirement is likely to be met in this case; even if the diploid cell should contain a single locus for interaction, the hybrid progenitor of the revertants would probably possess at least two such loci. The susceptibility to transformation of near-tetraploid BHK-21 variants suggests that the virus-sensitive loci of the cell need not be saturated for transformation to be established (4). Infection of the two revertant clones gave rise to retransformed colonies with a frequency of the same order as that of the normal parental lines. This suggests that the addition of viral (genetic) material is sufficient to restore the revertant cell to its original transformed condition.

The loss of tumorigenicity in the revertant clones is of particular interest, since this finding supports the idea that polyoma gene products are responsible for this property in transformed cells and that tumorigenictiy is not due to the selective transformation of cells with a higher tumor-producing capacity than the wild-type population.

If reversion is due to the loss of polyoma genes in association with chromosomes, one may ask how specific is this association. The interaction may be very specific; e.g., the viral genes may associate with one chromosome or even with a particular site on this chromosome. Then again, the specificity may be of a lower order, thus permitting the association of viral genes with any of a number of chromosomes. A third possibility is that association can take place with most or all chromosomes. Studies on the karyotype and retransformability of revertant clones may elucidate the nature of the cell-virus interaction.

The foregoing discussion is based on the tacit assumption that the maintenance of the transformed state depends on the continuous presence and functioning of viral genes in the cell. The detection of virus-specific ribonucleic acid proves that viral genes are indeed active in the transformed cell (1). It should be pointed out, however, that no direct demonstration has yet been given that there exists a viral function relevant for the maintenance of the transformed state. At least one mutation has been found which affects the ability of the virus to transform but does not interfere with the maintenance of the transformed state once it has been achieved (2). A crucial proof, such as a cell transformed by a mutant virus whose transformed phenotype is sensitive to conditions affecting the expression of the mutant viral gene, has not yet been obtained.

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