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Studies of the mechanism of spontaneous germline ecotropic provirus acquisition in mice

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SWR/J-RF/J hybrid mice spontaneously acquire new germline ecotropic proviruses at high frequency. We have performed ovarian transplantation and *in situ* hybridization studies to delineate the mechanism and developmental stage of germline provirus acquisition. In addition, we have developed a novel, efficient and simple method to introduce single copy proviruses into the mouse germline. The results reported here have direct implications for understanding how proviruses are acquired in the germline, for using murine leukemia viruses as insertional mutagens, and for using retroviral vectors to introduce foreign genes into the mouse germline.

Key words: germline infection/insertional mutagenesis/murine leukemia virus

Introduction

Causal associations between the integration of a mobile genetic element and induction of a mutant phenotype have been demonstrated in many organisms (Shapiro, 1973; Gridley *et al.*, 1987). A mobile genetic element acting as an insertional mutagen not only identifies genes of interest but also provides a molecular tag for isolation and characterization of those genes. In the mouse, endogenous murine leukemia proviruses are causally associated with several mutant phenotypes including alterations of coat color and embryonic lethality (Jenkins *et al.*, 1981; Jaenisch *et al.*, 1983; Soriano *et al.*, 1987). Because of the low frequency at which new proviruses are spontaneously acquired in the germline of most inbred mouse strains, relatively labor-intensive, technically-difficult procedures have been employed to introduce murine leukemia viruses (MuLVs) into the mouse germline. MuLVs have been introduced into the mouse germline either by infection of embryos *in vitro* or *in utero* (Jaenisch, 1976, 1980) or by infection of embryonic stem (ES) cells *in vitro* followed by incorporation of the infected cells into the germline of a chimeric mouse (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). The development of strains of mice that spontaneously acquire new germline proviruses at high frequency would provide a technically-simple experimental system for insertional mutagenesis. Potentially, such a system would enable the systematic isolation of genes of interest by virtue of the association between a MuLV integration and a mutant phenotype.

Recent reports indicate that the frequency of spontaneous acquisition of new germline ecotropic proviruses in SWR/J-RF/J hybrid mice is higher than previously reported in other inbred mouse strains (Rowe and Kozak, 1980; Langdon *et al.*, 1984; Jenkins and Copeland, 1985; Bautch, 1986; Panthier and Condamine, 1987). The frequency of provirus acquisition in SWR/J-RF/J hybrid mice is only 10- to 20-fold lower than the frequency of P-element acquisition in dysgenic crosses of *Drosophila melanogaster*. It should now be possible to develop strains of mice that spontaneously acquire new germline ecotropic proviruses at frequencies high enough to enable large-scale mutagenesis studies.

SWR/J-RF/J hybrid mice are produced by mating mice of two inbred mouse strains, SWR/J and RF/J. SWR/J mice carry no endogenous ecotropic murine leukemia proviruses (*Emv* loci) in their genome (McCubrey *et al.*, 1982; Jenkins *et al.*, 1982; Jenkins and Copeland, 1985). Mice of the RF/J strain carry three endogenous ecotropic murine leukemia proviruses; *Emv-1*, a defective provirus, and *Emv-16* and *Emv-17*, two non-defective proviruses capable of generating infectious virus *in vitro* (Jenkins *et al.*, 1982; Jenkins and Copeland, 1985; L.F.Lock, A.M.Buchberg, E.Keshet, N.A.Jenkins and N.G.Copeland, manuscript in preparation). *Emv-16* and *Emv-17* are closely linked (within 0.11 ± 0.11 cM) but not tandemly duplicated (Jenkins and Copeland, 1985). When *Emv-16* and *Emv-17* are backcrossed onto the SWR/J strain, new germline ecotropic proviruses are acquired at high frequency (Jenkins and Copeland, 1985; Bautch, 1986; Panthier and Condamine, 1987). As many as 75% of the progeny can carry newly acquired proviruses. A single mouse can carry as many as 10 or more new proviruses. Most of the new proviruses are present at less than one copy per diploid genome ($<0.04-0.74$ copies per diploid genome) in all tissues examined including the germline (Jenkins and Copeland, 1985; Bautch, 1986; Spence *et al.*, in press). Many of the newly acquired proviruses produced in SWR/J-RF/J crosses (designated SWR/J-RF/J endogenous provirus or *Srev* loci) are expressed and give rise to new germline proviruses at a high frequency (Spence *et al.*, in press). Moreover, one of the new proviruses (*Srev-5*) is associated with a recessive lethal phenotype demonstrating that the newly acquired proviruses can act as insertional mutagens (Spence *et al.*, in press).

The new germline proviruses observed in the progeny of SWR/J-RF/J hybrid mice could be acquired by extracellular virus infection, intracellular retroposition, DNA-mediated transposition, or other unknown mechanisms. It appears most likely that new germline proviruses are acquired by an extracellular infection mechanism because the acquisition of new germline proviruses in SWR/J-RF/J hybrid mice is dependent on virus production in the maternal environment (Jenkins and Copeland, 1985). New proviruses are observed only in the progeny of viremic SWR/J-RF/J hybrid female mice (Jenkins and Copeland, 1985). When viremia is blocked in SWR/J-RF/J hybrid mice by either genetic or non-

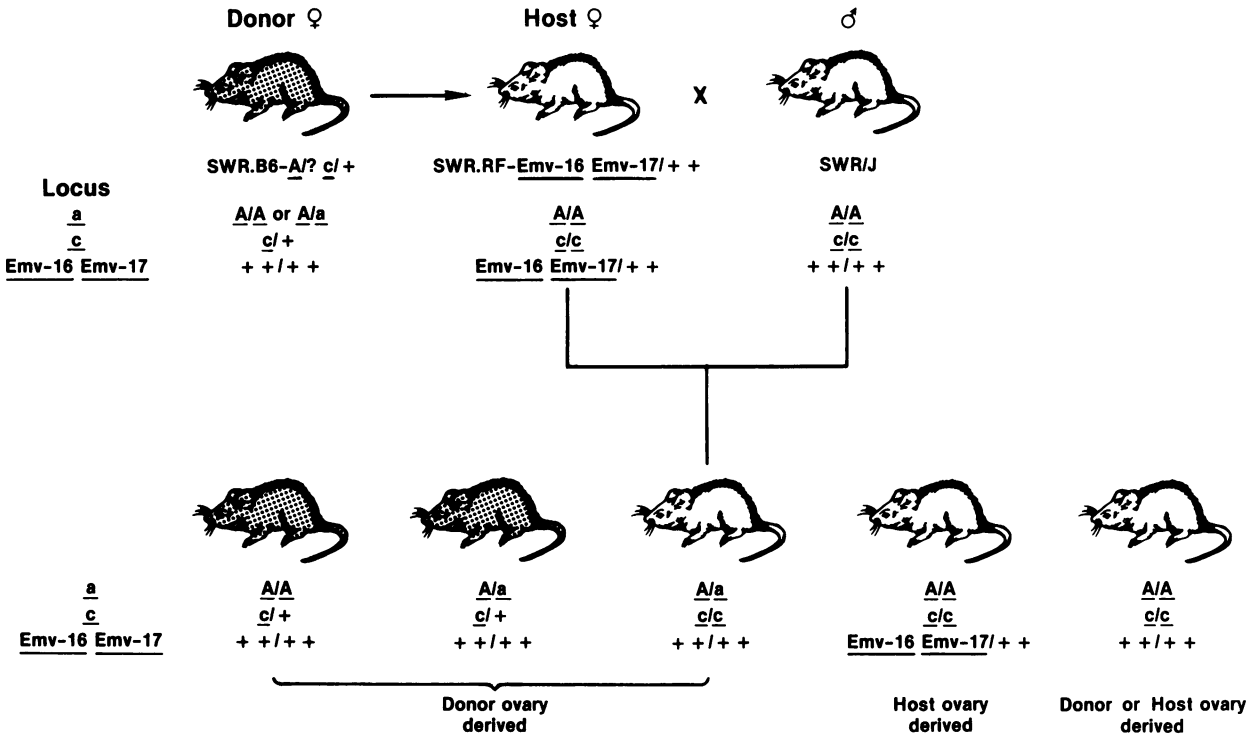

Fig. 1. Ovarian transplantations: genotypes and phenotypes of SWR.B6-*A*/?,*c*/+, SWR.RF-*Emv-16 Emv-17*/++, and SWR/J parental mice and their progeny. Ovaries from SWR.B6-*A*/?,*c*/+ mice were transplanted to SWR.RF-*Emv-16 Emv-17*/++ hosts (Jones and Krohn, 1960). Ovarian transplantation recipients were mated to SWR/J male mice. Five classes of progeny were distinguished by coat color, genotype at the agouti locus and the presence or absence of *Emv-16* and *Emv-17*. The donor mouse strain, SWR.B6-*a*/*a*,+/+, that would have enabled identification of the ovarian origin of all progeny was not available. Stippled mice represent mice with agouti coat color, whereas unstippled mice represent mice with albino coat color.

genetic factors, high-frequency acquisition of new germline proviruses is not observed (Jenkins and Copeland, 1985). In RF/J mice, high-titer virus production is blocked by genetic and non-genetic factors and new germline proviruses are not observed (Pincus *et al.*, 1971; Mayer *et al.*, 1978, 1980; Melamedoff *et al.*, 1983). Further, the new germline proviruses that are observed infrequently in AKR/J mice were found in the progeny of virus-positive females (Rowe and Kozak, 1980). The correlation between high-titer virus production in the maternal environment and high-frequency acquisition of new germline proviruses suggests that newly-acquired proviruses arise by extracellular virus infection of the female germline and/or embryos.

The objectives of the studies reported here are three-fold: (i) to determine the mechanism by which new germline proviruses are acquired in SWR/J−RF/J hybrid mice; (ii) to determine the developmental stage(s) at which new germline proviruses are acquired; and (iii) to develop efficient and convenient systems for insertional mutagenesis and for the introduction of foreign genes into the mouse germline. The ovarian transplantation experiments described here, along with *in situ* detection of ecotropic viral RNA in the female genital tract by cytohybridization, demonstrate that germline proviruses are acquired by extracellular virus infection and indicate that oocytes present in the adult ovary are the target of infection. Furthermore, exogenous administration of infectious ecotropic virus to newborn SWR/J mice is shown to result in germline infection and high-frequency acquisition of new germline proviruses. This capability constitutes an extremely simple, efficient method to introduce single copy, expressible proviruses into the mouse germline, either for insertional mutagenesis or the introduction of genes into the mouse germline.

Results

To determine the mode of provirus acquisition in SWR/J−RF/J hybrid mice, ovaries from genetically-marked SWR/J mice carrying no endogenous ecotropic proviruses (SWR.B6-*A*/?,*c*/+; see Figure 1) were transplanted to the ovarian bursa of SWR/J hosts carrying *Emv-16* and *Emv-17* (SWR.RF-*Emv-16 Emv-17*/++; see Figure 1). The ovarian transplantation recipients were mated to SWR/J male mice and the resultant progeny were analyzed for ecotropic proviral DNA content. The only source of ecotropic virus was the host female because neither the genetically-marked ovary donor nor the SWR/J male carry endogenous ecotropic proviruses (see Figure 1). If the progeny derived from the donor ovary acquire new proviruses, acquisition of new proviruses must occur by an extracellular infection mechanism. The genetically-marked SWR/J mice and the SWR/J mice carrying *Emv-16* and *Emv-17* were chosen for this study for several reasons. First, SWR/J mice carrying *Emv-16* and *Emv-17* acquire new proviruses at a high frequency (0.10 new proviruses per mouse; Spence *et al.*, in press). Second, the two SWR/J-based strains are identical at most, if not all, major and minor histocompatibility loci, thus allowing successful ovarian transplantation. Finally, the two mouse strains differ at two coat color loci, agouti and albino, facilitating discrimination of the progeny derived from the donor ovary from those derived from residual host ovary (see Figure 1).

Table I. New germline ecotropic proviruses are acquired by extracellular infection

Ovary of origin	Coat color	*a*	*c*	*Emv-16 Emv-17*	Total number of progeny born	Number of progeny with new proviruses	Number of new proviruses	Number of new proviruses / Total number of progeny
Donor	Agouti	*A/A*	*c/+*	*++/++*	5	1	7	1.0[b]
Donor	Agouti	*A/a*	*c/+*	*++/++*	1	1	3	
Donor	Albino	*A/a*	*c/c*	*++/++*	6	2	2	
Host	Albino	*A/A*	*c/c*	*Emv-16 Emv-17/++*	6	0	0	0
Donor or host	Albino	*A/A*	*c/c*	*++/++*	20	3	3	0.15
Control[a]	Albino	*A/A*	*c/c*	*Emv-16 Emv-17/++*	255	18	25	0.10

[a]Untreated SWR/J mice carrying *Emv-16* and *Emv-17*.
[b]Differs from control, $P = 0.0009$.

A total of 38 offspring were produced (Table I). Ecotropic proviruses were detected by Southern blot analysis of *Pvu*II-digested genomic DNA isolated from tail and other tissues using an ecotropic virus-specific DNA probe (Chattopadhyay *et al.*, 1980; Jenkins and Copeland, 1985; Siracusa *et al.*, 1987b). *Pvu*II cleaves twice within each provirus and generates a single detectable 3′ provirus-cell DNA junction fragment from each provirus present in the genome (see Figure 2, panel A). Four of the 12 confirmed donor ovary-derived progeny acquired new ecotropic proviruses (Table I). The two donor ovary-derived offspring that were agouti in coat color (Figure 2, panel B, lanes 3 and 4) acquired seven and three new proviruses, respectively, whereas two donor ovary-derived offspring that were albino in coat color each acquired a single new provirus (data not shown). The frequency of acquisition of new provirus in donor ovary-derived progeny was 1.0 new proviruses per mouse (Table I). The provirus−cell DNA junction fragments ranged in size from 3.0 to 7.4 kb and were easily distinguished from *Emv-16* and *Emv-17*, which generate fragments of 4.8 and 6.1 kb, respectively (Figure 2, panel B, lane 2). No new proviruses were acquired by six host ovary-derived progeny (Table I). The origin of the 20 remaining offspring could not be determined definitively (Table I). Three of these mice each acquired a single new ecotropic provirus (Figure 2, panel B, lanes 5 and 6; data not shown).

Newly-acquired proviruses were analyzed to detect gross rearrangements that might be present within these proviral loci. Genomic DNA digested with *Pst*I was hybridized with the ecotropic virus-specific DNA probe. *Pst*I cleaves once in each prototypic ecotropic proviral long terminal repeat to yield a single detectable fragment of ~8.2 kb from non-defective ecotropic proviruses (Figure 2, panel A). All newly-acquired proviruses examined yielded a single detectable fragment of ~8.2 kb, suggesting that no gross rearrangements occurred (data not shown).

All newly-acquired proviruses were present in DNA from the somatic tissues at less than one copy per cell as determined by visual comparison of the relative hybridization intensity of new proviruses to that of *Emv-16* and *Emv-17* that are present in genomic DNA at one copy per diploid genome (Figure 2, panel B, lanes 2−6). To determine the extent to which the new proviruses were present in the germline, all progeny that acquired new proviruses were mated to SWR/J mice and the proviral content of the resultant progeny was analyzed as previously described. Thirteen of the 15 newly-acquired proviruses (Table I) were transmitted to offspring. In all cases, the transmission frequency was <50% (5−30%). Thus, as previously observed in the

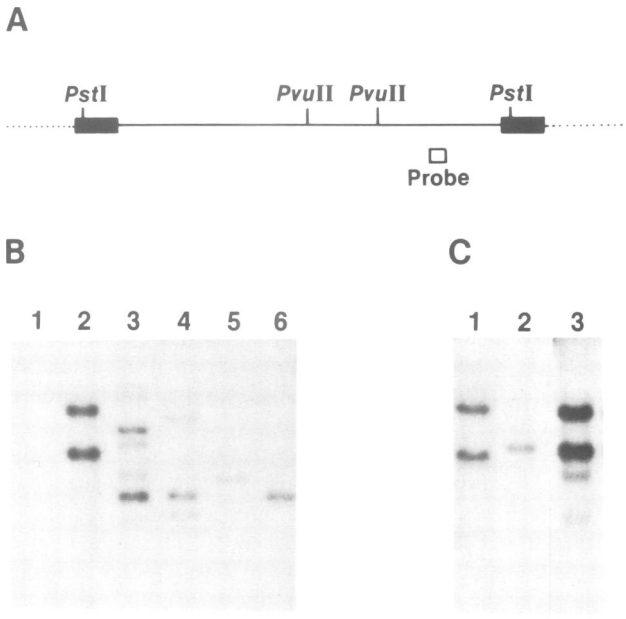

Fig. 2. Ecotropic proviruses present in parental and progeny genomes. **(A)** The prototypic ecotropic provirus showing the position of restriction endonuclease sites for *Pvu*II and *Pst*I and the position of the ecotropic virus-specific DNA probe used in **panels B** and **C**. Proviral DNA includes both the thin (——) and thick (▬) solid lines. The long terminal repeats (LTRs) are represented by the thick (▬) solid line. Cellular DNA is represented by the broken line (· · ·). **(B)** Genomic DNA isolated from the kidney was digested with *Pvu*II and hybridized to the ecotropic virus-specific DNA probe (Jenkins and Copeland, 1985; Chattopadhyay *et al.*, 1980). **Lane 1** is SWR.B6-*A/?,c/+*; **lane 2** is SWR.RF-*Emv-16 Emv-17/++*; **lanes 3** and **4** are donor ovary-derived offspring that are agouti in coat color; **lanes 5** and **6** are albino offspring of unknown ovarian derivation (Table I, as described in the text). **(C)** Genomic DNA isolated from the tail was digested with *Pvu*II and hybridized to the ecotropic virus-specific DNA probe (Jenkins and Copeland, 1985; Siracusa *et al.*, 1987b; Chattopadhyay *et al.*, 1980). **Lane 1** is SWR.RF-*Emv-16 Emv-17/++*. **Lane 2** is C57BL/6NCr mice carrying *nu*, the single hybridizing fragment represents the endogenous ecotropic provirus, *Emv-2* carried by this strain. **Lane 3** is a donor ovary-derived offspring that was albino in coat color (Table II). The smallest of the four hybridizing fragments in **lane 3** represents unintegrated linear viral DNA and not a newly-acquired provirus.

progeny of SWR/J−RF/J hybrid mice (Bautch, 1986; Jenkins and Copeland, 1985; Spence *et al.*, in press), the newly-acquired proviruses were present at less than one copy per diploid genome in both the somatic and germinal tissues.

Acquisition of new germline proviruses in offspring derived from genetically-marked SWR/J donor ovaries transplanted to the ovarian bursa of SWR/J hosts carrying *Emv-16* and *Emv-17* demonstrates that new germline proviruses are acquired by extracellular virus infection. Since the donor ovaries and the male carry no endogenous ecotropic proviruses, virus produced in the host environment must have infected cells derived from the donor ovary to give rise to newly-acquired proviruses in donor ovary-derived progeny. Interestingly, the frequency of acquisition of new proviruses in donor ovary-derived progeny was higher than the spontaneous frequency of acquisition of new proviruses in SWR/J mice carrying *Emv-16* and *Emv-17* (Table I; $P = 0.0009$). Several factors could be responsible for this difference. First, the relatively small sample size could account for the apparent difference. Second, the SWR.B6-*A*/? *c*/+ mice could be more susceptible to virus infection than SWR/J mice presumably as a result of genetic differences at or near the *Emv-16* and *Emv-17*, albino or agouti loci. Third, the ovarian transplantation procedure could have resulted in an increased susceptibility to infection. This appears unlikely, however, because the frequency of provirus acquisition in the host ovary-derived and unknown ovary-derived progeny did not differ from the spontaneous frequency in SWR/J mice carrying *Emv-16* and *Emv-17* (Table I), suggesting that the ovarian transplantation procedure did not increase the susceptibility to infection in these groups.

In addition to demonstrating that new proviruses are acquired by infection, the data also suggest the existence of a cell stage susceptible to virus infection either within, or derived from cells within, the adult ovary. The donor ovaries used in this study were obtained from 9- to 12-week-old adult females. In the adult ovary, the only germ cells present are oocytes, either arrested in first meiotic prophase or undergoing meiotic maturation in preparation for ovulation. Thus, the cell(s) susceptible to infection must include the first meiotic prophase arrested oocyte and/or cells derived from it. Oocytes in the adult ovary reside within follicles in various stages of growth. Primordial follicles consist of a single layer of granulosa cells surrounding an oocyte arrested in first meiotic prophase. Upon stimulation, oocytes within primordial follicles enter a growth phase in which a specialized cell layer, the theca, forms around the follicle; the granulosa cell layer thickens and forms a complete basal lamina; and an extracellular glycoprotein layer, the zona pellucida, begins to form around the growing oocyte. Once fully grown, the oocyte resumes meiosis in preparation for ovulation at which time the oocyte is released from the mature follicle. The cell stages that are potentially susceptible to virus infection would include oocytes before, during, or after ovulation and the cells of the early embryo.

To gain insight into which cell stage(s) are susceptible to infection, *in situ* hybridization was performed to determine whether ecotropic proviruses are expressed in the female genital tracts of SWR/J mice carrying *Emv-16* and *Emv-17* (SWR.RF-*Emv-16 Emv-17*/++) and the two parental strains, SWR/J and RF/J. Expression of ecotropic proviruses in the female genital tract could indicate the presence of a source of infectious virus in the vicinity of the potential target cells. Sections of ovary, oviduct and uterus were hybridized with an anti-sense RNA probe that specifically detects ecotropic-specific viral mRNA (Chattopadhyay *et al.*, 1980; Hogan *et al.*, 1986). In SWR/J mice carrying *Emv-16* and *Emv-17*, a distinctive distribution of ecotropic viral RNA was observed (Figure 3A, panels 1−4). In the ovary, RNA was detected in thecal cells around follicles in all stages of the growth phase. RNA was detected in lower amounts in some cells of the corpora lutea and the ovarian stroma. In contrast, detectable levels of ecotropic viral RNA were not apparent in the granulosa cells that immediately surround the oocyte, or in the oocyte itself. Transmission electron microscopic analysis indicated the presence of C-type virus particles, both free and budding, in the ovaries of SWR.RF-*Emv-16 Emv-17*/++ mice (data not shown). In the oviduct, ecotropic viral RNA was detected in some regions of the mucosal subepithelial connective tissue and the smooth muscle layer (Figure 3B, panels 1 and 2). RNA was not apparent in the mucosal epithelium. In the uterus, ecotropic viral RNA was detected in low amounts in the endometrial stroma but not the endometrial epithelium, uterine glands or myometrium (data not shown). Similar analysis of the parental strains, SWR/J and RF/J, revealed no detectable ecotropic viral RNA in the cells of the ovary, oviduct or uterus (Figure 3A, panels 5 and 6; Figure 3B, panels 3 and 4; data not shown). The presence of ecotropic viral RNA and budding C-type virus particles in the female genital tract indicates that there is a potential source of virus within the female genital tract of SWR/J mice carrying *Emv-16* and *Emv-17*. However, the data do not allow discrimination of the cell stage at which infection occurs since a potential source of infectious virus exists in the vicinity of oocytes in all stages of follicular growth and embryos.

Additional ovarian transplantation studies were performed to further investigate the cell stage(s) susceptible to virus infection. Ovaries from SWR/J mice carrying *Emv-16* and *Emv-17* (SWR.RF-*Emv-16 Emv-17*/++; see Figure 4) were transplanted to the ovarian bursa of C57BL/6NCr mice carrying the nude (*nu*) mutation (C57BL/6NCr-*nu*/*nu*; see Figure 4). The ovarian transplantation recipients were mated to SWR/J male mice, and the resultant progeny were analyzed for ecotropic provirus content. C57BL/6NCr mice carrying *nu* were chosen for several reasons. First, the immunodeficiency conferred by the nude mutation allowed for successful ovary transplantation despite differences at histocompatibilty loci. Second, ecotropic virus replication is genetically restricted in C57BL/6NCr mice carrying *nu* due to the presence of a restrictive allele at the *Fv-1* locus (*Fv-1*^{b}/*Fv-1*^{b}). *Fv-1* is the major locus in the mouse that controls ecotropic virus replication (Pincus *et al.*, 1971; Rowe and Hartley, 1972). N-tropic viruses, which include those derived from endogenous ecotropic proviruses such as *Emv-16* and *Emv-17*, do not efficiently replicate and spread in *Fv-1*^{b}/*Fv-1*^{b} cells either *in vitro* or *in vivo* (Pincus *et al.*, 1971; Rowe and Hartley, 1972). Thus, virus spread from the donor ovary to the host should be inhibited. Third, C57BL/6NCr mice carrying *nu* and SWR/J mice carrying *Emv-16* and *Emv-17* differ at the agouti and albino coat-color loci allowing discrimination of progeny derived from the donor and residual host ovaries (see Figure 4). Fourth, the *Emv-2* provirus carried by the C57BL/6NCr mice carrying *nu* is replication-defective and mice carrying *Emv-2* do not

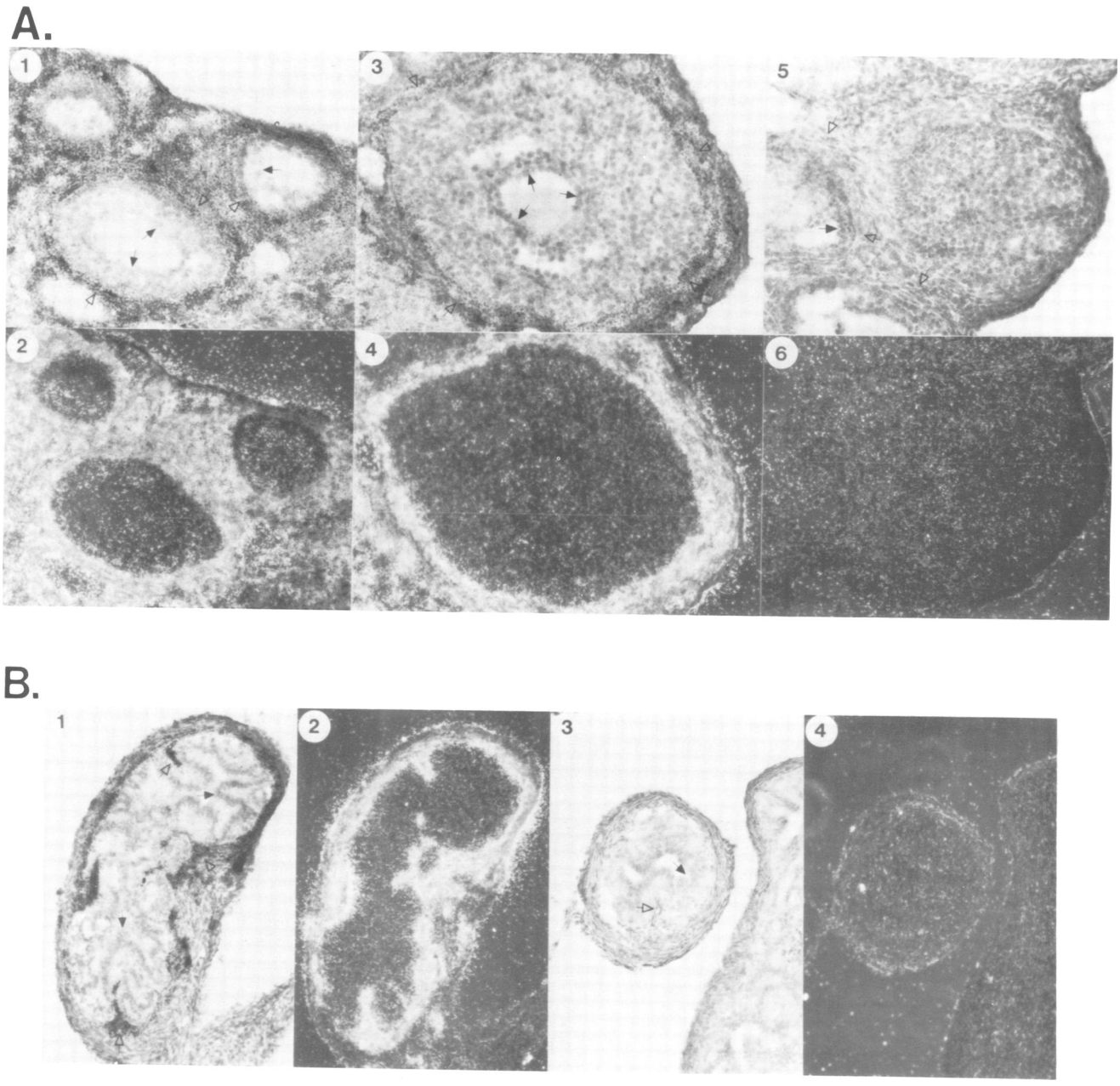

Fig. 3. Localization of ecotropic viral RNA in the female genital tract by *in situ* hybridization. (**A**) Photomicrographs of ovary sections hybridized to an ecotropic virus-specific RNA probe. Section of ovaries from SWR.RF-*Emv-16 Emv-17*/++ mice photographed in bright field (**panels 1,3**) and dark field (**panels 2,4**) and SWR/J mice photographed in bright field (**panel 5**) and dark field (**panel 6**) showing maturing follicles containing thecal cells (⇩) and granulosa cells (➧). **Panels 1–6**, ~200×. (**B**) Photomicrographs of oviduct sections hybridized to an ecotropic virus-specific antisense RNA probe. Sections of oviducts from SWR.RF-*Emv-16 Emv-17*/++ photographed in bright field (**panel 1**) and dark field (**panel 2**) and SWR/J mice in bright field (**panel 3**) and dark field (**panel 4**) showing mucosal epithelium (➧) and mucosal subepithelial connective tissue (⇩). **Panels 1–4**, ~200×.

spontaneously acquire ecotropic germline proviruses at detectable frequency (Jenkins *et al.*, 1982; King *et al.*, 1988). Since both the C57BL/6NCr mice carrying *nu* and the SWR/J male mice carry no replication-competent endogenous ecotropic proviruses and virus spread is suppressed in the C57BL/6NCr mice carrying *nu*, the donor ovary should be the only source of infectious virus. If progeny derived from the donor ovary acquire new germline proviruses, then infection must occur within, or very near, the donor ovary indicating that the oocyte is susceptible to infection.

A total of 41 offspring were produced from these matings (Table II). Of the 39 donor ovary-derived progeny produced, one mouse acquired one new provirus and one mouse acquired five new proviruses (Figure 2, panel C, lane 3; data not shown). The frequency of acquisition of new proviruses in donor ovary-derived progeny was 0.15 new proviruses per mouse (Table II). Genomic DNA from one of the donor ovary-derived offspring is shown in Figure 2, panel C, lane 3. The 4.0-kb provirus-cell DNA junction fragment was easily distinguished from the fragments generated by *Emv-16* and *Emv-17*, which were also present in the genome of this mouse. Analysis of *Pst*I-digested genomic DNA from all mice carrying new proviruses

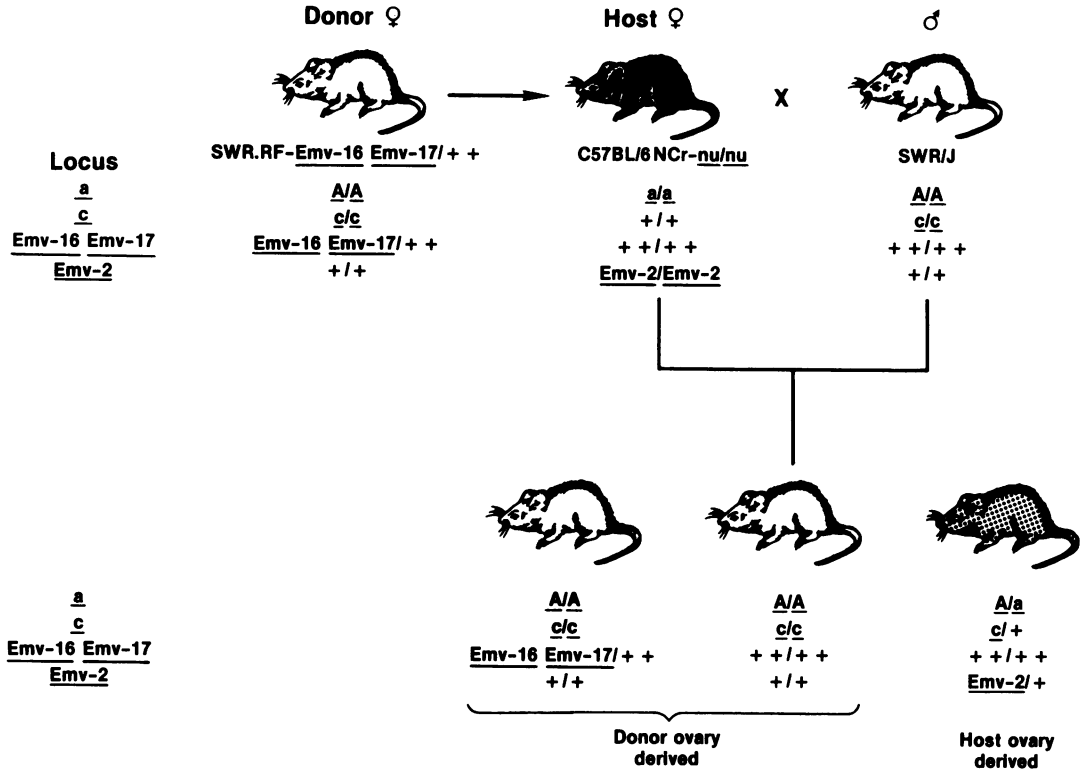

Fig. 4. Ovarian transplantations: genotypes and phenotypes of SWR.RF-*Emv-16 Emv-17*/++, C57BL/6NCr-*nu/nu*, and SWR/J parental mice and their progeny. Donor ovaries were obtained from SWR.RF-*Emv-16 Emv-17*/++ mice. The host mice were C57BL/6NCr-*nu/nu*. After ovarian transplantation, the mice were mated to SWR/J male mice. Three classes of progeny were distinguished by coat color and the presence or absence of *Emv-16*, *Emv-17* and *Emv-2*. Shaded mice represent mice with black coat color, stippled mice represent mice with agouti coat color, and unshaded mice represent mice with albino coat color.

Table II. New germline ecotropic proviruses can be acquired by extracellular infection of oocytes

Ovary of origin	Coat color	*a*	*c*	*Emv-16 Emv-17*	*Emv-2*	Total number of progeny born	Number of progeny with new proviruses	Number of new proviruses	Number of new proviruses / Total number of progeny
Donor	Albino	*A/A*	*c/c*	*Emv-16 Emv-17*/++	+/+	24	1	1	0.15[b]
Donor	Albino	*A/A*	*c/c*	++/++	+/+	15	1	5	
Host	Agouti	*A/a*	*c*/+	++/++	*Emv-2*/+	2	0	0	0
Control[a]	Albino	*A/A*	*c/c*	*Emv-16 Emv-17*/++	+/+	255	18	25	0.10

[a]Untreated SWR/J mice carrying *Emv-16* and *Emv-17*.
[b]Does not differs from control, $P = 0.68$.

revealed a single 8.2-kb fragment (data not shown), which suggests that the newly-acquired proviruses contain no gross rearrangements. The newly-acquired proviruses were present at less than one copy per diploid genome as assayed by visual comparison of the relative hybridization intensity of the new proviruses to that of *Emv-16* and *Emv-17* present at one copy per cell. Furthermore, when mice carrying newly-acquired proviruses were mated to SWR/J mice, the new proviral loci were transmitted to $<50\%$ of the offspring produced. No new proviruses were detected in the two host ovary-derived offspring produced in these matings (Table II).

Acquisition of new proviruses in the progeny of an ovary from an SWR/J mouse carrying *Emv-16* and *Emv-17* transplanted to the ovarian bursa of a C57BL/6NCr mouse carrying *nu* strongly suggests that the oocyte is susceptible to virus infection. The donor ovary is the only source of infectious ecotropic virus. Since virus spread is restricted in the host by the presence of a restrictive allele at the *Fv-1* locus, virus infection must have occurred within or near the donor ovary, suggesting that the oocyte was the target of infection. To confirm that virus spread from the donor ovary to the host oviduct did not occur, oviducts from a C57BL/6NCr host carrying *nu* that had produced donor ovary-derived progeny with new proviruses were analyzed by *in situ* hybridization with an ecotropic virus-specific antisense RNA probe. No evidence of ecotopic virus expression was observed confirming that virus spread from the donor ovary to the host oviduct did not occur (data not shown).

To investigate whether exogenously-administered infectious virus can also infect the germline, SWR/J newborn mice were injected subcutaneously with high-titer virus

Table III. New germline ecotropic proviruses are acquired following subcutaneous injection of virus into newborn SWR/J mice

Virus	Endogenous provirus encoding the virus	Virus stock (p.f.u./ml)	Total number of progeny born	Number of progeny with new proviruses	Number of new proviruses	Number of new proviruses[a] / Total number of progeny
16,17	*Emv-16, Emv-17*	4×10^5	154	4	4	0.03
AKV623	*Emv-11*	3×10^7	100	5	14	0.14

[a]The two groups do not differ, $P = 0.22$.

stocks. Since the relative contribution of the SWR/J strain and the *Emv-16*- and/or *Emv-17*-derived virus to the uniquely increased frequency of acquisition of new germline proviruses is not known, a virus other than that derived from *Emv-16* and *Emv-17* was also tested for its ability to infect the germline of SWR/J female mice. AKV623, an ecotropic virus derived from one of the endogenous ecotropic proviruses present in AKR/J mice, *Emv-11*, was chosen. This virus was chosen because it is similar to the *Emv-16*- and *Emv-17*-derived virus in many respects, but differs in that a higher titer of infectious virus can be produced *in vitro* due to a duplication of the enhancer present in the long terminal repeat (Rowe and Kozak, 1980; Lowy *et al.*, 1980; Van Beveren *et al.*, 1982; Jenkins *et al.*, 1982; Buckler *et al.*, 1982; Lock *et al.*, manuscript in preparation). At 6–9 weeks of age, all female mice infected with either the *Emv-16*- and *Emv-17*-derived virus or the AKV623 virus were viremic; they contained infectious virus in extracts of tail biopsies. Although the virus assays were not quantitative, females infected with the AKV623 virus appeared to have a higher titer of infectious virus in tail extracts than females infected with the *Emv-16*- and *Emv-17*-derived virus. *In situ* hybridization studies demonstrated that ecotropic viral RNA was present in the genital tract of females injected with either the *Emv-16*- and *Emv-17*-derived virus or the AKV623 virus in a pattern indistinguishable from that described for SWR/J mice carrying *Emv-16* and *Emv-17* (data not shown). Viremic females were mated to SWR/J males and the resultant progeny analyzed for newly-acquired proviruses as previously described. Four new proviruses were observed in 154 progeny of females infected with virus from *Emv-16* and *Emv-17* (Table III). Fourteen new proviruses were detected in 100 progeny of females infected with the AKV623 virus (Table III). The newly-acquired proviruses were present at less than one copy per diploid genome. When mice carrying new proviruses were mated in SWR/J male mice, the newly-acquired proviruses were transmitted to $<50\%$ of the offspring. Observation of newly-acquired germline proviruses in the progeny of SWR/J female mice infected with *Emv-16*- and *Emv-17*-derived and AKV623 viruses demonstrates that the mouse germline can be infected *in vivo* with ecotropic virus from an exogenous source. Preliminary results from restriction endonuclease mapping revealed no differences between *Emv-16*, *Emv-17* and *Emv-11* (Lock *et al.*, manuscript in preparation). The AKV623 virus differs from *Emv-11* in that it contains a duplication of the enhancer located in the long terminal repeat (Van Beveren *et al.*, 1982). This duplication probably accounts for the higher titer of virus produced *in vitro* and *in vivo* in the present study, but did not appear to have significantly increased the frequency of new germline proviruses in progeny.

Discussion

The results of the studies presented here (i) demonstrate that new germline ecotropic proviruses are acquired by extracellular virus infection, (ii) indicate that the oocyte is the target of infection, and (iii) demonstrate that the mouse germline can be infected by exogenous administration of infectious virus to newborn mice. The latter capability provides a technically simple, efficient method to introduce DNA into the mouse germline.

Endogenous ecotropic murine leukemia proviruses are present in the mouse germline in multiple non-allelic sites (*Emv* loci). A subset of *Emv* loci is contained in the genome of each inbred mouse strain (Lowy *et al.*, 1974; Stephenson *et al.*, 1976; Jenkins *et al.*, 1982). Analysis of the distribution of *Emv* loci and the known relationships among inbred strains suggest that most viral integrations occurred before inbreeding and are relatively stable. In some instances, however, new *Emv* loci have been observed (Rowe and Kozak, 1980; Buckler *et al.*, 1982; Herr and Gilbert, 1982; Steffan *et al.*, 1982; Langdon *et al.*, 1984). These new *Emv* loci are thought to have arisen by germline re-infection. The data presented here provides the definitive proof that new germline ecotropic proviruses are acquired by infection. Furthermore, the results indicate that the oocyte is the target of viral infection. These conclusions suggest that germline infection might occur when viremia is induced in the adult female by exogenous, infectious ecotropic virus. In accordance with the suggestion, newly-acquired germline proviruses were observed in the present study in the progeny of female SWR/J mice treated as newborns with infectious *Emv-16*- and *Emv-17*-derived virus. Similar results were also reported by Panthier *et al.* (1988).

Oocyte infection appears to be the predominant mechanism by which new proviruses are acquired. The frequency of acquisition by oocyte infection did not differ from the frequency of acquisition by all mechanisms (the frequency in donor ovary-derived progeny in Table II versus the spontaneous frequency of provirus acquisition in SWR/J mice *Emv-16* and *Emv-17*; $P = 0.68$). This suggests that oocyte infection is the predominant mechanism by which new proviruses are acquired. Moreover, infection of the alternative target cells, other germ cell stages or the cells of the early embryo, appears unlikely. Infection of the maternal germline before sexual dimorphism is established in the fetal gonads (about day 12.5 of gestation) appears unlikely because it would result in the acquisition of new germline proviruses in progeny of both male and female SWR/J–RF/J hybrid mice. New germline proviruses have not been observed in the progeny of male SWR/J–RF/J hybrid mice. Infection of mitotic germ cells in the female parent before entry into meiosis (about 13.5 days of gestation) also appears unlikely

because integration would occur before meiosis and result in new proviruses present in one copy per diploid genome in the progeny. New proviruses present in one copy per diploid genome have not been observed. Infection after fertilization appears unlikely because the zona pellucida is an effective barrier to retroviral infection of embryos infected *in vitro* (Baranska *et al.*, 1971; R.Jaenisch, personal communication). The results presented here suggest that oocyte infection is the predominant mechanism of provirus acquisition but cannot exclude the rare acquisition by infection of other germ cell stages or the cells of the early embryo.

Infection of the oocyte could occur at any stage during the growth and maturation of the oocyte. *In-situ* hybridization analysis of the ovaries of SWR/J mice carrying *Emv-16* and *Emv-17* in this and other studies suggests that infection probably occurs very early in follicular development or at the time of ovulation (Panthier and Condamine, 1987). Ecotropic-specific viral RNA was observed in the thecal, ovarian stromal and corpora luteal cells. RNA was not present at detectable levels in the granulosa cells. The ovarian cells in which ecotropic-specific viral RNA was observed are spatially separated from the oocyte by the granulosa cell layer, its associated basal lamina, and the zona pellucida, an extracellular glycoprotein layer that forms around the oocyte. Infection of the oocyte could occur if virus is able to traverse these potential barriers. Infection could also occur very early in follicular development prior to the complete formation of the granulosa cell layer and zona pellucida or at ovulation when part of the granulosa cell layer degenerates. Alternatively, virus present in the granulosa cell in amounts undetectable in this study could act as a direct source of infectious ecotropic virus. Analysis of the proviral content of mice derived from oocytes infected *in vitro* at different stages of growth and maturation, then fertilized *in vitro* and transferred to pseudopregnant females, will clarify the precise stage(s) at which the oocyte is susceptible to infection and, thus, further define the mechanism by which virus gains access to the oocyte.

Most newly-acquired proviruses observed in SWR/J−RF/J hybrid mice are present in <0.04 to 0.74 copies per diploid genome (Jenkins and Copeland, 1985; Bautch, 1986; Spence *et al.*, in press). Infection of the oocyte can result in newly-acquired germline proviruses present at less than one copy per diploid genome if virus integration is delayed until after the DNA synthetic phase of the cell cycle (S phase) in the zygote. Assuming random allocation of cells into embryonic and extra-embryonic cell lineages, integration between the S phase in the zygote, which begins about 4−6 h after fertilization, and the S phase in the two-cell stage embryo, which ends ~22−24 h after fertilization, would result in new proviruses present at about 0.5 copy per diploid genome (Abramczuk and Sawicki, 1975; Sawicki *et al.*, 1978). Integration is thought to require DNA synthesis (Varmus *et al.*, 1973; Khoury and Hanafusa, 1976). Since the mouse oocyte is arrested in meiosis until after fertilization, integration might be delayed until the S phase that occurs in the zygote. Integration at later developmental stages, nonrandom allocation of cells to embryonic and extra-embryonic cell lineages, or both, would result in proviruses present at more or less than 0.5 copy per diploid genome.

One or more unique circumstances could account for the unusually high frequency of acquisition of new germline proviruses observed in SWR/J−RF/J hybrid mice including characteristics of the SWR/J strain background and/or the *Emv-16* and *Emv-17* loci. The data presented here demonstrates that the *Emv-16*- and *Emv-17*-derived virus is not unique in its ability to infect the germline of SWR/J female mice. AKV623 virus, an ecotropic virus derived from *Emv-11*, was used to infect newborn SWR/J females. The frequency of new germline proviruses observed in progeny of these mice did not differ from that observed in the progeny of females infected with *Emv-16*- and *Emv-17*-derived virus. In contrast, the SWR/J strain background is important in the high-frequency acquisition of new germline proviruses (Bautch, 1986). When *Emv-16* and *Emv-17* were backcrossed onto a different mouse strain that is permissive for ecotropic virus replication, CBA/CaJ, a high frequency of new germline proviruses was not observed (Bautch, 1986). The frequency of provirus acquisition was greatly increased, however, when CBA/CaJ−RF/J hybrid mice were crossed with SWR/J mice (Bautch, 1986). Additionally, new germline proviruses are only rarely observed in other inbred mouse strains, even those in which high-titer virus is produced from *Emv-11* (Rowe and Kozak, 1980; Buckler *et al.*, 1982; Herr and Gilbert, 1982; Steffan *et al.*, 1982; Langdon *et al.*, 1984). The SWR/J strain background, but not the source of the infectious ecotropic virus, plays a role in the high-frequency acquisition of new germline proviruses.

The high frequency at which new proviruses are acquired in SWR/J−RF/J hybrid mice provides a unique opportunity to use MuLVs for insertional mutagenesis. An additional advantage is provided by the capability of infecting the germline by injection of infectious virus into newborn mice. This approach requires no special equipment nor technical expertise. A single SWR/J−RF/J hybrid or virus-infected female mouse could produce many offspring, each of whom could carry multiple independent integration events. Mice with new germline proviruses could be observed for phenotypic changes due to dominant mutations, intercrossed to screen for recessive phenotypic and/or lethal mutations, or crossed to a tester stock carrying recessive mutations to screen for mutations at virtually any locus. Isolation of mutations at specifically chosen genetic loci using this approach will require an increase in the frequency at which new proviruses are acquired to be practical. However, the frequency with which new proviruses derived from SWR/J−RF/J crosses (*Srev* loci) are associated with a recessive lethal phenotype appears high enough (~5%) that this system can easily be used to generate mammalian developmental mutations (Spence *et al.*, in press). Interestingly, the frequency of recessive lethal mutations induced by *Srev* proviruses is similar to that observed when embryos are infected with Mo−MuLV *in vitro* (Soriano *et al.*, 1987). If a stage-or cell-type specific bias exists in the sites of proviral integration, then the recessive lethal mutations induced using these approaches could identify a novel subset of genes that function in the oocyte and/or early embryo.

The capability to infect the mouse germline by introduction of infectious virus into newborn mice also is a novel method to introduce DNA into the mouse germline. Since maternal viremia is critical for germline infection, ecotropic retroviral vectors are being developed that retain their ability to produce

infectious virus upon incorporation of foreign DNA. Alternatively, modifications of the current method could enable the use of defective retroviral vectors to introduce foreign DNA into the mouse germline.

Materials and methods

Ovarian transplantations

SWR.B6-*A*/?, +/*c* were produced by repeated backcross (≥8 generations) of progeny from a cross of SWR/J-*A/A,c/c* and C57BL/6J-*a/a*, +/+ mice to SWR/J mice selecting, at each generation, for progeny heterozygous at the albino (*c*/+) and agouti (*A/a*) loci. SWR.RF-*Emv-16 Emv-17*/+ + mice were produced by repeated backcross (≥10 generations) of male SWR/J × RF/J mice to SWR/J female mice selecting, at each generation, for progeny heterozygous at *Emv-16* and *Emv-17*. C57BL/6NCr-*nu/nu* mice were obtained from Animal Production, National Cancer Institute–Frederick Cancer Research Facility. Ovarian transplantations were performed as described in Jones and Krohn (1960).

Coat color was assayed by visual inspection. The genotype at the agouti locus (*A/A* versus *A/a*) was determined by Southern blot analysis of genomic DNA isolated from the tail using a DNA probe that maps within 0.3 cM of the agouti locus (Siracusa *et al.*, 1987a, 1987b). Ecotropic proviruses were detected by Southern blot analysis of genomic DNA isolated from tail and other tissues using an ecotropic virus-specific DNA probe (Chattopadhyay *et al.*, 1980; Jenkins and Copeland, 1985; Siracusa *et al.*, 1987b). The Wilcoxon rank–sum test was used to analyze the data (Hollander and Wolfe, 1973). All probabilities are two-tailed.

In-situ hybridization

Ovaries, oviducts and uteri from SWR.RF-*Emv-16 Emv-17*/+ +, RF/J, and SWR/J mice were fixed in paraformaldehyde. Frozen thick sections (10 μm) were hybridized, washed and autoradiographed as described (Hogan *et al.*, 1986). The ecotropic virus-specific DNA probe described previously (Chattopadhyay *et al.*, 1980) was subcloned into a T7 promoter-containing plasmid and a ^{35}S-UTP-labeled single stranded anti-sense RNA was synthesized by standard procedures (Hogan *et al.*, 1986). The slides were exposed for 3–10 days, then developed, stained with Giemsa and photographed under bright and dark field optics.

Viruses

NIH 3T3 cells were cocultivated with spleen cells or transfected with DNA as described (Copeland and Cooper, 1979). Newborn SWR/J mice (<24-h old) were injected subcutaneously with 50–100 μl of virus stocks using a 1-ml tuberculin syringe equipped with a 27-gauge needle. Virus stocks and extracts of tail biopsies were assayed for the presence of infectious virus by XC plaque assay (Rowe *et al.*, 1970; Rowe, 1972).

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References

Abramczuk,J. and Sawicki,W. (1975) *Exp. Cell Res.*, **92**, 361–372.
Baranska,W., Sawicki,W. and Koproski,H. (1971) *Nature*, **230**, 591–592.
Bautch,V.L. (1986) *J. Virol.*, **60**, 693–701.
Buckler,C.E., Staal,S.P., Rowe,W.P. and Martin,M.A. (1982) *J. Virol.*, **43**, 629–640.
Chattopadhyay,S.K., Lander,M.R., Rands,E. and Lowy,D.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5774–5778.
Copeland,N.G. and Cooper,G.M. (1979) *Cell*, **16**, 347–356.
Gridley,T., Soriano,P. and Jaenisch,R. (1987) *Trends Genet.*, **3**, 162–166.
Herr,W. and Gilbert,W. (1982) *Nature*, **296**, 865–868.
Hogan,B., Constantini,F. and Lacy,E. (1986) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Hollander,M. and Wolfe,D.A. (1973) *Nonparametric Statistical Methods*. John Wiley and Sons, New York.
Hooper,M., Hardy,K., Handyside,A., Hunter,S. and Monk,M. (1987) *Nature*, **326**, 292–295.
Jaenisch,R. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1260–1264.
Jaenisch,R. (1980) *Cell*, **19**, 181–188.
Jaenisch,R., Harbers,K., Schnieke,A., Lohler,J., Chumakov,I., Jahner,D., Grotkopp,D. and Hoffman,E. (1983) *Cell*, **32**, 209–216.
Jenkins,N.A. and Copeland,N.G. (1985) *Cell*, **43**, 811–819.
Jenkins,N.A., Copeland,N.G., Taylor,B.A. and Lee,B.K. (1981) *Nature*, **293**, 370–374.
Jenkins,N.A., Copeland,N.G., Taylor,B.A. and Lee,B.K. (1982) *J. Virol.*, **43**, 26–36.
Jones,E. and Krohn,P. (1960) *J. Endocrinol.*, **20**, 135–146.
King,S.R., Berson,B.J. and Risser,R. (1988) *Virology*, **162**, 1–11.
Khoury,A.T. and Hanafusa,H. (1976) *J. Virol.*, **18**, 383–400.
Kuehn,M.R., Bradley,A., Robertson,E.J. and Evans,M.J. (1987) *Nature*, **326**, 295–298.
Langdon,W.Y., Theodore,T.S., Buckler,C.E., Stimpfling,J.H., Martin, M.A. and Morse,H.C.,III (1984) *Virology*, **133**, 183–190.
Lowy,D.R., Chattopadhyay,S.K., Teich,N.M., Rowe,W.P. and Levine, A.S. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 3555–3559.
Lowy,D.R., Rands,E., Chattopadhyay,S.K., Garon,C.F. and Hager,G.L. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 614–618.
Mayer,A., Duran-Reynals,M.L. and Lilly,F. (1978) *Cell*, **15**, 429–435.
Mayer,A., Struuck,F.D., Duran-Reynals,M.L. and Lilly,F. (1980) *Cell*, **19**, 431–436.
McCubrey,J., Horowitz,J.M. and Risser,R. (1982) *J. Exp. Med.*, **156**, 1461–1474.
Melamedoff,M., Lilly,F. and Duran-Reynals,M.L. (1983) *J. Exp. Med.*, **158**, 506–514.
Panthier,J.J. and Condamine,H. (1987) *Ann. Inst. Pasteur/Virol.*, **138**, 409–422.
Panthier,J.J., Condamine,H. and Jacob,F. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1156–1160.
Pincus,T., Hartley,J. and Rowe,W.P. (1971) *J. Exp. Med.*, **133**, 1219–1233.
Rowe,W.P. (1972) *J. Exp. Med.*, **136**, 1272–1285.
Rowe,W.P. and Hartley,J.W. (1972) *J. Exp. Med.*, **136**, 1288–1301.
Rowe,W.P. and Kozak,C.A. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4871–4874.
Rowe,W.P., Pugh,W.E. and Hartley,J.W. (1970) *Virology*, **42**, 1136–1139.
Sawicki,W., Abramczuk,J. and Blaton,O. (1978) *Exp. Cell Res.*, **112**, 199–205.
Shapiro,J.A. (1973) *Mobile Genetic Elements*. Academic Press, New York.
Siracusa,L.D., Russell,L.B., Eicher,E.M., Corrow,D.J., Copeland,N.G. and Jenkins,N.A. (1987a) *Genetics*, **117**, 93–100.
Siracusa,L.D., Russell,L.B., Jenkins,N.A. and Copeland,N.G. (1987b) *Genetics*, **117**, 85–92.
Soriano,P., Gridley,T. and Jaenisch,R. (1987) *Genes Dev.*, **1**, 366–375.
Spence,S.E., Gilbert,D.J., Swing,D.A., Copeland,N.G. and Jenkins,N.A. (1988/9) *Mol. Cell. Biol.*, in press.
Steffan,D.L., Taylor,B.A. and Weinberg,R.A. (1982) *J. Virol.*, **42**, 165–175.
Stephenson,J.R., Reynold,R.K., Tronick,R.R. and Aaronson,S.A. (1976) *Virology*, **67**, 404–414.
Van Beveren,C., Rands,E., Chattopadhyay,S.K., Lowy,D.R. and Verma, I.M. (1982) *J. Virol.*, **41**, 542–556.
Varmus,H.E., Vogt,P.K. and Bishop,J.M. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 3067–3071.

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