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Cell Biology

Temperature-sensitive tumorigenicity of cells transformed by a mutant of Moloney sarcoma virus

(*nude* mice/tumor regression/temperature shift)

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ABSTRACT **Normal rat kidney cells were nonproductively infected either with CP27, a mutant of Moloney sarcoma virus that is temperature-sensitive for maintenance of transformation, or with the parental wild-type virus. The nonproducer cells were inoculated into the tails of athymic *nude* mice that were subsequently incubated at 28 or 36°C. CP27-infected cells induced tumors only at 28°C, whereas cells infected with wild-type Moloney sarcoma virus were tumorigenic at both temperatures. Tumors induced at 28°C by wild-type virus-infected cells grew faster after shift of the mice to 36°C. In contrast, tumors induced by CP27-infected cells regressed upon shift to 36°C, indicating that continuous expression of viral functions is required for persistence and growth of the tumors. After regression, secondary tumor growth was observed late after upshift of temperature-sensitive tumors. Cells recovered from these late-appearing tumors were tumorigenic at the nonpermissive temperature, and tumors induced by these cells did not regress after upshift. Virus rescued from these recovered cells retained the temperature-sensitivity for focus formation, indicating that the occurrence of the phenotypically wild-type cells was due to host cell modifications rather than to reversion of the CP27 genome.**

RNA tumor viruses induce profound morphological and physiological changes in tissue culture cells (1). One change, which is of fundamental importance, is the acquisition, by these cells, of the ability to grow as tumors in susceptible animals.

Several mutants of RNA sarcoma viruses carrying temperature-sensitive (ts) lesions of transforming functions have been isolated. Temperature-shift experiments have shown that maintenance of transformation, as monitored by several different variables in tissue culture, requires continuous expression of viral transforming genes (1, 2).

The use of ts mutants *in vivo* has been limited because of the requirement of establishing different temperatures in animals. The effect of inoculating ts mutants of avian sarcoma viruses in chickens has been studied. In several cases, the mutants were found to have decreased tumorigenic potential (3–7), as expected, because the body temperature of chickens corresponds to the nonpermissive temperature of the mutants in tissue culture.

Different temperatures can be established in mice by utilizing the fact that the temperature of peripheral parts of homeothermic animals varies with the temperature of the environment. Normal rat kidney (NRK) cells transformed by a ts mutant of Moloney sarcoma virus were inoculated into the tissues of the tails of mice which were subsequently incubated at different temperatures. By this simple approach, we found that permissive and nonpermissive temperatures could be established in mice allowing full use of ts mutants for *in vivo* studies.

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MATERIALS AND METHODS

Virus, Cells, and Virus Assays. The origin of the Moloney sarcoma virus and the mutant tsCP27M-MSV has been described (8).

NRK cells were provided by Meloy Laboratories (NRK-1 is a clonal derivative of these cells). All NRK cells were grown at 33°C in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin and streptomycin).

10T1/2 cl. 8 cells obtained from C. A. Reznikoff were maintained as described (9). SC-1 cells were obtained from W. Rowe, and the mink cells were the Mv-1-Lu (ATCC no. CCL64) strain. IC-3T3 cl. 19 cells (10) were obtained from P. J. Fischinger. All cells lines and cell strains were tested (11) and found to be free from mycoplasma.

For virus assays, cells were plated in medium containing Polybrene (2 μg/ml) and were infected on the following day. Focus titer was determined 6–8 days after infection. Leukemia virus was assayed by an immune staining technique for p30 (12) using peroxidase-conjugated antiglobulin from Nordic Immunological Laboratories, Tilburg, The Netherlands.

Mice and Determination of Tumor Volumes. Female 5- to 6-week-old BALB/c *nu/nu* mice bred at the Fibiger Laboratory were used. They were in their 24th backcross generation and were kept under minimal disease conditions. During the experiments, the mice were placed in temperature-regulated incubators.

Cells were given fresh medium the day before inoculation. Trypsinized cells were pelleted, resuspended in tissue culture medium, and injected into the tissue of the tails via a 0.65- or 0.70-mm needle.

Tumor sizes were determined by making the following measurements with a slide caliper: l, length of the tumor; w, width of the tumor; d_1, diameter of the tail distal to the tumor; d_2, diameter of the tail proximal to the tumor; d_t, maximal diameter of the tail and tumor.

The volumes of the tumors were calculated by assuming an ellipsoid shape:

$$\text{Volume} = 1.047 \times h \times l \times w$$

The height (h) was calculated as:

$$h = d_t - \frac{d_1 + d_2}{2}$$

in which the last term represents an estimate of the diameter of the tail at the center of the tumor, with the assumption that no tumor had been present.

In animals in which two tumors appeared, the diameters of

Abbreviations: ts, temperature-sensitive; wt, wild type; NRK, normal rat kidney.

the tails at the centers of the tumors were estimated by linear interpolation of the diameters proximal and distal to the tumors.

Explantation of Cells from Tumors. Tumors measured 0.4–1.4 cm^3 and were growing exponentially when explanted. Mice were killed by cervical dislocation, and tumor tissue was removed aseptically. The tissue was passed through an 0.3-mm-mesh sieve and transferred to tissue culture. Remaining cell aggregates dissociated during subsequent trypsinizations.

Isolation of Nonproducer Transformants. Foci of transformed cells were isolated 11–15 days after infection of NRK-1 cells with CP27 or the parental Moloney sarcoma virus. The cell strains used in this study were derived from different Petri dishes containing one to five foci.

Assays for infectious virus were performed on supernatants collected 24 hr after seeding at 5×10^5 cells per ml. The supernatants were centrifuged for 10 min at $10^3 \times g$, passed through 0.45-μm Millipore filters, and frozen at −80°C. Cocultivation was performed by seeding 2.5×10^4 cells together with 10^5 SC-1 cells. The cultures were passaged four times, and supernatants were collected from semiconfluent cultures and treated as above.

RESULTS

ts Tumorigenicity of Cells Transformed by a Transformation-Defective Mutant. CP27 is a mutant of Moloney sarcoma virus that is ts for maintenance of transformation in tissue culture (8). The focus titer is decreased to 1/200th–1/25th at 39°C, and the few foci that appear at this temperature are smaller than the foci that appear at 33°C (13).

In order to determine the tumorigenic potential of cells transformed by the CP27 or wt virus, nonproducer-transformed cell strains were isolated from transformed foci of NRK-1 cells. Testing by several assays showed that no infectious virus was produced by any of the cell strains. Undiluted supernatants tested on SC-1, NRK-1, and mink cells did not induce antigens related to the major structural protein, p30, as determined by an immune staining technique (12). None of the supernatants induced transformed foci on 10T1/2 cells. Finally, as a sensitive test for production of ecotropic virus, the transformed cell strains were cocultured with SC-1 cells and the supernatants were tested for ability to induce p30-related antigens in SC-1 cells. Again, no infectious virus was detected.

NRK-1 cells transformed by CP27 or wt Moloney sarcoma virus were round and grew partly suspended at 33°C. At 39°C, the CP27-infected cells had a flat appearance, although the cultures contained somewhat more rounded cells than were observed in cultures of untransformed NRK-1 cells.

Transforming virus was rescued from the nonproducer cell strains by cocultivation with 10T1/2 cells infected with leukemia virus derived from IC-3T3 cells. Virus rescued from all the CP27-infected cell strains retained the temperature sensitivity for focus formation (data not shown).

To test the tumorigenic potential, cells were inoculated into *nude* mice. Because these mutant mice lack a functional thymus, tumorigenic cells of nonmurine origin may grow as tumors in these animals (14, 15). Cells were inoculated subcutaneously into the tissues of the tails, and the mice were incubated at either 28 or 36°C.

Mice inoculated with ts or wt virus-transformed cells developed tumors when kept at the lower temperature, and there were no significant differences in latency periods (Table 1). Tumors always appeared locally and grew progressively. In mice kept at the higher temperature, no tumors were induced by cells transformed by CP27 whereas tumors appeared with even shorter latency periods with cells transformed by wt virus. Clearly, NRK cells transformed by the ts mutant CP27 showed temperature sensitivity for tumor formation.

Effect of Shift to Higher Temperature. In tissue culture, cells transformed by CP27 can be converted to the untransformed state by increasing the incubation temperature. The corresponding experiment can easily be performed *in vivo* with the present system, and we investigated the consequences for tumor growth of shift to nonpermissive conditions.

Mice were incubated at 28°C after 5×10^5 cells were inoculated into the tails. The volumes of the tumors were determined daily after they had reached measurable sizes. Growth of tumors was exponential up to volumes of about 1–3 cm^3, and growth rates of tumors induced by wt or ts cell strains were similar at the permissive temperature. When tumors reached 0.12 cm^3, every other mouse was shifted to 36°C, and the remaining mice were left at 26°C.

Tumors induced by five ts cells strains (NP-3, 4, 7, 9, and 11) regressed after shift to 36°C (Figs. 1 and 2) and the decrease in size was evident 1 or 2 days after the shift. The effect of upshift was tested in at least two mice with tumors induced by each cell strain. The degree of regression varied; all tumors decreased at least 50% in size, and many were barely visible 1–2 weeks after the shift.

The observed decrease in tumor size was presumably due to cell death. Histological examination of tumors induced by two

Table 1. Tumorigenicity of transformed NRK cells in tails of *nude* mice incubated at 28 or 36°C

Cells	Cell strain	At 28°C: No. with tumor/ total no.	At 28°C: Mean latency, days	At 36°C: No. with tumor/ total no.	At 36°C: Mean latency, days
Untransformed	NRK-1	0/12	—	1/10	31
ts virus-transformed	NP-3	4/5	8	0/4	—
	NP-4	4/6	11	0/6	—
	NP-7	5/6	10	0/6	—
	NP-9	6/6	10	0/5	—
	NP-11	6/6	10	0/5	—
wt virus-transformed	NP-103	5/6	7	6/6	5
	NP-104	6/6	7	6/6	5
	NP-107	5/6	9	6/6	8
	NP-109	6/6	10	6/6	5

Six mice per group; 5×10^4 transformed cells or 5×10^5 NRK-1 cells were inoculated into each mouse. The data for NRK-1 are from two different experiments. Mice were scored as negative for tumor formation only if they survived for 20 days or more; the mean survival time was 40 days. The mice died spontaneously with signs of the wasting syndrome (16). All tumors induced by transformed cells appeared within 15 days after inoculation. The latency was defined as the time interval between inoculation of cells and first appearance of visible tumors.

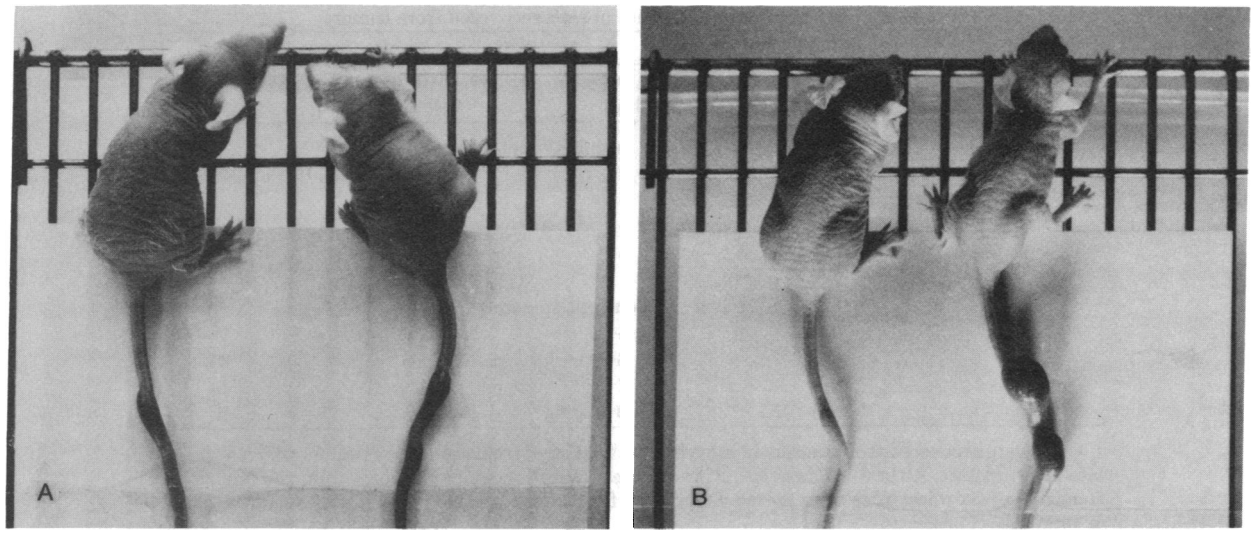

FIG. 1. Effect of shift to nonpermissive temperature. The mice were inoculated with either the ts cell strain NP-11 (left mouse) or the wt cell strain NP-103 (right mouse), and the mice were initially kept at 28°C. (*A*) The mice at the time of shift to 36°C. (*B*) The same mice at 4 days after shift. The volumes of the tumors were 25% and 700% the volumes in *A*, respectively.

of the ts cell strains revealed extensive pyknosis and death of the tumor cells (Fig. 3).

Tumors induced by wt cell strains did not regress after upshift. In 11 mice with tumors induced by three wt virus-transformed cell strains (NP-103, −107, and −109), faster growth rates were observed in all cases, after the shift to 36°C (Fig. 2).

Late Appearance of Phenotypically wt Tumors After Upshift of ts Tumors. Following regression of tumors after the shift to 36°C, secondary tumor growth was observed. With three cell strains, the recurrent tumors reached their original size about 25 days after shift of temperature; with one cell strain, this occurred after 13 days. The growth rates of the recurrent tumors corresponded to the growth rates of tumors induced by wt virus-transformed cells.

To characterize further the late-appearing tumors, cells from such tumors were transferred to tissue culture. The cells proved to be tumorigenic in mice kept at 28°C as well as in mice kept at 36°C (Table 2), and no tumor regression was seen after shift of these mice from 28°C to 36°C. In contrast, cells recovered from ts tumors in mice that were kept constantly at 28°C retained the temperature sensitivity for tumor induction, and regression was always seen after upshift. These results confirm that the appearance of recurrent tumors late after regression was due to growth of phenotypically wt cells.

One obvious possibility was that these cells were generated by reversion of the viral mutation. However, virus rescued from explanted tumors, including the ones referred to in Table 2, retained the temperature sensitivity for focus formation, indicating that this was not the case.

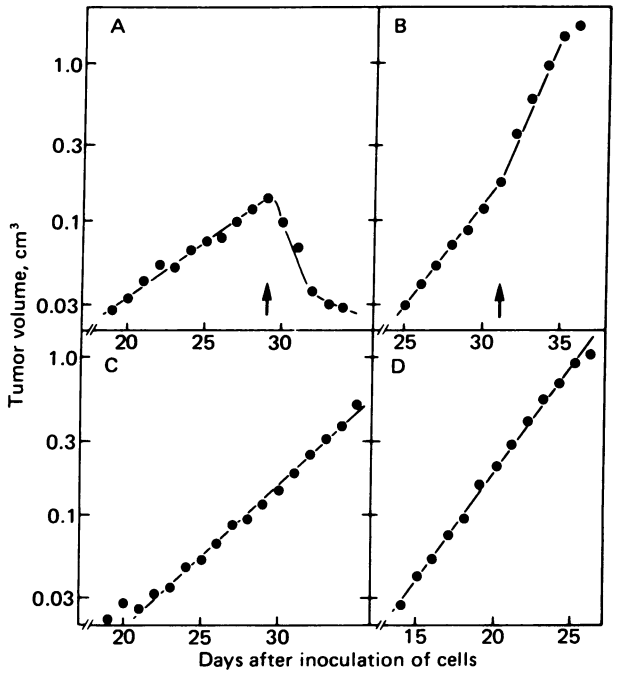

FIG. 2. Tumor growth during shift to nonpermissive temperature. In this experiment tumors were induced at the permissive temperature by the ts cell strain NP-11 (*A* and *C*) or the wt cell strain NP-103 (*B* and *D*). At the times indicated by the arrows in *A* and *B*, the mice were shifted to the nonpermissive temperature.

DISCUSSION

Characterization of ts mutants of oncogenic viruses and transformed cells generally has been limited to studies in tissue culture. Because tumorigenicity is a fundamental aspect of transformation, we wished to study the tumorigenic properties of cells transformed by a ts mutant of a transforming virus at

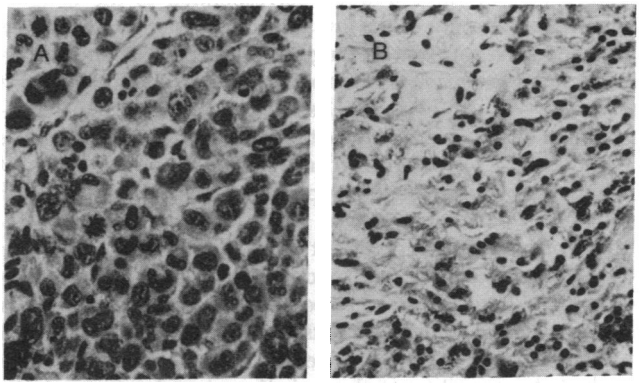

FIG. 3. ts tumors before and after shift to nonpermissive temperature. The tumors were induced by NP-11. (*A*) Specimen taken at the time of shift. (*B*) Specimen taken 2 days later. (Hematoxylin and eosin; ×240.)

Table 2. Tumorigenicity in mice of cells recovered from tumors

Original tumor induced by	At 28°C		At 36°C	
	No. with tumor/ total no.	Mean latency, days	No. with tumor/ total no.	Mean latency, days
	Cells tumors grown at permissive temperature			
NP-9	5/5	6	1/6	34
	6/6	7	0/6	—
NP-11	6/6	7	0/6	—
	6/6	6	0/6	—
	Cells from recurrent tumors			
NP-9	6/6	5	6/6	3
	4/4	6	6/6	4
NP-11	6/6	7	6/6	5
	4/5	9	5/5	5

Cells were recovered from tumors in mice kept at 28°C and from secondary tumors which appeared late after upshift. After 6–15 days in culture 10^5 cells were inoculated into each mouse. Each group consisted of six mice; mice were scored as negative for tumor formation only if they survived 20 days or more.

both permissive and nonpermissive temperatures. A simple procedure for studying the influence of temperature is to inoculate the cells into the tails of mice and to incubate the mice at different temperatures. Temperature-shift experiments are easily performed, and possible regional differences in tumor growth (17) are of no consequence because the different temperatures are established at the same site in the animals.

We have previously found that inoculation of CP27, a mutant of Moloney sarcoma virus, elicited a ts tumor response in normal BALB/c mice (13). However, we have found that the CP27 lost the ts trait upon multiplication *in vivo*. Nevertheless, tumors did not appear at the nonpermissive temperature, presumably due to an anti-tumor immune response in these mice (18).

We thought that the mutant might be stabilized if it were incorporated as a nonreplicating provirus in the tumor cells. Therefore, nonproducer cells were isolated and inoculated into the tails of nude mice. CP27-transformed cells induced tumors in mice incubated at 28°C but not in mice incubated at 36°C, whereas cells transformed by wt virus induced tumors at both temperatures. Clearly, the CP27-transformed cells were ts for induction of tumors. The latency period for appearance of wt tumors at the higher temperature was reduced, probably due to a direct effect of the temperature on the growth rates of the tumor cells. It cannot be ruled out, however, that physiological adjustments of the mice to the environmental temperature might also influence the tumor growth.

The tumorigenic potential of several ts mutants of avian sarcoma viruses have been studied in chickens. The body temperature of these animals corresponded to the nonpermissive temperature of the mutants. Inoculation of ts virus has been reported to result in longer latency periods and in formation of fewer tumors compared to the corresponding wt viruses (3–7). The reason that some tumors do form after inoculation of ts mutants may be that the transforming functions are not completely suppressed at the temperature of the site of inoculation. This especially applies to the use of the wing web which may have temperatures lower than the body temperature. Formation of wt revertant virus, either by back mutation or by recombination with endogenous sarcoma-specific sequences (19), might also be responsible for formation of tumors. In one study (6), some ts mutants were found to induce as many tumors as did wt virus. However, the data are difficult to evaluate because it is not clear whether virus dosage was taken into account.

The tumorigenic potential of ts mutants has also been investigated by inoculation of virus into the chorioallantoic membrane of embryonated eggs. Permissive and nonpermissive temperatures were established by incubating the eggs at different temperatures. Using this approach, Biquard and Vigier (20) found that a mutant of Rous sarcoma virus was ts for pock formation.

In a recent study (21), cells transformed by several mutants of avian sarcoma viruses were found to induce tumors in the chorioallantoic membrane at a nonpermissive temperature. This is surprising because pock formation induced by free mutant virus was ts and because many of the mutants have previously been reported to have clearly decreased tumorigenic potential in animals (3, 5). It remains to be established if the results from the study of this system reflect an ability of mutant-infected cells to grow as tumors in animals under nonpermissive conditions.

The present system offers the possibility of performing temperature-shift experiments. Animals were inoculated with transformed cells, and tumors were established at the permissive temperature. When the temperature was raised to the nonpermissive level, tumors induced by cells transformed by ts virus regressed. This indicated that expression of viral function is continuously required for maintenance of tumor growth and for survival of cells in tumors. The results are in accordance with the results from *in vitro* studies with ts mutants, which have demonstrated that maintenance of numerous characteristics of transformation in tissue culture also depends on continuous expression of viral functions (1, 2).

The reason for the extensive cell death that occurred after shift of ts tumors to the nonpermissive temperature is not clear. It is possible that the circulatory system in the tumors is insufficient to meet the nutritional demands of the cells in the untransformed state. However, it should be noted that the tumors contained well-developed capillary networks both before and after the shift. Alternatively, the cell death may be due to regulatory mechanisms that hinder the survival of aggregates of untransformed cells in the animal, and the phenomenon may be related to the observation that many untransformed cell lines die after inoculation into animals (22).

A seemingly similar situation exists with hormone-dependent mammary tumors. Just as growth of the virally induced tumors reported here requires continuous stimulus from viral transforming functions, the growth of hormone-dependent tumors requires continuous stimulus from hormonal factors, and discontinuation of hormone treatment results in tumor regression (23, 24).

Secondary growth of tumors was seen at various time inter-

vals after regression of ts tumors. This was shown to be due to growth of phenotypically wt cells after the upshift. The cells still contained ts virus genome, suggesting that changes in cellular functions were responsible for generation of these cellular variants. It is possible that the variants were formed by spontaneous transformation. In a control experiment, one mouse developed a tumor after inoculation of large doses of NRK-1 cells, indicating that the cells may have a small but significant rate of spontaneous transformation. At the time of shift, the tumors contained more cells than could be inoculated in the control experiments. Therefore, tumors of spontaneously transformed cells are more likely to form after shift than in the control experiments.

Graf and Beug (25) have found a high frequency of phenotypically wt cells in cultures of NRK cells transformed by ts mutants of avian sarcoma viruses. Similar to our findings, they found that in some cases these cells contained mutant virus. They suggested that the altered phenotype was due to a host cell modification affecting the expression of the virally induced transformation.

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