GENETIC FACTORS INFLUENCING C-TYPE RNA VIRUS INDUCTION*

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There is now considerable evidence that spontaneous leukemias in mice are caused by C-type RNA viruses. These viruses appear to be capable of existing in the animal in an unexpressed form for many generations. In inbred mouse strains such as C58 and AKR, in which virus can be detected early in life, leukemia develops at high frequency in young animals (1, 2). In other strains such as BALB/c or C57BL/6, virus is first detectable in older mice; in these strains leukemia appears at a much lower frequency in late life (2, 3) or after exposure to X-irradiation or chemical carcinogens (3, 4). Some strains such as NIH Swiss have a very low incidence of leukemia, and C-type viruses have yet to be isolated.

In tissue culture, evidence that an endogenous C-type viral genome can exist in an unexpressed state in mouse cells was presented in studies of BALB/c and random-bred Swiss mouse embryo cells (5). Certain cell lines derived from virus-negative primary embryos were shown to produce virus spontaneously after many generations in culture. More recent studies have shown that activation of virus production can be greatly enhanced by treatment of mouse cells with certain chemical and physical agents (6, 7); these studies demonstrate that C-type viruses are present in an unexpressed form in all AKR (6, 7) and BALB/c cells (8, 9).

The genetic factors influencing the induction process in tissue culture might be expected to be important in the expression of virus and development of leukemia in the animal. In the present studies, we have investigated some of these factors in cells from high and low leukemia strains and their F_1 hybrids.

Materials and Methods

Mice.—C57BL/6N, NIH/N, and BALB/cN mice were obtained from the colonies of the National Institutes of Health (N)[1] and the C58/J strain from the Jackson Laboratory (J), Bar Harbor, Maine. F_1 hybrids were bred in our laboratory. The symbols for these strains have been abbreviated as follows: C57BL, N, B, and C58. The F_1 hybrids are designated with the female parent listed first.

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[1] *Abbreviations used in this paper:* B, BALB/cN strain mice; DTT, dithiothreitol; IdU 5-iododeoxyuridine; ME, mouse embryo; MuLV, murine leukemia virus; N, NIH/N strain, mice; PFU, plaque-forming units; TTP-^{3}H, methyl-thymidine triphosphate-^{3}H.

Cells.—Cells were grown in T-75 flasks (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). The contact-inhibited, continuous cell line, NIH/3T3, has been described previously (10). Mouse embryo (ME) cultures were prepared by fine mincing and disaggregation of embryos in the 16th–18th day of gestation; all embryos from a single mother were pooled.

For virus induction experiments confluent ME cultures were treated with 20 μg/ml of 5-iododeoxyuridine (IdU) (Calbiochem, Los Angeles, Calif.) for 24 hr and transferred every 2–3 days at 2×10^6 cells/T flask. In comparisons between the kinetics of virus induction from different embryo cultures, care was exercised to maintain similar cell density and viability.

Virus Assays.—Virion-associated reverse transcriptase activity was measured in tissue culture fluids. Reaction mixtures were incubated at 37°C for 60 min and contained, in 0.05 ml: 0.05 M tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.8; 0.06 M potassium chloride; 0.002 M dithiothreitol (DTT); 2×10^{-4} M manganese acetate; 0.02 A ^{260} polyriboadenylic·oligodeoxythymidylic acid$_{(12-18)}$; 2×10^{-5} M TTP-^{3}H (5000 cpm/pmole); and 0.1% (v/v) Triton X-100. DNA synthesis was measured as described previously (11). The standard error for replicate assays of a given virus stock was ± 15%. The specificity of the reaction for detection of murine leukemia virus (MuLV) was shown by inhibition of enzyme activity by antibody directed against MuLV reverse transcriptase (11).

The biologic activity of C-type viruses was titrated by either the XC plaque test (12) or by an end-point titration method using the polymerase assay. The latter method is described in detail elsewhere (13).

RESULTS

Kinetics of Virus Induction from Primary Embryo Cultures.—For quantitation of the induction of virus from primary or secondary mouse embryo cell cultures, virus-associated reverse transcriptase activity was assayed in the supernatants of IdU-treated cultures. In each case, polymerase activity was demonstrated to be viral by means of the marked inhibitory effect of antiserum previously shown to be specific for mammalian RNA C-type viral enzymes (11). In some instances, the induced virus could be grown up to high titer; in these situations the properties of the viruses were examined in more detail.

In the first series of studies, a comparison was made between the inducibilities of B and N embryo cells and of the (N × B)F_1 hybrid. As shown in Fig. 1 *a*, induction of virus from B embryo cells exhibited a time-course similar to that reported previously for continuous clonal lines derived from this strain (8). Virus production reached a peak by 3–4 days and then declined. In general, the amount of virus activated from primary or secondary B embryo cultures was somewhat lower than from the continuous lines. Nonetheless, the results show that endogenous C-type viruses can be activated from diploid mouse embryo cells as well as from continuous aneuploid B mouse cell lines. As shown in Fig. 1 *a*, virus induction from N cells was not detected under experimental conditions which gave positive results with B cells. Whether this reflects the lack of an endogenous C-type virus in N cells or the lack of ability to detect such a virus under the present experimental conditions is not yet resolved. (N × B)F_1 hybrid embryo cells were clearly inducible (Fig.

1 *a*); the kinetics and magnitude of virus production were indistinguishable from those with B embryo cells.

Experiments were also performed to determine whether virus could be acti-

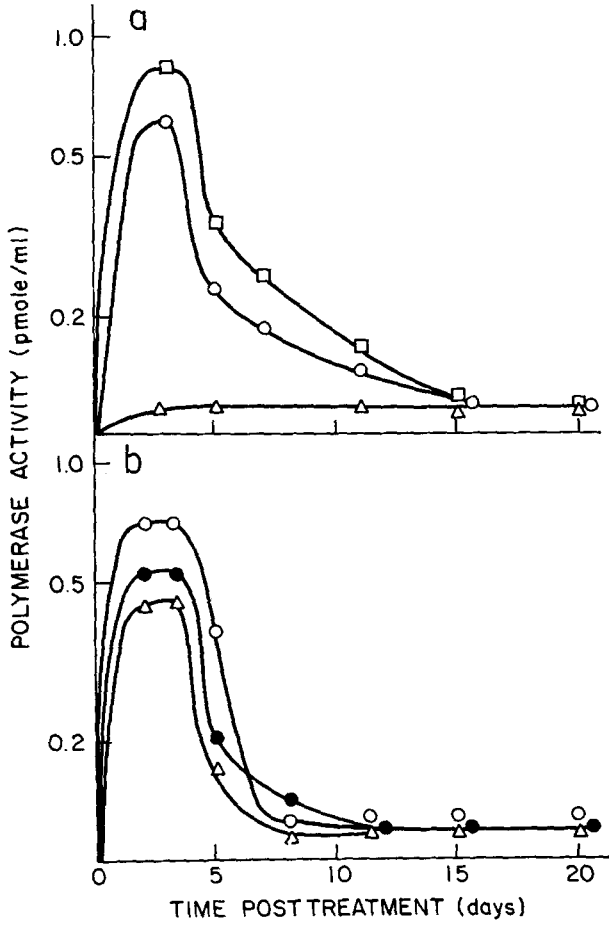

FIG. 1. (*a*) Kinetics of induction of MuLV from secondary mouse embryo cultures. Cells were exposed to 20 μg/ml IdU for 24 hr, washed, and incubated at 37°C in medium. The cell densities were maintained relatively constant by subculture every 2–3 days. Viral enzyme activity was measured in supernatants at appropriate times as described in Materials and Methods. Each point represents the mean value of triplicate assays. B, ○; N, △; (B × N)F_1, □ (*b*) C57BL, ○; (C57BL × B)F_1, △; (C57BL × N)F_1, ●.

vated from C57BL embryo cells. This low leukemic strain was of interest in view of previous reports of activation of virus in vivo by X-irradiation or treatment with chemical carcinogens (4). As shown in Fig. 1 *b*, virus was induced from C57BL cells by IdU, and the time-course of virus activation resembled that seen with B embryo cells. The kinetics of virus activation from both (C57BL ×

N)F_1 and (C57BL × B)F_1 hybrid cultures were also similar. The fact that each of the F_1 hybrids with N was inducible indicated that virus inducibility was inherited as a dominant genetic characteristic.

Mice of the C58 strain have a high susceptibility to spontaneous leukemia, and replicating virus can be detected in primary embryo cells of this strain (14). The influence of C58 genetic properties in F_1 hybrids with either B, N, or C57BL strains was investigated. In order to avoid possible contamination of embryos with growing virus present in the C58 adult, the female used in each cross was of the low leukemia incidence strain. As shown in Fig. 2, with embryo cultures of each of the three hybrids, (B × C58)F_1, (N × C58)F_1, and (C57BL × C58)F_1, the magnitude of the initial burst of virus production after treatment with IdU was similar to that observed with B or C57BL embryo cells. With each of these F_1 hybrids, however, the induced virus was able to persist. Thus, it appeared that not only virus inducibility but also the ability of the activated virus to persist were inherited as dominant traits.

Biologic Properties of Induced Viruses.—Previous reports have shown that MuLV's can be classified into three host range groups: (*a*) those that grow much better on B cells than on N cells (B-tropic), (*b*) those that grow preferentially on N cells (N-tropic), and (*c*) those that grow equally well on both prototype strains (NB-tropic) (15). N and C58 strains have been classified as N-type, while B and C57BL strains are B-type in virus susceptibility (14, 15). Pincus et al. (16, 17) have recently demonstrated that cells of F_1 hybrids involving a cross between N and B type cells are resistant to both N- and B-tropic leukemia viruses, indicating that resistance to infection by either virus is dominant. In the present studies (N × B)F_1, (C57BL × C58)F_1, and (B × C58)F_1 embryo cell cultures were each hybrids of an (N × B)-type cross. In one hybrid, (N × B)F_1, only the initial burst of virus synthesis was seen. However, in the two other (N × B)-type hybrids, the induced viruses were readily able to persist. These findings suggested that genetic factors in addition to the (N × B) type must influence whether or not C-type virus can become established in the cells from which it is activated.

It was, thus, of interest to examine some of the characteristics of induced viruses. The biologic activity of each virus was standardized on the basis of its viral reverse transcriptase activity since, in general, the amount of enzyme activity of a virus preparation correlates with the number of physical particles (13). Two distinct classes of virus were distinguishable by the efficiency with which they could be transmitted to new cells. The class of efficiently growing induced viruses included those activated from each F_1 hybrid involving C58. These viruses were characterized by their ability to transmit readily to new cells in the initial burst of virus production after IdU treatment (Table I). The XC plaques formed by such viruses were large and were typical of the kind formed by laboratory strains of MuLV. It should be noted that each virus with the above properties was able to persist after induction.

As shown in Table I, biologic activity of viruses induced from B, C57BL, or (N × B)F_1 cells was not initially detectable, and these viruses did not persist under the present experimental conditions. As was previously shown for continuous lines of BALB/c cells (8), it was possible to establish the viruses induced

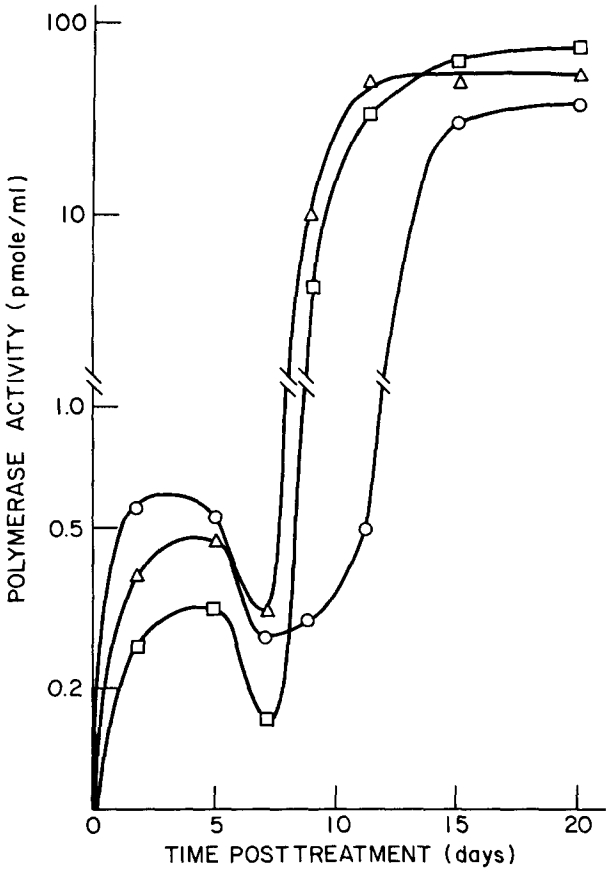

FIG. 2. Kinetics of induction of MuLV from secondary mouse embryo cultures of F_1 hybrids involving C58. IdU treatment and viral enzyme assays were as described in Fig. 1. (N × C58)F_1, △; (B × C58)F_1, □; (C57BL × C58)F_1 ○.

from B and (N × B)F_1 cells by cocultivation with the continuous line of NIH/3T3 cells. The viruses grown by this method formed only small diffuse XC plaques, and their efficiency of transmision to new cells was approximately 100-fold lower than that of more biologically active induced viruses. It should be noted that host range was not a variable among the activated viruses, since each was more infectious for N than for B cells. These results indicate that induced viruses have characteristic genetically determined properties, and these

properties are closely correlated with their ability to persist after chemical activation.

Spontaneous Virus Activation In Vitro and In Vivo.—The above results predicted that whatever the frequency of spontaneous activation in F_1 hybrid cells, virus detection would be more likely in those cases where the induced virus was biologically active (i.e. initially transmitted readily to new cells). To test this hypothesis, the frequency of detection of spontaneous virus activation from

TABLE I

Biologic Properties of Induced Viruses

Embryo cultures from which viruses were induced*		XC plaque morphology‡	Tropism§	XC plaques/pmole polymerase‖	Polymerase-Inducing units/pmole
B	Initial	N.D.	—	<1.0	<1.0
	Late	S	N	0.5	0.2
C58	Initial	L	N	108	118
	Late	L	N	110	115
N × B	Initial	N.D.	—	<1.0	<1.0
	Late	S	N	0.6	0.3
B × C58	Initial	L	N	130	115
	Late	L	N	125	140
N × C58	Initial	L	N	105	110
	Late	L	N	113	100

* The properties of the induced viruses were examined both at the peak of the initial burst of virus synthesis after induction (initial) and after growth to high titer (late). With the B and (N × B)F_1 embryo cultures, in which induced viruses did not persist, late stocks of virus were obtained by cocultivation with NIH/3T3.

‡ XC plaques were classified as either large (L) or small (S). N.D. means not detectable.

§ The host range of each induced virus was tested by titration on NIH/3T3 and BALB/3T3 cells by the polymerase-inducing assay. An N-tropic virus was defined as one which titrated at least 50-fold higher on NIH/3T3 than on BALB/3T3 cells.

‖ Each induced virus was titrated for infectivity on NIH/3T3 cells by the polymerase-inducing assay and by the XC plaque assay. The results are standardized on the basis of picomoles per milliliter of polymerase activity for each virus preparation.

the different strains of embryo cells was examined. When mass cultures of F_1 hybrids involving C58 were passaged at high cell number, virus production was invariably detectable within 3 or 4 wk of explantation of the embryo. It was only possible to maintain such cultures free of detectable virus by transfer at low cell number and constantly eliminating those cultures that became positive for virus as measured by XC plaque and polymerase assays. In contrast, spontaneous virus activation was not detectable under any conditions with cells of low leukemia incidence strains including B, C57BL, and the (C57 × B)F_1 hybrids.

Spontaneous virus appearance was also studied in the animal. Spleen cells were obtained from weanlings of C58, C57BL, N, B, and the appropriate F_1

hybrids; the cells were tested for virus production by an amplification method involving cocultivation with NIH/3T3 cells. After 6–7 days, tissue culture fluids were assayed for viral polymerase activity and for XC plaque formation. From the results (Table II) it is clear that leukemia virus was recovered only from spleen cells of C58 or F_1 hybrids involving C58. It was with these same strains that spontaneous virus production was detected in vitro. These results confirm and extend those of previous in vivo studies which showed complete penetrance of virus expression in F_1 hybrids involving high and low leukemia incidence strains (18, 19, and W. P. Rowe, personal communication). Thus, the genetic factors

TABLE II

*Recovery of Infectious MuLV from Spleen Cultures of F_1 Hybrid and Parental Mouse Strains**

Mouse strain	Viral polymerase activity pmole/ml	XC plaque formation $\log_{10}$ PFU/ml
Parent		
N	<1	Neg
B	<1	Neg
C57BL	<1	Neg
C58	4×10^3	5.7
Hybrid		
(N × B)F_1	<1	Neg
(C57BL × N)F_1	<1	Neg
(C57BL × B)F_1	<1	Neg
(B × C58)F_1	5×10^3	5.3
(N × C58)F_1	3×10^3	5.7
(C57BL × C58)F_1	3×10^3	5.6

* 10^7 nucleated spleen cells from 4–5-wk old mice were incubated with subconfluent monolayers of NIH/3T3 cells for 7 days. Tissue culture fluids were assayed for polymerase activity and were titrated for infectivity on NIH/3T3 cells by the XC plaque test.

controlling detection of spontaneous virus activation in vitro also appear to be involved in the expression of virus in vivo.

DISCUSSION

The discovery of a reverse transcriptase (20, 21) in RNA tumor viruses has led to the development of very sensitive (13) and specific (11) methods for detection of virus. These techniques have resulted in the demonstration that murine C-type viruses can be activated from established, clonal lines of virus-negative B embryo cells under conditions where the induced virus may be undetectable by standard biologic techniques (8). The present report extends these studies to show that C-type viruses can be activated from primary or secondary B embryo cells and also from embryo cells of several other strains of mice including C57BL, and F_1 hybrids of B, C57BL, and C58.

Genetic factors affecting virus induction could be studied in F_1 hybrids of mouse strains having different patterns of virus activation. In F_1 hybrids between inducible strains such as B, C57BL, or C58, and a strain (N) which itself was not detected as inducible under the same experimental conditions, activation of virus was detected in each case. Thus, inducibility of virus was inherited as a dominant genetic characteristic. This result argues against the presence of a diffusible "repressor" to induction in N cells. The fact that the biologic properties of viruses activated from (C58 × N)F_1 and (B × N)F_1 hybrids were distinguishable and were characteristic of the viruses of their respective parents suggests that the structural gene(s) of C58 and B endogenous viruses were themselves present in the F_1 hybrids with N.

The ability of activated viruses to persist was also found to be a genetically determined characteristic. Viruses activated from F_1 hybrids between C58 and either N, B, or C57BL initially formed large XC plaques and had high plaque-forming unit (PFU)/physical particle ratios. It was this class of virus that persisted after the initial phase of induction and also became detectable following spontaneous activation in culture and in vivo. With each of the low leukemia incidence strains and in F_1 hybrids between these strains the induced viruses failed to persist at detectable levels after chemical treatment; similarly, spontaneous activation was not observed in vitro or in the animal. These results indicate that at least one genetic factor responsible for the high incidence of leukemia in C58 mice and in hybrids involving C58 (22, 23) is the greater biologic activity of the endogenous virus of the high leukemia incidence strain. Further studies are clearly required to elucidate other genetic factors influencing the induction process and to determine the relationship between virus expression and leukemia development in the animal.

SUMMARY

Genetic factors were studied which affect the inducibility of C-type RNA viruses from embryo cultures of mouse strains with high and low incidence of spontaneous leukemia. Virus was inducible by chemical treatment from secondary embryo cultures of several inbred strains. Both virus inducibility and the capacity of the virus to persist in cells from which it was activated were found to be inherited as dominant genetic characteristics. The results show that the factors controlling virus activation and persistence in culture also play an important role in spontaneous virus expression in the animal.

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