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Elimination of the Sarcoma Genome from Murine Sarcoma Virus Transformed Cat Cells

Abstract. *Cat cells transformed by Moloney murine sarcoma virus contain virus-specific sequences in their RNA and DNA. Cloned, spontaneous revertant cell lines derived from clones of these cells had no evidence of the sarcoma genome in the cell RNA or DNA as judged by RNA-complementary DNA or DNA-complementary DNA hybridizations. This is apparently the first report of loss of a transforming genome in a revertant cell line.*

Reversion of virus transformed cells is a rare event but has been observed in a number of systems including Rous sarcoma virus transformed hamster cells, polyoma and SV40 transformed hamster and mouse cells, and Moloney murine sarcoma virus (MSV) transformed mouse cells (*1*). Revertant cells can be obtained from every subclone of the sarcoma virus transformed mouse cells (*1*). This suggests that the capacity for reversion is inherent in all MSV transformed cells in culture. Clones of MSV transformed cat cells were obtained by the so-called "single-hit" infection of a cat cell line with the feline leukemia pseudotype of MSV [MSV(FeLV)] as described (*2*). The cloned 8C transformed cat cell line had a high rate of spontaneous reversion with loss of expression of murine p30 antigen and no rescuable sarcoma virus (*3*). This system was chosen because MSV contains murine leukemia virus (MuLV) sequences that are present in normal mouse cells, making revertant mouse cells more difficult to study (*4, 5*). We now report evidence for the loss of a transforming sarcoma genome in these revertant cells.

Because of the loss of the MuLV group-specific antigen of p30 in the revertants we began by examining the quantity of virus-specific RNA in these cells (*3*). With the use of an MSV and Moloney MuLV virus complex (*6*) (the ratio of biological activity of MSV to MuLV was 4 : 1) in an endogenous reverse transcriptase reaction with actinomycin D, a labeled DNA transcript complementary to $\geq$ 70 percent of the 65*S* viral RNA was made (Fig. 1a). This DNA was hybridized with cellular RNA, and the hybrids were analyzed by hydroxylapatite chromatography (*7*) by varying the cellular RNA and viral RNA concentrations in hybridizations of RNA and complementary DNA (cDNA). The 65*S* homologous viral RNA hybridized with 83 percent of the probe (cDNA) (Fig. 1a). The positive controls consisted of MSV transformed cat cell lines. Cell line P521 is a cloned MSV transformed cat cell line infected with and producing MSV and FeLV. The P521 cell RNA hybridized with 77 percent of the probe, with a half-saturation value (the RNA concentration of half-maximal hybridization with cDNA) of 10 μg of cell RNA per 0.1 ml of reaction mixture (Fig. 1a). Cell line 81 is a subclone of the 8C cat cell line transformed by MSV with rescuable MSV but no replicating ecotropic oncornavirus. Although its RNA also hybridized with 74 percent of the cDNA probe, higher concentrations of cell RNA were required (Fig. 1a). In the RNA-cDNA hybridization curves of Fig. 1a, the final degree of hybridization reflects the total genome content; the half-saturation value of RNA gives the copy number for the genome (Table 1). There were fewer copies of the viral genome in the cell line 81 RNA. The negative control was the normal cat strain CCC, the normal cat cell line from which all other lines were derived (*2*). Only 4 percent hybridized with the probe. The cat cell revertants 8CR4, 8CR7, and 8CR10 were derived from the

Table 1. Murine sarcoma–leukemia specific information in cat cell lines. The percentage of genome present as RNA is calculated as the maximal hybridization shown on the RNA-cDNA curve divided by the maximal hybridization of the probe with 65*S* homologous RNA (83 percent) minus the percentage of hybridization without RNA (4 percent) from each. The fraction of the cell RNA that is viral is the ratio of the half-saturation concentration of the viral RNA (0.03 μg/0.1 ml) to the half-saturation concentration of cell RNA. (The half-saturation viral RNA concentration was 0.03 μg/0.1 ml.) The percentage of genome present as DNA is the maximal hybridization on the DNA-cDNA curve divided by the maximal hybridization of probe with MSV-MuLV infected mouse cell DNA (80 percent). The number of viral copies per haploid genome is the $C_0t_{1/2}$ of the cell DNA reannealing divided by the $C_0t_{1/2}$ of the probe-cell hybridization (*20*). The percentage of genomes in the DNA and RNA are minimal values because the probe has murine leukemia–specific sequences not expected to hybridize with the DNA's and RNA's containing only MSV.

Cell line	Description	DNA genomes		RNA	
		In cell DNA (%)	Copies per haploid cell (No.)	In cell RNA (%)	Cell RNA that is viral (10^{-2} %)
P521	Cat cell line producing MSV(FeLV) and FeLV	72	16	92	30
81	MSV transformed cat cell line	38	3	89s	5
FeLV-81	FeLV infected 81 cell	64	8	85	6
CCC	Normal cat cell line	12	0	4	<.3
818	Revertant from 81	11	0	5	<.3
8CR7	Revertant from 8C	17	0	1	<.3
8CR4	Revertant from 8C	9	0	0	<.3
8CR10	Revertant from 8C	12	0	0	<.3
F49-1	Normal human amnion cell line	6	0		
Calf thymus	(Worthington)	2	0		
	No DNA	2	0		

8C. The revertant 818 was derived from 81. Hybridization of the RNA's of these revertants with the probe was from 5 percent to none at all, an indication that MSV specific sequences were not present in revertant cell RNA. This absence could be due to either transcriptional control in these revertants or actual loss of the DNA viral genome.

To ascertain whether there was actual loss, the probe was hybridized to cellular DNA; and the hybrids were analyzed again by hydroxylapatite chromatography and confirmed with digestion with nuclease S1 (*8*). The DNA was made from cells by two different methods—extraction with 8*M* urea phosphate buffer (*9*), or extraction by a modification of the Thomas procedure (*10*) (Fig. 1b). On repeated experiments there was an error of ± 5 percent on the curves for DNA-cDNA hybridization. The amount of sarcoma genome was determined by varying the time of incubation in cell DNA-cDNA hybridizations. As an internal control the cell DNA reassociation was monitored by optical methods in the same solution (*11*). The cell DNA reassociated at least 80 percent in all cases, without a correction for hypochromicity. The cellular DNA from mouse cells producing Moloney MSV and MuLV viruses hybridized with 89 percent of the DNA probe, indicating that the DNA extraction method was adequate. Hybridization of calf thymus DNA with the probe was 3 percent. The cell DNA from two cat cell lines containing MSV showed hybridization with the probe. For the purposes of comparison we considered the hybridization of MSV-MuLV cDNA with mouse cells producing MSV and MuLV as being 100 percent. Then the P521 cell DNA hybridized with 75 percent of the probe and the 81 cell DNA hybridized with 40 percent of the probe. These results were obtained with four different preparations of 81 cell DNA. The 81 cell line was tested for a rescuable MSV genome at the beginning and at the end of the experiment by further subcloning experiments. Murine sarcoma virus was rescued from nine out of ten and nine out of nine subclones of cell line 81 before and after these studies.

The same probe showed 12 percent relative homology with the DNA from the uninfected CCC cat cell line DNA. A similar low degree of hybridization was observed with DNA extracted from revertant cells (Fig. 1b). The 12 percent hybridization shown by CCC and revertants was further studied by thermal elutions from hydroxylapatite (*12*). The DNA-cDNA hybrids had limited structure and melted by 70°C. Nuclease S1 digested these hybrids to a 1 percent background. Further, by changing the incubation temperature for DNA-cDNA hybridizations from 70° to 63°C, the CCC and revertant hybridizations rose to 35 percent, but the hybrids also had limited structure because they melted at low temperature and were 99 percent digestible with nuclease S1. At the same time, S1 digested the mouse cell DNA hybrid producing the homologous virus to 44 percent, the P521 hybrid to 41 percent, the FeLV-81 to 37 percent, and the 81 hybrid to 22 percent.

Both the percentage of the genome present and the relative copy number were calculated from these data (Table 1). The probe was an accurate copy of the viral RNA. We obtained maximal protection—70 to 75 percent at a molar ratio of 2 : 1 of probe DNA to 60*S* RNA labeled in vitro with ^{125}I (*13*). It therefore seems unlikely that a major portion of the MSV genome escaped detection.

Positive strand viral RNA can be hybridized with cell DNA to reveal negative strand viral sequences in the cell. Using the ^{125}I-labeled 65*S* RNA from MSV-MuLV, we hybridized it with cell DNA and analyzed the extent of hybridization by protection of the [^{125}I]RNA labeled from ribonuclease T1 digestion (*14*). The results quantitatively confirmed the hybridizatons obtained with the DNA probe. Hence, the negative strand viral DNA sequences in the cell have the same amount of the genome as the positive strand sequences tested with the ^{3}H-labeled DNA probe.

The results show that (i) the sarcoma transformed cat lines have acquired de novo viral sequences in the RNA and

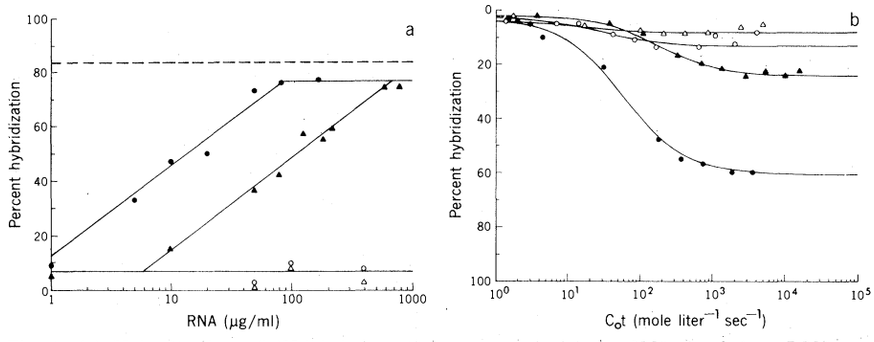

Fig. 1. (a) Hybridization of ^{3}H-labeled murine sarcoma–leukemia DNA (probe) to RNA extracted from cat cell lines. The virus was obtained from the supernatant of a S + L– clone of Moloney MSV transformed 3T3FL cells infected with Moloney leukemia virus (*6*). The virus had a fourfold excess of sarcoma to leukemia, as judged by biological assays. The supernatant was concentrated 2000-fold by polyethylene glycol precipitation. The pellet was resuspended in phosphate buffered saline. This material was banded through sucrose, and kept for 45 minutes at 37°C in an endogenous reaction mixture with actinomycin D (100 μg/ml) and 10^{-5}*M* [^{3}H]TTP (thymidine triphosphate) (50 c/mmole, New England Nuclear) (*21*). Protein was removed from the DNA product with sodium dodecyl sulfate (SDS), chloroform, and phenol; the DNA product was treated with alkali at 90°C for 5 minutes and concentrated on hydroxylapatite in 0.3*M* phosphate buffer. The [^{3}H]-DNA probe protected 70 percent of the ^{125}I-labeled 65*S* Moloney sarcoma–leukemia RNA at a 2 : 1 molar ratio of DNA to RNA, with 5 percent background. Ribonuclease T1, heated to 80°C for 10 minutes, was used in double-strength saline sodium citrate at 37°C for 30 minutes at a concentration of 200 unit/ml in the protection experiment. Cell RNA was extracted with the SDS and hot phenol method (*22*). Varying amounts of cell RNA or viral 65*S* RNA (or no RNA) were incubated in 0.2 ml of 0.2*M* phosphate buffer with the ^{3}H-labeled DNA probe (1000 count/min). After 16 hours, annealed samples were diluted in 3 ml of 0.12*M* phosphate buffer and fractionated on 1-cm^{3} hydroxylapatite columns (15 cm^{3} if more than 50 μg of RNA) and washed with 0.14*M* and 0.3*M* phosphate buffer at 50°C. The washings were precipitated with trichloroacetic acid (TCA). The radioactivities (count/min) in the TCA precipitate of the 3*M* buffer wash divided by the total radioactivity in both washes represents the percent hybridization. The maximum hybridization of the 65*S* RNA to the labeled probe is shown by the dotted line. ●, Annealing of MSV-MuLV ^{3}H-labeled DNA to cell RNA of P521; ▲, annealing to 81; ○, annealing to CCC; and △, annealing to 8CR4. (b) Hybridization of murine sarcoma–leukemia [^{3}H]DNA (probe) to DNA extracted from cat cell lines. The probe was prepared as described for (a). The cell DNA was extracted from cell pellets as described by Thomas (*10*). The DNA was boiled in alkali, neutralized, and pressure-dialyzed. The cell DNA was then sonicated, boiled with the probe in one-tenth strength saline sodium citrate, and incubated at 70°C in mixtures (total volume of 0.3 ml) containing 4 to 6 mg of cell DNA per milliliter, 5×10^{-5} μg of probe (1000 count/min) per 40 μg of cell DNA, 0.1*M* tris buffer (*p*H 7.4), 0.75*M* NaCl, 0.2 m*M* EDTA, and 0.05 percent SDS. Portions containing 40 μg of cell DNA were removed and fractionated on hydroxylapatite columns (1 cm) with 0.14*M* phosphate buffer and 0.3*M* phosphate buffer at 50°C. Absorbance was measured at 260 nm, and the radioactivity in the fractions precipitated by TCA was determined. The ratio of the absorbance in the 0.3*M* phosphate buffer fraction or the number of counts per minute to the total absorbance or counts per minute gave the percent hybridization for the cell DNA or probe-cell DNA, respectively, C_0t values (C_0 is the concentration of cellular DNA in moles per liter and t is the time in seconds) were calculated (*20*) as $[A_{260}/(\text{ml} \times 2)] \times$ hours and corrected to a monovalent cation concentration of 0.18*M*. Annealing of MSV-MuLV [^{3}H]DNA to cell DNA follows the same notation as in (a), and the hybridization curves were plotted with a least squares, computer-determined second-order fit.

DNA of the cell; (ii) revertants have lost this information from both cellular RNA and DNA; (iii) fewer copies of the MSV genome exist per cell in the RNA of a sarcoma-positive subclone than in the MSV-producing cat cell P521; and (iv) the MSV transformed cat cell 81, which has MSV and a complete sarcoma RNA copy, does not have an apparent complete MSV copy in its DNA. The proportion of MSV-specific DNA in cell DNA apparently increases after FeLV infection. This increase was not due to FeLV infection alone since only 17 percent of the CCC-FeLV cell DNA was hybridized as judged by fractionation on a hydroxylapatite column; the thermal elution profile of the hybrid was unstructured and the hybrid was 99 percent digestible by nuclease S1. An increase in copies in 81-FeLV cells may be responsible for the apparent increase in genome content (*15*).

There may be multiple mechanisms of control leading to in vitro reversion including repression of transcription, loss of chromosomes containing an integrated virus, and plasmid segregation. Revertants of hamster kidney cells transformed by Rous sarcoma virus have the provirus in the chromosomal DNA, but virus-specific RNA is reduced (*11*).

Polyoma and SV40 virus transformed cells have a high rate of spontaneous reversion with an imbalance of chromosome number and makeup and a tendency to transform back with retention of the viral genome (*17*). Both polyoma transformed and revertant hamster cells have eight viral DNA equivalents in the cell DNA (*18*). Here again reversion may be due to control, perhaps by chromosomal factors (*17*), of the expression of the viral genes concerned with the maintenance of transformation. Within the sensitivity of our methods (> 0.1 DNA copy per haploid genome and > 0.003 percent of the total cellular RNA), the cat cell system described above represents the only revertant cell that has lost the transforming viral genome. Several properties of the transformed cat cells, including the high rate of reversion and, as demonstrated, the loss of the genome from revertants, suggest that the genome may exist as a plasmid in cell line 81 (*19*). Although the mechanisms of eviction of a transforming virus genome are not known, an understanding of such phenomena may represent an alternative approach to the control of malignancy.

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12. Thermal elutions were performed with the hybrids (5000 count/min) from the incubations described for Fig. 1b. The hybrid was diluted with 0.12*M* phosphate buffer and passed through a hydroxylapatite column (1 cm^3), washed with 0.12*M* phosphate buffer at 2°C increments from 50° to 100°C. The P521 and 81 hybrids had a high T_m, an 86°C component, and an unstructured component of 10 to 15 percent which melted between 50° and 70°C. The CCC and revertant hybrids had only the unstructured component.
13. We thank D. Colcher for iodination of 65*S* RNA.
14. ^{125}I-Labeled 65*S* hybridized MSV-MuLV RNA (2400 count/min equal to 0.012 μg) was boiled 10 minutes with 4 mg of sheared cell DNA (made as described in the legend of Fig. 1b) and incubated in 0.3 ml of 0.3*M* phosphate buffer at 63°C. At various times 50-μl portions (4000 count/min) were removed and divided equally into two 1-ml volumes of double-strength sodium saline citrate buffer. One portion was treated with 200 units of ribonuclease T1 (Calbiochem). Both were incubated for 30 minutes at 37°C, precipitated with trichloroacetic acid, and filtered; the radioactivity in the precipitate was counted; the proportion of radioactivity remaining with ribonuclease treatment represents the percentage of hybridization of the RNA. With no DNA, protection was 5 percent; CCC and revertants protected 9 percent; 81 protected 20 percent; P521 protected 44 percent; and 3T3 mouse cells producing MSV-MuLV protected 61 percent.
15. The lower final hybridization with 81 cell DNA may be related to the low copy number in these cells. Reconstruction experiments using nine-tenths CCC cell DNA and one-tenth P521 cell DNA revealed three copies per haploid genome. Fifty percent rather than 75 percent of the probe hybridized with the diluted P521 DNA.
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A Cytoplasmic Factor Promoting Oocyte Maturation: Its Extraction and Preliminary Characterization

Abstract. *Cytoplasm of maturing amphibian oocytes possesses a factor that induces germinal vesicle breakdown. This factor was extracted from* Rana pipiens *eggs and assayed by microinjection into* Xenopus laevis *oocytes. The activity of this factor is Mg-dependent, Ca-sensitive, and associated with heat-labile protein. Centrifugation on a sucrose density gradient revealed that the factor exists in three different molecular sizes.*

In Amphibia, fully grown ovarian oocytes that have been arrested at meiotic prophase resume meiosis when exposed to progesterone in vitro as well as when normally ovulated in vivo (*1, 2*). The resumption of meiosis, called meiotic maturation, is signaled by nuclear membrane breakdown (germinal vesicle breakdown). Since such breakdown is not induced when progesterone is injected into the oocyte (*3, 4*), a cytoplasmic factor, produced in the oocyte in response to externally applied progesterone, has been postulated as the direct cause. Indeed, cytoplasm taken from oocytes matured in the presence of progesterone induces germinal vesicle breakdown when injected into untreated ovarian oocytes (*2–8*). This cytoplasmic factor, the maturation-promoting factor (MPF), is not species-specific among amphibians (*6*), nor does it appear to be cell-type specific, in that it induces similar changes in nuclei from somatic cells when they are injected into cytoplasm of fully mature oocytes (*9*).

Table 1. Frequency of germinal vesicle breakdown (GVBD) in recipient oocytes injected with extract on the day of extraction. Each extract was injected into 15 *Xenopus* oocytes at 80 nl per oocyte.

Addition to extraction medium	Ca^{2+}		Mg^{2+}		EGTA		EDTA	
	Total	GVBD	Total	GVBD	Total	GVBD	Total	GVBD
0 m*M*	30	30	30	30	45	45	30	30
1 m*M*	30	3	15	15	30	30	15	13
2 m*M*	30	1	15	15	45	45	15	5
5 m*M*	30	0	30	30	30	30	30	0
10 m*M*	30	0	30	30	30	30	15	0