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Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus

(*in vitro* infection of 4–8 cell embryos with exogenous Moloney leukemia virus/leukemia/genetic transmission/DNA annealing kinetics/gene amplification)

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ABSTRACT **Mice were infected with the exogenous Moloney leukemia virus (M-MuLV) at two different stages of development. Either newborn mice (which can be considered as essentially fully differentiated animals) or preimplantation mouse embryos (at the 4–8 cell stage) were infected with M-MuLV. In both cases, animals that had developed an M-MuLV-induced leukemia were obtained. Two lines of evidence indicate that infection of preimplantation embryos, in contrast to infection of newborns, can lead to integration of the virus into the germ line.**

1. Viremic males of the first backcross generation (N-1 generation) transmitted the virus to 50% of their offspring (N-2 generation) when mated with uninfected females. Likewise, a 50% transmission was observed from viremic N-2 and N-3 males to the next generations.

2. Molecular hybridization experiments revealed that viremic N-1 and N-2 animals carried one copy of M-MuLV per diploid mouse genome equivalent in all "non-target" organs tested.

Together, both experiments indicate that the exogenous M-MuLV can be converted to an endogenous virus after infection of preimplantation embryos. The available evidence suggests that M-MuLV integrated into the germ line at one out of two possible integration sites. Thus, viremic backcross animals are heterozygous for a single Mendelian locus carrying the *M-MuLV* gene. During leukemogenesis an amplification of the M-MuLV from one copy to a maximum of four copies per diploid mouse genome equivalent takes place in the tumor tissues.

The genetic information of endogenous C-type viruses is present in all somatic and germ cells of all individuals of many species, and these virus-related genes are transmitted genetically according to Mendelian expectations (1–5). In contrast, exogenously infecting C-type viruses are not transmitted genetically and thus are not part of the normal genetic complement of an animal (6, 7). Whereas it is reasonable to assume that exogenous viruses evolved from endogenous viruses (7–9), the converse event, namely the conversion of exogenous viruses into endogenous viruses, seems to be very rare. However, recent evidence suggests that the transfer of an exogenous virus into the germ line of another species can occur during evolution (1, 10).

Prior to this study, the conversion of an exogenous into an endogenous virus had not been observed under defined laboratory conditions. Thus, leukemic mice infected *in utero* or after birth with leukemia virus never transmitted the virus genetically, i.e., from the leukemic father to the offspring (11, 12). In this instance, it is important to distinguish genetic transmission from the congenital transmission (13) which occurs from leukemic mothers to the majority of their offspring by infection through the placenta or the milk. The apparent resistance of the germ line to virus infection may be explained by the "organtropism" of murine leukemia viruses, i.e., the observation that only certain "target" tissues, such as spleen or thymus cells, are susceptible to infection with leukemia viruses, whereas other "non-target" tissues cannot be infected (14, 15). Therefore, integration of an exogenous virus into the germ line, a "non-target" tissue, should be a very rare event.

We have succeeded in overcoming the "organtropism" restriction of exogenous Moloney leukemia virus (M-MuLV) by infecting mice at the 4–8 cell preimplantation stage (14). The rationale of this approach was that infection of animals at this early stage of embryonal development, before any differentiation into "target" and "non-target" cells has taken place, should not be restricted by the "organtropism" of a virus. Leukemic mice obtained in this way indeed carried M-MuLV-specific sequences in all organs tested. I report here that infection of preimplantation embryos with M-MuLV can lead to germ line integration of the virus and to genetic transmission according to Mendelian expectations.

MATERIALS AND METHODS

Virus. M-MuLV clone no. 1 was grown, purified, and tested by the XC assay as described (16–18).

Preparation and Purification of M-MuLV-specific cDNA. Virus-specific DNA probes were prepared from purified M-MuLV stocks in the presence of 100 μg/ml of actinomycin D by the endogenous transcriptase reaction (16). [^{32}P]dCTP at a specific activity of 100 Ci/mmol (New England Nuclear) was used as radioactive precursor.

Because up to 60–70% of M-MuLV cDNA anneals to DNA extracted from uninfected mice (3, 14), the sensitivity of detecting M-MuLV-specific DNA sequences in a given mouse DNA preparation would be greatly enhanced by removing those sequences which are homologous to normal mouse DNA ("common" sequences). This was partly achieved by hybridizing cDNA of M-MuLV (6 ng) to RNA (1.1 mg) extracted from liver and spleens of highly leukemic AKR mice for 5 hr in 1 M NaCl at 68°. The rationale of this procedure was that sequences that are homologous to some of the "common" sequences of M-MuLV are expressed in leukemic AKR animals (Berns and Jaenisch, in preparation). The cDNA sequences that did not anneal with AKR RNA were eluted at 0.14 M PO_4^{-3} from hydroxyapatite columns (19) and used for hybridization. This DNA had a specific radioactivity of 80 to 160 $\times$ 10^6 cpm/μg and sedimented at 5.5–6 S in alkaline sucrose gradients. It annealed to 92% to M-MuLV 60–70S RNA, only up to 23% to DNA extracted from uninfected animals (Fig. 2), and up to 8% to calf thymus DNA.

Isolation and Infection of Mouse Embryos. Four to eight cell stage BALB/129 embryos were isolated, treated

Abbreviation: M-MuLV, Moloney leukemia virus.

with Pronase, infected with M-MuLV, and transplanted to foster mothers as described (14).

Extraction of Mouse DNA. Mouse tissues were removed and extracted by the Kirby method (20). The nucleic acids were precipitated with ethanol and banded in ethidium bromide–CsCl gradients (60,000 rpm for 14 hr in the Beckman no. 75 Ti rotor). The dye was removed with isopropanol, and the nucleic acids were sonicated and boiled for 10 min in 0.2 M NaOH. After neutralization the CsCl was removed. The purified DNA sedimented at 5.5–6 S in alkaline sucrose gradients.

Molecular Hybridization. DNA·DNA hybridizations were carried out as described previously (14, 21). Hybrid formation was monitored by resistance to S1 digestion (21). The mouse DNA was in a 4×10^7-fold excess over the ^{32}P-labeled M-MuLV cDNA by mass ratio. The results are corrected to standard annealing conditions (19).

Detection of M-MuLV in Mouse Sera. Mouse serum was tested for infectious M-MuLV by the XC assay (18) and for p30 by the radioimmunoassay (17). Animals were considered to be virus-positive when two independent bleedings gave positive results with both methods.

RESULTS

Infection of Mouse Embryos with M-MuLV and Transmission of Virus to the Next Generation. Mouse embryos at the 4–8 cell stage were infected as described in *Materials and Methods*. Out of 140 blastocysts transplanted to foster mothers, 45 mice were born and their serum tested for the presence of M-MuLV by radioimmunoassay for viral protein P30 and by the XC assay. At 4 weeks of age, three animals (one male and two females) were virus-positive by these criteria (10^3–10^4 XC plaque-forming units/ml; 1–3 μg/ml of p30), and the male (no. 339) was bred at 10 weeks of age with uninfected BALB/c females to yield N-1 animals (first backcross generation). The sera of all progeny animals were tested by XC assay and radioimmunoassay for the presence of M-MuLV. To this date, of 160 progeny produced, eight were positive for infectious M-MuLV at 3–4 weeks of age, and all eight animals developed a Moloney-specific leukemia (14) at a later age. Fig. 1 shows the percentage of viremic offspring in consecutive litters fathered by male no. 339. Whereas four of the first 25 offspring were viremic, this number dropped to 0 of 28 in the most recent litters tested. These observations suggest either that a fraction of the sperm of male no. 339 carried integrated M-MuLV sequences, and that this fraction decreased with age, or else that the offspring was congenitally infected from the father via the mother. Earlier observations seem to rule out the latter explanation because viremic and leukemic males infected *in utero* or after birth with M-MuLV do not transmit the disease to their offspring (11–13). If, on the other hand, germ line transmission had occurred, two testable predictions should be fulfilled: (*i*) the number of M-MuLV-specific DNA copies in different viremic N-1 animals and in all organs of individual animals (except in "target" organs, see later) should be constant per mouse genome equivalent; and (*ii*) viremic N-1 animals mated to uninfected females should transmit the virus according to simple Mendelian expectations to the next generation.

Mendelian Transmission of M-MuLV. Viremic N-1 males derived from male no. 339 (Fig. 1) were bred with uninfected BALB/c females to yield N-2 animals. The sera of these animals were tested for the presence of M-MuLV. Positive males were again selected and bred with females to give N-3 animals. The fourth backcross generation (N-4) was derived similarly from positive N-3 males. Table 1 summarizes the breeding results.

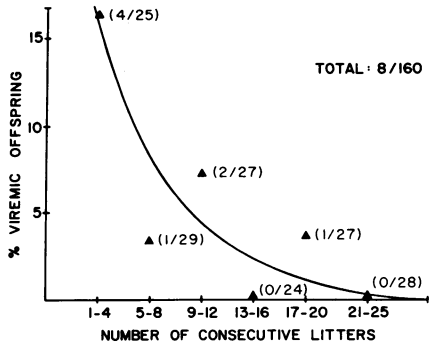

FIG. 1. Genetic transmission of M-MuLV to N-1 generation from viremic male infected at the preimplantation stage. Male no. 339 was infected with M-MuLV at the 4–8 cell stage and mated with uninfected BALB/c females when he was 10 weeks old. Serum of the progeny were tested for M-MuLV. The last five litters tested were fathered when the animal was 10–12 months old.

As pointed out above (Fig. 1), male no. 339, infected at the 4–8 cell preimplantation stage, transmitted M-MuLV to only 5% of his offspring. In contrast, his viremic sons, grandsons, and great grandsons transmitted M-MuLV to 50% of their offspring. These data strongly favor the hypothesis that M-MuLV was integrated into the germ line of these mice and transmitted genetically to their offspring. The results, together with the hybridization data shown below, suggest that the males tested are heterozygous for one genetic locus carrying the *M-MuLV* genome.

It should be mentioned that viremic daughters of male no. 339 transmitted the disease to almost 100% of their offspring when mated with uninfected males. However, genetic transmission (as opposed to congenital infection) occurred in only 50% of their offspring, as can be shown by molecular hybridization experiments with DNA extracted from "non-target" organs (see next section) or by further breeding experiments (data not shown).

Table 1 also contains breeding data of viremic males infected with M-MuLV at birth. None of these animals transmitted the virus to the offspring confirming earlier observations (9–11).

M-MuLV-Specific DNA Sequences in N-1 and N-2 Animals. The first 25 progeny of viremic male no. 339 infected at the preimplantation stage were tested for infectious virus in their sera (Fig. 1) as well as for M-MuLV-specific sequences in some of their organs (liver, kidneys, and spleen). Only the four animals that expressed infectious virus in their sera (Fig. 1) carried M-MuLV-specific sequences in their DNA, whereas in the other 21 virus-negative animals no M-MuLV-specific sequences could be detected (data not shown). These results suggest that M-MuLV did not behave as a recessive gene.

Fig. 2 shows the annealing kinetics of ^{32}P-labeled M-MuLV cDNA with DNA extracted from six different organs of a 4-week-old viremic N-2 animal that was not yet leukemic. Percentage of hybrid formation (Fig. 2A) or the reciprocal of the fraction of DNA remaining single-stranded (Fig. 2b) is plotted as a function of C_0t [initial concentration of total DNA (moles of nucleotide/liter) × time (seconds)]. DNA extracted from a liver of an uninfected animal and DNA extracted from a highly leukemic spleen of an animal infected with M-MuLV after birth were included as con-

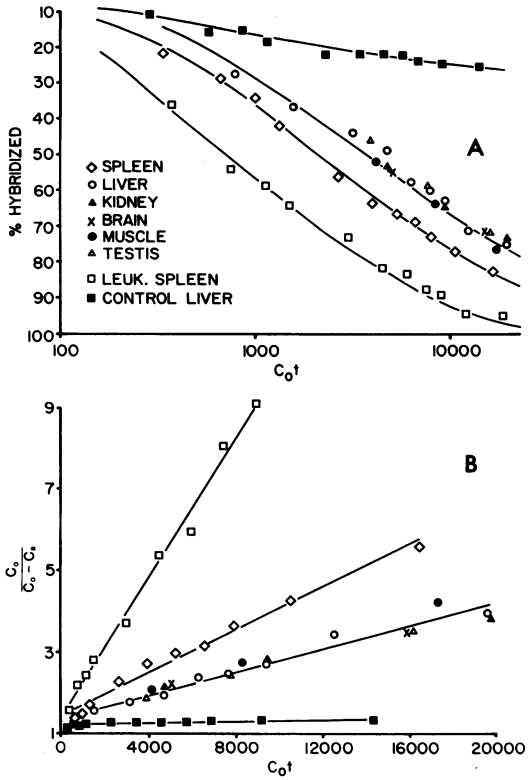

FIG. 2. Association kinetics of ^{32}P-labeled M-MuLV cDNA with mouse cellular DNA from various organs of an N-2 animal derived from male no. 339, of DNA from a highly leukemic spleen, and of control DNA. Organ DNA of a viremic N-2 animal was extracted at 4 weeks of age, as described in the *text*. ^{32}P-Labeled M-MuLV cDNA was purified as described in *Materials and Methods*, and 0.25 ng/ml of this probe was annealed to 10 mg/ml of mouse DNA in 1 M NaCl, 0.01 M Tes (*N*-tris[hydroxymethyl]methyl-2-aminoethansulfonic acid) at pH 7.0, 1 mM EDTA at 68° (14). Included as controls are DNA extracted from a highly leukemic spleen of an M-MuLV-infected mouse and from a liver of an uninfected animal. Percentage hybridized (A) or the reciprocal of the fraction remaining single-stranded (B) is plotted as a function of $C_o t$ (14). The maximum observed hybridization was normalized to 100%. The lines in B are plotted by means of a "least square fit" analysis. C_o, fraction of single-stranded DNA at time 0; C_a, at different times.

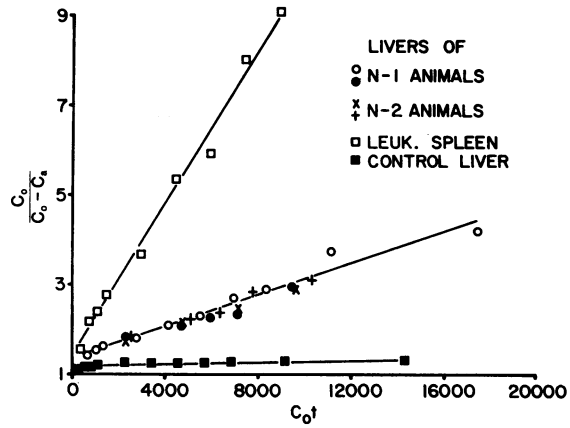

FIG. 3. Annealing of ^{32}P-labeled M-MuLV cDNA with liver DNA from N-1 and N-2 animals derived from male no. 339. Hybridization conditions and plotting of data are as in Fig. 2B. Included as controls are DNA from a highly leukemic spleen and from a liver of an uninfected animal.

trols. Whereas, 23% of the M-MuLV cDNA annealed to the DNA from uninfected animals, up to 92% annealed to DNA from the highly leukemic spleen. As shown in Fig. 2, M-MuLV cDNA annealed with DNA extracted from "non-target" organs (liver, kidneys, brain, muscle, and testes) with identical kinetics in each case, but at a rate about four times slower than that observed for DNA extracted from the highly leukemic control spleen. The DNA extracted from the spleen ("target" organ) of the experimental N-2 animal annealed at an intermediate rate. Fig. 3 shows hybridization experiments with DNA extracted from livers of four different viremic N-1 and N-2 animals. Again, these DNA preparations annealed with M-MuLV cDNA with identical kinetics at a rate four times slower than control DNA extracted from the highly leukemic spleen. In Fig. 4, annealing kinetics of DNA extracted from a highly leukemic N-1 animal (the father of no. 901-3 and 901-10 of Table 1) are shown. In this experiment, nonpurified M-MuLV cDNA (see *Materials and Methods*) which contained 65% sequence homology to DNA of uninfected mice was used. Therefore, a two component kinetics (3, 14) was obtained. Again, the "non-target" organ DNA annealed with identical kinetics at a rate four times slower than DNA extracted from the highly leukemic spleen of this animal.

The $C_o t_{1/2}$ value [half value of initial concentration of total DNA (moles of nucleotide/liter) × time (seconds)] of these annealing kinetics was calculated. The DNA from "non-target" organs of Figs. 2, 3, and 4 hybridized with a $C_o t_{1/2}$ of 6800–7200 and the highly leukemic spleen DNA in Figs. 2 and 3 and the spleen DNA of the N-1 animal in Fig. 4 with a $C_o t_{1/2}$ of 1500–1700. Because unique cell DNA reanneals with a $C_o t_{1/2}$ of 3200–3500 under our annealing conditions, I calculate that one-half copy of M-MuLV per haploid (or one copy per diploid) mouse genome equivalent is present in the "non-target" organs of viremic N-1 and N-2 animals derived from male no. 339. During leukemogenesis this number increases in the target organs up to a maximum number of two copies per haploid mouse genome (Figs. 2 and 4).

As a control, DNA was extracted from organs of a young mouse infected at birth with M-MuLV and hybridized as

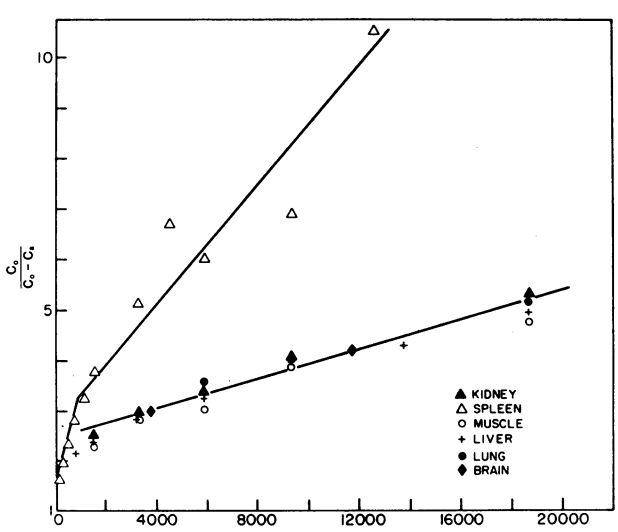

FIG. 4. Annealing of ^{32}P-labeled M-MuLV cDNA with DNA extracted from a highly leukemic N-1 animal derived from male no. 339. Hybridization conditions are as described in Fig. 2B, except that the M-MuLV cDNA was not purified by hybridization to AKR RNA (see *Materials and Methods*).

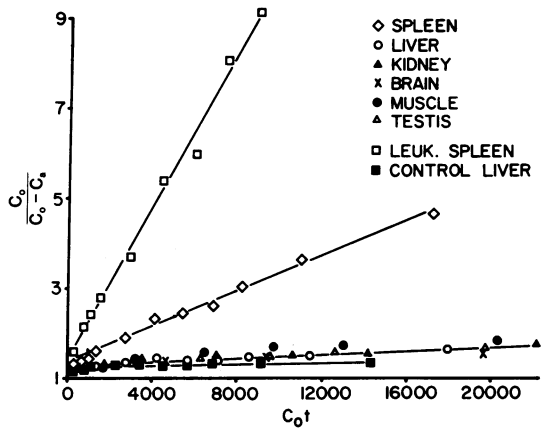

FIG. 5. Annealing of ^{32}P-labeled M-MuLV cDNA with DNA extracted from an animal infected after birth with M-MuLV. When sacrificed at 2 months of age, this animal was viremic but not yet leukemic (spleen slightly enlarged). Hybridization and plotting of data are as in Fig. 2B.

above (Fig. 5). This animal was as viremic (by XC assay and radioimmunoassay) as the animals described in Figs. 2 and 3, but not yet leukemic. The kinetics show no evidence of M-MuLV sequences in the "non-target" organs, but approximately one copy per haploid mouse genome in the slightly enlarged spleen. When highly leukemic animals, infected at birth, were analyzed, the maximum number of two copies per haploid mouse genome was detected in the highly enlarged spleen or thymus, whereas no M-MuLV copies were detected in "non-target" organs (data not shown).

DISCUSSION

The animal described in this paper (male no. 339) was infected at the 4–8 cell preimplantation stage with M-MuLV after removal of the zona pellucida. Such an infection cannot occur *in vivo* because the zona pellucida is lost only at the blastocyst stage before implantation. It is likely that incubation of embryos with virus leads to infection of only a fraction of the blastomeres (see below). Because preimplantation embryos are nonpermissive for virus expression (14), virus integrated in one blastomere should not be able to infect other previously uninfected blastomeres, thus possibly giving rise to mosaic animals. When bred with uninfected females, male no. 339 transmitted the virus in a non-Mendelian manner, with a decreasing frequency of viremic offspring with increasing age (Fig. 1). A similar phenomenon has been observed in allophenic mice with germ line mosaicism, where age-dependent selection for one marker took place during spermiogenesis (22). The results in Fig. 1 suggest that male no. 339 may similarly be a "germ line" mosaic carrying M-MuLV integrated into a fraction of his sperm (about 16% at 2–4 months of age), a fraction that was decreasing with age. It is not clear what percentage of germ cells carried integrated M-MuLV at earlier developmental stages because it is possible that selection against spermatogonia carrying M-MuLV occurred by some immunological or other mechanism which may have started soon after birth. The majority of mice obtained from M-MuLV infected preimplantation embryos is nonviremic at young age (ref. 14 and this paper). Molecular hybridization experiments with somatic "non-target" tissues of these virus-negative mice suggest that mosaicism is not restricted to the germ line cells, but that in any organ a variable percentage of cells may carry M-MuLV sequences (Jaenisch, unpublished observation) similar to the mosaicism observed in allophenic mice (23). This somatic mosaicism observed in mice infected at the preimplantation stage is in sharp contrast to the situation found after genetic transmission of M-MuLV.

Table 1. Genetic transmission of M-MuLV

Male no.	Mode of infection with M-MuLV	Transmission of M-MuLV to backcross generations: No. of viremic offspring	%
339	4–8 cell stage embryo	8/160	5
921	N-1 of 339	21/39	54
984	N-1 of 339	16/30	53
901-3	N-2 of 339	14/31	45
901-10	N-2 of 339	28/59	48
901-10-5	N-3 of 339	10/22	45
		Total 89/181	49
1	Infected as newborns with M-MuLV	0/35	0
2	Infected as newborns with M-MuLV	0/29	0
4	Infected as newborns with M-MuLV	0/32	0
9	Infected as newborns with M-MuLV	0/25	0
11	Infected as newborns with M-MuLV	0/46	0
		Total 0/167	

Male no. 339 was bred with uninfected BALB/c females to give the first backcross generation (N-1 generation; Fig. 1). Virus-positive N-1 animals were identified as described in the *text* and bred with uninfected females to give the second backcross (N-2 generation). N-3 and N-4 animals were obtained similarly. As controls, viremic males infected as newborns with M-MuLV were bred with BALB/c females.

Integration of M-MuLV into the germ line was indicated by genetic and molecular hybridization experiments. The breeding data of N-1, N-2, and N-3 males derived from male no. 339 (Table 1) strongly suggest that these animals are heterozygous for one locus responsible for M-MuLV synthesis. The presence and expression of viral DNA in 50% of N-2 and N-3 animals suggests that the integrated virus behaved essentially like a Mendelian dominant gene.

The hybridization data (Figs. 2–4) show that one-half copy of M-MuLV per haploid mouse genome was present in all "non-target" organs of all viremic offspring tested, confirming that these animals are heterozygous for the *M-MuLV* locus. During leukemogenesis, an increase in the number of M-MuLV specific sequences was observed in the "target" organs (spleen and thymus). Young animals with beginning disease showed a slight amplification (Figs. 2 and 5), whereas animals with advanced leukemia showed up to two copies of M-MuLV per haploid mouse genome (Fig. 4). This number is the maximum we have observed in any leukemic animal or tissue culture cell that produces virus. Therefore, it seems possible that the mouse genome has a total of two integration sites for M-MuLV and that the viremic progeny of male no. 339 carry the *M-MuLV* gene integrated into their germ line at only one out of two possible sites. The animal described previously (14) seemed to have the maximum of two M-MuLV copies per haploid genome integrated into all somatic tissues, and therefore no amplification was observed in the tumor tissues. An increase in the number of virus copies is also seen in animals with progressing leukemia after infection as newborns (Fig. 5; refs. 14 and 15). The amplification of virus copies found in leukemic

spleens or thymus might therefore reflect the degree of infiltration of a given spleen or tumor with lymphoma cells which carry the maximum of four copies of M-MuLV per diploid cell. It is not known if the observed amplification of M-MuLV sequences in lymphoma cells is due to further integration of the virus into the mouse genome, if it is the result of selective chromosomal duplication in transformed cells (24), or if it represents unintegrated proviral DNA. The latter possibility seems to be very unlikely because animals have been found with two virus copies per haploid genome in tumor and nontumor tissues, suggesting that no further amplification beyond this maximum number can occur. An amplification of Gross virus sequences is also observed in tumors of leukemic AKR mice (Berns and Jaenisch, in preparation).

The results described in this paper are consistent with the hypothesis that the exogenous M-MuLV can be established in mice as an endogenous virus after infection of preimplantation embryos. Thus, it should be possible to map genetically the integration site and compare it to the integration sites of other viruses with a different or similar organtropism.

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