Characterization of Morphologic Revertants of Murine and Avian Sarcoma Virus-Transformed Cells

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Morphologic revertants which contain avian or murine sarcoma viruses have previously been isolated at low frequency from clonal lines of transformed mammalian cells. In the present study, these lines have been further characterized. They are indistinguishable from nontransformed parent cell lines with respect to parameters such as saturation density and colony formation in depleted medium or on monolayers of contact-inhibited cells. The rate of glucose uptake had also reverted to normal. The malignant potential of one of the revertant lines was examined and found to be markedly reduced compared to that of the corresponding transformed cells. The differences in the susceptibilities of revertant cells to retransformation by the same or other oncogenic viruses suggest that different cellular genes may be involved in expression of transformation by various tumor viruses.

Transformation of cells in tissue culture by murine and avian sarcoma viruses generally results in a stable association of the sarcoma virus with the morphologically altered cell. Temperature-sensitive mutants of avian (6, 14, 23) and murine (20) sarcoma viruses have been isolated which induce transformation of cells at the permissive temperature; however, the transformed cells become morphologically normal at the nonpermissive temperature. These studies demonstrate that viral genetic functions are clearly required for maintenance of the transformed state. The question as to what cellular functions might be required for expression of transformation has recently led to the isolation of morphologic revertants containing normal sarcoma viruses (22). These cell lines were isolated from clonal lines of avian and murine sarcoma virus-transformed mammalian cells. They were selected in microtiter and occurred at very low frequency (less than one per 1.2×10^3 subclones examined).

MATERIALS AND METHODS

Media. Cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co.) in 50-mm petri dishes (Falcon Plastics, Los Angeles). Growth-factor depleted medium was prepared as previously described (13).

Cells and viruses. The cell lines used included

BALB/3T3 (3) and a line of normal rat kidney cells (9). Sarcoma virus transformants included a Kirsten murine sarcoma virus (KiMSV) nonproducer NRK clone (K-NRK) (5) and a Schmidt-Ruppin Rous sarcoma virus (SR-RSV) nonproducer NRK clone (SR-NRK) (22). A B77 avian sarcoma virus non-producer clonal line of BALB/3T3 (B77-BALB) was kindly provided by Peter Vogt. Morphologic revertants of each of the respective transformed lines are designated K-NRK-R, SR-NRK-R, and B77-BALB-R (22). The methods used to isolate these revertant lines and the frequencies of revertants in transformed cell lines have been described in detail previously (22). The viruses used were Rauscher leukemia virus (R-MuLV) (5), an R-MuLV pseudotype of KiMSV, (KiMSV[R-MuLV]), and the small plaque variant of simian virus 40 (SV40) (3).

Virus assays. The transformation assays for MSV (5) and SV40 (3) have been reported.

Tumorigenicity studies. Cells for inoculation were trypsinized, washed twice by centrifugation, and suspended in serum-free medium. Syngeneic BALB/c mice, 6 to 8 weeks old, were injected subcutaneously in the interscapular region with 0.2 ml of cell suspension per animal.

Radioimmunoprecipitation assay. The methods for measurement of intracellular levels of avian group-specific antigen by radioimmunoprecipitation are described in detail elsewhere (19; J. R. Stephenson, R. Wilsnack, and S. A. Aaronson, *manuscript in preparation*).

Cellular ^{14}C -glucose uptake. Rates of ^{14}C -glucose uptake were measured by the method of Hatanaka and Hanafusa (10).

RESULTS

Growth characteristics of sarcoma virus-containing morphologic revertants. Transformed cells in culture generally can be distinguished from their normal counterparts by quantitative differences in growth properties such as saturation density (4) and colony-forming efficiency (CFE) on monolayers of contact-inhibited cells (4). They can also be distinguished by their ability to divide in medium partially depleted of factors necessary for growth (13). Studies were undertaken to determine whether the morphologic revertants, K-NRK-R, SR-NRK-R and B77-BALB-R, had reverted to the nontransformed state with regard to any of these properties. As shown in Table 1, each of the nontransformed control cell lines and the revertant lines had saturation densities of 6.0×10^4 to 11.2×10^4 cells per cm^2 . In contrast, all of the parent lines of morphologically transformed cells had saturation densities of greater than 4×10^5 cells per cm^2 . The doubling time of each revertant was compared to that of its transformed counterpart. Although SR-NRK-R and K-NRK-R had slightly decreased generation times compared to that of the parent line, the B77-BALB-R line had a doubling time equal to that of its control (Table 1).

The CFE's of the different cell lines were compared after growth in either regular medium or growth-factor depleted medium, or on monolayers of contact-inhibited cells. Under conditions of growth on a plastic surface in

regular medium, the CFE's of the cell lines ranged from 20 to 75%. In depleted medium, the CFE of each morphologically transformed line was approximately 50% of its CFE in complete medium. In contrast, both normal and revertant cell lines showed a very marked reduction in CFE in depleted medium (Table 1). The ability of each cell line to form colonies on confluent monolayers of BALB/3T3 even more clearly distinguished the morphologically transformed and revertant cell lines. In each case, both the control cells and the revertant lines were at least 3 logs less efficient at colony formation on confluent monolayers than in petri dishes, whereas the transformed lines showed very similar CFE's under both growth conditions. Thus, in each of the growth parameters investigated, the sarcoma virus-containing revertant cell lines were similar to normal cells and strikingly different from the morphologically altered cells from which they had been isolated.

Tumorigenicity of B77-BALB and B77-BALB-R. Previous studies have shown that in vitro cell growth characteristics such as saturation density and the ability to form colonies on monolayers of contact-inhibited cells correlate well with in vivo tumorigenicity (4). It was of interest, therefore, to compare the tumor-forming abilities of virus-transformed and virus-containing morphological revertant cell lines which showed such great differences in in vitro growth properties. As shown in Table 2, there was a striking difference in the tumor-forming ability of B77-

TABLE 1. Growth properties of normal, transformed, and morphological revertant cell lines

Cell line	Saturation density ($\times 10^{-5}$) ^a total cells/ cm^2	Doubling time (hr) ^b	Colony-forming efficiency (%) ^c		
			CM	DM	Monolayer
NRK	0.8	21	45	0.9	<0.01
K-NRK	4.5	20	75	48	72
K-NRK-R	0.7	27	48	25	0.03
SR-NRK	4.2	21	42	12	41
SR-NRK-R	0.6	29	45	0.4	<0.01
BALB/3T3	0.7	25	24	1.0	<0.01
B77-BALB	4.2	24	21	10	37
B77-BALB-R	1.1	24	22	2.0	<0.01

^a Maximum number of cells obtained when cells were inoculated into 20-cm² petri dishes at an initial saturation density of 5×10^5 cells/ cm^2 and then incubated at 37 C under conditions where medium containing 10% calf serum was changed every 3 days.

^b Doubling times were determined by culturing cells at 37 C at an initial saturation density of 5×10^5 cells/ cm^2 . Cells from duplicate plates were then trypsinized, and cell counts were made at 24-hr intervals.

^c Cells were plated at 10-fold dilutions in petri dishes in complete medium (CM), growth factor-depleted medium (DM), or on confluent monolayers of BALB/3T3. Colony formation was determined after 10 days of incubation at 37 C. Colony-forming efficiencies are expressed as: (number of colonies formed divided by the number of cells plated) $\times 100$.

TABLE 2. Comparison of the tumorigenicity of BALB/3T3, B77-BALB, and B77-BALB-R cell lines

Cell dose	Fraction of tumor-bearing mice ^a		
	BALB/3T3	B77-BALB	B77-BALB-R
10 ⁴	0/5	2/5	0/5
10 ⁵	0/5	5/5	0/5
10 ⁶	0/5	5/5	0/5
10 ⁷	0/5	5/5	2/5

^a Syngeneic BALB/c mice were inoculated at the indicated cell doses subcutaneously in the interscapular region. Results are expressed as the fraction of tumor-bearing mice at 8 weeks postinjection.

BALB and B77-BALB-R cell lines. The transformed line, B77-BALB, was approximately 1,000-fold more malignant than the revertant line, B77-BALB-R.

Comparison of ¹⁴C-glucose uptake rates with normal, transformed, and revertant cell lines. Previous studies have indicated that an increased rate of ¹⁴C-glucose uptake is characteristic of cells productively transformed by MSV and RSV (10, 11). The glucose uptake of each of the cell lines was determined. As shown in Table 3, MSV-transformed nonproducer BALB/3T3 cells had a 10-fold increase in incorporation of ¹⁴C-glucose compared to control BALB/3T3 cells. This finding indicates that the previously reported increased glucose uptake of MSV-transformed cells (11) does not require the presence of replicating MuLV. The avian sarcoma virus-transformed NRK and BALB/3T3 cell lines also exhibited an elevated glucose uptake, as has previously been reported for avian virus-transformed cells (10). It is of interest that the glucose uptake of each of the revertants was similar to that of the control cell lines (Table 3). This result further indicates that this biochemical parameter is associated with the expression of transformation in mouse fibroblast cell lines.

Avian gs antigen levels in transformed and revertant cell lines. The level of cell-associated avian group-specific (gs) antigen was tested by the radioimmunoprecipitation assay in avian sarcoma virus-transformed and revertant mammalian lines to determine whether the block to expression of the transformed state in the transformed cells was associated with a block in avian gs antigen expression. As shown in Table 3, the concentration of avian gs antigen in SR-NRK cells was 95 ng/mg of total cellular protein and was 23 ng/mg in the B77-BALB cells. The

NRK and BALB/3T3 control lines had no detectable avian gs antigen. The revertant lines each contained a concentration of avian gs antigen similar to that of its morphologically transformed counterpart. These results indicate that the block to expression of transformation in the revertant avian sarcoma-transformed lines is independent of avian gs antigen expression.

Susceptibilities of morphologic revertants to retransformation by tumor viruses. The availability of morphologic revertants produced by different tumor viruses but derived from the same clonal lines of nontransformed cells made it possible to ask whether revertant cells containing one sarcoma virus might be resistant to retransformation by the same virus. As shown in Table 4, the susceptibility of K-NRK-R cells to transformation by KiMSV (R-MuLV) was strikingly reduced to a level of 10⁻⁴-fold less than that of control NRK cells. The few foci that did develop following infection of the K-NRK-R line were very small and consisted of cells which appeared to be only partially altered morphologically. That the resistance of the cells to focus formation must be related to decreased susceptibility to a transformation function of the murine sarcoma virus was implied by the fact that this revertant line was as sensitive as NRK cells to infection by R-MuLV, the helper virus which provided the envelope for the sarcoma virus in these studies. Infectivity of MuLV was measured by the polymerase induction assay (21). That the block to retrans-

TABLE 3. Biochemical properties of normal, transformed, and morphological revertant cell lines

Cell lines	Avian gs antigen (ng/mg of protein) ^a	¹⁴ C-glucose uptake (counts per min per μ g of protein) ^b
NRK	<5	0.37
K-NRK	<5	4.87
K-NRK-R	<5	0.56
SR-NRK	95	1.61
SR-NRK-R	89	0.26
BALB	<5	0.15
B77-BALB	23	0.81
B77-BALB-R	20	0.50

^a Avian gs antigen determinations were performed on sonically treated whole cells as described by Stephenson et al. (*manuscript in preparation*). The concentrations of total cellular protein were measured by the method of Lowry (15).

^b Cells were incubated in medium containing ¹⁴C-glucose at 0.2 μ Ci/ml for 20 min at 37 C. Incorporation of ¹⁴C-glucose was determined by the method of Hatanaka and Hanafusa (10).

TABLE 4. *Relative susceptibilities of normal and revertant cell lines to retransformation by SV40 and KiMSV (R-MuLV)*

Cell lines on which viruses were assayed	Virus titer		
	R-MuLV (PIU/ml) ^a	KiMSV (R-MuLV) (FFU/ml) ^b	SV40 (transformation frequency) ^c
NRK	0.8×10^5	1.5×10^6	NT ^d
K-NRK-R	0.6×10^5	2.0×10^1	NT
SR-NRK-R	0.5×10^5	0.7×10^6	NT
BALB/3T3	0.9×10^5	0.6×10^6	4.5%
B77-BALB-R	0.6×10^5	1.6×10^6	3.2%

^a PIU, Polymerase-inducing units.

^b FFU, Focus-forming units.

^c SV40 transformation frequencies are expressed as the percentage of the total colonies which were morphologically transformed.

^d NT, Not tested.

formation of K-NRK-R cells by KiMSV was specific for that virus was suggested by the fact that the morphological revertant line, SR-NRK-R, which contained the avian sarcoma virus, SR-RSV, was as susceptible to focus formation by KiMSV(R-MuLV) as were NRK cells. In other studies, it was found that B77-BALB-R, the revertant line of BALB/3T3 cells containing the B77 strain of avian sarcoma virus, was as sensitive as normal BALB/3T3 to transformation by the deoxyribonucleic acid (DNA) tumor virus, SV40. These experiments all suggest that cellular factors which may block expression of transformation by one tumor virus do not necessarily influence the action of other ribonucleic acid (RNA) or DNA tumor viruses.

DISCUSSION

In the present report, morphologic revertants of mammalian cells transformed by avian and murine sarcoma viruses have been characterized. Each of the revertant lines studied was found to have biological properties very similar to those of the respective nontransformed control cell lines. Each had reverted to normal in parameters such as contact-inhibition of cell division and the ability to divide in growth factor-depleted medium. Glucose uptake, a biochemical function that is altered in RNA virus-transformed cells (10, 11), was also found to be normal in each of the revertant cell lines. Two of the lines, B77-BALB-R and SR-NRK-R, clearly contain avian sarcoma viral genetic information, since avian gs antigen could be detected at levels similar to those found in the corresponding morphologically transformed

lines. These revertant cells also show a very low frequency of spontaneous re-reversion (*unpublished observations*). In the case of K-NRK-R, superinfection of the cells with leukemia virus results in rescue of normal KiMSV (22). It would appear, therefore, that each of the cell lines studied has undergone phenotypic reversion to normal morphology as a result of alteration of a cellular genetic function (either positive or negative regulation) required for expression of transformation.

Morphological flat revertants of SV40 and polyoma virus-transformed cells have been obtained by selective kill of the least contact-inhibited cells (16), by passage in tissue culture at low cell density (17), or by exposure to plant lectins such as concanavalin A (8; B. Ozan, *personal communication*). The viruses present in these lines have been found to be normal implying that, with DNA virus transformation also, cellular factors must influence expression of the transformed state. Karyological analysis of DNA virus-transformed flat revertant and transformed re-revertant cell lines have suggested that expression of transformation depends on a balance between chromosomes that carry factors for expression and chromosomes that carry their suppressors (7, 12). It should be of interest to determine whether similar alterations in karyotype occur in RNA tumor virus-transformed cells after reversion and spontaneous re-reversion.

Previous studies have demonstrated that a growth property of cells in tissue culture termed contact-inhibition provides an excellent method of predicting the ability of cells to produce tumors *in vivo*; in the absence of other variables, less contact-inhibited cells are more tumorigenic (4). The present finding that a contact-inhibited revertant line of avian sarcoma virus-containing cells has a markedly decreased tumorigenicity compared to the parent line, which lacks growth regulation *in vitro*, confirms and extends those studies. The present results lead to the conclusion that those cellular factors which can impair expression of the transformed state of sarcoma virus-containing cells in tissue culture are likely to play an important role in reducing the malignant potential of the cells *in vivo*.

The KiMSV-containing morphological revertant cell line was markedly resistant to retransformation by KiMSV. This was not due to an inability of the virus to enter the cells, since the MuLV helper virus which provided the envelope for the sarcoma virus was shown to enter and replicate equally well

in revertant and control cells. Each of the other revertant lines was as susceptible as the nontransformed control cells to transformation by KiMSV. Recently, SV40-transformed cells have been isolated which contain normal SV40 virus but are temperature-sensitive for the expression of transformation (18). These cellular mutants are resistant to retransformation by SV40 at the nonpermissive temperature (39°C) but as susceptible to transformation by MSV as nontransformed control cells (H. C. Renger, *Nature* (London), *in press*). In a reciprocal experiment in the present studies, a morphologic revertant containing avian sarcoma virus was found to be susceptible to SV40 transformation. All of the above evidence suggests that different cellular genes may be required for expression of transformation by different sarcoma viruses. Alternatively, the results obtained in these studies may be explained by the presence of virus-specific repressors in the cellular mutants examined.

Studies such as cell fusion may help to resolve these two possibilities by establishing whether complementation of genetic functions can result in full expression of the transformed state. A better understanding of cellular genetic factors which can influence the *in vitro* and *in vivo* alterations imposed upon cells by tumor viruses may eventually lead to ways of controlling the malignant potential of these viruses.

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