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Acceleration of Transformation of Rat Embryo Cells by Rat Type C Virus

SURAIYA RASHEED,* AARON E. FREEMAN, MURRAY B. GARDNER, AND ROBERT J. HUEBNER

Department of Pathology, University of Southern California School of Medicine, Los Angeles, California 90033, Childrens Hospital of Akron, Ohio 44301, and Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland 20014*

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Sprague-Dawley and Fischer rat embryo cells became spontaneously transformed about 20 passages after release of endogenous ecotropic type C virus (SD-RaLV and F-RaLV). The virus-producing transformed cells showed loss of contact inhibition, increased growth rate, and tumorigenicity in vivo. Exogenous infection of other Fischer rat embryo cultures in early passage with SD-RaLV and F-RaLV markedly accelerated their rates of transformation.

In another paper (18) we described the isolation and characterization of endogenous type C viruses, designated SD-RaLV and F-RaLV, from Sprague-Dawley (SD-1) and Fischer (F-1) rat embryo cell cultures, respectively. The viruses had typical type C morphology, 60-70S RNA, RNA-directed DNA polymerase (RDP), and interspecies- and rat leukemia virus (RaLV)-specific p30 antigen, and were neutralized by RaLV antisera. Both SD-RaLV and F-RaLV propagated efficiently in their cells of origin as well as in cells of other rat strains.

The SD-1 and F-1 cells were fibroblastic and contact inhibited at the subculture levels, passage 13 and 23, respectively, when RaLV was first detected. These same cells showed a progressive increase in rate of growth, loss of contact inhibition, and morphological transformation after approximately 20 further in vitro passages. Here we describe the properties of the transformed cells and the virus produced by them; we also show an acceleration in the rate of transformation of various rat embryo cultures exogenously infected with SD-RaLV and F-RaLV.

MATERIALS AND METHODS

Cell cultures. Two random-bred Sprague-Dawley (SD-1 and SD-2) and two random-bred Fischer (F-1 and F-2) rat embryo cultures were obtained from Flow Laboratories, Inc. (Rockville, Md.), and three inbred Fischer (F344) rat embryo cultures were obtained from Microbiological Associates, Inc. (Bethesda, Md.). Each of these embryo cultures was from a different pregnant rat. The cells were grown in minimum essential medium with Earle salts supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, and 50 μg of gentamicin per ml or 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of Kantrex (fungicide) per ml. The cells were subcultured once a week using either 0.1% trypsin containing 0.2 mg of EDTA per ml in Hanks balanced salt solution or 0.25% trypsin in complete medium.

Virus assays. Techniques for infecting the cells and for virus assay by complement fixation (CF), radioimmunoassay, rescue of mouse sarcoma virus genome, electron microscopy, [^{3}H]uridine uptake, RDP activity, and neutralization have been described in another paper (18). The transplant tumor-bearing animals were bled periodically, and their sera were tested by CF and neutralization for antibody to RPL-RaLV.

Growth curve and doubling time. The rate of growth was determined in the RaLV-infected and uninfected cultures by plating a known number (10^5 or 10^6) of cells per 75-cm^2 flask and counting and replating the same number at each subculture every week until the end of the experiment.

For doubling time, 24 dishes were plated at 2.5×10^4 cells per 60-mm^2 dish in complete medium and incubated at 37 C in a humidified atmosphere containing 5% CO_2 in air. At 24, 48, 72, and 96 h one dish was trypsinized, cells were counted, and five other dishes were stained and counted. The doubling time was determined by plotting average cell number against time.

Plating efficiency and cell cloning. The uninfected and RaLV exogenously infected cultures at the same passage level were trypsinized and plated at a density of 50 and 500 cells per 100-mm^2 dish; after 8 days the dishes were stained and colonies counted. For cloning efficiency the cell suspension was diluted to contain approximately 5 cells/ml (10 cells/ml was not satisfactory), and 0.1 ml was seeded in each well of Falcon Microtest II plates. The plates were repeatedly viewed microscopically during the first 48 h to identify wells receiving only one cell.

Inoculation of cells in vivo. Eight different groups of 30 to 40 Sprague-Dawley rats and one group of newborn Fischer rats were inoculated subcutaneously with 4×10^6 to 5×10^6 cells as listed in Table 1. The subcutaneous and metastatic tumors

TABLE 1. *Inoculation of rat cells in newborn Sprague-Dawley rats*

Inoculum	In vitro passage no.	Cell morphology	Endogenous RaLV released	Tumor incidence (no. positive/no. inoculated)
Saline			−	0/30
Fischer rat embryo cells (F-1)	17	Fibroblastic (nontransformed)	−	0/30
Fischer rat embryo cells (F-1)	29	Fibroblastic	+	0/30
Fischer rat embryo cells (F-1)	74	Transformed	+	30/30[a]
Fischer rat embryo cells (F-2)	6	Fibroblastic	−	0/30
Sprague-Dawley rat embryo cells (SD-1)	14	Fibroblastic	+	0/30
Sprague-Dawley rat embryo cells (SD-1)	31	Fibroblastic	+	0/30
Sprague-Dawley rat embryo cells (SD-1)	48	Transformed	+	30/30
Sprague-Dawley rat embryo cells (SD-2)	5	Fibroblastic	−	0/30

[a] A 100% incidence of tumors was also obtained in newborn Fischer rats inoculated with F-1 transformed cells at passage 89.

were removed after 15 to 20 days and transplanted to other weanling rats and also grown in vitro.

RESULTS

The spontaneous release of endogenous RaLV in SD-1 and F-1 rat embryo cultures was first confirmed at passages 13 and 23, respectively (18). At this point, both cultures were fibroblastic and contact inhibited but, after approximately 20 further passages, they began to show a progressive increase in growth rate with concomitant increase in the virus production. The SD-1 cells at passages 33 to 40 and F-1 cells at passages 43 to 50 exhibited altered morphology and loss of contact inhibition, and became permanently transformed cell lines, designated SD1-T and F1-T, respectively.

Properties of the transformed cells. The F1-T cells were a mixed population of small, rounded, or polygonal cells with large hyperchromatic nuclei and comparatively small cytoplasmic mass (Fig. 1). The SD1-T cells were elongated and spindle shaped with well-marked cytoplasmic boundaries (Fig. 2A). The transformed cells in both cultures grew to high saturation densities, and the population doubling time was reduced to 11 h, compared to 25 h in the nonvirus-producing cultures (Table 2). Both transformed cell lines produced RaLV as evidenced by the presence of budding and free type C particles, RDP activity in the culture fluids, and RaLV-specific p30 antigen in the cultured cells (Table 2).

Several single cell clones were derived from the F1-T cell line. Each of these clones produced RaLV, but three were morphologically distinguishable from the mixed parent cell line. One clone was comprised of small and compact round cells piled up in dense colonies (Fig. 1D); the second clone was mainly spindle cells (Fig. 1E), and the third clone consisted of large epithelioid cells (Fig. 1F).

Inoculation of cells in vivo. Eight groups of newborn rats were inoculated as listed in Table 1. The transformed cells, SD1-T and F1-T, produced tumors in 100% of the newborn syngeneic and allogeneic rats, but the nonvirus-producing and the virus-producing but nontransformed SD-1 and F-1 cultures failed to grow in vivo. The tumors were metastatic to lung, and killed the rats within 3 to 4 weeks. The primary tumors were transplantable to young adult or weanling syngeneic and allogeneic rats which also died with metastatic tumors after 3 to 4 weeks. Although the SD1-T and F1-T transplant tumors contained RaLV p30 antigen by CF in high titer (1:320), there was no systemic infection with RaLV based upon lack of RaLV p30 antigen or infectious virus in the corresponding spleens. Sera of weanling Fischer rats bearing F1-T transplant tumors, collected 32 to 43 days postinoculation, contained high-titered ($\geq$1:160) CF antibody to banded RaLV antigen. Sera from Sprague-Dawley rats bearing SD1-T or F1-T tumors also showed, by MSV (F-RaLV) focus reduction, RaLV neutralizing antibody in high titer (1:320). The first generation transplant tumors contained higher-titered RaLV CF p30 antigen and induced higher titers of RaLV neutralizing and CF antibody than did the later serial transplants.

The in vitro grown transplant tumors were morphologically similar to the inoculated cells and were positive for RaLV gs antigen, RDP, and type C particles (Table 2).

Properties of the virus. The transformed cell lines, SD1-T and F1-T, and each of the in vitro grown transplant tumors produced RaLV. The virus released by these cells had the same properties as described for the RaLV originally isolated from the fibroblastic nontransformed SD-

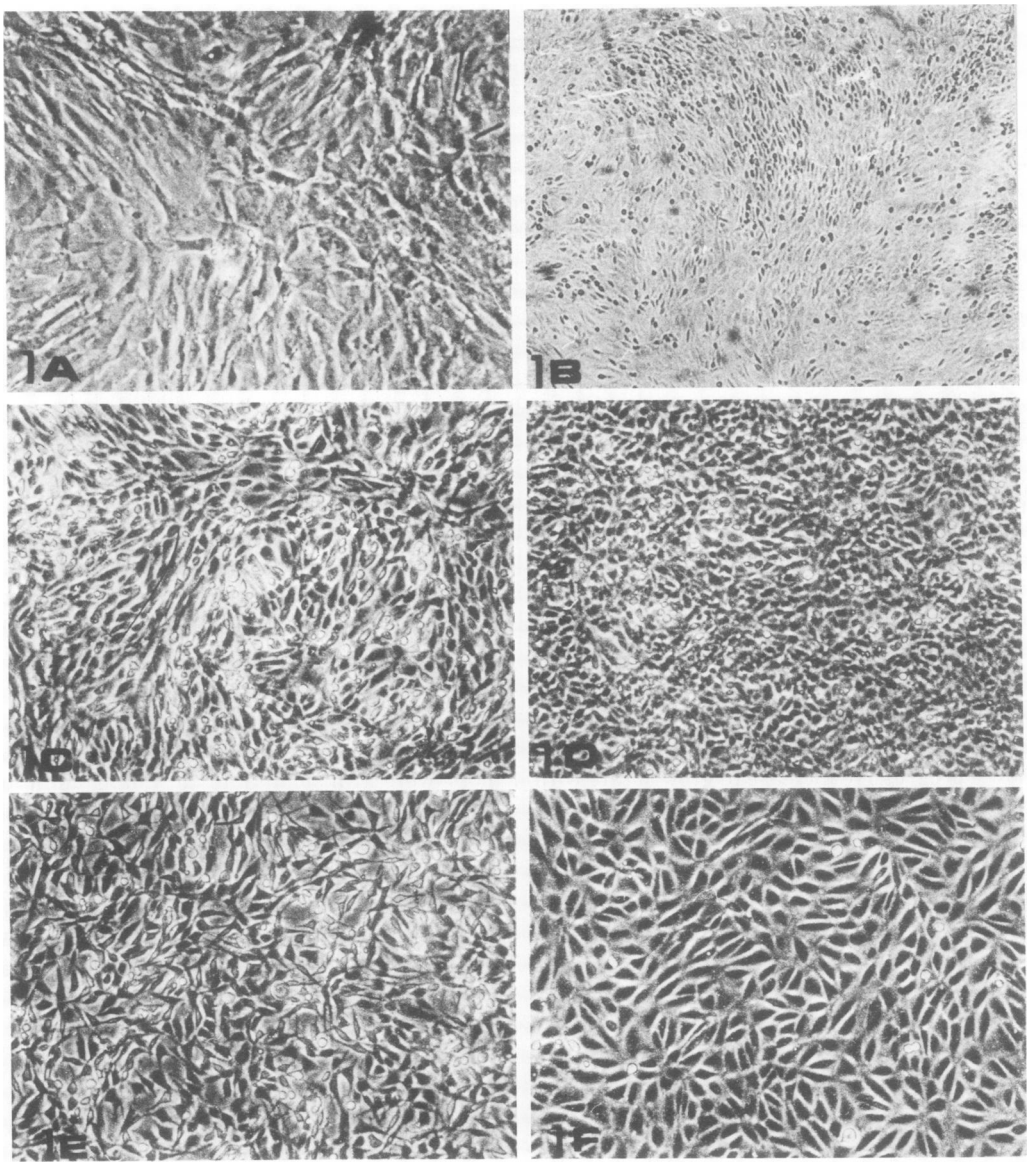

FIG. 1. *Sequence of transformation in Fischer rat embryo cultures (F-1). Unstained, phase contrast. (A) Fibroblastic, contact-inhibited, nontransformed cells spontaneously releasing endogenous RaLV, passage 29. ×60. (B) Initiation of transformation, passage 42. Note the dark, refractile, and rounded cells. ×25. (C) Fully transformed rat embryo culture (mixed cell population), passage 49. ×60. (D-F) Clonal cell lines derived from a transformed Fischer rat embryo culture, passage 86. Photographs taken 24 h after separately plating an equal number of three different cell types. (D) Clone I, rounded, piled-up cells, passage 90. ×60. (E) Clone II, spindle-shaped cells showing criss-cross pattern of growth, passage 90. ×60. (F) Epithelioid, comparatively large cells, passage 90. ×60.*

1 and F-1 cultures (18). These viruses also showed a restricted host range and replicated only in cells of homologous and heterologous rat strains but not in cultures of 20 other human and animal species tested (See footnote *a*, Table 1, and ref. 18). One of the Fischer viruses from a transplant tumor, tested by infecting Fischer cells (F-2) with serial 10-fold virus dilutions showed, at 20 days, a titer of $10^{2.9}$ mean (50%) tissue culture infective doses/ml, by CF and RDP activity in the infected cells. Virus produced before transplantation (passage 45) had the same infective titer.

SD-RaLV and F-RaLV isolated from trans-

formed cells and from the in vitro grown transplant tumors rescued the MSV genome from Ki-MSV transformed nonproducer NRK cells. The titer of the MSV (F-RaLV) pseudotype on NRK was $10^{4.4}$ focus-forming units/ml.

Exogenous infection of other rat cells. Various rat cells were exogenously infected with SD-RaLV and F-RaLV in two separate laboratories (A.E.F. and S.R.) (Table 3). Two different Fischer embryo cultures infected with F-RaLV and F1-T RaLV showed evidence of virus replication at 20 days by the presence of RDP in the culture fluids and RaLV p30 antigen in cells by radioimmunoassay. After an additional 10 to 12

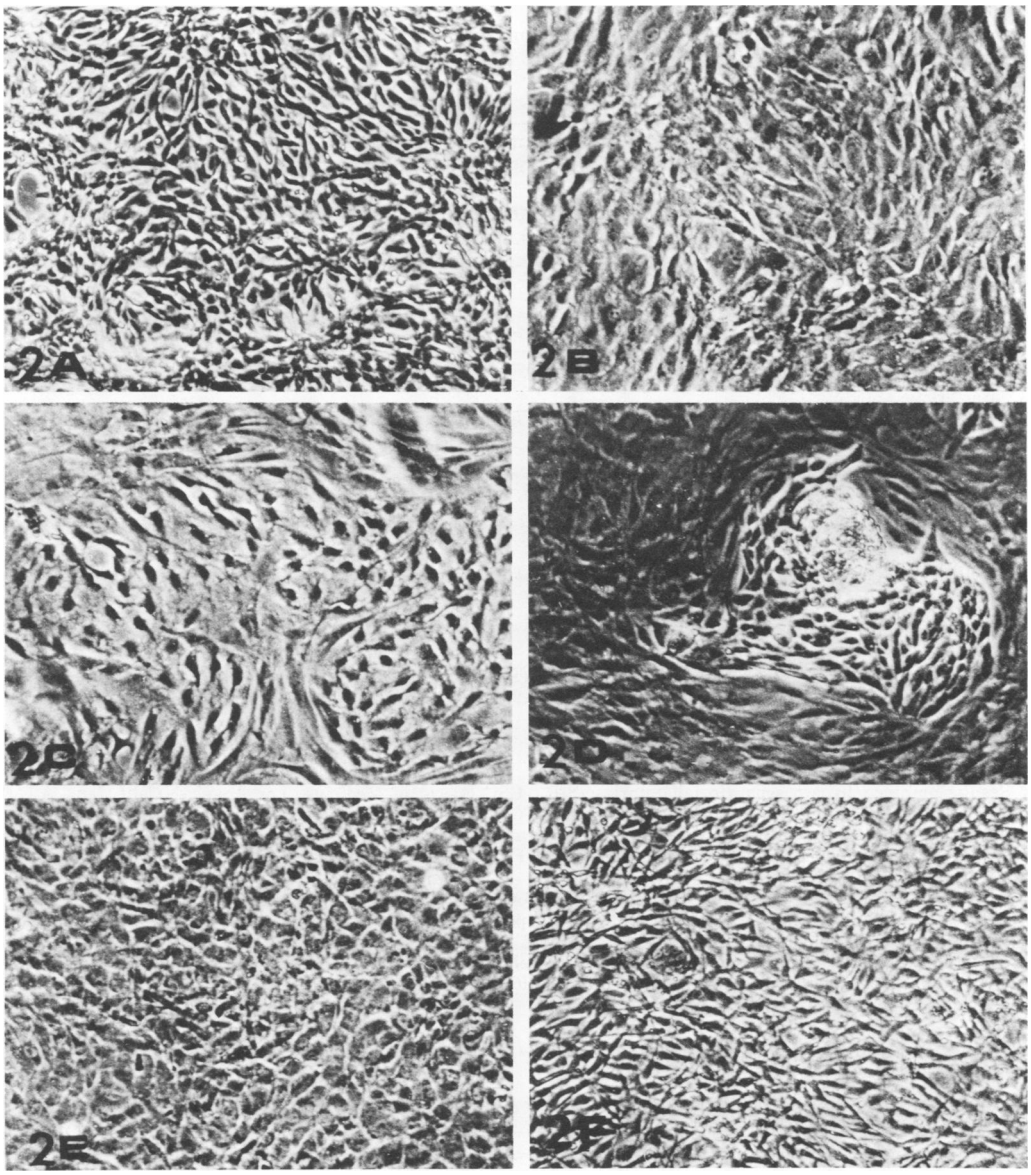

FIG. 2. *(A) Sprague-Dawley rat embryo cultures (SD-1) spontaneously transformed, passage 77. ×60. (B) Fischer rat embryo cultures (F-1) uninfected, passage 20. ×60. (C) F-1 cultures (same as in Fig. 2, panel B) at passage 20, exogenously infected with F-RaLV at passage 4, showing morphologically altered, refractile cells interspersed with nontransformed, contact-inhibited cells. ×60. (D) Same culture as in Fig. 2C, after one additional week in vitro. Colonies of piled-up transformed cells are clearly evident. ×60. (E) Normal uninfected Fischer rat embryo culture (F-3), passage 36. (F) Fischer rat embryo cultures (F-3) exogenously infected with SD-RaLV at passage 1. Transformation occurred at passages 19 to 20, photograph taken at passage 36. ×60.*

TABLE 2. *Comparison of "normal" and transformed cells*[a]

Characteristic	Normal uninfected cells	Transformed cells
Morphology	Spindle-shaped fibroblastic	Rounded, polygonal hyperchromatic nuclei
Electron microscopy	No type C virus particles	Numerous budding type C virus particles
Rat-specific p30 antigen[b]	No p30 antigen	RaLV p30 antigen
Chromosomes	Diploid	Polyploid
Population doubling time	25 h	11 h
Population density[c]	0.5×10^6 cells/cm^2	1.8×10^6 cells/cm^2
Contact inhibition[d]	Yes	No
Plating efficiency (%)	25	100
Growth in vivo (%)	0	100

[a] F1-T or SD1-T cell lines and their transplant tumors grown in vitro.
[b] Detected by CF and radioimmunoassay using highly specific guinea pig antiserum against isoelectric focus-purified p30 protein of RaLV.
[c] Density of transformed cells represents the number of cells present at the time the control uninfected cells reached a growth plateau, i.e., showed no further increase in cell population.
[d] Decrease in rate of cellular growth as an increasing proportion of cell surface comes in contact with other cells.

TABLE 3. *Exogenous infection of Fischer rat embryo cultures with SD-RaLV and F-RaLV*[a]

Characteristic	Embryo I F-1		Embryo II F-2		Embryo III F-3		Embryo IV F-4		Embryo V F-5	
	TCC	RaLV	TCC	RaLV	TCC	RaLV	TCC	RaLV	TCC	RaLV
Passage level of cells at time of infection	6	6	4	4	1	1	1	1	1	1
Infecting virus	0	F-RaLV (44)	0	F1-T-RaLV[b] (74 + 1)	0	SD-RaLV (35)	0	SD-RaLV (35)	0	SD-RaLV (35)
RDP[c] in culture fluid	201 (10)	63,799 (10)	0 (6)	1,588 (6)	0 (18)	159,540 (18)	0 (18)	2,340 (18)	0 (18)	33,090 (18)
RaLV-specific p30 antigen	0	+	0	+	0	+	0	+	0	+
Cell morphology	F (14-28)[d]	T (14-16)	F (15-33)	T (15-20)	F (19-33)	T (19-20)	F (19-33)	T (19-20)	F (19-33)	T (19-20)
Population density per cm^2 at time transformation appeared	6×10^5	8×10^5	5×10^5	7.5×10^5	6.2×10^5	9.3×10^5	2.8×10^5	4×10^5	3.8×10^5	5.6×10^5

[a] Results obtained in two separate laboratories (S.R., USC School of Medicine, Los Angeles, Calif., and A.E.F., Childrens Hospital of Akron, Akron, Ohio). Abbreviations: TCC, Tissue culture control (uninfected); RaLV, RaLV-infected cells from the same embryo culture and at same passage level as TCC controls. The number in parentheses indicates the passage level of cells at which infecting virus was harvested; F, fibroblastic, contact-inhibited, and nontransformed cells; T, transformed and piled-up cells.
[b] RaLV isolated from first passage in vitro grown tumor of Fischer rat transformed cells.
[c] RNA-directed DNA polymerase activity (counts per min per ml) in culture fluid. The number in parentheses represents the passage level of the cells tested.
[d] The numbers in parentheses represent the passage levels of cells showing the respective cell morphology. The TCC remained fibroblastic even when carried for an additional 10 to 15 passages.

passages in vitro an increased rate of growth was observed in the RaLV-infected cells but not in the uninfected control cultures at the same passage levels. The cells also became refractile and rounded, and showed areas of criss-cross orientation (Fig. 2B-D).

Three separate Fischer rat embryo cultures were also exposed to SD-RaLV (Table 3). The virus-exposed cells showed RDP activity and rat gs antigen, but the uninfected control cultures remained negative for virus. The SD-RaLV-infected cells showed an increased rate of growth compared to the uninfected cultures and transformed after 19 to 22 in vitro passages (Fig. 2E-F), whereas the uninfected control cells remained fibroblastic, even when carried for an additional 10 to 11 passages (Table 3).

DISCUSSION

Spontaneous transformation of rat embryo cells has been reported for many years (9, 13, 23, 24, 27, 28, 30). Transformation of chemically treated (17, 19, 31) or long-term cultures of

murine leukemia virus-infected rat embryo cells has also been described (2, 12, 20). However, only recently has it been possible to relate transformation with expression of endogenous RaLV (3,4, 8, 15, 22). Freeman et al. (4) showed that Fischer rat embryo cells did not transform spontaneously until 60 to 90 subpassages, at which time they also expressed endogenous RaLV gs antigen. However, transformation at earlier passage levels could be induced in these cells by the addition of exogenous MuLV, together with chemical carcinogens (4–7, 21). In the SD-1 and F-1 cultures, the spontaneous release of endogenous RaLV at relatively early passage levels, passage 13 and passage 23, respectively, and the ensuing transformation at passage 35 and passage 45, therefore, represents a telescoping of the usual phases characterizing "aging" rat embryo cell cultures (4).

An important property is the ability of the endogenous RaLV released by SD-1 and F-1 cultures to exogenously infect rat cells, i.e., ecotropism, whereas in most of the previously described rat embryo cell cultures the endogenous RaLV genome, if activated spontaneously or with chemical carcinogens, was either only partially expressed as gs (p30) antigen (3, 4, 22) or, when fully expressed as type C virus, was apparently noninfectious for other rat cells (8, 14, 15, 25). Although not yet proven, some of these viruses may show xenotropic behavior (16). The ecotropic nature of the SD-RaLV and F-RaLV, therefore, permitted exogenous infection of other nonvirus-producing Fischer rat embryo cell cultures in early passage (passages 2 to 6); these cells then transformed without added chemicals after another 15 to 22 passages in vitro. In line with previous reports (5–7, 10, 12, 21), our initial results also indicate that Fischer embryo cells (F-2) exogenously infected with F-RaLV and treated at passage 12 with 1 μg of 3-methylcholanthrene per ml for 6 days transform after only 4 to 5 further passages.

In the first experiments it was impossible to know whether release of endogenous RaLV was the cause of the transformation event, since noninfected controls at the same passage levels were not available. However, the release of endogenous RaLV in the SD-1 and F-1 cultures for 20 passages preceding transformation and the transformation of rat embryo cells in early passage by exogenous infection with RaLV in the absence of transformation in the uninfected cultures even at higher passage levels (Table 3) strongly suggest that RaLV is responsible for the transformation event. The molecular mechanism by which leukemia viruses transform cells or cause leukemia is not known. Contrary to the sarcoma viruses, which are generally defective and transform fibroblasts within 5 to 7 days, the leukemia viruses are nondefective and do not normally transform fibroblasts. Our findings suggest that the ecotropic RaLV strains that we have isolated may, indeed, transform rat embryo cells, but only after several cellular and viral replication cycles. Rat embryo fibroblasts, productively infected with the RaLV which was harvested from the transplant cultures, still require 15 to 20 further passages before transformation. Thus, we have no evidence so far to suggest rescue of a rat sarcoma genome or R-35-like rat virus (1) from in vitro and in vivo passage of RaLV. Whether the RaLV-induced transformation is attributable to viral oncogenes (11) or to derepression or transduction of rat cellular oncogenes (26, 29) by replicating RaLV is yet unknown. Analysis of precloned early passage embryo cells and in vitro dose-response and virus neutralization studies now in progress should help determine the role of RaLV in cell transformation.

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Addendum in Proof

Recently, we have observed that although the high passage (over 90) transformed cells released up to 10^9 particles/ml by EM negative stain and ROP activity of 90,000 to 10,000 counts/ml in culture fluid, the infective virus titer has decreased significantly. Properties of these particles are presently under investigation.

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