Brief Definitive Report

ALLELISM AND LINKAGE STUDIES OF MURINE LEUKEMIA VIRUS ACTIVATION GENES IN LOW LEUKEMIC STRAINS OF MICE*

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Endogenous ecotropic murine leukemia viruses (MuLV) found in low virus strains of mice differ from those found in high virus strains in both their genetic location and stability. Many investigators (1–7) have demonstrated that high virus strains contain multiple ecotropic proviruses, some of which are not allelic among substrains. However, low virus strains of similar genetic origin appear to contain a single ecotropic provirus that is allelic among divergent substrains (8, 9, Jenkins et al., personal communication).

Recently (10), we demonstrated that low virus strains of mice also contain genes that enhance the induction or expression of ecotropic MuLV in certain F_1 hybrid mice. This increase in MuLV induction, as compared to the response of either parental strain, occurs both in iododeoxyuridine (IUdR)-treated tissue cultures and spontaneously in spleen cells (J. McCubrey and R. Risser, manuscript submitted for publication). We determined that BALB/c and C57BL/6 (B6) strains each contain one genetic element that, in combination, enhance virus induction, and we denoted the BALB/c locus as *Inc-1* and the B6 locus as *Inb-1*. The enhanced virus induction phenotype is dominant, and the interaction for virus expression is seen in all possible combinations, even when the F_1 hybrid is heterozygous at the *Fv-1* locus, a locus known to regulate MuLV replication.

To relate these induction genes to known proviruses, we determined their position in the mouse genome. Ihle and co-workers (11) and Kozak and Rowe (12) mapped the ecotropic virus-inducing gene of BALB/c mice to chromosome 5, 24 centimorgans (cM) from phosphoglucomutase (*Pgm-1*). Ihle and colleagues (11) also determined that the virus-inducing gene of BALB/c and C3H/He mice are allelic, whereas those of C3H/He and B6 are not (8). Here, we demonstrate that *Inc-1* is allelic among mouse strains A/J, BALB/c, C3H/He, and SEC and maps to chromosome 5 close to *Cv-1*. *Inb-1* is allelic between strains B6 and C57BR and maps to chromosome 8, presumably near *Bv-1*, the endogenous ecotropic virus-inducing gene of C57BL/10 mice (6).

Materials and Methods

Mice. A/J, C57BR/cdJ, C3H/HeJ, C57BL/6BJ, SEC/ReJ, and SWR/J were purchased from The Jackson Laboratory, Bar Harbor, ME. All F_1 and backcross progeny were bred at McArdle Laboratory, University of Wisconsin, Madison, WI, under standard breeding conditions.

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TABLE I

Allelism Tests for Inc-1 in BALB/c-related Mice

Cross	Number of embryos with *Inc-1*$^+$, *Inb-1*$^+$ phenotype	Number of embryos with *Inc-1*$^-$, *Inb-1*$^+$ phenotype	95% confidence interval		
			Lower limit	to	Upper limit
(BALB/c × A/J) × B6	52/52	0/52	6.9 cM	to	0 cM
(BALB/c × C3H/HeJ) × B6	54/54	0/54	6.6 cM	to	0 cM
(BALB/c × SEC) × B6	43/43	0/43	8 cM	to	0 cM

* Statistical analysis of allelism and linkage studies. Presented in Tables I–IV are the error intervals for the allelism tests and the SE for the linkage tests. The confidence intervals for the allelism tests are calculated from the formula:

$$\text{limit}_{\text{upper}} = \frac{(2NP + t^2) + [(2NP + t^2)^2 - 4NP^2 (N + t^2)]^{1/2}}{2 (N + t^2)},$$

where N is the number of mice examined, P is the observed proportion in a binominal distribution, and t is the confidence level figure. The lower limit is the same as the above formula, but the quantity under the square root is subtracted rather than added. Because no recombinants were observed in the allelism tests, the confidence intervals are merely a function of the desired confidence level and the number of mice examined. The lower limit is a statistical estimate of the furthest possible distance between the two genes under study.

The SE in the linkage tests is calculated by the formula $s = \left[\frac{P\,(1\text{-}P)}{N}\right]^{1/2}$, where P is the proportion of A in a binominal population and N is the sample size.

Cell Culture. All cell culture techniques have been described (10). Briefly, 2.5×10^5 secondary embryo cells or tail biopsy fibroblast cells derived from segregating crosses were plated and induced for 48 h with 20 μg/ml of IUdR. 10^5 SC-1 cells were then added to each culture; cultures were continued for 11 d without trypsinization and then developed in the ultraviolet-XC plaque test. BALB/c, B6, and (BALB/c × B6)F_1 cells show median titers of 0.2 (N = 44), 0.5 (N = 40), or 8 (N = 55) syncytia per 2.5×10^5 induced cells, respectively, in this *in situ* virus-induction assay (10). In crosses where *Inc-1* segregated, e.g., (BALB × SWR)F_1 × B6, individual cultures that showed 0–1 syncytia *in situ* were scored as *Inc-1*$^-$; individual cultures that showed greater than three syncytia per culture were scored as *Inc-1*$^+$. The segregation of *Inb-1* was scored in a similar fashion in crosses where it segregated, e.g., BALB/c × (BALB × B6)F_1.

Isozyme Assays. Extracts from tissues of mice previously typed for *Inc-1* or *Inb-1* were scored for inheritance of various alleles of *Pgm-1*, *Gus*, and *Es-1*. The typing of enzymes was carried out exactly as described (6, 8, 12, 13).

Results

Allelism Tests of Inc-1 in BALB/c-related Mice. Previously, we demonstrated that A/J, BALB/c, C3H/He, and SEC mice each carry a gene that in combination with a gene of B6 or C57BR increases the frequency of cells that produce ecotropic MuLV after IUdR induction. Thus, BALB/c or B6 cells show a median titer of ≤0.5 MuLV-induced syncytia *in situ* per 2.5×10^5 IUdR-treated cells, whereas (BALB/c × B6)F_1 cells yielded a median titer of eight syncytia *in situ* per 2.5×10^5 treated cells (10). Cultures prepared from hybrids within a family of related mouse strains showed no increase in the frequency of cells that produce MuLV, i.e., cells from (BALB/c × A)F_1, (BALB/c × SEC)F_1, and (BALB/c × C3H/He)F_1 mice all gave the same response as the parental cells, or 0–1 syncytia per 2.5×10^5 treated cells.

Therefore, we sought to determine by classical genetic techniques whether A/J, C3H/He, and SEC strains have a locus allelic to *Inc-1* of BALB/c (10). BALB/c mice were mated to A mice, and this F_1 was mated to B6. Similar tests were performed

TABLE II

Allelism Test for Inb-1 in B6 and C57BR

Cross	Number of embryos with *Inc-1*$^+$, *Inb-1*$^+$ phenotype	Number of embryos with *Inc-1*$^-$, *Inb-1*$^+$ phenotype	95% confidence interval		
			Lower limit	to	Upper limit
(B6 × C57BR) × BALB/c	70/70	0/70	5.2 cM	to	0 cM

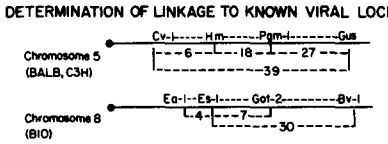

FIG. 1. Location of *Cv-1* and *Bv-1* on chromosomes 5 and 8, respectively, and relevant genetic markers.

TABLE III

Linkage of Inc-1 with Pgm-1 and Gus-1 (Chromosome 5)

Genetic cross:		(BALB/c × SWR)F$_1$ (*Inc-1*$^+$, *Pgm-1*^{a}, *Gus-1*^{a} × *Inc-1*$^-$, *Pgm-1*^{b}, *Gus-1*^{b})		× B6 × (*Inb-1*$^+$, *Pgm-1*^{a}, *Gus-1*^{b})
Progeny class	Inheritance of BALB/c allele			Number of mice
	Inc-1	*Pgm-1*	*Gus*	
Parental (nonrecombinant)	+	+	+	20
Parental (nonrecombinant)	−	−	−	15
Recombinant (*Inc-1-Pgm-1*)	+	−	−	6
Recombinant (*Inc-1-Pgm-1*)	−	+	+	5
Recombinant (*Pgm-1-Gus*)	+	+	−	6
Recombinant (*Pgm-1-Gus*)	−	−	+	13
Double recombinant	+	−	+	4
Double recombinant	−	+	−	1
Percent recombinant	*Inc-1-Pgm-1* = 16/70 = 23 cM ± 5.0 cM			
	Inc-1-Gus = 30/70 = 43 cM ± 5.9 cM			
	Pgm-1-Gus = 24/70 = 34 cM ± 5.6 cM			

Linkage of *Inc-1* with *Pgm-1* (chromosome 5, 24 cM from *Cv-1*)*

Genetic cross:	(BALB/c × SWR) (*Inc-1*$^+$, *Pgm-1*^{a} × *Inc-1*$^-$, *Pgm-1*^{b}	× B6 × (*Inb-1*$^+$, *Pgm-1*^{a})
Number of offspring with:	*Pgm-1*$^{a/a}$	*Pgm-1*$^{a/b}$
Inc-1$^+$	43	12
Inc-1$^-$	14	43

$$\frac{\text{Number of recombinant mice}}{\text{Total number of mice}} : 26/112 \text{ (23 cM ± 4 cM)}$$

* Some of the mice could not be types for *Gus* and *Pgm-1*, however, 42 additional mice were typed for *Pgm-1* and *Inc-1*, as presented in this subtable.

with C3H/He and SEC (in place of the A strain). If the genes were allelic, 100% of the mice should be positive for enhanced virus induction and resemble the (BALB/c × B6)F$_1$. If the loci were not allelic and not closely linked, 75% of the mice should be positive for enhanced virus induction, and 25% should be parental-like. Intermediate values would indicate linkage but not allelism of the two elements.

The results presented in Table I clearly indicate that the *Inc-1* loci in BALB/c, A/J, C3H/He, and SEC are tightly linked. No recombinants were recovered in the

TABLE IV

Linkage of Inb-1 with Es-1 (Chromosome 8, 33 cM from Bv-1)

Genetic cross:	BALB/c ($Inc\text{-}1^+$, $Es\text{-}1^b$) ×	(BALB/c ($Inc\text{-}1^+$, $Es\text{-}1^b$ ×	B6)F_1 ($Inb\text{-}1^+$, $Es\text{-}1^a$)
Number of offspring with:			
	$Es\text{-}1^{a/b}$		$Es\text{-}1^{b/b}$
$Inb\text{-}1^+$	32		18
$Inb\text{-}1^-$	11		41
Number of recombinant mice / Total number of mice:	29/102 (28%)		
	28 cM ± 4.4 cM		

149 cultures examined, a result entirely consistent with the hypothesis that these genes are at identical locations in these strains.

Allelism of Inb-1 in B6 and C57BR Mice. Because B6 and C57BR mice both possess an *In* locus that will enhance virus induction in combination with a gene BALB/c and other members of the *Inc-1* family, we sought to determine whether these *Inb* loci are allelic in B6 and C57BR strains. B6 mice were mated to C57BR, and this F_1 was crossed to BALB/c mice. For reasons similar to those presented above, 100% of the mice should be positive when these *Inb-1* loci are allelic. The data presented in Table II indicate that B6 and C57BR are allelic for *Inb-1*.

Linkage of Inc-1 to Pgm-1 in BALB Mice. Kozak and Rowe (12) and Ihle and coworkers (11) have shown that the BALB/c ecotropic virus-inducing gene *Cv-1* is linked to *Pgm-1* on chromosome 5 (Fig. 1). Because it is quite possible that *Inc-1* is linked to or identical with *Cv-1*, we examined the linkage of *Inc-1* with *Pgm-1* and *Gus-1*. Since BALB/c and B6 mice are $Pgm\text{-}1^a$, a third strain, SWR, ($Pgm\text{-}1^b$) had to be used to test for segregation of *Pgm-1*. The results of this experiment, presented in Table III, indicate that *Inc-1* is ~24 cM from *Pgm-1* and is loosely linked to *Gus-1*. Therefore, *Inc-1* is on the centromeric side of *Pgm-1* at approximately the position Kozak and Rowe (12) reported for *Cv-1*.

Linkage of Inb-1 to Es-1 in B6 Mice. Kozak and Rowe (6, 9) determined that the ecotropic virus-inducing gene of C57BL/10 mice is linked to *Es-1* (esterase-1) on chromosome 8 (Fig. 1). Therefore, we analyzed the linkage of *Es-1* to *Inb-1*. Table IV presents the results of these linkage experiments. We found that *Inb-1* is ~30 cM from *Es-1*. However, we did not perform the appropriate three-point cross to determine whether *Inb-1* and *Bv-1* are on the same side of *Es-1*.

Discussion

Studies with high virus strains of mice indicate that the proviruses in these strains are located at diverse positions in the mouse genome (1, 5, 7). Our studies indicate that some genes involved in the induction of MuLV in low virus strains of a related pedigree are allelic and probably have not changed position in at least 60 yr of inbreeding. This result is significantly different from AKR proviruses that have been observed to reinsert in additional chromosomal locations during the construction of the NFS-*Akv-1* congenic strain that is <10 yr old (3). Presumably, in low virus strains there is a lower probability of infection of germ-line cells and therefore of virus reintegration.

We determined that *Inb-1* and *Inc-1* are linked to markers known to be linked to

Bv-1 and *Cv-1*, the respective ecotropic virus-inducing genes of B6 and BALB/c mice. Given that our *Inc-1* linkage data is virtually identical with the map distances between the virus-inducing genes and biochemical markers observed by Kozak and Rowe (12) for the BALB/c virus-inducing gene *Cv-1*, it seems reasonable to predict that *Inb-1* will be found in the proximity of the C57BL virus-inducing gene *Bv-1* (6). It is reasonable to propose from our data that the genes governing induction phenotype (*In* loci) are tightly linked to MuLV structural elements, and this unit is conserved among inbred strains of related pedigree.

However, even though we observed no recombinants in the allelism tests, a statistical analysis of the data indicates the genes of A/J and SEC that govern induction phenotype could be as far as 8 cM from the BALB/c *Inc-1* locus, a genetic distance that corresponds to $\sim 5 \times 10^6$ base pairs. Moreover, statistical analysis of the *Pgm-1* linkage data indicates a comparable uncertainty in the linkage of *Inc-1* to *Pgm-1*. From these results, the only valid conclusion is that *Inc-1* is near the BALB/c ecotropic virus-inducing gene *Cv-1*, and *Inc-1* may or may not be identical to *Cv-1*. Likewise, there is considerable uncertainty in the allelism of *Inb-1* loci in C57BR and B6 mice, and we do not know for certain that *Inb-1* is tightly linked to *Bv-1* because a flanking marker was not readily available in the *Inb-1* linkage tests.

Nucleic acid hybridization studies by Horowitz (unpublished data) in this laboratory and by Jenkins and colleagues (personal communication), who compared the size of cellular-ecotropic proviral DNA junction fragments produced by digestion with restriction endonucleases, indicate that inbred strains that carry *Inc-1* have a single ecotropic provirus with characteristic cellular flanking sequences, and inbred strain that carry *Inb-1* have a single ecotropic provirus with different flanking cellular sequences than those of *Inc-1* mice. Because the provirus-cell DNA junction fragments are the same among a family of related mouse strains, these results substantiate our hypothesis that proviruses and their controlling elements are allelic within low virus strains of mice of a common geneological origin. Analysis of the segregation of proviral sequences with *In* loci in recombinant inbred strains and segregating generations should further resolve question of identity of proviral sequences and *In* loci.

The phenotypes associated with a particular ecotropic proviral locus, i.e., pattern of spontaneous and induced expression, and enhanced virus induction in combination with other proviruses, might be explained by two different hypotheses. The site of provirus integration might be important in provirus expression, as originally proposed by Cooper and Temin (13) and further substantiated by Jaenisch et al. (14). One interpretation of studies of Jaenisch and of our studies of virus induction and expression in low leukemic mice (10) is that low virus strains of mice have their proviruses integrated in positions that are restrictive for virus expression, whereas high virus strains have their proviruses in positions permissive for expression. An equally likely possibility is that minor changes within proviral sequences determine their unique biological properties.

Summary

Previously, we identified two genes, termed *Inc-1* and *Inb-1*, that interact to enhance ecotropic murine leukemia virus induction in low virus strains of mice. Mice related to BALB/c in origin carry a locus termed *Inc-1*, whereas mice related to B6 carry an *Inb-1* locus. Mice that carry both *Inc-1* and *Inb-1* yield 10- to 50-fold more virus-

producing cells than parental strains on induction with halogenated pyrimidines in vitro and demonstrate enhanced murine leukemia virus production in vivo. Here, we show that mice related to BALB/c in origin, i.e., A, C3H/He, and SEC, have an *Inc-1* locus that is allelic with that of BALB/c. The C57BR mouse strain has an *Inb-1* locus that is allelic with that of B6, located on chromosome 8, 30 cM from *Es-1*. We also show that the *Inc-1* locus of BALB/c mice is located on chromosome 5, 24 cM from *Pgm-1* and 43 cM from *Gus*. Kozak and Rowe (6, 8) and Ihle and co-workers (3) have shown that the ecotropic virus-inducing genes in BALB/c and B10 mice are located on chromosomes 5 and 8, respectively, with similar distances from the previously mentioned biochemical markers. Our data are consistent with two possibilities: *Inc-1* and *Inb-1* are part of the virus-inducing genes *Cv-1* and *Bv-1*, respectively, or *Inc-1* and *Inb-1* are tightly linked regulatory genes.

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