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Low rates of proviral integration in SWR/J–RF/J hybrid mice

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Summary

A high frequency of proviral acquisition has previously been reported in the offspring of SWR/J–RF/J hybrid mice. In the present study, it was investigated whether this proviral acquisition would be useful for large-scale insertional mutagenesis studies. A population of SWR/J–RF/J hybrid mice with a predominantly SWR/J background was created. Lines of mice with such a background and partially congenic for two active proviruses from the RF/J strain were generated (the insert lines). Control lines were derived from mice which had no proviral loci but had an otherwise similar genetic background. DNA samples of mice in the insert lines were screened for the appearance of new proviral loci by Southern hybridization. The rate of proviral acquisition, calculated from the observed number of new proviral loci was 0·023 new proviruses per mouse. This rate is lower than found in previous studies and too low for large-scale insertional mutagenesis studies. A sensitivity experiment indicated that there was adequate detection of new proviral loci.The number of segregating proviruses was consistent with the number of newly acquired proviruses actually detected. Two additional crosses between mice in the insert lines and SWR/J mice were performed. The rate of proviral acquisition was greatly increased when SWR/J females were initially mated to insert mice, but remained unchanged when SWR/J males were used. This suggested that mice in the insert lines had acquired a maternally transmitted factor, which was suppressing viral expression and thus reducing the rate of proviral acquisition.

1. Introduction

Endogenous ecotropic murine leukemia viruses (MuLVs) are a well-studied family of retroviruses with a relatively homogenous structure. They are present in many inbred strains of mice at copy numbers of up to 10 per haploid genome (Jenkins *et al.* 1982). Expression of these proviruses varies greatly with the inbred strain and the age of the mice. In high viraemic strains e.g. AKR, germline acquisition of new ecotropic proviruses can occur, presumably due to viral infection (Rowe & Kozak, 1980).

Spontaneous acquisition of new proviruses has also been observed in many of the progeny of hybrid females derived from the SWR/J and RF/J strains (Jenkins & Copeland, 1985). The SWR/J strain does not carry any MuLV proviral loci (Jenkins *et al.* 1982), but is permissive for the expression of most MuLVs (Jolicoeur, 1979). RF/J mice have three MuLV proviral loci, designated *Emv*-1, *Emv*-16 and *Emv*-17 but this strain is not permissive for MuLV expression. *Emv*-16 and *Emv*-17 are very tightly linked (Buchberg *et al.* 1986) and segregate with the high viraemia phenotype in backcrossed mice. Proviral acquisition occurred in both somatic and germ-line cells of the offspring of hybrid females heterozygous for *Emv*-16 and *Emv*-17. In the original study, approximately 0·35 new proviruses per mouse were observed in somatic cells (Jenkins & Copeland, 1985).

A series of crosses of RF/J mice with SWR/J or CBA/CaJ (another virus-negative strain) indicated that the rate of proviral acquisition depends on the genetic background and confirmed that the SWR/J–RF/J strain combination is particularly suitable (Bautch, 1986). In another study, 18 separate lines of mice, each starting with a single newly acquired proviral locus, were derived from SWR/J–RF/J hybrid mice (Spence *et al.* 1989). Lines which expressed the virus acquired new proviral loci at a similar rate to that observed by Jenkins & Copeland (1985) and Bautch (1986), suggesting that such a system should be useful for insertional mutagenesis studies.

Spontaneous proviral acquisition in SWR/J–RF/J

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hybrid mice is a particularly attractive technique for generating insertional mutations because there is no need for the technically sophisticated manipulation of eggs, blastocysts or embryos (reviewed in Gridley *et al.* 1987). Also, it has recently been observed that embryonic stem-cell derived mouse lines containing many retrovirus vector insertions showed considerably higher variation for quantitative traits than insert-free controls (Keightley *et al.* 1993), suggesting that the insertion of retroviral vectors may be useful for studying genes affecting quantitative traits. The aim of the present study is to investigate the potential of using proviral acquisition in lines of SWR/J–RF/J hybrid mice for such a purpose. Lines of mice with a mainly SWR/J background but containing the *Emv*-16 and *Emv*-17 proviruses were established by crosses between SWR/J and RF/J mice. The rate of new proviral acquisition and the frequency of transmission of newly acquired proviruses through the germ-line were measured.

2. Materials and methods

(i) *Derivation of insert and control lines of mice*

Lines of SWR/J–RF/J hybrid mice partially congenic for *Emv*-16 and *Emv*-17 (insert lines) or lacking MuLV proviral loci (control lines) were derived by the scheme shown in Fig. 1. SWR/J and RF/J mice were obtained from the Jackson laboratory in 1989. Male offspring of a cross between SWR/J females and RF/J males were mated with SWR/J females. Then, for four generations, females heterozygous for *Emv*-16 and *Emv*-17 were repeatedly backcrossed with SWR/J males. This created a starting population of SWR/J–RF/J hybrid mice with a predominantly SWR/J background so that most individuals will be permissive for viral expression (see discussion). Also the backcrossing would provide a relatively homogenous background against which to measure quantitative variation. Mice in the starting population were crossed *inter se* and the progeny screened by Southern hybridization for the presence of MuLV proviruses. Mice lacking MuLV proviruses were used to form the control lines. Mice which appeared to be homozygous for *Emv*-16 and *Emv*-17 from the intensity of the appropriate bands were used to form the insert lines.

Insert and control mice were maintained in a number of separate lines. Each line comprised five mating pairs of mice from a family selected at random each generation. If there were insufficient individuals in the selected family, substitutes from an additional

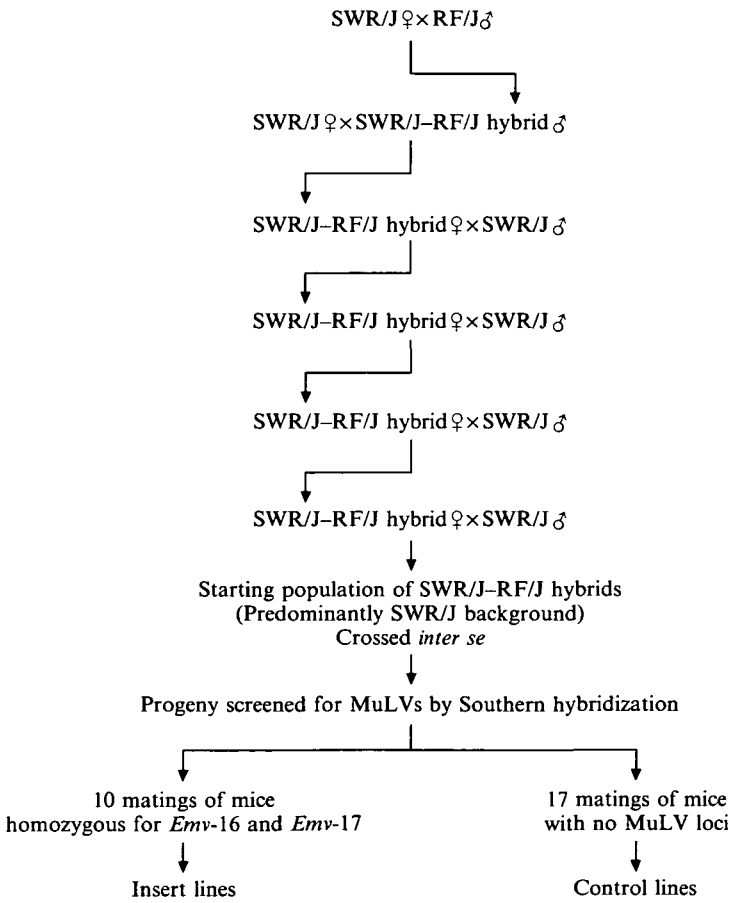

Fig. 1. Derivation of insert and control lines of SWR/J–RF/J hybrid mice from crosses between SWR/J and RF/J mice.

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were used but full-sib mating was maintained. New lines were formed by occasionally making an additional set of five mating pairs from a line. By the fifth generation after subdivision of the insert and control mice, there were 18 insert lines and 5 control lines.

(ii) *Detection of new proviruses in somatic cells*

Mouse tail biopsies (~ 20 mg) were digested in 400 μl of 200 μg ml^{-1} proteinase K, 0·3 M sodium acetate, 10 mM Tris (pH 7·9), 1 mM-EDTA, 1 % SDS and then extracted by slow rotation for 10 min with saturated phenol, then 1:1 saturated phenol:chloroform and finally chloroform. The DNA was precipitated by adding 2·5 volumes of ethanol and resuspended in TE buffer (pH 8·0). Approximately 10 μg of DNA was digested to completion with *Pvu*II or *Hind*III. *Pvu*II and *Hind*III cut the MuLV proviruses investigated in this study approximately 3 kb and 2·5 kb respectively from the 3′ end. The probe hybridization site is within this section. With each enzyme, a single cell DNA–proviral DNA junction fragment containing approximately 3 kb or 2·5 kb of viral DNA plus various lengths of function DNA is produced for each proviral locus.

Electrophoresis of digested DNA was performed at approximately 2 V/cm for 17 h in 20 cm 0·7% agarose-TBE gels. The DNA was transferred to a charged nylon membrane (Hybond–N+, Amersham Ltd., United Kingdom) by capillary blotting with 0·4 M-NaOH for 24 h. A 330 base-pair *Sma*I fragment from plasmic pC6 (Panthier & Codamine, 1987), specific to ecotropic MuLV DNA sequences, was used to synthesize DNA probes. About 20 ng of the fragment in low-melting point agarose was labelled with ^{32}P by random oligonucleotide primer extension (Feinberg & Vogelstein, 1983). The labelled probe was hybridized to the membrane-bound DNA in 1 mM-EDTA, 0·5 M-$NaHPO_4$ (pH 7·2), 1 % SDS (Church & Gilbert, 1984), with 100 μg ml^{-1} denatured herring sperm DNA for 17 h at 68 °C. The labelled membranes were washed three times at 68 °C for 30 min in 1 mM-EDTA, 40 mM-$NaHPO_4$ (pH 7·2), 1 % SDS and autoradiographed for up to 14 d at −70 °C using an intensifying screen. Fragments different in size from *Emv*-16 and *Emv*-17, which hybridized to the probe, were scored as new somatic proviruses.

(iii) *Transmission of proviruses through the germ-line*

Proviruses which have been transmitted through the germ-line can be identified because a provirus, detected by the above methods, should be inherited by a proportion of the offspring of the mouse in which the provirus was first observed and in these offspring the provirus should have a copy number of one per cell. The frequency of transmission of all newly observed proviruses was measured.

(iv) *Estimation of the rate of acquisition of somatic proviruses from the number of germ-line proviruses*

Proviruses can be considered as belonging to two categories: 1. Newly observed proviral loci resulting from new infection of an individual, often occurring at a copy number of less than one per cell (somatic proviruses). 2. Proviruses, acquired in previous generations, which have been transmitted through the germ-line and so should be segregating amongst the members of the line (germ-line proviruses). Estimates of the rate of acquisition of germ-line proviruses (μ) were made by Monte Carlo simulation of a line of mice inheriting proviruses acquired in previous generations and gaining new ones. Five mating pairs per line were simulated, as in a line in the actual experiment. Proviruses were assumed to segregate independently. Each new offspring had a diploid genome formed by firstly inheriting proviruses from its parents and then gaining new germ-line proviruses. The number of new proviruses gained per mouse was a random variable sampled from the Poisson distribution with parameter (μ). Each new provirus was assigned to a new locus in the genome.

Two different models were considered. In the first, offspring with proviruses were preferentially mated and if additional individuals were required, one of the five mating pairs was selected at random and its offspring mated. All matings were between full-sibs. This models what should occur in the actual experiment if all mice with proviruses were used for mating. The second model was the same as above except that there were no preferential matings of individuals with proviruses. This models what would occur in the experiment if matings were at random.

With either model, for a given μ, many replicates were simulated, each time noting the final number of germ-line proviruses. The likelihood of producing an observed number of proviruses is the fraction of times that the simulation produced that final number. The likelihood as a function of μ was maximized. Support limits were values of μ which gave a natural log likelihood of two less than that given by the maximum. The maximum likelihood estimate of the rate of acquisition of somatic proviruses is simply the maximum likelihood estimate of rate of acquisition of germ-line proviruses divided by the average experimentally observed transmission frequency.

3. Results

(i) *Sensitivity of detection of new proviruses*

Newly observed proviruses hybridized at a variety of intensities up to one half of that of *Emv*-16 and

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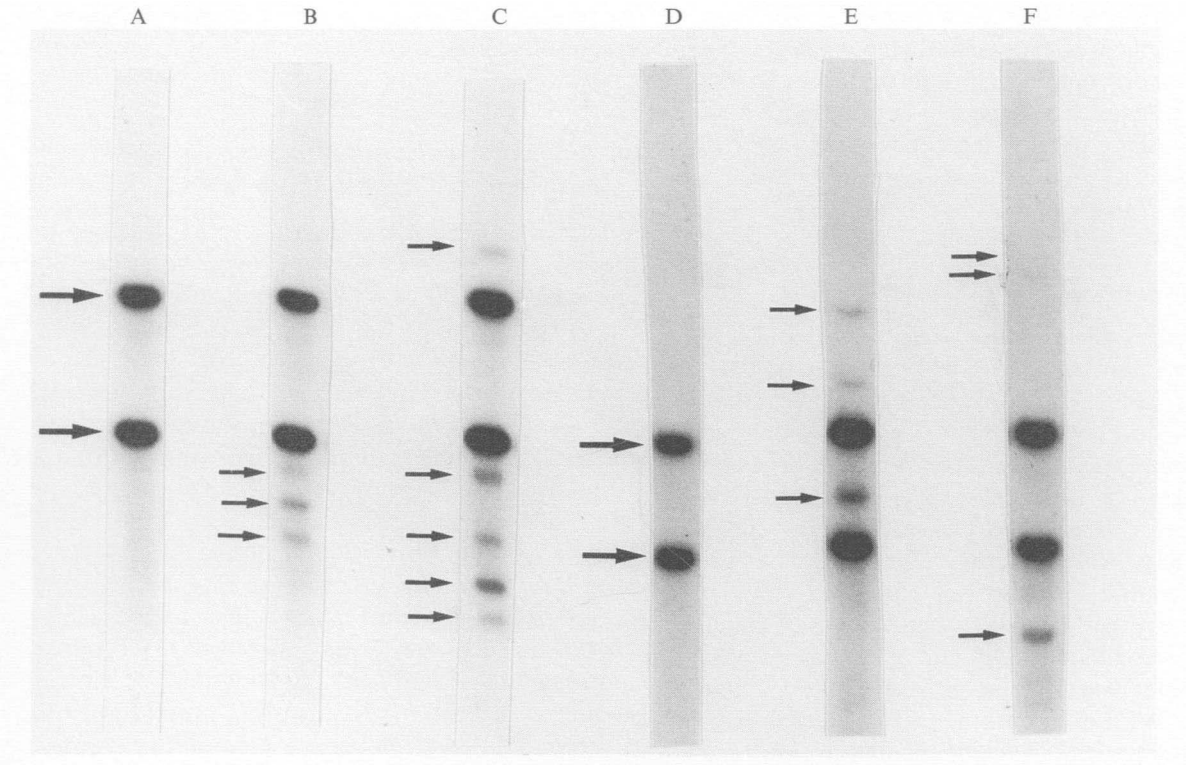

Fig. 2. Representation of proviruses in tail genomic DNA from SWR/J–RF/J hybrid mice. *Pvu*II (A–C) or *Hind*II (D–F) digested tail genomic DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with the [^{32}P]-labelled probe. The fragments corresponding to *Emv*-16 and *Emv*-17 are indicated by large arrows in lanes A and D. New proviral loci are indicated by small arrows in lanes B, C, E and F.

Emv-17 i.e. at copy numbers of up to one per cell. Therefore, to determine the lower limit at which a new provirus could be detected, the DNA of an individual known to be heterozygous for an provirus was diluted by adding appropriate amounts of DNA from an individual lacking this provirus. The total DNA concentration and so the concentration of *Emv*-16 and *Emv*-17 were kept the same at each dilution. It was found that the provirus could consistently be detected at a dilution factor of 0·1, indicating that new proviruses at copy numbers of 0·1 per cell or greater should be detectable. This would seem to be a reasonable level of sensitivity since it has been reported that the intensity of all new fragments observed representing proviruses was between 10 and 100% of the intensity of the intensity of *Emv*-16 and *Emv*-17 in the heterozygous state (Bautch, 1986).

(ii) *Acquisition of new proviruses in somatic cells*

Tail biopsies of mice in the insert lines were screened for newly acquired proviruses by Southern hybridization. After digestion of DNA samples with *Pvu*II, *Emv*-16 and *Emv*-17 appeared as 4·6 kb and 6·1 kb hybridizing fragments respectively (Fig. 2, lane A, large arrows). With *Hind*II, two fragments of size 2·8 kb and 3·9 kb were observed (Fig. 2, lane D, large arrows), although which fragment corresponded to *Emv*-16 or *Emv*-17 is not known. All DNA samples were first, digested with *Pvu*II. To check that newly observed fragments represented proviral loci, the DNA was then digested with *Hind*II which should produce different sized fragments from those found with *Pvu*II. For example, lanes B and E of Fig. 2 show results for the same mouse but digested with *Pvu*II and *Hind*II, respectively. The three new fragments detected with *Pvu*II (small arrows) also occur as different sized fragments after digestion with *Hind*II and so represent new proviral loci. Similarly, lanes C and F show the pattern for a different individual. Five new fragments were observed after *Pvu*II digestion (lane C) and at least three are visible after *Hind*II digestion (lane F). The absence of the other two fragments in lane F may be due to fragments co-migrated with themselves or with *Emv*-16 or *Emv*-17 after digestion with *Hind*II. In this case all five fragments observed after *Pvu*II digestion were so well defined that it was decided to score all of them as new proviral loci. Mice which appeared to have new proviral loci were preferentially used for mating in order to increase the fixation rate.

The frequencies of mice with at least one new provirus and the average number of new proviruses per mouse were 0·015 and 0·023, respectively (Table 1). For comparison, the results of four other previously published studies are also given in Table 1. There is a large range of values of the frequency of mice with new proviruses (0.080–0·30) and even larger range

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Table 1. *Rates of acquisition of somatic proviruses in SWR/J–RF/J hybrid mice*

Results from indicated generations of insert lines, additional crosses, or other previous studies	Mice with new proviruses/ total analysed	Frequency of mice with new proviruses	New proviruses/ total analysed	Average number of new proviruses per mouse
Generation 4	5/717	0·007	12/717	0·017
Generation 5	13/519	0·025	17/519	0·030
Generation 6	9/619	0·015	14/619	0·023
Total of generations 4, 5 and 6	27/1855	0·015	43/1855	0·023
*(SWR/J♀ × SWR/J–RF/J♂) × SWR/J♂	10/119	0·084	14/119	0·12
*(SWR/J–RF/J♀ × SWR/J♂) × SWR/J♂	1/46	0·022	1/46	0·022
Jenkins and Copeland 1985	20/103	0·19	36/103	0·35
Bautch 1986	47/586	0·080	82/586	0·14
Panthier and Codamine 1987	22/73	0·30	130/73	1·8
Spence *et al.* 1989	18/234	0·077	25/234	0·11

* In these crosses SWR/J mice were initially mated to SWR/J–RF/J mice from the insert lines as indicated and the female offspring were subsequently mated to SWR/J males.

of average numbers of new proviruses per mouse (0·11–1·8). The frequencies of mice with new proviruses in the four previous studies were significantly different ($P < 0·005$, variance test for homogeneity of the binomial distribution (Snedecor & Cochran, 1989)). However, the frequency observed in the present study was less than a fifth of even the lowest of the previously reported values and was significantly different from each value tested separately ($P < 0·005$). This could be caused by the mice in the insert lines being less susceptible to viral infection than the mice used in the other studies. One factor which affects susceptibility to viral infection which is of great importance in this experiment is maternal resistance factor (MRF) (see discussion). The mice used to form the insert lines should be MRF− because only SWR/J females were used in the initial cross (Fig. 1) and MRF has not been detected in SWR/J mice. To test whether the insert lines had developed a maternal resistance factor, SWR/J females were mated with SWR/J–RF/J hybrid males from generation 6 of the insert lines producing hybrid offspring which should be MRF−. These were then mated with SWR/J males and the offspring were screened for new proviruses. As a control experiment, SWR/J–RF/J hybrid females were mated with SWR/J males producing offspring which were again mated with SWR/J males and the progeny were scored for new proviral loci. The results of these crosses are shown in Table 1.

When SWR/J females were initially used i.e. (SWR/J♀ × SWR/J–RF/J♂) × SWR/J♂, the frequency of mice with new proviruses and the average number of new proviruses per mouse were greatly increased and were comparable to values from the four previous studies. The frequency of mice with new proviruses in this cross was significantly different from the overall frequency observed in generations 4–6 ($P < 0·005$). However, the control cross yielded very similar values to those observed in generations 4–6. These results suggest that the mice in the insert lines have, at an unknown time before generation 6 developed a maternally transmitted factor, thus reducing their susceptibility to new viral infection.

(iii) *Frequency of transmission of proviruses through the germ-line*

In previous studies most newly acquired proviruses were observed to be transmitted into the germ-line at a frequency of less than one: range 0·05–0·35 (average 0·22) (Bautch, 1986); range 0·06–0·46 (average 0·19) (Spence *et al.* 1989). The germ-line transmission of 15 proviruses acquired by individuals in generations 4 and 5 of the insert lines was investigated. Only 6 of the 15 proviruses were observed to be transmitted at frequencies ranging from 0·18 to 0·38 with an average value of 0·28 (Table 2). These values are similar to those found in the other studies. However, nine of the 15 proviruses did not appear to be transmitted and including these results reduces the average transmission frequency to 0·11. In some cases lack of transmission may be due to mice with new proviruses producing so few offspring that by chance none received the provirus.

(iv) *Estimation of the rate of acquisition of somatic proviruses from the number of germ-line proviruses*

The observed rate of acquisition of somatic proviruses in the insert lines (Table 1), may be an underestimate because of new proviruses occurring at too low an intensity to be detected. Segregating proviruses which have been transmitted into the germ-line should not suffer from problems of poor detection because they all occur at a copy number of one per cell. The number of germ-line proviruses depends on the rate of acquisition of newly occurring somatic proviruses and the transmission frequency and so can be used to make a semi-independent check that there was

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Table 2. *Germ-line transmission of newly acquired proviral integrations*

Integration number	Mating in which integration observed	*Proportion transmitted	Transmission frequency
1	A	4/12	0·33
2	B	0/14	0
3	B	0/14	0
4	B	0/14	0
5	C	3/8	0·38
6	C	2/8	0·25
7	C	0/8	0
8	D	9/39	0·23
9	E	0/4	0
10	E	0/4	0
11	F	0/2	0
12	F	0/2	0
13	G	5/15	0·33
14	H	0/11	0
15	H	2/11	0·18

* The number of offspring which received the integration by germ-line transmission, divided by the total number of offspring of the individual in which the integration was first observed.

adequate detection of newly occurring somatic proviruses. A maximum likelihood estimate of the rate of acquisition of germ-line proviruses was inferred from the number present in the insert lines after six generations.

In generation 6 of the insert lines, four out of the 18 lines each had one germ-line provirus. With a model of preferential mating, the maximum likelihood estimate of the rate of acquisition of germ-line proviruses was 0·001, with lower and upper support limits of 0·0002 and 0·0026, respectively. The corresponding rate of acquisition of somatic proviruses, obtained by dividing this rate by the average observed transmission frequency (including cases where there was no transmission), i.e. 0·11, is 0·0091. The rate of acquisition of somatic proviruses actually observed in the insert lines was 0·023. This indicates that the observed rate of acquisition was not only sufficient to explain the number of germ-line proviruses found but actually predicts that there should be 2·5 times more, suggesting that detection of newly occurring proviruses was adequate. The most likely explanation for too few germ-line proviruses being produced is that some mice with new proviruses selected for mating failed to reproduce whereas in the model all mice are assumed to reproduce. The extreme case of poor selection would be random mating and for comparison the simulation was repeated with a model of random mating. In this case the maximum likelihood estimate of the rate of acquisition of germ-line proviruses was 0·006, with lower and upper support limits of 0·0017 and 0·013, respectively. The corresponding rate of acquisition of somatic proviruses was 0·055 which is about twice the experimentally observed value. This result indicates that the observed rate of acquisition of somatic proviruses only becomes too low when completely random mating is assumed, which is obviously not the case in the experiment, and so presumably detection of new proviruses must have been adequate.

4. Discussion

The aim of this study was to investigate whether the spontaneous acquisition of new MuLV proviral integrations in SWR/J–RF/J hybrid mice could be useful for large-scale insertional mutagenesis studies of quantitative traits. A high frequency of proviral acquisition has been observed in these mice in four previous studies (Jenkins & Copeland, 1985; Bautch, 1986; Panthier & Codamine, 1987; Spence *et al.* 1989). However, the frequency of mice with new proviruses and the average number of new proviruses per mouse observed in the insert lines were less than a quarter of the lowest values reported in these four other studies (Table 1). The reduced rate in the insert lines is unlikely to be due to inadequate detection of newly acquired proviral integrations, since it was found that proviruses with copy numbers greater than 0·1 per cell were detectable. Also, a maximum likelihood simulation indicated that the number of newly occurring integrations observed was sufficient to account for the number of segregating, germ-line integrations. It should be taken into consideration that although the insert lines had a lower rate of proviral acquisition than the other studies, the rates found in these studies varied significantly. The mechanism by which new integrations occur may partly account for the wide range of observed rates of proviral acquisition.

The mechanism and developmental stage of provirus acquisition has been investigated by ovarian transplantation and *in situ* hybridization (Lock *et al.* 1988). This demonstrated that new proviruses are acquired by extracellular virus infection, the oocyte being the target of infection. Investigation of the expression of MuLVs in the genital tract of SWR/J–RF/J mice using *in situ* hybridization (Panthier & Codamine, 1987) showed that MuLV genomic sequences can be transcribed at an easily detectable level in some cells of the ovary. The expression takes place mainly in thecal cells and to a lesser extent in stromal cells but no transcription was observed in fallopian tubes or uterus tissues. This suggests that infection may occur predominantly during oogenesis rather than in very early embryos. Proviral integration probably takes place after the first round of DNA replication in the egg and before the segregation of the germline since new integrations usually occur at a copy number of less than 0·5 per cell and are often transmitted in the germline. The erratic

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frequency of proviral acquisition might be explained by infectious MuLVs having to travel from the thecal cells, where they are liberated, all the way through the follicle before they can infect the oocyte.

Also, there are several factors that can affect susceptibility to viral infection (Lilly & Duran-Reynals, 1985). The allele present at the *Fv-1* locus and maternal resistance factor are of great importance in this study. The *Fv-1*^{nr} allele of RF/J mice is genetically dominant and has the capacity to suppress virus expression (Mayer *et al.* 1980). The allele present in the SWR/J strain, *Fv-1*^{n}, is permissive for the expression of most ecotropic MuLVs (Jolicoeur, 1979). During the derivation of the starting population RF/J mice were backcrossed five times against SWR/J mice (Fig. 1). This should ensure that the mice in the starting population predominantly have the permissive *Fv-1*^{n} allele and so should allow MuLV expression leading to further infection. Maternal resistance factor is thought to be caused by antiviral antibodies being transferred via milk from mother to offspring (Melamedoft *et al.* 1983; Mayer *et al.* 1980; Duran-Reynals, 1985). These antibodies prevent virus expression in the young mice until their native antiviral immune response has become active and itself makes antibodies against virus particles. These antibodies are then later transmitted via milk to the next generation. Once this sequence of events has been established it should persist indefinitely. MRF has been detected in RF/J mice (Mayer *et al.* 1980) but is not present in SWR/J mice, presumably because the SWR/J strain does not carry any endogenous ecotropic proviral loci (Jenkins *et al.* 1982).

Only SWR/J females were used in the initial cross during the derivation of the insert lines (Fig. 1), which should prevent MRF from being transmitted to the SWR/J–RF/J hybrid mice. However, the results of the two test crosses between SWR/J and SWR/J–RF/J hybrid mice (see Table 1) suggest that mice in the insert lines developed a maternally transmitted factor before generation 6. It is conceivable that if mice in a MRF− strain were to start producing antibodies to MuLVs the strain would permanently become MRF+. Indeed, this has been achieved with the MRF− strains DBA/2 and St/b by hyperimmunizing females with virus-containing extracts (Melamedoft *et al.* 1983), and in some AKR females by repeated inoculation of a goat antiserum to a MuLV glycoprotein (Scharw *et al.* 1981). However, without such manipulation, AKR mice have continued to be highly viraemic for many generations but have remained MRF−. During 45 years of inbreeding 14 different loci have become fixed in seven sublines of AKR mice (Steffen *et al.* 1982). Assuming two generations per year, this represents a haploid mutation rate of 0·022. The equivalent rate in the insert lines is simply the average number of new proviruses per mouse multiplied by the transmission frequency i.e. 0·0025. Therefore, the appearance of a maternally transmitted factor in the insert lines is unlikely to be caused solely by MuLV expression, as AKR mice have remained MRF− over a long time period even though they have a much greater rate of expression. The true nature of the maternally transmitted factor in the insert lines and how it arose are unknown.

In conclusion, lines of SWR/J–RF/J hybrid mice were established by crosses between SWR/J and RF/J mice. The rate of proviral acquisition was lower than expected from the results of four other studies (Jenkins & Copeland, 1985; Bautch, 1986; Panthier & Codamine, 1987; Spence *et al.* 1989). The reason for this would appear to be because that the SWR/J–RF/J hybrid mice have developed a maternally transmitted factor. The rate of proviral acquisition observed in the insert lines would be too low for large-scale insertional mutagenesis studies.

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