Induction of Murine C-Type Viruses from Clonal Lines of Virus-Free BALB/3T3 Cells

Abstract. *Each clone of BALB/c mouse embryo cells that has been tested can be induced to form C-type virus. The individual cells therefore contain a complete copy of the genetic information for making the murine RNA tumor viruses.*

Most strains of mice carry C-type RNA tumor viruses; these viruses have been shown to have an etiologic role in the development of their naturally occurring leukemias (*1*). In certain inbred strains, virus is not easily detected. However, after the animals are exposed to radiation and certain chemical carcinogens, leukemia viruses have been induced to appear (*2*). Furthermore, in cell culture many long-term, established mouse cell lines produce C-type viruses (*3*).

When cell lines derived from BALB/c (*4*) and randomly bred Swiss mouse embryos (*5*) were established in cell culture, it was found that early in their life in vitro the cells were free of detectable virus. After they had "spontaneously" transformed and acquired malignant properties in culture, some of the lines began to release C-type virus (*6*). Because the cells had been viably frozen during the course of their development into established lines, they could be thawed and systematically tested for virus production. The pattern for a given subline was reproducible; virus production developed only in those lines that had lost normal control of cellular growth. The above studies, along with many genetic and sero-epidemiological observations (*7*), raised the possibility that the genetic information for making a complete murine C-type virus might be present in an unexpressed form in every mouse cell (*6–8*). It was clear that direct proof of this hypothesis would require the specific manipulation in tissue culture of virus-free cell lines derived from clones started from single cells, with the resultant release of complete virus (*6*).

Subsequent studies revealed that clonal lines of BALB/3T3, after being exposed to high doses of radiation, did not contain virus in tissue culture but did contain virus after they had formed tumors in animals (*9*). These and similar studies with hamster embryo cells (*10*) could not resolve the question of whether the virus came from the inoculated cells or from the animal in which the tumors had developed. Rowe *et al.* (*11*) have isolated lines of cells derived from AKR mice, a strain with a very high incidence of leukemia. Although most of the lines produced large quantities of leukemia viruses, some remained free of virus during many successive subcultures. Clonal lines derived from them also remained free of virus and then "spontaneously" released virus. The probability of virus production could be increased by various experimental procedures such as infection with SV40 virus and exposure to x-irradiation (*11*); bromodeoxyuridine (BrdU) and iododeoxyuridine (IdU) were found to be extremely efficient inducers (*12*).

With the development of very rapid and sensitive methods (*13*) for detecting C-type virus production using the reverse transcriptase assay (*14*) and an antibody that specifically inhibits the viral enzyme (*15, 16*), it has become possible to search for very small amounts of virus in tissue culture fluids. Using these methods we have investigated the spontaneous production and the induction of virus from several well-characterized clonal lines of mouse cells. The results show that each BALB/c clone tested can be induced to release C-type viruses. These findings provide strong evidence that the genetic information for virus production is present in every mouse cell and that this information can be transmitted vertically from cell to progeny cell for many hundreds of generations without being expressed.

Table 1. Time course of appearance of reverse transcriptase in culture fluids of BALB/3T3 clone A31 after exposure to BrdU. After treatment with BrdU for 24 hours, cells were washed carefully and fresh medium was added. At 24-hour intervals the medium was collected and virus particles were pelleted by centrifugation at 40,000*g* for 90 minutes. The potential virus-containing pellets were resuspended in one-hundredth of the original volume and assayed for viral enzyme (*16*).

After treatment (days)	Polymerase* (pmole/ml of supernatant)	
	30 μg/ml BrdU	300 μg/ml BrdU
0	< 0.1	< 0.1
1	< 0.1	< 0.1
2	0.1	0.2
3	3.8	6.3
4	10.1	10.5
8	1.4	2.7
14	0.2	NT

* Each reaction mixture was incubated at 37°C for 60 minutes and contained in 0.05 ml: 0.04*M* tris HCl, *p*H 7.8; 0.06*M* KCl; 0.002*M* dithiothreitol; $2 \times 10^{-5}M$ [^{3}H]thymidine triphosphate (5000 count min^{-1} pmole^{-1}); 0.02 A_{260} poly(rA) • oligo(dT)$_{12-18}$; 0.1 percent (by volume) Triton X-100; $2 \times 10^{-4}M$ manganese acetate; and 0.01 ml of concentrated supernatants. Incorporation in the absence of added template was 0.1 to 0.2 pmole and was subtracted from each value.

The derivation of the cell lines and culture conditions used have been described (*4, 5*). Clonal lines were derived from mass cultures of BALB/3T3 and BALB/3T12-1 (*4*). In neither of these mass culture lines has virus ever spontaneously appeared. Virus-negative clonal lines were also derived from BALB/3T12-4 before this mass culture line had become virus-positive (*6*). One particular BALB/3T3 clone, A31, has been in culture for over 500 generations and has been used in a great many laboratories for transformation and growth control studies. An SV40-transformed clone of BALB/3T3 (subclone T2) and a subclone, R4, that lost contact inhibition and became tumorigenic after exposure to x-irradiation (1500 rads), were also tested. Subclones of BALB/3T3 clone A31 transformed by the Moloney (M) or Kirsten (K) strains of murine sarcoma virus (MSV) and containing the sarcoma genome in a nonproducing state (*17*) were also examined. The 3T6 clone 7 (*5, 6*) derived from random-bred Swiss mice was recloned and also examined. Most of the methods that can be used to detect viruses of the C-type group were applied to the cell lines described here. By available criteria our clonal lines have shown no evidence of C-type virus production (*17*).

Subconfluent BALB/3T3 cultures in 50-mm plastic petri dishes (Falcon) were exposed to potential inducing agents. Although a number have worked, BrdU and IdU (*12*) have been the most efficient. The time course of induction of virion-associated polymerase in response to BrdU is shown in Table 1. There was no evidence of polymerase activity in supernatants of untreated cultures. However, by the second day, a small amount of enzyme activity was observed in the treated cultures (Table 1). Polymerase activity in supernatants reached a maximum between 3 and 4 days after the cells were exposed and

Table 2. Inhibition of induced polymerase activity by antibody to the murine virus reverse transcriptase.

Serum	Protein added (μg)	Polymerase (% of control)*
BALB/3T3 clone A31		
Control serum	0	100
	2	88
	5	79
	10	60
Antiserum	2	5
	5	3
	10	<1
BALB/3T12-4 clone 7		
Control serum	2	76
	5	71
	10	73
Antiserum	2	10
	5	2
	10	<1

* In the absence of added antibody, the incorporation with the induced clone A31 supernatant was 12,200 count/min, and with BALB/3T12-4 clone 7 it was 5700 count/min. Reaction mixtures were as in Table 1.

then fell off, so that by 14 days after exposure viral polymerase was barely detectable; this was not due to cell killing, which under the conditions was minimal. Exposure to 300 μg of BrdU per milliliter has in some experiments permitted detection of enzyme activity somewhat earlier than with 30 μg/ml.

Cellular DNA polymerases, as well as the murine viral enzyme, can utilize synthetic templates, such as polyriboadenylate · polydeoxythymidylate [poly(rA) · poly(dT)]. In order to identify the polymerase activity induced as viral reverse transcriptase, an antibody that inhibits viral but not cellular DNA polymerases was used (*15, 16*). Polymerase activity induced from BALB/3T3 and a BALB/3T12 clone (see below) were inhibited by antiserum, whereas the control (serum from the same rabbit before it was immunized) exhibited only a small, nonspecific effect on the reaction at comparable concentrations. No viral reverse transcriptase has been detected in extracts of uninduced BALB/3T3 cells (*16*).

A number of clonal lines derived from BALB/3T3 and BALB/3T12 mass cultures were tested for virus induction by BrdU. All BALB/c–derived clones treated were inducible (Table 3).

Clone A31 has been transformed by SV40 and by MSV; variants that have lost contact inhibition of growth have also been isolated after exposure to x-irradiation. As with the parent clone, each subclone tested (Table 3) showed viral polymerase activity in tissue culture fluids 3 to 4 days after treatment with BrdU. When the reverse transcriptase assay was used, C-type virus was also induced from a clone of random-bred Swiss 3T6 mouse embryo cells.

In most studies with clonal lines of BALB/c cells, viral enzyme induction followed a time course similar to that shown in Table 1, with an early peak of virus production and then a progressive decline to a level below detection. Because of our earlier studies, in which virus spontaneously appearing in the BALB/3T12-4 mass culture was found to propagate much better in NIH/Swiss than in BALB/c embryo cells (*6*), NIH/3T3 cells (*18*) were added to certain BrdU-treated BALB/c lines in order to enhance the infectivity of the induced virus. When cell-free tissue culture fluids from these mixed cultures were transmitted to new NIH/3T3 or BALB/3T3 cells, evidence of murine leukemia virus was detected by the XC plaque test (*19*); however, the plaques were often small and difficult to score. Plaque formation was at least 100 times more efficient on NIH/3T3 than on BALB/3T3. Another demonstration of the transmissibility of the virus induced from three BALB/c clonal lines is shown in Table 4. In each case, transmission to NIH/3T3 cells was much more efficient than transmission to BALB/3T3 cells.

In some mass cultures of BALB/c and random-bred Swiss embryo cells, C-type virus appeared spontaneously after prolonged culture (*6*). There were two possible explanations of those results. The first was a chronic, low-level virus infection in the original primary embryo culture which could not be detected by the methods available. Under this hypothesis, the virus could have persisted in a carrier state because there always were a few infected cells in the population; the virus would be maintained by horizontal spread from cell to neighboring cell. The second explanation was that virus production began spontaneously in previously virus-free cells during the course of establishment of the cell lines. By this model transmission of viral information could be entirely "vertical," from cell to progeny cell without the need for the virus to ever be extracellular. In the first model, virus information should be present only in a small fraction of the cells at any one time. The second model predicts a potential for making C-type virus in each and every cell (*6–8*). In our present experiments, however, and in those of Lowy *et al.* (*12*) each of a large number of virus-negative clonal lines of AKR and BALB/c embryo cells were induced to make C-type virus. These findings provide strong support for the second model.

Studies of mass cultures of chick embryo cells have shown naturally occurring C-type viruses. Weiss, Hanafusa, and others have shown that chick embryo cultures previously thought to be virus-free also harbor a C-type virus (*20*). Some evidence derived from the study of hamster cells continuously cultured over a long period suggests that the C-type virus indigenous to this species may also be activated (*10*). The viruses induced from BALB/c clones have tissue culture and biochemical

Table 3. Induction of virus from clonal lines of mouse cells.

Test cultures	Virus production	
	"Spontaneous"	After induction
BALB/c embryo series		
BALB/3T3 clone A31		
(100 generations)	−	+
(> 500 generations)	−	+
Transformed by SV40 (subclone T2)	−	+
MSV nonproducer (subclone K234)	−	+
MSV nonproducer (subclone M85)	−	+
"Radiation" (subclone R4)	−	+
BALB/3T12-1		
Clone 1	−	+
Clone 4	−	+
Clone 6	−	+
Clone 9	−	+
BALB/3T12-4		
Clone 2	−	+
Clone 6	−	+
Clone 7	−	+
Random-bred Swiss embryo series		
3T6 clone 7	−	+

Table 4. Transmission of viruses induced from BALB/c clonal lines to new mouse cell cultures. After the cells were treated with 100 μg of BrdU per milliliter for 24 hours, they were washed and then cocultivated with an equal number of NIH/3T3 cells. Two weeks later filtered (0.45 nm, pore size) culture fluids were transmitted to new BALB/3T3 or NIH/3T3 cultures, and supernatant viral polymerase was assayed 7 to 10 days later as described in the legend to Table 1.

Virus from:	Polymerase activity (pmole/ml of supernatant) transmitted to:	
	NIH/3T3	BALB/3T3
BALB/3T3		
Clone A31	13	0.2
Subclone R4	340	<0.1
BALB/3T12-4		
Clone 7	210	1.6

properties of murine C-type viruses. We do not yet know if the virus induced from normal BALB/3T3 is different in any of its in vivo or in vitro properties from those viruses induced from "transformed" cells.

The mouse cell lines described here may be comparable to lysogenized bacterial cells. The nature of the control of expression of virus genetic and oncogenic information remains to be determined. The ability of agents such as BrdU to induce the formation of C-type viruses in well-characterized clonal lines should permit study of the regulation of endogenous tumor virus information. Such studies have obvious implications for chemical and viral carcinogenesis.

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Chemosterilant Action of Anthramycin: A Proposed Mechanism

Abstract. *The activity of anthramycin and structurally related analogs as chemosterilants of the housefly,* Musca domestica *L., correlates closely with the action of these compounds as inhibitors of* Escherichia coli *RNA polymerase. Since inhibition of RNA polymerase by anthramycin reflects binding of this antibiotic to the DNA primer required for enzyme activity, we propose that the interaction of anthramycin with DNA may also account for its action as a chemosterilant.*

The use of sterilized insects (*1*) has received considerable attention as an effective and safe means of insect control (*2*). The release of sterile males into the general population has already proved effective in controlling the screwworm fly in the southwestern region of the United States and in preventing the entrance of the Mexican fruit fly into southern California (*3*). Sterilization for these purposes is achieved by exposure of insects to gamma irradiation or by treatment with specific chemical agents. The latter approach, known as chemosterilization (*4*), has been proposed as a general means by which vertebrate, as well as invertebrate, populations can be controlled.

Insect chemosterilants include alkylating agents, antimetabolites, and various miscellaneous compounds (*5*). Although alkylating agents may induce chromosomal aberrations (*6*), the biochemical mechanisms by which chemosterilants affect fertility in insects has not been established.

Barnes *et al.* (*7*) recently described

Table 1. Effect of anthramycin and its derivatives on fertility and mortality of adult houseflies. Groups of ten newly emerged males or eight females were injected with various doses of the test compounds dissolved in a mixture of acetone and dimethylsulfoxide (1 : 1). The treated males or females were then caged with eight untreated virgin females or ten males of the same age. In all experiments, one or more batches of eggs were collected and their hatchability was scored as previously described (*13*).

Compound injected*	Dose (micrograms per fly)	Male injected			Female injected		
		Mortality† (%)	Eggs collected	Hatching (%)	Mortality† (%)	Eggs collected	Hatching (%)
None		0	1230	98			
I	0.25	0	740	5	0	103	79
	.50	10	362	0	0	0‡	
	1.00	100			100		
II	0.25	10	858	9	13	0‡	
	.50	30	300	1	13	0§	
	1.00	80	295	0	62	0§	
III	0.25	10	725	23	0	0‡	
	.50	20	137	0	38	0§	
	1.00	90	85	0	88	0§	
IV	0.25	0	527	53	0	480	79
	.50	0	638	43	0	377	74
	1.00	0	512	4	0	0‡	
V	0.25	0	332	92	0	541	82
	.50	0	509	93	0	371	88
	1.00	0	658	69	0	323	77
VI	0.25	0	567	94	0	456	96
	.50	0	744	91	0	659	93
	1.00	0	740	95	0	396	90
VII	1.00	0	483	96	0	287	90
VIII	0.25	0	452	94	13	515	85
	.50	0	662	86	0	486	88
	1.00	0	513	86	0	491	98
IX	0.25	0	536	85	0	642	88
	.50	20	672	86	0	665	84
	1.00	0	343	92	0	312	83

* Roman numerals refer to the structural formulas in Fig. 1. † Measurement at 48 hours. ‡ The female survivors were dissected 1 week after injection and their ovaries were found atrophied. § All injected females died before the date for egg collection (6 days after injection).