JOURNAL OF VIROLOGY, Nov. 1986, p. 693–701
0022-538X/86/110693-09$02.00/0

Vol. 60, No. 2

Genetic Background Affects Integration Frequency of Ecotropic Proviral Sequences into the Mouse Germ Line

VICTORIA L. BAUTCH

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received 25 April 1986/Accepted 18 July 1986

Germ line acquisition of ecotropic proviruses occurs at a high frequency in the progeny of SWR/J-RF/J hybrid mice carrying two genetically linked RF/J ecotropic proviral loci, *Emv-16* and *Emv-17* (N. A. Jenkins and N. G. Copeland, Cell 43:811–819, 1985). To determine if genetic background affects proviral integration frequency, I analyzed a series of crosses in which the two RF/J proviral loci were transferred onto different provirus-negative background strains. Unlike SWR/J-RF/J hybrid progeny, few CBA/CaJ-RF/J hybrid mice were identified that carried new germ line proviral loci. These results indicate that genetic factors other than the linked RF/J proviral loci contribute to the increased frequency of germ line provirus integration seen in the SWR/J-RF/J hybrids. The frequency of proviral acquisition appeared to increase when females carrying *Emv-16*, *Emv-17*, and at least one new proviral locus were further backcrossed, suggesting that integration frequency can be increased by genetic manipulation. The breeding data are consistent with the hypothesis that virus from the mother infects the egg or the early embryo. Analysis of the transmission frequency and cosegregation patterns of new proviral loci indicated that (i) viral integration occurs after the first round of DNA replication and before the germ line is set aside during embryogenesis, with a majority of viral integrations occurring at the two-cell stage of development, and (ii) independent viral integrations can occur in the same or in different cells of the embryo.

Exogenous DNA can integrate into vertebrate genomes and cause insertional mutations that either activate or inactivate gene expression (5, 12, 22, 35, 38). Integration of both retroviral and microinjected DNA into the mouse germ line can result in heritable mutations that are usually recessive and lethal, although nonlethal insertional mutations have been described (3, 8, 14, 21, 37, 39). Most insertional mutations recovered in mice have resulted from experimental manipulation of embryos; dilute mice, however, carry a recessive coat color mutation that has been bred for many years and is caused by integration of an ecotropic provirus at the *d* locus (3).

Ecotropic proviruses are found at a number of different heritable loci in many but not all inbred strains of mice (10, 13, 33). Because ecotropic proviruses can give rise to virus capable of infecting murine cells, these ecotropic proviral loci probably arose from infection of mouse germ line cells. The finding that the highly viremic AKR strain of mice fixes new germ line proviral loci at a higher frequency than do nonviremic mice supports the hypothesis that expressed ecotropic proviral loci can promote germ line integration of new loci (1, 6, 20, 23, 32). Analysis of congenic lines carrying expressed AKR ecotropic proviral loci indicates that new loci arise from maternally derived virus (26).

Jenkins and Copeland (9) have recently documented the acquisition of new germ line ecotropic proviral sequences in mice carrying two genetically linked ecotropic proviral loci, *Emv-16* and *Emv-17* (where *Emv* means endogenous ecotropic murine leukemia proviral locus), after transfer of these loci from the RF/J strain of mice onto the SWR/J background. Although RF/J mice exhibit only a low level of viremia that occurs late in life (16), these mice carry a single dominant locus responsible for viremia that occurs early in life in hybrids between RF/J and other strains. This locus segregates with the *Emv-16* and *Emv-17* proviruses and not with another proviral locus, *Emv-1*, that is also found in the RF/J strain (18). The viremic phenotype may be suppressed on the RF/J background because these mice carry a semirestrictive allele (*Fv-1^{nr}*) at the *Fv-1* locus (15), which controls the permissiveness of cells to infection by many ecotropic viruses; RF/J females also transmit a nongenetic maternal resistance factor to their progeny that prevents infection by ecotropic viruses (2, 16, 17). The SWR/J strain is negative for ecotropic proviral loci and carries a permissive allele (*Fv-1^{n}*) at the *Fv-1* locus (10, 11). Jenkins and Copeland (9) have shown that female carriers of *Emv-16* and *Emv-17* that had been backcrossed for two, three, or four generations (the N2, N3, and N4 backcross generations, respectively) onto the SWR/J background give rise to progeny that have sustained new proviral integrations in both somatic and germ line tissue. More than 50% of the progeny in some litters acquired new proviruses, and the frequency of these integration events appeared to be substantially higher than the estimated frequency of germ line integration in AKR mice.

As part of an evaluation of this approach to generating insertional mutants in mice, I determined that genetic factors affect embryonic proviral acquisition. A series of crosses between the RF/J strain and two ecotropic virus-negative strains of mice, SWR/J and CBA/CaJ, were analyzed. The results described here indicate that the genotype of the virus-negative strain influences the frequency of acquisition of new ecotropic proviruses. Moreover, the data indicate that most integrations occur early in embryogenesis, but after the first round of DNA replication, and that multiple integrations can occur in the same or different embryonic cells.

MATERIALS AND METHODS

Mice. All inbred mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, and all animals were maintained at Cold Spring Harbor Laboratory.

DNA preparation. Genomic DNA from tail biopsies of 2-week-old mice was prepared as follows. Tail pieces (0.5 to

1.0 cm) were digested in 50 mM Tris hydrochloride (pH 7.4)–100 mM NaCl–50 mM disodium EDTA–1.0% SDS (sodium dodecyl sulfate) containing 400 μg of proteinase K (Merck & Co., Inc., Rahway, N.J.) per ml in a total volume of 0.5 ml. No homogenization was necessary if tails were incubated overnight at 55°C. After extraction with phenol and with phenol-chloroform (1:1), high-molecular-weight DNA was precipitated by the addition of an equal volume of isopropanol. The DNA was spooled with a small glass rod and rinsed in 70 and 100% ethanol. The DNA was air dried briefly and dissolved into 0.5 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM disodium EDTA. The DNA concentration was determined by assaying the optical density of representative samples, and most preparations yielded 30 to 60 μg of DNA.

DNA analysis. A portion of each DNA preparation (5 to 10 μg) was digested in 200 μl of appropriate buffer with 10 to 20 U of restriction enzyme (New England BioLabs, Inc., Beverly, Mass., or International Biotechnologies, Inc., New Haven, Conn.) for 12 to 20 h. The addition of spermidine to a final concentration of 4.0 mM was necessary to achieve complete digestion with some of the restriction enzymes (e.g., *Hin*dIII). The digested DNAs were precipitated with 2 volumes of ethanol after the addition of ammonium acetate to a final concentration of 2.5 M. The DNA was rehydrated in agarose gel loading buffer (0.025% bromphenol blue, 0.025% xylene cyanol, 2.5% Ficoll-400 [Pharmacia Fine Chemicals, Piscataway, N.J.], 0.8% SDS). After separation by electrophoresis through a 0.8% agarose gel in TAE buffer (50 mM Tris hydrochloride (pH 8.05), 20 mM sodium acetate, 1.0 mM disodium EDTA), DNA was transferred to nitrocellulose filters by the method of Southern (31). Filters were hybridized with a restriction fragment isolated from pAKV-4 (7), an ecotropic provirus-specific probe containing 367 base pairs of AKV DNA in the *env* gene. The fragment was made radioactive by nick translation (24). Hybridization was for 36 h at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution (4)–20 mM $NaHPO_4$ (pH 6.8)–0.1% SDS–10 mM disodium EDTA–100 μg of denatured salmon sperm DNA per ml. After hybridization the filters were washed for 45 min in three changes of 2× SSC–0.1% SDS at room temperature and for 90 min in three changes of 0.2× SSC–0.1% SDS at 55°C. The filters were exposed to x-ray film (X-AR5; Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen for approximately 7 days.

RESULTS

Experimental design and detection of new proviral loci. To generate ecotropic proviral integrations into the mouse germ line, three master crosses were set up and propagated by a further series of crosses. For the first cross RF/J males were mated with females of the CBA/CaJ strain. The CBA/CaJ strain was chosen because it was available at the time the experiments were started and because it appeared to be a good candidate to be permissive for proviral acquisition. This is because the CBA/CaJ strain, like the SWR/J strain, is ecotropic provirus negative and carries a permissive allele at the *Fv-1* locus (*Fv-1*^{n}) (10, 11). In all cases F1 progeny were mated with CBA/CaJ mice to begin backcrossing, and mice of the N2, N3, and N4 backcross generations carrying *Emv-16* and *Emv-17* were set up in mating with CBA/CaJ mice. Because these hybrids exhibited a very low frequency of proviral acquisition (see below), a comparison of integration frequencies was made by establishing a second series of crosses involving RF/J males and SWR/J females. Offspring carrying *Emv-16* and *Emv-17* were selected for mating with SWR/J mice. A third series of crosses was set up with mice carrying *Emv-16* and *Emv-17* on a hybrid background to determine if the RF/J strain carried loci that suppress germ line proviral acquisition. Mice of the second backcross generation (N2) onto the CBA/CaJ background and carrying *Emv-16* and *Emv-17* were mated with SWR/J mice. Offspring were selected for further mating with SWR/J mice as described above.

Detection of the endogenous ecotropic RF/J proviral loci (*Emv-1*, *Emv-16*, and *Emv-17*) and new proviral loci was accomplished as follows. Genomic DNA prepared from tail clips was digested with the restriction enzyme *Pvu*II. This enzyme generates distinct virus-cellular DNA junction fragments containing *env* gene sequences of each RF/J provirus (18). After separation by agarose gel electrophoresis and transfer to nitrocellulose filters, restriction fragments were visualized by hybridization to the *env* gene probe (see Materials and Methods). *Pvu*II digestion products complementary to the probe but differing in size from those produced by the endogenous loci were scored as new proviral loci.

Most of the new loci were also detected by digestion of the DNA with at least one other restriction enzyme (data not shown), thus reducing the possibility that the *Pvu*II fragments were artifacts resulting from partial DNA digestion. Proviral loci represented by fragments comigrating with fragments corresponding to *Emv-16* and *Emv-17* (4.6 and 6.0 kilobases [kb]) were not scored unless present in animals that did not carry these endogenous loci. New proviral loci transmitted to progeny were assayed by digestion of DNA with *Pst*I to check for gross deletions. *Pst*I digestion of intact ecotropic proviruses generates an 8.2-kb DNA fragment because there is a single *Pst*I site in each flanking long terminal repeat. Only 3 of the 40 loci analyzed generated *Pst*I fragments of altered mobility, suggesting that most new loci do not carry gross deletions (data not shown).

Ecotropic provirus acquisition with different strain combinations. The three master crosses tested for acquisition of new proviral loci are shown in Fig. 1. In all breeding schemes, only the N2, N3, and N4 animals carrying *Emv-16* and *Emv-17* were used in subsequent crosses. The results shown in Fig. 1 are pooled from several individual crosses of each genotype and are not pedigrees of single breeding pairs. For example, Fig. 1B shows that initially SWR/J females were mated with RF/J males. The F1 progeny were used in two reciprocal matings. The cross involving SWR/J females (S♀) and F_1 males (F1♂) produced 113 N2 offspring with no new proviral loci. The N2 offspring of this cross were also set up in reciprocal crosses. N2 females (N2♀) were mated to SWR/J males (S♂), and testing of 402 N3 progeny identified 54 new proviral loci, whereas testing of 46 offspring of SWR/J females and N2 males identified no new proviral loci.

Reciprocal crosses were set up for two reasons. The first was to test the hypothesis that only female carriers of *Emv-16* and *Emv-17* could produce offspring with new proviral loci. This situation prevailed in all crosses tested, and the only crosses productive for new loci involved female carriers of *Emv-16* and *Emv-17* (e.g., Fig. 1B, lines 4 and 5). Reciprocal crosses were also used to carry *Emv-16* and *Emv-17* through the male germ line. Because the RF/J maternal resistance factor to viremia was retained by some females with RF/J fathers (2), the possibility that female carriers of the endogenous loci could suppress proviral

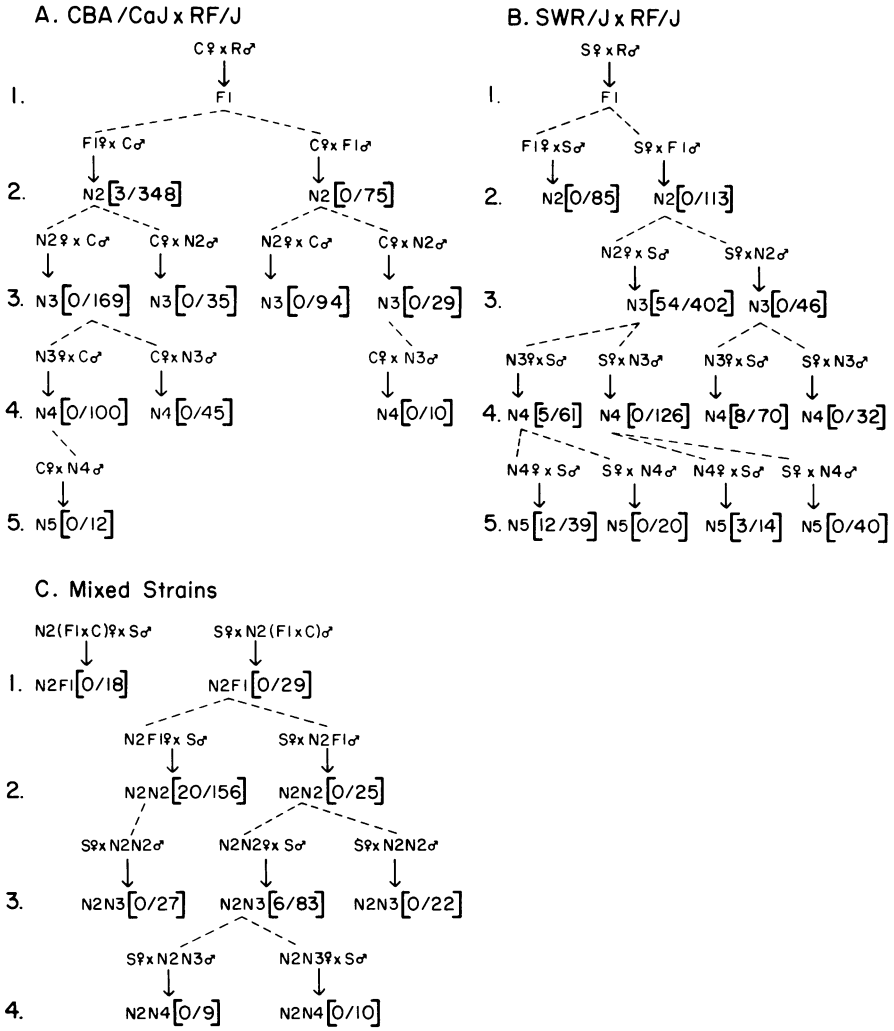

FIG. 1. Genetic crosses tested for proviral acquisition. The parental genotypes are shown at the top of each breeding scheme, the genotypes of the resulting progeny are shown by an arrow to the line below, and crosses involving offspring are identified by a dashed line connecting the progeny genotype to the subsequent cross. The numbers of new proviral loci scored relative to the total number of progeny tested are indicated in brackets next to the appropriate genotype. By convention, the maternal genotype is always listed first. Abbreviations: C, CBA/CaJ; R, RF/J; S, SWR/J.

acquisition in their grandchildren was tested. The data in Fig. 1 indicate that this is not the case because after the first backcross (N2) generation, the sex of the grandparent has little or no effect. For example, the analysis of N4 offspring (Fig. 1B, line 4) shows that new proviral loci were identified in those progeny whose grandmother (N2♀) was a carrier (5/61) and in those progeny whose grandfather (N2♂) was a carrier (8/70).

A tabulation of data for each of the crosses productive for ecotropic proviral integrations is shown in Table 1. In all

TABLE 1. Genotype distribution of new proviral integrations

Genotype of progeny	Parental genotypes	No. of positive mice/ total analyzed	% Positive mice	No. of integrations/ total analyzed	Frequency of integrations	Location in Fig. 1[a]
N2	F1 × C[b]	3/348	0.9	3/348	0.01	A2
N3	N2 × S[c]	26/402	6.5	54/402	0.13	B3
N4	N3(N2×S)[d] × S	5/61	8.2	5/61	0.08	B4
N4	N3(S×N2) × S	3/70	4.3	8/70	0.11	B4
N5	N4(N3×S) × S	10/39	25.6	12/39	0.31	B5
N5	N4(S×N3) × S	3/14	21.4	3/14	0.21	B5
N2N2	N2F1 × S	11/156	7.0	20/156	0.13	C2
N2N3	N2N2 × S	6/83	7.3	6/83	0.07	C3

[a] Letters refer to the breeding scheme; numbers refer to the line within the breeding scheme containing each set of progeny data.
[b] C, CBA/CaJ.
[c] S, SWR/J.
[d] Genotypes of the grandparents, where significant, are shown in parentheses. Fig. 1 contains full pedigrees.

TABLE 2. Litter distribution of new integrations in N3 progeny[a]

N2 mouse no.	No. positive mice/no. of mice in litter:												No. of positive offspring/ total offspring	Total integrations
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12		
9497	0/8	0/2	0/5	0/7	3/7	0/1							3/30	13
9498	1/7	1/6	1/6	2/7	1/4	2/7	0/3	0/3	0/1				8/44	16
9501	0/5	2/6	0/7	0/7	0/8	ND[b]	0/5						2/38	3
9989	0/9	0/8	0/9	ND	ND	ND	0/7	0/7	0/6	0/4	1/6	3/7	4/63	10
10287	ND	0/6	0/3	1/7	1/8								2/24	2
10292	ND	0/7	0/6	0/6	0/7								0/26	0
10293	0/8	1/7	0/7	0/6	0/1								1/29	1
11322	0/4	0/4	0/7	0/7	0/8	2/6							2/36	2
11323	0/2	0/7	ND	0/6	0/4	0/3	0/8	ND	0/7	0/2			0/39	0
11325	1/3	0/6	ND	0/7	1/6	ND	0/5	0/5	2/4				4/36	7
11326	0/3	0/5	0/10										0/18	0
Other[c]	0/19												0/19	0
Total	2/68	4/64	1/60	3/60	6/53	4/17	0/28	0/15	2/18	0/6	1/6	3/7	26/402	54

[a] These N3 progeny are from N2(S♀XF1♂) females and SWR/J males.
[b] ND, Not determined.
[c] Three females, each bearing a single litter.

cases both the percentage of positive mice from each cross and the frequency of proviral acquisition (total integrations/total mice analyzed) are displayed. The percentage of positive mice may be relevant to the mode of proviral acquisition, whereas the integration frequency is relevant to an evaluation of this method in generating insertions. These numbers differ because many mice contained multiple integrations; on average, each mouse that scored positive had two new loci.

Examination of the data presented in Fig. 1 shows that proviral acquisition frequency was affected by the virus-negative strain onto which the RF/J loci *Emv-16* and *Emv-17* were crossed. Most of the crosses tested with CBA/CaJ as the ecotropic virus-negative recipient strain did not score positive for new proviral loci (Fig. 1A). The one positive cross involved mating F1 females (F1♀) with CBA/CaJ males (C♂). Three new proviral loci were identified in an analysis of 348 N2 progeny (Fig. 1A, line 2), with a frequency of 0.01 integrations per mouse (Table 1). Not only was the frequency of proviral acquisition very low, but the three new loci were all scored in the progeny of one female among the 38 females set up in this particular mating scheme. In contrast, several of the crosses tested with SWR/J as the virus-negative recipient strain were positive for new proviral loci, and the acquisition frequency was consistently higher than that observed with CBA/CaJ mice (Fig. 1B, Table 1). Proviral acquisition was seen in N3, N4, and N5 backcross progeny descended from the pool of N2 animals with SWR/J mothers (S♀) and F1 fathers (F1♂).

Because proviral acquisition in both crosses was low or not detected in the F1 and N2 backcross generations, the RF/J strain may contain loci that suppress proviral acquisition until these loci are lost by backcrossing. If this is the case, crosses begun with mice carrying *Emv-16* and *Emv-17* on a CBA/CaJ-RF/J hybrid background might show embryonic proviral acquisition after fewer backcross generations. This possibility was tested by establishing the mixed strain cross shown in Fig. 1C. The cross was started by mating SWR/J animals to both male and female N2 backcross progeny of the genotype (F1♀ × C♂) (Fig. 1A, line 2). The 20 new proviral loci were identified in analysis of 156 N2N2 progeny of the mixed strain cross (Fig. 1C, line 2). These mice are the second backcross generation onto the SWR/J background. This positive result differed from the negative result obtained in analyzing the second backcross generation onto the SWR/J background from the SWR/J × RF/J mating (Fig. 1B, line 2). These results indicate that the RF/J background is less permissive than the CBA/CaJ background for proviral acquisition.

A comparison of proviral acquisition among the productive crosses seen in Table 1 shows that the N3 and N4 backcross progeny of the SWR/J × RF/J cross and the N2N2 and N2N3 progeny from the mixed strain cross produced from 4.3 to 8.2% positive mice, whereas the N5 backcross progeny from the SWR/J × RF/J cross produced 21.4 and 25.6% positive mice in two different crosses. The exceptionally high rate of provirus acquisition in the latter crosses may be due to further backcrossing onto the SWR/J background or to the fact that all N4 mice mated had already sustained one new proviral integration. Mice in all other crosses either carried no new proviral loci or, in the case of N3(N2♀ × S♂) females giving rise to N4 progeny (Table 1, line 3), were a combination of animals with and without new proviral loci. These results suggest that the percentage of progeny mice sustaining proviral integrations can be increased by genetic manipulation. The relative contributions of backcrossing and selection to the increased integration frequency, however, cannot be determined from the data.

Litter distribution of proviral integrations. To determine if there is any pattern to proviral acquisition among individual mothers, the litter distribution of proviral integrations in one of the sets of productive crosses was examined in detail. Proviral acquisition by litter for 11 N2 females (N2♀) crossed to SWR/J males (S♂) are shown in Table 2. These N3 offspring (N2♀ × S♂) are the animals whose pooled data is shown in Fig. 1B, line 3. Eight of these females had at least one offspring positive for new integrations, whereas three females produced only negative offspring (no. 10292, 11323, 11326). All of the eight females with positive offspring had both positive and negative litters, and in no case were all members of a litter positive. Thus, a permissive maternal genotype is not sufficient to ensure that all progeny will acquire new proviral loci. Examination of the total number of positive offspring from all 1st through 12th litters shows no obvious pattern of increase or decrease of integration efficiency by litter (see totals at bottom of Table 2).

Germ line transmission of new proviral integrations. The autoradiographs resulting from hybridization of the *env* gene

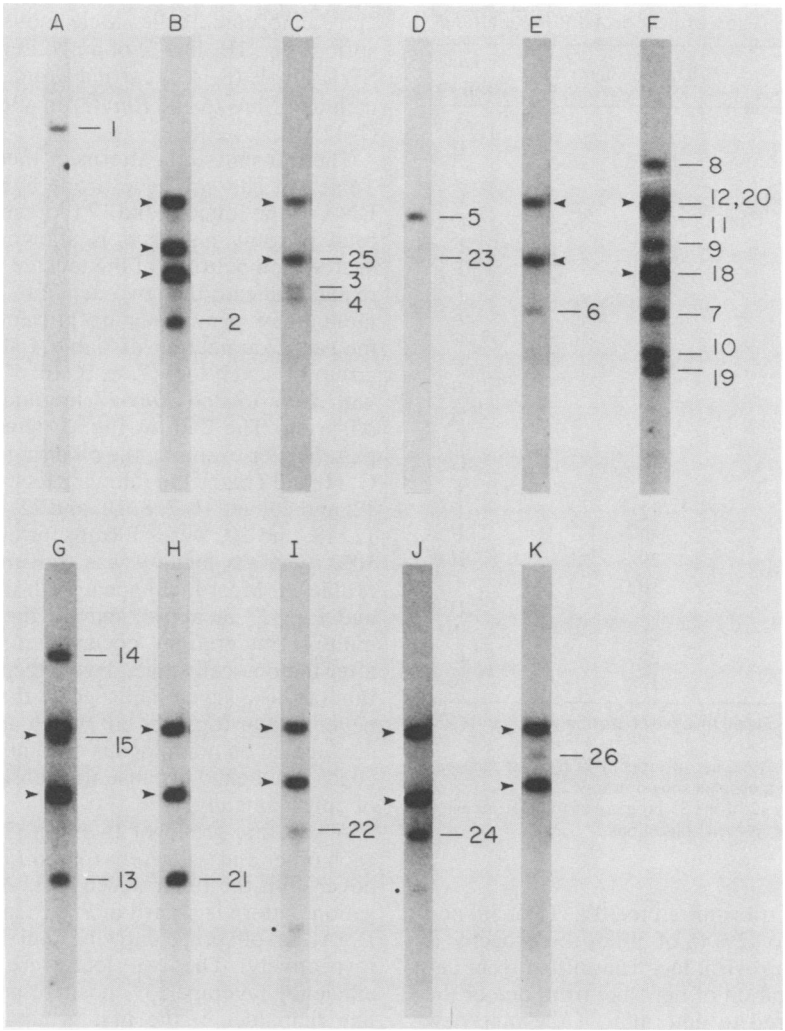

FIG. 2. Representation of new ecotropic proviral loci in tail genomic DNA. *Pvu*II-digested tail genomic DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with the [^{32}P]labeled *env* gene probe. Each lane contains DNA from a different founder mouse carrying at least one new proviral fragment, which is identified by a number (the numbering system is described in the text). The fragments corresponding to the endogenous loci (*Emv-1*, *Emv-16*, *Emv-17*) are marked by arrows when present in the lane and have sizes of 4.0, 4.6, and 6.0 kb, respectively, relative to size markers (λ DNA digested with *Hin*dIII). The dots to the left of lanes I and J identify hybridizing sequences that were not transmitted to progeny. Exposures were for 7 days at −70°C with an intensifying screen.

probe to tail DNA from each of 11 founder mice analyzed for transmission frequency are shown (Fig. 2). To estimate the representation of each new locus in tail DNA, the relative intensities of bands corresponding to new loci were compared visually with bands corresponding to *Emv-16* and *Emv-17*. These endogenous loci should be present at one copy per diploid genome. For example, Fig. 2C shows bands in the tail DNA of founder mouse C that correspond to loci 3 and 4, and both loci appear to be approximately half the intensity of the upper endogenous band representing *Emv-17*. The bands representing new loci appeared to have from 10 to 100% of the intensity of the endogenous bands.

An analysis of the germ line transmission of newly acquired proviral integrations is shown in Table 3. Each founder mouse was set up in mating with a mouse of a provirus-negative strain (CBA/CaJ or SWR/J), and the progeny were monitored for new loci. Each locus was given a number as it was transmitted to progeny (numbers 16 and 17 were not assigned to avoid confusion with *Emv-16* and *Emv-17*). To date, 40 new proviral loci have been transmitted through the germ line. Although 111 new loci were produced, some mice died before breeding or did not breed; only two loci carried in mice for which enough progeny were collected to permit analysis (more than 15 progeny) were not transmitted. Analysis of the transmission frequency of 24 representative loci (Table 3) shows that the frequencies range from 6 to 35%, with an average transmission frequency of 22%. These frequencies are lower than the 50% maximum expected for a provirus present at one copy per cell, which is found for the heterozygous loci *Emv-16* and *Emv-17*.

Comparison of Fig. 2 and Table 3 allows an estimation of the relative representation of each new locus in the somatic tail tissue and in the germ line. In most cases the representation of a new locus in tail DNA corresponded to the frequency of germ line transmission. For example, the band representing locus 6 of founder mouse E (Fig. 2, lane E) is approximately half the intensity of the bands representing *Emv-16* and *Emv-17*, and this locus was transmitted to 32% (8 of 25) of the progeny. A few exceptions were noted, however. Bands representing loci 10 and 19 (Fig. 2F) are of equal intensity in the autoradiograph of tail DNA of founder

TABLE 3. Germ line transmission of new proviral integrations

Locus[a]	Proportion transmitted[b]	% Transmission	Lane display[c]
1	4/17	23	A
2	3/23	13	B
3	9/35	26	C
4	4/35	11	C
5	19/55	35	D
6	8/25	32	E
7	14/50	28	F
8	16/50	32	F
9	10/50	20	F
10	15/50	30	F
11	7/50	14	F
12	16/50	32	F
13	3/17	18	G
14	1/17	6	G
15	1/17	6	G
18	16/50	32	F
19	6/50	12	F
20	15/50	30	F
21	4/14	29	H
22	1/10	10	I
23	3/55	5	D
24	1/4	25	J
25[d]	3/12	25	C
26	3/18	17	K

[a] Numbers 16 and 17 were not assigned to avoid confusion with *Emv-16* and *Emv-17*.
[b] No. of progeny with new locus/total no. of progeny of founder mouse.
[c] Tail genomic DNA of founder mouse as shown in Fig. 2.
[d] Locus 25 was analyzed in only 12 of the 35 progeny of founder mouse C because it comigrates with *Emv-16* in *Pvu*II digestions.

mouse F, yet locus 10 was transmitted to 30% (15 of 50) and locus 19 was transmitted to 12% (6 of 50) of the progeny.

Segregation of nine new proviral loci transmitted from one founder mouse. The segregation of new loci from one of the founder mice was monitored to determine if the nine new proviral integrations present in this mouse occurred in one or more embryonic cells. Male mouse 11526 was the N3 offspring (Fig. 1B, line 3) of an N2 female (N2♀) crossed to an SWR/J male (S♂). It carried a total of 11 proviral loci, which included *Emv-16* and *Emv-17* in addition to the nine new loci (7–12, 18, 19, 20).

The transmission patterns of these loci are shown (Fig. 3, Table 4). The *env* gene probe hybridization pattern of tail DNA of the founder and 17 representative progeny of the 50 total progeny analyzed is shown in Fig. 3, whereas the segregation patterns of the loci are tabulated in Table 4. The results indicate that two separate populations of cells containing new proviruses are present in the germ line of this mouse. Examination of Table 4 shows that in general one group of seven loci (7, 8, 9, 10, 12, 18, 20) appeared along with *Emv-16* and *Emv-17* in random combinations in the offspring. The data in Fig. 3 demonstrate this segregation pattern. For example, the offspring whose DNAs are in lanes G, H, and I carry the folowing loci: (G) 9 and 18; (H) 7, 9, 10, 12, and 20; and (I) 7, 8, 10, and 12. Six of these loci, 7, 8, 10, 12, 18, and 20, were all transmitted at a frequency of about 30%, whereas locus 9 was transmitted to 20% of progeny (Table 3). Most loci appear with the others or with *Emv-16* and *Emv-17* at approximately the frequencies expected if multiple integrations occurred in a single embryonic cell after the one-cell stage. Two exceptions to random segregation of these seven loci suggest that (i) loci 7 and 18, which never appear together but which appear with the other loci, are located on homologous chromosomes and (ii) loci 12 and 20 may be linked because they cosegregate with a frequency of approximately 85%.

A separate group of two loci (11 and 19) appeared with each other and with *Emv-16* and *Emv-17* in the progeny, but never with the other seven loci. An example of this segregation pattern is shown in Fig. 3, lanes L and P, which show DNAs of offspring carrying loci 11 and 19 and 19 alone, respectively. The two loci were transmitted at a lower efficiency (average 13% positive progeny) through the germ line than loci in the first group (Table 3). These results indicate that multiple viral integrations occurred in at least

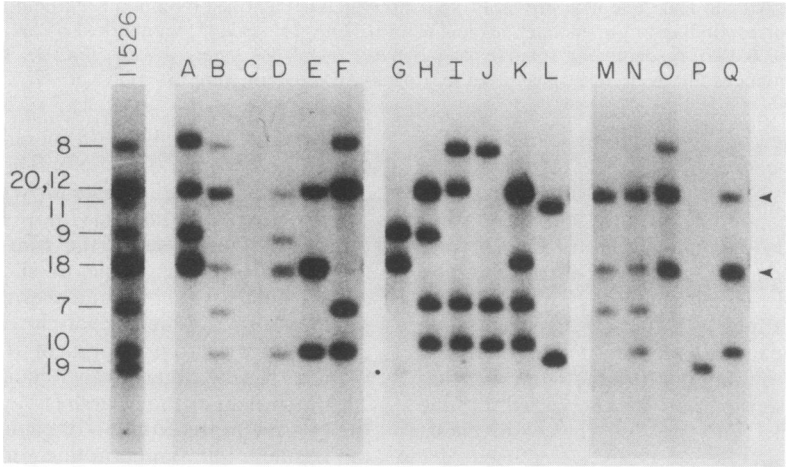

FIG. 3. Segregation of nine new ecotropic proviral loci from one founder mouse. Tail genomic DNAs of the founder mouse (lane 11526) and 17 progeny (lanes A through Q) were digested with *Pvu*II, separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized with the [^{32}P]labeled *env* gene probe. The nine new loci of the founder mouse are indicated by numbers to the left of lane 11526. The positions of the two fragments corresponding to the endogenous proviral loci (*Emv-16* and *Emv-17*) present in some of these mice are marked by arrows to the right of the lanes and have sizes of 4.6 and 6.0 kb, respectively, relative to size markers (λ DNA digested with *Hin*dII). Several loci have comigrating *Pvu*II restriction fragments (new loci 12, 20, and *Emv-17*; new locus 18 and *Emv-16*) that produce bands of greater hybridization intensity relative to other bands in the same lane; these loci were distinguished by use of other restriction enzymes. The dot to the left of lane G indentifies hybridizing sequences that were not transmitted to progeny. Exposures were for 7 days at −70°C with an intensifying screen.

TABLE 4. Segregation of new proviral loci in 50 progeny of mouse 11526[a]

Locus	Locus									
	7	8	9	10	11	12	18	19	20	*Emv-16*, *Emv-17*
Emv-16, *Emv-17*	6[b]	6	4	9	2	8	9	1	8	(22)[c]
20	8	8	5	7	0	13	7	0	(15)	
19	0	0	0	0	2	0	0	(6)		
18	0	7	7	6	0	7	(16)			
12	9	8	5	8	0	(16)				
11	0	0	0	0	(7)					
10	8	6	5	(15)						
9	3	5	(10)							
8	9	(16)								
7	(14)									

[a] Six progeny carried no ecotropic proviral loci, whereas two progeny carried only *Emv-16* and *Emv-17*.
[b] Number of times locus at the top of the column has been found in the same offspring as locus in the far left column. A total of 50 progeny were scored.
[c] Numbers in parentheses refer to number of times each locus was transmitted in analysis of 50 progeny.

two different cells of the embryo and that members of these lineages were both recruited to the germ line of mouse 11526.

DISCUSSION

I examined some of the genetic parameters that influence the integration frequency of ecotropic proviral sequences into the DNA of early mouse embryos. Indirect evidence suggested that certain hybrid strain backgrounds might be permissive for embryonic proviral acquisition (26). Jenkins and Copeland (9) have recently documented this phenomenon and have defined a permissive strain combination. They have shown that proviral loci *Emv-16* and *Emv-17* of the RF/J strain are important for embryonic proviral acquisition and that other proviral loci capable of expressing virus do not substitute for the RF/J loci (N. Jenkins and N. Copeland, personal communication).

The data presented here indicate that other genetic factors also contribute to embryonic proviral acquisition and specifically that the virus-negative background onto which the RF/J proviral loci are crossed is important. Crossing *Emv-16* and *Emv-17* onto the SWR/J background for three generations results in a higher frequency of integration events (0.13 per mouse) than crossing *Emv-16* and *Emv-17* onto the CBA/CaJ background for three generations (none detected in 94 mice). Both strains lack endogenous ecotropic proviral loci and carry an allele at the *Fv-1* locus (*Fv-1*^{n}) that is permissive for infection by the ecotropic RF/J virus (10, 11). Therefore, other loci that differ between the two strains must affect embryonic proviral acquisition. The second backcross onto the SWR/J background starting with N2 animals of the genotype F1♀ × C♂ was positive for proviral acquisition, in contrast to the second backcross onto the SWR/J background starting with RF/J animals. This finding indicates that at least one genetic locus that differs between the RF/J and CBA/CaJ strains affects the efficiency of proviral acquisition. One locus that may affect proviral acquisition in this case is *Fv-1* because RF/J and CBA/CaJ carry different alleles; RF/J has the semipermissive *Fv-1*^{nr} allele whereas CBA/CaJ has the permissive *Fv-1*^{n} allele.

Further evidence that genetic factors contribute to this phenomenon is that matings set up with N4 females carrying at least one new proviral integration in addition to *Emv-16* and *Emv-17* showed an increased percentage of offspring with new integrations (see Table 1). This increase is probably not caused by the presence of the new loci per se because to date none of the females carrying new loci in the absence of *Emv-16* and *Emv-17* have had offspring with additional new loci (unpublished results). This finding, therefore, provides additional evidence that genetic factors are involved in this phenomenon and suggests that the efficiency of proviral acquisition may be increased by genetic manipulation.

Several lines of evidence support the hypothesis that infection rather than DNA-mediated transposition is the mechanism whereby new proviral loci are generated. The establishment of viremia in an animal is known to involve both genetic and epigenetic factors (25, 27); the data presented here show that embryonic proviral acquisition is affected by genetic factors. The litter distribution of integrations (Table 2) shows that a permissive maternal genotype is not sufficient to ensure that all progeny will acquire new proviral loci, and other genetic or epigenetic factors must determine whether an integration event will actually occur in a permissive situation. The same backcrossing protocol that relieves suppression of viremia in other RF/J hybrid mice (18) promotes embryonic proviral acquisition, further supporting the hypothesis that proviral acquisition and viremia are linked.

The virus infecting the oocyte or embryo probably originates from the mother. The probability that an embryo will acquire a new provirus is determined at least in part by the maternal genotype, because only female carriers of *Emv-16* and *Emv-17* gave rise to progeny with new loci. The presence of *Emv-16* and *Emv-17* in the embryonic genome is not correlated with the probability that the genome will acquire a new provirus, thus ruling out the possibility that embryonic cells produce virus which reinfects cells in the same embryo (unpublished results). If maternal infection is responsible for proviral acquisition, a correlation may exist between viremia in the blood or tail tissues of females and proviral acquisition that can be used to increase the embryonic proviral integration frequency by selection.

The new proviral loci detected in these experiments are transmitted through the mouse germ line. Because all of the loci were transmitted at less than 50% (heterozygous) efficiency, I conclude that integration of proviral sequences in most cases occurred after the onset of DNA replication in the zygote. In general the representation of a new locus in tail DNA corresponds to the frequency of germ line transmission, indicating that most integration events occur before the embryonic germ line is set aside and that infected cells can contribute equally to both somatic and germ line tissue.

It is possible that infection and proviral integration are separate events occurring at different times; that this may be the case is suggested by the fact that the zona pellucida, an effective barrier to virus infection in vitro (28), surrounds mouse embryos during the preimplantation stages when provirus integration is probably occurring.

The two separate cosegregation patterns of nine loci in progeny from founder mouse 11526 indicate that proviral integrations can occur in two different embryonic cells. The fact that both lineages are represented in the germ line indicates that at least two distinct cells from the early embryo contributed to the germ line of this mouse. This finding is consistent with analyses of mouse embryos and chimeras, which have indicated that 10 to 200 cells are recruited from a larger pool of embryonic cells to form the germ line (19, 29, 30).

Because recruitment leads to formation of the mouse germ line, it is impossible to determine exactly from transmission data the timing of the integration events during development. Nevertheless, the correspondence between somatic representation and germ line transmission and the average transmission frequency of 22% suggests that integration may often occur at the two-cell stage of embryogenesis. Addition of the average transmission frequencies (30 and 13%) of the two separate cell populations contributing to the germ line of mouse 11526 approximates heterozygous (50%) transmission, supporting this hypothesis. Analysis of transmission frequencies of multiple loci that do not cosegregate in four additional founder mice revealed that in three cases the combined frequencies also approximate 50% (unpublished results). It is thought that cellular DNA synthesis is required for proviral integration (36); therefore, the data indicating viral integration at the two-cell stage are consistent with the model that integration occurs after cellular DNA synthesis is activated during embryogenesis. Although proviral integrations occurring at successively later stages in development would be more difficult to detect by both the tail DNA assay and by transmission to progeny, I only identified one locus not detected by the tail DNA assay of founder animals in analysis of over 800 progeny of founder mice. This finding suggests that most germ line integration events occur early during development.

Integration of these new proviruses into the mouse germ line may result in insertional mutations leading to activation or inactivation of host genes. Although retrovirus integration appears to be random with respect to nucleotide sequence (34), recent investigations of targeted mutagenesis by retroviruses in culture suggest that integration may not be completely random (12). If proviral integration into actively transcribed DNA is favored, then detectable mutations recovered from this method of generating insertions might involve genes active early in mouse development because most proviral integrations occur at this time. It is not known, however, whether any bias of proviral integration will favor or disfavor disruption of gene expression. Therefore, choice of a large target size may increase the probability of detecting retroviral insertion mutations in mice and allow for an estimation of the efficiency of this form of mutagenesis. To this end, I assayed the new loci for all visually detectable phenotypes in mice; none were detected in founder animals or mice carrying heterozygously the 40 independent proviral loci (unpublished results), indicating that these insertion events did not generate visually detectable dominant mutations. I am currently attempting to make these 40 loci homozygous to screen for recessive phenotypes and for recessive lethal mutations.

ACKNOWLEDGMENTS

I thank N. Copeland, W. Herr, and N. Jenkins for invaluable discussions and for critical reading of the manuscript. I thank L. Silver, in whose laboratory this work was initiated. I thank W. Herr and J. Sambrook for encouragement; H. Fox, D. Hanahan, M. Krangel, P. Mains, and N. Sarvetnick for many helpful discussions; W. Herr for the pAKV-4 clone; S. Grant for statistical analysis; E. Krecko, S. Panzer, and S. Toda for technical assistance; D. Lukralle, C. Jackson, and C. O'Loughlin for animal care; M. Goodwin for typing the manuscript; and D. Greene, M. Ockler, and J. Roberts for art and photography.

This work was supported by a Public Health Service grant to L. Silver (HD16091) from the National Institutes of Health and by a grant from the Monsanto Corporation to Cold Spring Harbor Laboratory. The author is the recipient of a National Institutes of Health Postdoctoral Fellowship.

LITERATURE CITED

1. **Buckler, C. E., S. P. Staal, W. P. Rowe, and M. A. Martin.** 1982. Variation in the number of copies and in the genomic organization of ecotropic murine leukemia virus proviral sequences in sublines of AKR mice. J. Virol. **43**:629–640.
2. **Chen, S., F. D. Struuck, M. L. Duran-Reynals, and F. Lilly.** 1980. Genetic and non-genetic factors in expression of infectious murine leukemia viruses in mice of the DBA/2 × RF cross. Cell **21**:849–855.
3. **Copeland, N. G., K. W. Hutchison, and N. A. Jenkins.** 1983. Excision of the DBA ecotropic provirus in dilute coat-color revertants of mice occurs by homologous recombination involving the viral LTRs. Cell **33**:379–387.
4. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Comm. **23**:641–646.
5. **Frankel, W., T. A. Potter, N. Rosenberg, J. Lenz, and T. V. Rajan.** 1985. Retroviral insertional mutagenesis of a target allele in a heterozygous murine cell line. Proc. Natl. Acad. Sci. USA **82**:6600–6604.
6. **Herr, W., and W. Gilbert.** 1982. Germ-line MuLV reintegrations in AKR/J mice. Nature (London) **296**:865–868.
7. **Herr, W., and W. Gilbert.** 1983. Somatically acquired recombinant murine leukemia proviruses in thymic leukemias of AKR/J mice. J. Virol. **46**:70–82.
8. **Jaenisch, R., K. Harbers, A. Schnieke, J. Lohler, I. Chumakov, D. Jahner, D. Grotkopp, and E. Hoffman.** 1983. Germline integration of Moloney murine leukemia virus at the *Mov-13* locus leads to recessive lethal mutation and early embryonic death. Cell **32**:209–216.
9. **Jenkins, N. A., and N. G. Copeland.** 1985. High frequency acquisition of ecotropic MuLV proviruses in SWR/J-RF/J hybrid mice. Cell **43**:811–819.
10. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1982. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J. Virol. **43**:26–36.
11. **Jolicoeur, P.** 1979. The *Fv-1* gene of the mouse and its control of murine leukemia virus replication. Curr. Top. Microbiol. Immunol. **86**:68–122.
12. **King, W., M. D. Patel, L. I. Lobel, S. P. Goff, and M. C. Nguyen-Huu.** 1985. Insertion mutagenesis of embryonal carcinoma cells by retroviruses. Science **228**:554–558.
13. **Lowy, D. R., S. K. Chattopadhyay, N. M. Teich, W. P. Rowe, and A. S. Levine.** 1974. AKR murine leukemia virus genome: frequency of sequences in DNA of high-, low-, and non-virus-yielding mouse strains. Proc. Natl. Acad. Sci. USA **71**:3555–3559.
14. **Mark, W. H., K. Signorelli, and E. Lacy.** 1986. An insertional mutation in a transgenic mouse line results in developmental arrest at day 5 of gestation. Cold Spring Harbor Symp. Quant. Biol. **50**:453–463.
15. **Mayer, A., M. L. Duran-Reynals, and F. Lilly.** 1978. *Fv-1* regulation of lymphoma development and of thymic ecotropic and xenotropic MuLV expression in mice of the AKR/J × RF/J cross. Cell **15**:429–435.

16. **Mayer, A., F. D. Struuck, M. L. Duran-Reynals, and F. Lilly.** 1980. Maternally transmitted resistance to lymphoma development in mice of reciprocal crosses of the RF/J and AKR/J strains. Cell **19**:431–436.
17. **Melamedoff, M., F. Lilly, and M. L. Duran-Reynals.** 1983. Suppression of endogenous murine leukemia virus by maternal resistance factor. J. Exp. Med. **158**:506–514.
18. **McCubrey, J., J. M. Horowitz, and R. Risser.** 1982. Structure and expression of endogenous ecotropic murine leukemia viruses in RF/J mice. J. Exp. Med. **156**:1461–1474.
19. **McLaren, A.** 1978. Reproduction in single-sex chimeras, p. 125–134. *In* L. B. Russell (ed.), Genetic mosaics and chimeras in mammals. Plenum Publishing Corp., New York.
20. **Moore, J. L., and H. W. Chan.** 1982. Identification of ecotropic proviral sequences in high- and low-ecotropic-virus-producing mouse strains. J. Virol. **43**:1038–1045.
21. **Palmiter, R. D., T. M. Wilkie, H. Y. Chen, and R. L. Brinster.** 1984. Transmission distortion and mosaicism in an unusual transgenic mouse pedigree. Cell **36**:869–877.
22. **Payne, G. S., J. M. Bishop, and H. E. Varmus.** 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature (London) **295**:209–214.
23. **Quint, W., W. Quax, H. van der Putten, and A. Berns.** 1981. Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. J. Virol. **39**:1–10.
24. **Rigby, P. W., M. Diekmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
25. **Rowe, W. P.** 1973. Genetic factors in the natural history of murine leukemia virus infection. Cancer Res. **33**:3061–3068.
26. **Rowe, W. P., and C. A. Kozak.** 1980. Germ-line reinsertions of AKR murine leukemia virus genomes in *Akv-1* congenic mice. Proc. Natl. Acad. Sci. USA **77**:4871–4874.
27. **Rowe, W. P., and T. Pincus.** 1972. Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. J. Exp. Med. **135**:429–436.
28. **Sawicki, W., W. Baranska, and H. Koprowski.** 1971. Susceptibility of unfertilized and fertilized mouse eggs to simian virus 40 and maloney sarcoma virus. J. Natl. Cancer Inst. **47**:1045–1051.
29. **Searle, A. G.** 1978. Evidence from mutable genes concerning the origin of the germ line, p. 209–224. *In* L. B. Russell (ed.), Genetic mosaics and chimeras in mammals. Plenum Publishing Corp., New York.
30. **Snow, M. H. L., and M. Monk.** 1983. Emergence and migration of mouse primordial germ cells, p. 115–135. *In* A. McLaren and C. C. Wylie (ed.), Current problems in germ cell differentiation. Cambridge University Press, Cambridge.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
32. **Steffan, D. L., B. A. Taylor, and R. A. Weinberg.** 1982. Continuing germ line integration of AKV proviruses during the breeding of AKR mice and derivative recombinant inbred strains. J. Virol. **42**:165–175.
33. **Stephenson, J. R., R. K. Reynolds, S. R. Tronick, and S. A. Aaronson.** 1975. Distribution of three classes of endogenous type-C RNA viruses among inbred strains of mice. Virology **67**:404–414.
34. **Varmus, H. E.** 1982. Form and function of retroviral proviruses. Science **216**:812–820.
35. **Varmus, H. E., N. Quintrell, and S. Ortiz.** 1981. Retroviruses as mutagens: insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. Cell **25**:23–26.
36. **Varmus, H., and R. Swanstrom.** 1984. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Wagner, E. F., L. Covarrubias, T. A. Stewart, and B. Mintz.** 1983. Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line. Cell **35**:647–655.
38. **Wolf, D., and V. Rotter.** 1984. Inactivation of p53 gene expression by an insertion of Moloney murine leukemia virus-like DNA sequences. Mol. Cell. Biol. **4**:1402–1410.
39. **Woychik, R. P., T. A. Stewart, L. G. Davis, P. D'Eustachio, and P. Leder.** 1985. An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. Nature (London) **318**:36–40.