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Independent Segregation of Loci for Activation of Biologically Distinguishable RNA C-Type Viruses in Mouse Cells

(genetic controls/inducible virus/BALB/c strain)

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ABSTRACT **Two genetic loci for induction of RNA C-type virus in BALB/c mouse embryo cells segregate independently in backcross embryo cell lines. The viruses at these loci are shown to be biologically distinguishable. The first, described previously, codes for activation of a virus that grows preferentially in NIH Swiss embryo cells. The newly detected locus codes for activation of a virus that grows poorly, if at all, in NIH Swiss or BALB/c cells, but replicates well in a rat cell line. Its serologic properties are different from those of viruses of the two major serologic subgroups of murine leukemia virus. The present findings provide support for the hypothesis that the loci for virus induction in BALB/c cells represent genetic information for C-type viruses and that the endogenous viruses are subject to different cellular genetic controls.**

RNA C-type viruses that occur *in vivo*, either spontaneously (1) or after exposure to physical agents (2), are etiologically linked to leukemias of mice. Studies of the association of these viruses with mouse cells in tissue culture has led to the demonstration that they exist in an unexpressed form in all cells (3, 4). More recent studies indicate the presence of genetic loci for virus induction in cells of several inbred strains of mice (5, 6). It has been possible to distinguish two biologic classes of inducible mouse C-type virus (5). A very infectious virus can be activated from cells of C58 and AKR mouse strains, which have a high incidence of leukemia, (5, 6). This class of virus can be activated at two genetic loci for inducibility in AKR cells (6) and multiple (more than two) loci in C58 cells (7). In the BALB/c strain, which has a low incidence of leukemia a class of endogenous virus has been identified that is much less infectious than those obtained from the other two strains (4,5). A single genetic locus has been detected at which this virus can be activated (8).

The demonstration of multiple loci for virus inducibility in C58 cells raised the possibility that additional loci might be present in cells of other mouse strains. Viruses at such loci must either be defective (i.e., noninducible) or have markedly different biologic properties from those of endogenous viruses thus far detected. In the present studies, the search for other classes of endogenous C-type viruses has led to the isolation and characterization of an inducible virus of BALB/c cells with host range and serologic characteristics distinct from those of any previously described endogenous mouse C-type virus. Its inducibility locus is shown to segregate independently of that of a previously detected locus for BALB/c virus activation.

Abbreviations: Ki, Kirsten; MSV, murine sarcoma virus; MuLV, murine leukemia virus.

METHODS

Cells were grown in Dulbecco's modification of Eagle's medium. Many of the cell lines used have been described elsewhere and include the continuous mouse cell lines, BALB/3T3 (9) and NIH/3T3 (10), and the rat NRK line (11). Isolation of the nonproducer lines transformed by Kirsten murine sarcoma virus (KiMSV), K-BALB, K-NIH, and K-NRK, has been reported (12). Primary New Zealand Black (NZB) mouse embryo cells were provided by T. Pincus, Cornell University. Secondary cultures of individual embryos of NIH Swiss (N), BALB/c (B), and of the F_1 hybrids, $(NxB)F_1$, and backcross generation, $Nx(NxB)F_1$, have been described (5,8).

Viruses. Standard strains of mouse leukemia virus (MuLV) used included clonal stocks of Rauscher (R)- and Kirsten (Ki)-MuLV (12). Isolation of a C58 induced C-type virus has been described (7). MuLV pseudotypes of KiMSV were obtained by superinfection of K-NRK or K-NIH cells with the appropriate strain of MuLV (12).

Biologic Assays. The methods used in our laboratory for induction of C-type virus have been described (4, 5). MSV was titrated by a focus-forming assay (12), while MuLV was assayed either by the XC plaque test (13) or by measurement of virion-associated activity of RNA-directed DNA polymerase in supernatants of infected cells. The latter method has been termed the polymerase-induction assay (14). The radioimmunoassay for MuLV gs antigen has also been described (15). Virus neutralization assays were performed by a focus-reduction method (16). Rat sera with antibodies to the Friend–Moloney–Rauscher (FMR) and AKR type-specific antigens of MuLV were from rats carrying a transplantable MSV(R-MuLV) or AKR lymphoma, respectively (17). These sera were supplied by R. Wilsnack, Huntingdon Labs. Sera from NZW/NZB mice were provided by A. Gazdar, NCI. All sera were heat-inactivated at 56° for 30 min and filtered before use.

RESULTS

BALB/c mouse cells contain an inducible C-type virus that is poorly infectious (4, 5). A sensitive method for its detection was developed by use of a line of BALB/3T3 cells nonproductively transformed by MSV (18). Rescue of the sarcoma virus genome by endogenous MuLV induced by IdU is used as a marker for endogenous helper virus. The latter virus confers its host range and serologic properties to the induced MSV genome.

TABLE 1. *Alteration in host range of virus activated from K-BALB cells*

Days after chemical treatment	MSV titer (log FFU/ml) on		Ratio (log FFU/ml)
	NIH/3T3	NRK	
3	2.1	3.5	−1.44
10	0.0	0.0	N.A.
20	3.7	2.1	+1.6
30	3.9	2.2	+1.7

3×10^5 K-BALB cells were treated with IdU (30 μg/ml) for 24 hr. Culture fluids were collected at various times after treatment, passed through a 0.45-μm filter, and assayed for focus formation on polybrene (2 μg/ml)-treated NIH/3T3 and NRK cells. *N.A.* means not applicable. *FFU*, focus-formation units.

As shown in Table 1, early in the course of activation, the induced sarcoma virus transmitted much more efficiently to NRK than to NIH/3T3 cells. As previously reported (4), after the initial virus peak there was a decline in infectious virus. However, we found that if K-BALB cells were maintained in exponential phase by continued transfer, infectious virus often again became detectable. This virus was able to persist, but had a titer at least 30- to 100-fold higher in NIH/3T3 than in NRK cells (Table 1). The above findings suggested that there may have been a marked change in the host range of a single induced virus. Alternatively, the induced virus population may have consisted of more than one virus, one of which grew preferentially in NRK cells and the other in NIH/3T3 cells.

Segregation of a New Endogenous Virus of BALB/c Cells. The previously reported inducible virus of BALB/c cells grows preferentially in NIH Swiss compared to BALB/c cells (4, 5), and according to convention is termed N-tropic (19). In genetic studies of virus induction with cell lines derived from embryos of inducible $(NxB)F_1$ hybrids backcrossed to the noninducible N strain, a genetic locus was detected at which this virus was inducible (8). In those studies to facilitate detection of any induced N-tropic MuLV, the cell lines were cocultivated with NIH/3T3 cells after chemical treatment. In the present study, the same series of backcross embryo cell lines was tested to determine whether there was a locus in BALB/c cells for induction of an endogenous virus that could grow preferentially in NRK cells.

Individual embryo lines of parental N and B cells, $(NxB)F_1$ hybrids, and the $Nx(NxB)F_1$ backcross generation were exposed to 20 μg/ml of IdU for 24 hr and cocultivated with either 10^5 NRK or NIH/3T3 cells. Parallel IdU-treated embryo cultures were passaged in the absence of cocultivation. Culture fluids were assayed for virion-associated of RNA-directed DNA polymerase at 24-hr intervals for the first 5 days and subsequently at weekly intervals. As shown in Table 1, each B parental and $(NxB)F_1$ hybrid line showed an early burst of virus production at 3–4 days after exposure to IdU. Further, virus production persisted when these lines were cocultivated with either NIH/3T3 or NRK cells. In contrast, N embryo cells released neither detectable virus acutely nor after cocultivation. In induction studies with the $Nx(NxB)F_1$ backcross lines, 19/41 (49%) released detectable virus at 3–4 days after exposure to IdU. With 21/41 (51%) of the lines, the induced virus grew to high titer after cocultivation with NIH/3T3. These same lines had been shown to contain an inducible N-tropic virus (8). The present results indicate that virus activated from each of these lines was also able to persist when the cells were cocultivated with NRK cells.

Unexpectedly, there was no correlation between cells showing an early virus peak and those from which virus persisted after cocultivation with NIH/3T3. An excellent correlation was found, however, between those lines with an early virus peak and those that yielded persisting virus *only* when cocultivated with NRK cells (Table 2). There were nine such lines (22%) in this class. The viruses activated from the other 10 lines, which showed an early virus peak, were able to establish themselves when cocultivated with NIH/3T3 as well as NRK cells. This finding was explained by the fact that these lines also contained the N-tropic endogenous virus. The third group, containing 27% of the backcross lines, showed no detectable early virus peak, but virus established after cocultivation with both N and NRK cells. These cell lines all contained the locus for inducibility of an N-tropic virus (8). In repeated experiments, the remaining 27% of the backcross lines were not inducible. All viruses that had grown up after cocultivation with NRK cells were tested for the presence of mouse gs antigen by radioimmunoassay and found to be positive. These results confirmed that each isolate was mouse C-type in origin. The above results are those expected for the independent segregation in $Nx(NxB)F_1$ backcross embryos of two endogenous viruses of BALB/c cells, one that grows in both NIH/3T3 and NRK cells and the other that replicates only in NRK cells. The previously detected N-tropic virus (8) will subsequently be referred to as BALB virus-1 and the newly detected NRK-tropic virus as BALB virus-2.

Host Range and Infectivity of BALB Viruses 1 and 2. Representative viruses, induced from the appropriate Nx-

TABLE 2. *Independent segregation of two loci for virus induction of BALB/c cells*

Embryo culture	Virus detected 72 hr after treatment	Virus persistence on		Observed	
		NIH 3T3	NRK	No.	%
N	−	−	−	0/5	0
B	+	+	+	5/5	100
$(NxB)F_1$	+	+	+	5/5	100
$Nx(NxB)F_1$	+	−	+	9/41	22
	+	+	+	10/41	24
	−	+	+	11/41	27
	−	−	−	11/41	27

Secondary embryo cell cultures containing about 3×10^6 cells were exposed to IdU (20 μg/ml) for 24 hr. They were transferred to three recipient plates each at 10^6 cells per plate to which was added 10^5 NIH/3T3 or NRK cells or medium only. Cells were maintained at 37° for 8 weeks with weekly subculture (8). Supernatant fluids were tested for RNA-directed DNA polymerase activity at 24-hr intervals for the first 5 days and subsequently at weekly intervals as described (5, 8). The data are the results of duplicate experiments.

(NxB)F_1 backcross lines, were grown up for further characterization. Each was used to prepare an MuLV pseudotype of MSV. The virus isolates were standardized to contain equivalent amounts of MuLV gs antigen, and hence physical particles (7). As shown in Table 3, BALB virus-1 transmitted more efficiently to NIH/3T3 than to NRK cells, while BALB virus-2 grew much more efficiently in NRK cells. In fact, transmission of BALB virus-2 to NIH/3T3 cells was not detected. Neither of the viruses showed detectable infectivity for BALB/3T3 cells. The infectivity of a representative C58 endogenous virus is also shown in Table 3 for comparison. Its polymerase-inducing ability as well as the focus-forming activity of its MSV pseudotype was between 10- and 500-fold better per physical particle than that of either of the two BALB/c endogenous viruses. Thus BALB virus-2 like the previously described N-tropic BALB virus-1, was much less infectious than an endogenous virus of the C58 strain which has a high incidence of leukemia.

Distinguishing Serologic Characteristics of BALB/c Endogenous Viruses. The serologic properties of the induced BALB/c viruses were studied by use of antisera that neutralized the two major subgroups of MuLV, Friend–Rauscher–Moloney (FMR) and Gross-type (17). Further, since the host range of BALB virus-2 was reminiscent of that reported for a naturally occurring MuLV of the NZB strain (20), neutralization tests were also performed with sera obtained from NZB mice. As shown in Table 4, a dilution of antiserum to R-MuLV that completely neutralized R-MuLV had little inhibitory effect on either a representative Gross-type virus (Ki-MuLV) or on NZB-MuLV. This antiserum was also ineffective against either of the inducible BALB/c viruses or C58 endogenous virus. Antiserum to ARK-MuLV almost completely neutralized Ki-MuLV but did not significantly inhibit R-MuLV. Both BALB virus-1 and virus-2, C58 endogenous virus, and NZB-MuLV, were markedly inhibited by this antiserum at the same dilution.

TABLE 3. *Host ranges of BALB/c endogenous viruses 1 and 2*

Induced virus*	Virus transmission assayed by: Polymerase-induction assay†(log PIU/ml) NRK	NIH/3T3	BALB/3T3	Focus-formation‡ (log FFU/ml) NRK	NIH/3T3	BALB/3T3
BALB virus-1	<0.0	1.5	<0.0	1.1	3.1	<0.0
BALB virus-2	1.0	<0.0	<0.0	3.0	<0.0	<0.0
C58	2.0	3.5	N.T.	4.5	5.8	N.T.

* KiMSV pseudotypes of each induced virus were obtained by cocultivation of virus-producing cells with a nonproducer cell line transformed by KiMSV. Each was standardized by radioimmunoassay to contain 1 ng/ml of MuLV gs antigen.

† Polymerase-inducing activity was measured by assay of RNA-directed DNA polymerase of 100-fold concentrates of tissue culture fluids of cells infected 7 days earlier with serial 10-fold virus dilutions. The titer in PIU/ml represents the end-point for polymerase induction (14). *PIU*, polymerase-induction units.

‡ Transmission of sarcoma virus was assayed at 7 days by the focus assay on polybrene (2 μg/ml)-treated cells. The results are the mean of three experiments. *N.T.* means not tested. *FFU*, focus-forming units.

TABLE 4. *Serologic properties of BALB/c induced viruses*

Virus	% Reduction in focus-formation after treatment with the antiserum* against: R-MuLV	AKR-MuLV	NZB-MuLV
MuLV pseudotype of KiMSV			
R-MuLV	100	10	25
Ki-MuLV	<5	99	<5
NZB-MuLV	20	95	95
BALB virus-1	15	100	<5
BALB virus-2	20	100	100
C58	12	100	<5
BALB/3T3 Induced Virus			
Early (3 days)	20	100	90
Late (20 days)	20	100	<5

* Neutralization tests were performed by the focus-reduction method. Around 100 FFU of each MuLV pseudotype of KiMSV were exposed to neutralizing antisera for 30 min at 37° and then assayed on polybrene (2 μg/ml)-treated NRK or NIH/3T3 cells. The number of MSV foci was scored at 7 days. Antisera to R-MuLV was used at a final dilution of 1:300; to AKR-MuLV at 1:100; and to NZB-MuLV at 1:50. The results are the means of three experiments.

The above results established that both BALB virus-1 and virus-2 were Gross-type, but did not distinguish between them. Neutralization tests with sera obtained from NZB mice (Table 4), however, provided a clear distinction between these two viruses. NZB serum completely inhibited BALB virus-2 but had little or no effect on BALB virus-1. At the same dilution of this serum, NZB-MuLV was effectively neutralized.

The induction studies with K-BALB cells described above suggested that the early activated virus contained a majority of BALB virus-2 while the virus that eventually persisted consisted primarily of BALB virus-1. Neutralization tests were, therefore, performed with "early" (3 days) nad "late" (20 days) induced virus from K-BALB cells. As shown in Table 4, the early induced virus clearly resembled BALB virus-2 in serologic properties; it was Gross-type and was markedly inhibited by NZB serum. In contrast, the late induced virus was serologically similar to BALB virus-1 in that neither was neutralized by NZB serum, and both were Gross-type.

Regulation of the Persistence of Induced BALB Virus-2. The Fv-1 locus (21) helps to regulate the expression of endogenous BALB:virus-1 (5, 8). Studies were performed to determine whether this locus also plays a role in regulation of BALB:virus-2. Of the nine Nx(NxB)F_1 backcross lines containing inducible virus-2 but not virus-1, five were previously classified as Fv-1^{nb} and four as Fv-1^{nn} in genotype (8). When these cell lines were exposed to IdU and passaged in the absence of cocultivation with either NIH/3T3 or NRK cells, the activated virus failed to persist in any of the nine lines. In contrast, with each of five backcross embryo lines that was Fv-1^{nn} in genotype and contained BALB:virus-1 but not

virus-2, the induced virus was able to persist in the absence of cocultivation. These latter results confirm our previous findings (8).

DISCUSSION

After the demonstration that all mouse cells contain inducible RNA C-type viruses (3, 4), subsequent genetic experiments have indicated the presence of specific loci for virus inducibility in cells of several strains, including BALB/c (8), AKR (6), and C58 (7). The fact that multiple loci could be detected in cells of one strain, C58, suggested that additional ones might exist in cells of the other strains (7). The methods used previously to isolate induced viruses of BALB/c cells had been optimum for detection of viruses able to grow well in NIH Swiss embryo cells. Viruses that grow preferentially in those cells comprise the majority of natural MuLV isolates (19). It was predicted that viruses at other loci in BALB/c cells must be either defective (i.e., not activatable) or markedly different from N-tropic MuLV in their biologic properties. During induction studies with MSV-transformed BALB/c nonproducer cells, the possibility of a second endogenous BALB/c virus with tropism for NRK cells was suggested. The availability of a large number of genetically well-characterized Nx(NxB)F_1 backcross embryo cell lines (8) made this hypothesis testable.

The present studies demonstrate the existence of a new endogenous virus of BALB/c cells and define its biologic characteristics. This virus is inducible from parental BALB/c cells but not from NIH Swiss embryo cells. The pattern of virus persistence in Nx(NxB)F_1 backcross embryo lines and the biologic properties of induced viruses from these individual lines clearly indicate that the locus for induction of this newly identified virus segregates independently from that of a previously detected N-tropic virus of BALB/c cells (8).

Major differences were detected in the cellular regulation of the two endogenous viruses. After chemical treatment, much more BALB virus-2 was released than BALB virus-1. That this was the case was shown by the finding that the early induced virus from K-BALB cells was predominantly virus-2 in its serologic type. Further, only in backcross cell lines containing virus-2 was early virus induction high enough to be detectable by the viral polymerase assay. The abilities of the viruses to persist after activation also differed markedly. The capacity of BALB virus-1 to replicate after induction was previously shown to be strongly influenced by the Fv-1 regulatory locus (5, 8). In the present studies, there was no indication of genetic factors that allowed persistence of BALB virus-2. Thus, if the inability of BALB virus-2 to persist after activation is due to the Fv-1 or to other undefined loci, both NIH Swiss and BALB/c cells appear to have nonpermissive alleles.

The biologic properties of BALB virus-2 resemble those of a naturally occurring MuLV found in the NZB mouse strain (20). Both NZB-MuLV and BALB virus-2 transmit to and replicate in NRK cells but are unable to infect NIH Swiss or BALB/c mouse embryo cells. Since BALB virus-2 and NZB-MuLV are similar in their host range and serologic properties and distinct from other MuLV isolates, these viruses appear to form a new subgroup of mouse leukemia virus. Viruses induced from BALB/c cells have recently been studied by immunoelectronmicroscopy. A fraction has been found that can be labeled by antibody to a mouse myeloma virus (22). Whether this antiserum will detect cross-reactivity with one of the endogenous viruses isolated in the present studies awaits further investigation.

From the present studies and our previous findings (5, 8), three biologically distinguishable endogenous mouse C-type viruses have been identified. Loci at which each can be activated segregate in backcross embryo cell lines with a common strain, NIH Swiss. Cells of the latter strain are not inducible under the same experimental conditions. These results, along with those of recent biochemical studies indicating the presence of C-type viral-specific DNA within the mouse cell genome (23), indicate that the loci detected for virus induction may represent mouse C-type viral genetic information. The two biologically distinguishable endogenous viruses of BALB/c cells each appear to be subject to different cellular genetic controls, yet neither is able to replicate efficiently in BALB/c cells. The relationship of these findings to the mechanisms of leukemia development *in vivo* has yet to be determined.

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