

# MUTANTS OF NONPRODUCER CELL LINES TRANSFORMED BY MURINE SARCOMA VIRUS

## II. RELATIONSHIP OF TUMORIGENICITY TO PRESENCE OF VIRAL MARKERS AND RESCUABLE SARCOMA GENOME

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The preceding paper described the induction and isolation of cell mutants derived from a single clone of virus-transformed, nonproducer (NP)<sup>1</sup> cells (1). This paper presents the characterization of these mutants with regard to the relationship between tumorigenesis and presence of sarcoma virus genome.

### *Materials and Methods*

*General.*—Cell lines including those produced by 5'-bromodeoxyuridine (BrdU) mutagenesis were described in the previous paper (1). The complement-fixation (CF) test for mouse gs antigen has been described previously (2). The assay of RNA-dependent DNA polymerase in culture fluid by use of the synthetic polymer, rA:dT<sub>12</sub>, was previously reported (3). Rescue of the sarcoma genome by superinfection with helper virus was done as previously described (4). A more sensitive rescue technique, using Sendai virus to fuse cells replicating helper virus and test cells was also employed (5). The XC test for mouse Type C virus was performed as described by Rowe et al. (6).

*Chromosome Analysis.*—Growing cells were treated for 4–18 h with 0.2 µg/ml of colchicine. Cells arrested in metaphase were removed by shaking, then centrifuged at 1,000g for 5 min, resuspended in 1% sodium citrate for 20 min at 37°C, recentrifuged, and finally fixed in freshly prepared glacial acetic acid:methanol (1:3). Chromosome preparations were made from the suspension and air dried. After staining with Giemsa and mounting, well-spread metaphases were photographed and the chromosome number determined.

### RESULTS

*Detection of Viral Markers. Mouse Type C Virus gs Antigen.*—Mouse gs antigen was not detectable by the CF test in any of the virus negative mutant cells. In contrast, the M-57-1 and M-58-4 cell lines which were found to shed virus, contained gs antigen in cellular and culture fluid extracts (Table I).

*Biological Activity of Type C Particles.*—The Type C particles shed from M-57-1 and M-58-4 cells contained gs antigen and RNA-dependent DNA polymerase activity, induced syncytium formation when plated with XC cells, and showed focus forming activity on both NIH Swiss and BALB/c mouse embryo cells (Table I and Fig. 1). In general higher titers were obtained on

<sup>1</sup> Abbreviations used in this paper: BrdU, 5'-bromodeoxyuridine; H(MSV), Harvey strain of murine sarcoma virus; MuLV, murine leukemia virus; NP, nonproducer.

BALB/c cells. These results indicated that the Type C particles shed from M-57-1 and M-58-4 were infectious sarcoma viruses with no clear indication of either N- or B-tropism (7).

*Rescue of Sarcoma Genome from the Virus-Free Mutants.*—As shown in Table I, the nontumorigenic cell line M-43-2 did contain a rescuable sarcoma genome, while the tumorigenic mutant M-50 did not yield sarcoma virus in repeated rescue attempts by direct superinfection. With the more sensitive fusion rescue

TABLE I  
*Presence of Viral Markers in the Cell Mutants*

Cell type	Cell line	gs Antigen		RNA-DNA polymerase	Sarcoma genome rescue	
		Cell	Culture fluid	Culture fluid	–Helper	+Helper
Normal	B/3T3	0*	0‡	0,	0	0¶
Tumorigenic	NP	0	0	0	0	100
A	M-50	0	0	0	0	0
B	M-57-1	32	4	1,312	76	—
C	M-43-2	0	0	0	0	2,600
D	M-58-4	32	4	855	340	—

\* Number shows dilution of monospecific mouse gs antiserum demonstrating 4+ CF reaction with 2 U of complement against 50  $\mu$ g of cell extract protein.

‡ The same CF test against particles from 25 $\lambda$  or  $\mu$ l of 20-fold concentrated culture media.

§ Assay was performed as described previously (3). Average CPM from three to five plates by the following methods: 0.1 ml of culture fluid after low centrifugation was incubated with an equal volume of reaction mixture at 37°C for 30 min. The reaction mixture was then treated with cold 10% trichloroacetic acid and the acid insoluble material was collected on Millipore filter papers (Millipore Corp., Bedford, Mass.); the radioactivity was determined in an LS-250 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

|| Focus-forming units per milliliter culture media after 5 day infection on NIH Swiss mouse embryo fibroblasts.

¶ The test cells were infected with 10<sup>6</sup> endpoint dilution of RLV. 3 days postinfection, culture medium was filtered through a 0.45  $\mu$  HA Millipore filter and 0.5 ml of the filtrate was put on NIH Swiss mouse embryo fibroblasts. 5 days later the foci were counted.

technique, the M-50 cell line did yield sarcoma virus, but at levels  $\frac{1}{25}$  that of parental NP cells. Preparations were assayed on both BALB/c and NIH Swiss cell lines with equivalent results.

*Superinfection.*—The nonshedding mutants, M-50 and M-43-2, were superinfectable by the Harvey strain of murine sarcoma virus [H(MSV)] or murine leukemia virus (MuLV). H(MSV) superinfection caused typical transformation in these mutant cells (Fig. 2). Although MuLV infected M-43-2 cells shed infectious sarcoma virus, they became flatter in appearance.

*Chromosome Analysis.*—The number of chromosomes was determined for each of the cell lines; frequency distributions compared to their progenitor cells are shown in Table II. BALB/3T3 has a subtetraploid mode (91% of the cells)

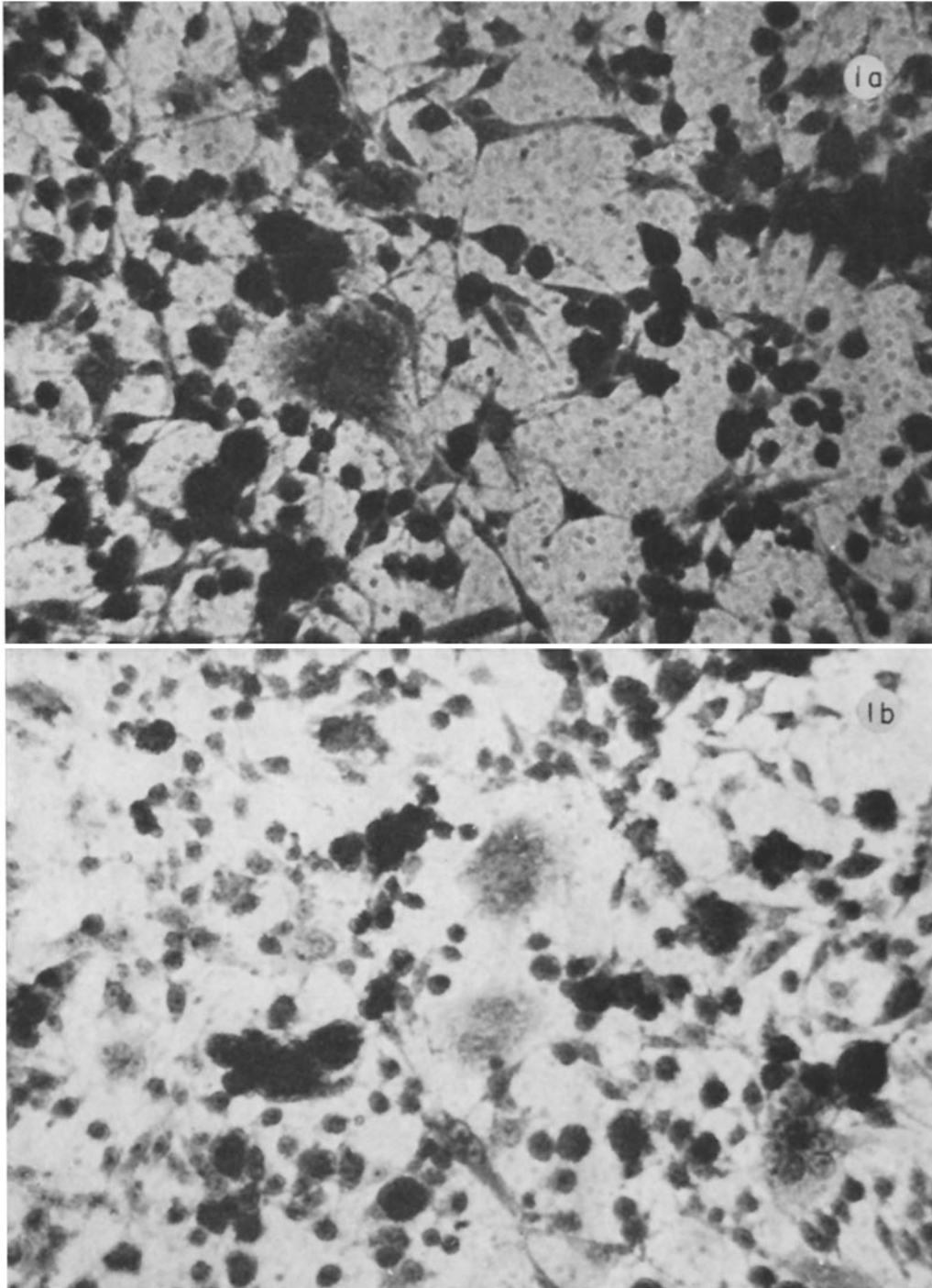


FIG. 1. XC cell syncytium formation with mutant cells shedding Type C viruses. (a) XC cell syncytium formation with M-57-1 cells. (b) XC cell syncytium formation with M-58-4 cells. Magnification 1,400 $\times$ .

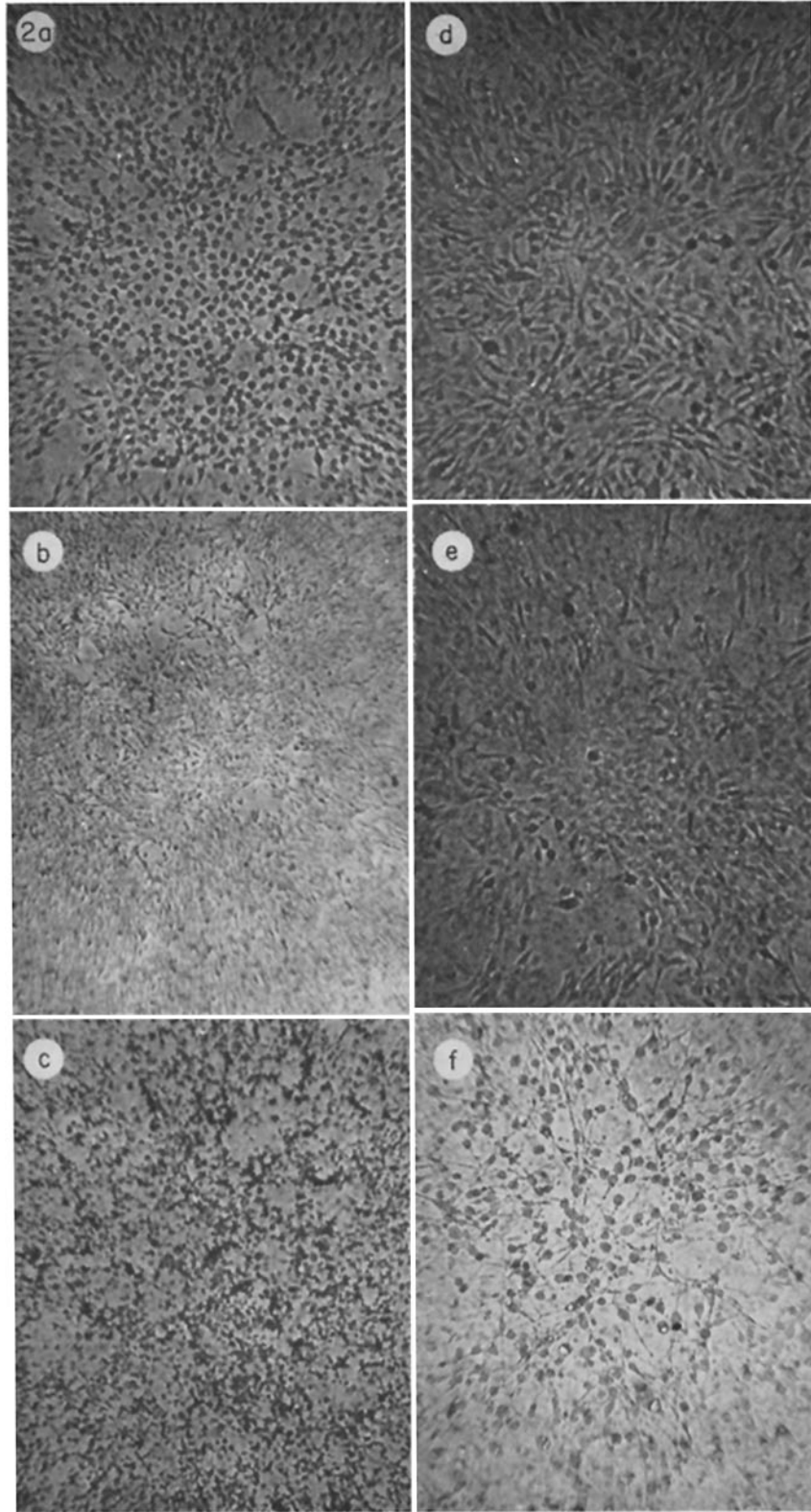


FIG. 2. Morphological changes of mutant cells after superinfection by leukemia and sarcoma viruses. (a) M-50 cells; (b) M-50 cells plus RLV; (c) M-50 cells plus (MSV), Five days postinfection; (d) M-43-2 cells; (e) M-43-2 cells plus RLV; (f) M-43-2 cells plus H(MSV), Five days postinfection. Magnification 218 $\times$ .

TABLE II  
*Chromosome Analysis of BrdU-Induced Cell Mutants*

Cell lines	Passage no.	Modal no.	Frequency distribution of chromosome no.					Endo- redupli- cation	
			>70	71-80	81-90	91-100	101-120		121-140
B/3T3	>100	74	9						
				%					
NP	>100	84	5	25	50			20	
	>100	84	2	29	54	6	2	7	
	>100	84		14	72	9		5	
M-50	30	91	8		38	38	8	8	
	45	86	9	9	60	22			
M-57-1	35	78	14	59	27				
	50	71							
M-43-2	30	138					27	65	
	45	135					5	86	
M-58-4	30	76	7	79	7				

The details are described in Methods. 40-60 cells were counted to get the chromosome number.

with a stemline of 70-75 chromosomes and a modal chromosome number of 74. The progenitor NP cells contain a stemline of 81-90 chromosomes with a modal number of 84. The modal number of the M-50 cell line after 45 passages was found to be 86, essentially similar to the modal number of the progenitor NP cells, while the modal numbers of M-57-1 after 50 passages (71), and M-58-4 after 30 passages (76) are closer to that found for the normal progenitor BALB/3T3 cells. One remarkable difference is the modal number of 135 found for M-43-2 cells after 45 passages. All cell lines contained at least one large acrocentric and one metacentric chromosome (data not shown).

*Stability of the Viral Markers.*—The relationship between the sarcoma genome and the maintenance of the transformed state was shown by studies of the M-50 cell line. This line was highly tumorigenic but did not contain a rescuable genome by usual superinfection techniques. However, rescue was obtained using a more sensitive fusion rescue method (5). To test for the stability of the non-rescue property (by direct superinfection), one of the tumors derived from the M-50 cells was explanted and assayed. This line, designated M-50T, behaved as the parental line except that it was possible to rescue the sarcoma genome from M-50T by superinfection. The possibility of a partial genetic reversion or alteration of some control at the phenotypic level may account for this result.

Tumor derived from the M-57-1 cell line retained the properties of the parental cell. When explanted in vitro, these tumors continued to produce a sarcomagenic virus.

The M-43-2 cell line was generally nontumorigenic and did not shed virus, however, cells inoculated at passage 15 produced a small static tumor in one mouse after a 30 day latent period. This tumor line, M-43-2T, when explanted began to shed sarcoma virus in similar fashion to the M-57-1 cells. From the chromosome analysis it was clear that the tumor was derived from the M-43-2 cell line (modal number 135 each).

One of the nontumorigenic mutant virus producing cell lines, M-58-4, became tumorigenic after long-term passage in vitro. At passage 48,  $10^6$  cells produced an 80% tumor incidence in 14 days, while  $10^4$  cells produced a 20% incidence in 36 days. Thus, the high passage cell designated M-58-4-48 is similar to M-57-1, but has a lower oncogenic potential. The available mutants in group C (virus shedding, nonmalignant) are early passages (10-30) of M-58-4 and M-58-2, which has still not produced tumors at high passage levels.

#### DISCUSSION

Activation of an N-tropic sarcoma virus by BrdU from the progenitor NP cell line used in these studies has been reported by Aaronson (8). Results with M-57-1 and M-58-4 cell lines are in agreement with this observation except the tropism of the induced virus appeared to be NB-tropic; however, the cells used for our assays were not tested with standard N and B viruses. Stephenson et al. (9) reported that natural reversion of NP cells was quite rare, while reversion of S+L- cells appeared quite often (10). S+L- cells contain gs antigen, shed Type C particles with deficient RNA-dependent DNA polymerase activity, and do contain a rescuable sarcoma genome (11-13).

Recently, Fischinger et al. (10, 14) reported that all of the flat revertants isolated from cloned transformed mouse 3T3 cells lacked a rescuable sarcoma genome and did not produce Type C particles. The M-50 cell line partially corresponds to this type of flat revertant which can be isolated without any mutagenic treatment (10, 14). The M-50 line is however as tumorigenic as its NP cell progenitor. The tumorigenicity of flat revertants has not been reported (10, 14). In contrast to the M-50 type of flat mutant, the M-43-2 cell line has an easily rescuable sarcoma genome, but thus far does not produce tumors in vivo.

Several papers have described a relationship between chromosome constitution and the occurrence of flat revertants (15-19). Reversion of transformed cells to those exhibiting growth control was associated with a large increase in chromosome number, while reversion back to lack of growth control resulted in a return to the original chromosome complement (15, 16, 19). Results obtained with the M-43-2 cell line are consistent with this type of reversion since it had a modal number of 135 chromosomes, was flat in appearance, and nontumorigenic in vivo.

Suppression of tumorigenicity by BrdU treatment of mouse melanoma cells has been reported (20). This however, does not appear to be a necessary consequence of BrdU treatment of all tumor cell types, since in the present studies,

variant cell lines retaining oncogenic potential were obtained. These included both particle producing and nonproducing derivatives.

It is worthwhile to note that rescuability of sarcoma genome does not directly correlate with tumorigenicity. Although ability to rescue the sarcoma gene certainly proves the existence of the genome in cells, it does not guarantee phenotypic expression of the genome. It is possible that the rescuable sarcoma gene in M-43-2 cells is subject to some cellular control mechanism which prevents the expression of malignant properties. On the other hand, the M-50 cell line is tumorigenic, but possesses a sarcoma genome which was not rescuable by superinfection. Its presence was however shown by fusion-rescue, but still with relatively low yield. A tumor cell line (M-50T) derived from M-50 did possess a sarcoma genome rescuable by direct superinfection. The mechanism of control of the "rescue" phenotype is at present obscure; however, it does not appear to be based on virus exclusion since helper MuLV did replicate in M-50 cells.

Further evidence for cellular control of a potentially malignant cell containing the sarcoma genome was obtained with the nontumorigenic mutants M-58-4 and M-43-2. In both cases, tumorigenic mutants could be derived from these lines; in one case, after high cell passage (M-58-4) *in vitro*, and in the other from an individual small static tumor obtained with the cell line (M-43-2).

The cell lines described in these studies should be useful for identification of cellular control mechanisms operative in regulation of oncogenicity of cells carrying a sarcoma virus genome.

#### SUMMARY

Tumorigenic and nontumorigenic mutants induced by a single 5'-bromodeoxyuridine (BrdU) treatment of a nonproducer (NP) tumorigenic cell line were isolated and characterized. Among the cloned derivatives were examples of virus-free and sarcoma virus-producing cell lines. Oncogenicity did not correlate with production of virus or ease of rescue of the sarcoma genome. All lines, including nononcogenic derivatives, retained the sarcoma genome.

Phenotypic reversion of some cell mutants was observed after *in vivo* inoculation or long term *in vitro* cultivation. The M-50T cell line, obtained from a tumor induced by M-50 cells, had a sarcoma genome rescuable by direct superinfection; this was only achieved with parental M-50 cells by a cell fusion rescue technique. The M-43-2T cell, obtained from a single small static tumor induced by otherwise nononcogenic M-43-2 cells, shed sarcoma virus and became tumorigenic. M-58-4-48 became tumorigenic after passage 48 of the M-58-4 line, which was originally nontumorigenic. These observations of phenotypic reversion demonstrate that the presence of the sarcoma gene in cells is an essential but not sufficient condition of tumorigenesis.

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