Infection of Developing Mouse Embryos with Murine Leukemia Virus: Tissue Specificity and Genetic Transmission of the Virus

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Abstract

The tissue specificity of Moloney leukemia virus (M-MuLV) was studied by infecting mice at two different stages of development. Either newborn mice which can be considered as essentially fully differentiated animals were infected with M-MuLV or preimplantation mouse embryos were infected *in vitro* at the 4–8 cell stage, a stage of development before any differentiation has taken place. After surgical transfer to the uteri of pseudopregnant surrogate mothers, the latter developed to term and adult mice. In both cases, animals were obtained that had developed an M-MuLV induced leukemia.

Molecular hybridization tests for the presence of M-MuLV-specific sequences were conducted on DNA extracted from different tissues of leukemic animals to determine which tissues were successfully infected by the virus. Mice which were infected as newborns carried M-MuLV-specific DNA sequences in "target tissues" only, i. e., thymus, spleen, lymph nodes or in organs infiltrated by tumor cells, whereas "non-target tissues" did not carry virus-specific sequences. In contrast, when leukemic animals derived from M-MuLV-infected preimplantation embryos were analyzed, virus-specific sequences were detected in target tissues as well as in non-target tissues, such as liver, kidney, brain, testes and the germ line.

To study the expression of the viral DNA integrated in target and non-target organs, RNA was extracted from different tissues of an animal infected at the preimplantation stage. Fifty to 100 times more M-MuLV-specific RNA was detected in tumor tissues than was found in non-target organs. Since all organs contained the same amount of virus-specific DNA, these results indicate that the integrated virus genome can be differentially expressed in different tissues. The organ-tropism of RNA tumor viruses is discussed in view of these findings.

Mice that were infected at the preimplantation stage were found to have M-MuLV integrated into their germ line. Virus transmission from the father to the

offspring occurred according to simple Mendelian expectations. Molecular hybridization tests revealed that in the animals studied, the virus was integrated into the germ line at only one out of two or three possible integration sites. During the development of leukemia amplification of this virus copy was observed in the target tissues only, but not in the non-target tissues.

Introduction

In principle, two different classes of RNA tumor viruses can induce murine leukemia: endogenous and exogenously or horizontally infecting C-type viruses. The genetic information of endogenous viruses is present in all somatic and germ cells of all animals in a given mouse strain and the endogenous virus is transmitted genetically according to Mendelian expectations (1). One of the best characterized examples of an endogenous oncogenic leukemia virus is found in the AKR strain of mice with a high incidence of leukemia (2). Virus production in AKR mice is controlled by two dominant loci, one of which has been mapped on linkage group I (3). This locus has been shown to represent the structural gene of the AKR virus by molecular hybridization techniques (4).

In contrast, the genetic information of horizontally infecting or exogenous viruses is not transmitted genetically from the father to the offspring (5, 6, 7). This is readily understood since infection of newborn animals with leukemia virus leads to integration of viral DNA into a few "target tissues" only whereas most other tissues, notably the germ line, do not become infected (8, 9). One of the goals of this work was to study the basis of this "organ-tropism" of murine leukemia viruses.

The second aim of the experiments described in this paper was to obtain mice that carry an exogenously infecting virus in their germ line. This would allow us to study genetic transmission of an exogenous virus, to map its integration site and to compare this site with the known loci of endogenous viruses.

One way to obtain an animal carrying exogenous virus genes in every cell including the germ line would be to infect the animals at a very early stage of embryonic development before any differentiation has taken place, for example at the preimplantation stage. At this stage of development, cellular infection might not be restricted by the organ-tropism of a given virus and therefore all cells of the embryo might become successfully infected. An animal derived from such an infected embryo should contain virus information in each cell and the expression of the virus information and of virus-induced oncogenesis should depend on regulatory events in individual cells which, in turn, might be influenced by the differentiated state of a given cell. Indeed, it has been shown recently that adult mice derived from blastocysts infected with SV40 DNA carried SV40-specific sequences in some of their organs (10, 11).

We report here the successful infection and development of Moloney virus (M-MuLV) infected embryos into mature adult mice and the experimental recovery of M-MuLV-specific DNA sequences from the tissues of some of these animals. Our experiments indicate chromosomal integration and genetic transmission of M-MuLV. Some of the experiments described below have been published recently (9).

Materials and Methods

1. *Virus*-M-MuLV clone No. 1 was grown and purified as described (12, 13). Virus stocks were titered by endpoint dilution using the XC plaque test (14).

2. *cDNA*-Virus-specific DNA probes were prepared from purified M-MuLV stock in the presence of 25 or 100 μg/ml actinomycin D (12). ^{32}P-labeled dCTP and/or TTP at a specific activity of 100 Ci/mm (NEN) was used as radioactive precursor. The DNA synthesized had a specific radioactivity of 60–120 x 10^6 cpm/μg and sedimented between 5.5 and 6S in alkaline sucrose gradients. It annealed up to 90 % to M-MuLV 60–70S RNA.

3. *Isolation and infection of mouse embryos* – Four-eight cell stage embryos were isolated from BALB/c females mated with 129J males and the zona pellucida was removed with pronase (15). The embryos were infected with 10^8 PFU/ml of M-MuLV in medium containing 2 μg/ml polybrene for 5 hr and subsequently cultured in medium for 24 hr. At this time, they were surgically transplanted to the uterine horns of pseudopregnant ICR foster mothers (15).

4. *Extraction of nucleic acids* – Mouse tissues were removed and extracted as described (9, 10). In some experiments, the nucleic acids were extracted by the Kirby method (16), the DNA was banded in ethidium bromide CsCl gradients, sonicated and boiled in 0.2 M NaOH for 10 min. The DNA used for hybridization sedimented with approximately 6S in alkaline sucrose gradients. RNA was purified as described previously (12).

5. *Molecular hybridization* – DNA-DNA and DNA-RNA hybridizations were carried out as described previously (9, 12). The cell DNA was in a 2–10 x 10^6 fold excess over the ^{32}P-labeled M-MuLV cDNA. Input radioactivity was 400–800 cpm per experimental point.

6. *Histology* – Sections of the major organs were fixed, sectioned and stained by standard histological techniques (14). Each organ was then evaluated for the extent of lymphomatous infiltration.

7. *Serum analysis* – Mice were bled from the retro-orbital plexus and the serum was analyzed for p30 (14).

Results

1. *Infection of preimplantation mouse embryos with M-MuLV.*

Preimplantation mouse embryos at the 4–8 cell stage were infected with M-MuLV as described in Materials and Methods, washed extensively and incubated in medium at 37°. After 24 hr the virus-infected embryos, as well as the uninfected control embryos, had developed to the blastocyst stage (32–64 cell stage). At this time, they were washed again in medium and transplanted to foster mothers to insure further development *in utero* (see next section).

In order to determine whether infectious virus could be detected on blastocysts, two types of experiments were performed. First, the blastocysts were co-cultivated with BALB/c or NIH Swiss 3T3 cells for six days, the cultures were passed two or three times and all the tissue culture supernatants were tested for M-MuLV by the XC cell assay. Five separate attempts to recover infectious virus were negative. Second, infected blastocysts were fixed, sectioned and prepared for electron microscopy. No C-type particles were observed in five embryos examined.

These observations suggest that the input M-MuLV used for the primary infection of the 4–8 cell embryos did not survive in infectious form during the *in vitro* cultivation period and that the infected embryos did not produce detectable virus at the preimplantation stage.

a) *Induction of leukemia after M-MuLV infection:* Of 29 embryos that were infected at the 4–8 cell stage with M-MuLV and transferred to foster mothers, 15 developed to term and into apparently healthy young mice. The survival rate to birth was therefore 50 % and is comparable to the survival rate of uninfected embryos (50–70 % in this laboratory).

At two months of age, the animals were bled and the serum tested for the presence of murine p30 protein by radioimmune assay. Whereas 14 animals were negative in this test (less than 0.03 µg/ml serum), one mouse showed a high level of 1.9 µg p30 per ml serum. Two weeks later this animal was sacrificed. Autopsy revealed a typical lymphatic leukemia with enlarged spleen and lymph nodes.

The foster mothers used in this experiment were bled four and twelve weeks after delivery and tested for murine p30. The serum contained less than 0.03 µg/ml p30, suggesting that no virus infection of the mother via the embryos was detectable.

b) *Histology and isolation of virus:* Histological examinations of sections of different organs revealed massive infiltration of lymph nodes, spleen and kidneys with lymphoma cells, intermediate infiltration of the thymus and relatively little infiltration of the liver. The lung, brain and testes did not show any tumor cell

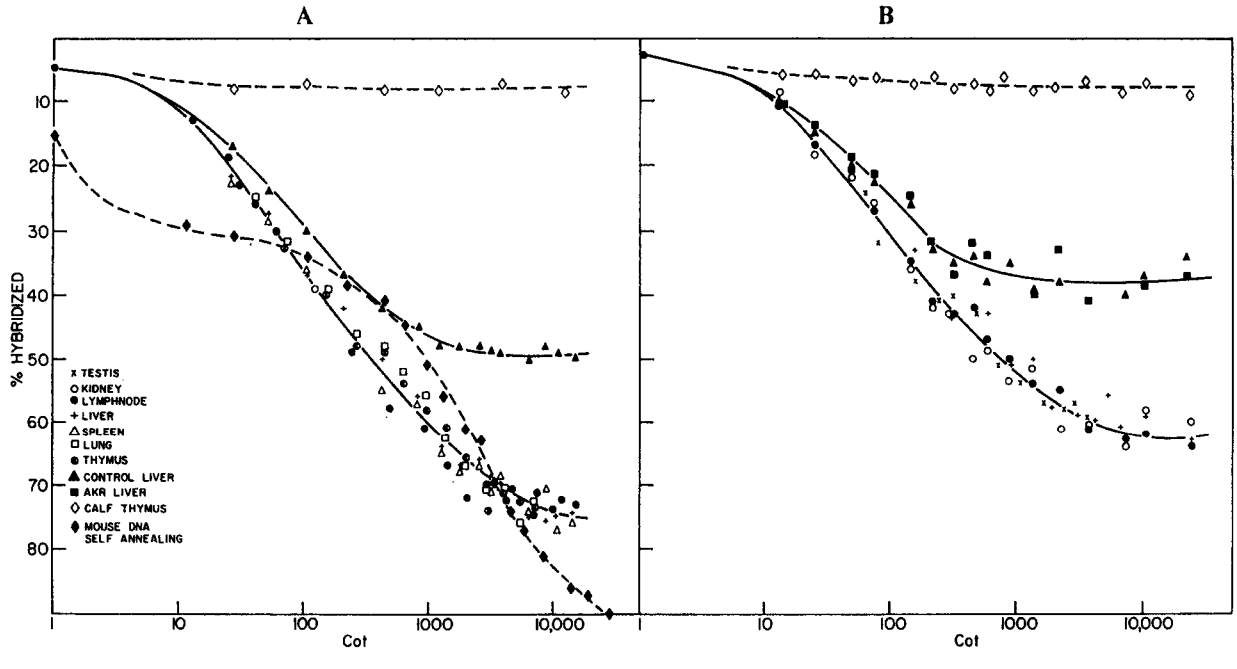

Fig. 1: Association kinetics of ^{32}P-labeled M-MuLV cDNA with mouse cellular DNA derived from various organs of the mouse infected at the 4–8 cell stage with M-MuLV, from control mice (BALB/c 129 and AKR) or from calf thymus. The reaction mixture containing 0.5 ng/ml of ^{32}P-labeled M-MuLV cDNA (1.2×10^8 cpm/µg), 4 mg/ml mouse or calf thymus DNA in 0.01 M Tris-HCl, pH 7.0, 1 mM EDTA, 1M NaCl, was heat denatured and incubated at 68°. Per cent hybridization is plotted as a function of Cot, corrected to standard annealing conditions (25). The cDNA probes used annealed to 80 % (A) or 70 % (B) to M-MuLV 60–70S RNA.

infiltration (Table 1). The lymphoid tumors and infiltrates were composed of sheets of uniform large lymphoblasts.

The serum was tested for infectious virus on B and N type cells. Virus was found and titered equally well on both test cells and the TCID 50/ml serum was 2×10^4 on B cells and 3×10^4 on N cells. Virus recovered from infected BALB/c cells also titered equally well on NIH Swiss and B type cells, indicating that the virus isolated was N-B tropic similar to the infecting M-MuLV.

c) *M-MuLV specific sequences in mouse DNA:* The presence of M-MuLV specific sequences in cellular DNA was determined by annealing the M-MuLV cDNA probe with DNA extracted from various tissues of the M-MuLV-infected mouse, control BALB/129 and AKR mice. The reassociation kinetics are shown in Figs. 1a and b. In Fig. 1a, up to 75 % of the cDNA probe (up to 63 % in Fig. 1b) hybridized to the DNA extracted from the different tissues of the M-MuLV-infected mouse; with BALB/c 129 control or AKR DNA, the maximum hybridization observed was 50 % (37 % in Fig. 1b) and essentially no hybridization was detected to calf thymus DNA. The different maxima of hybridization observed for the same cellular DNA preparations in Figs. 1a and b are attributable to different cDNA probes used with different levels of maximal hybridization to virion 60–70S RNA. In Fig. 1a the reassociation kinetics of total mouse cell DNA is also plotted.

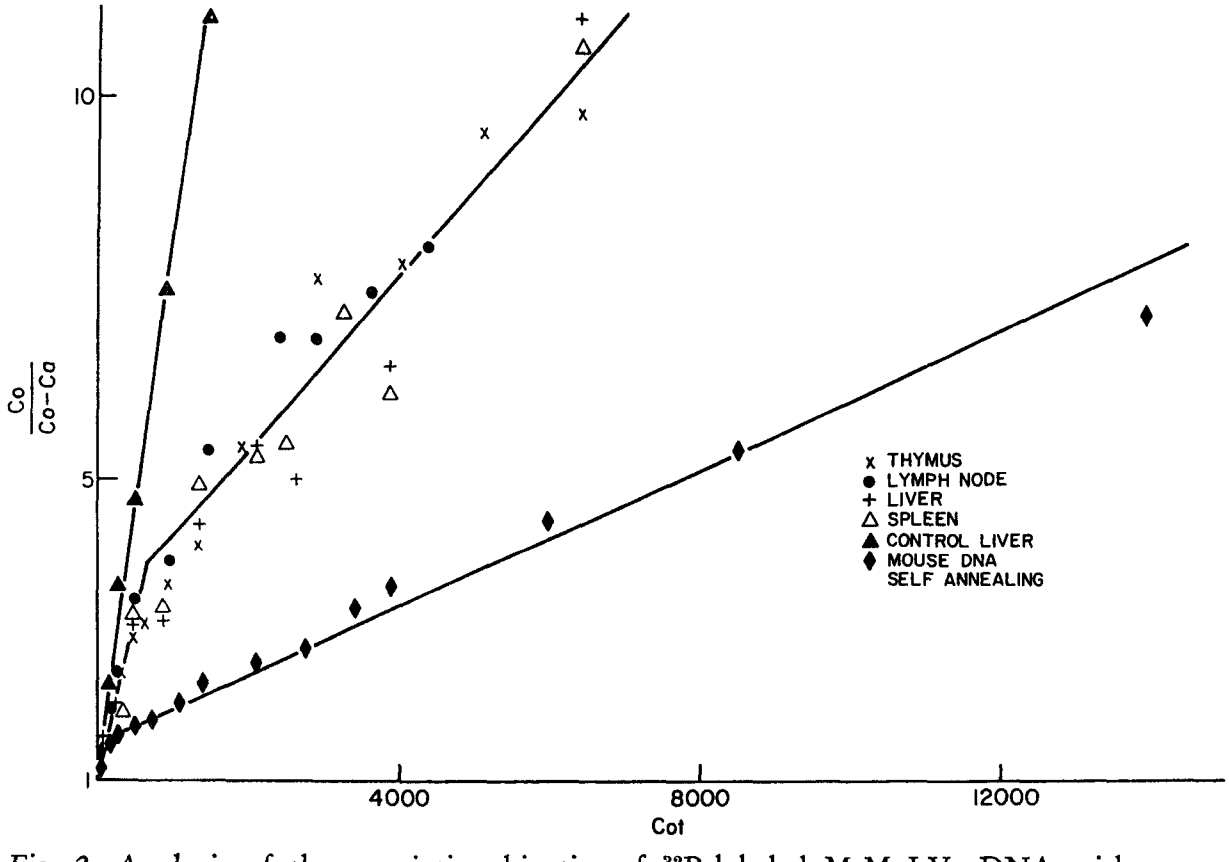

Fig. 2: Analysis of the association kinetics of ^{32}P-labeled M-MuLV cDNA with mouse DNA by the method of Wetmur and Davidson (17). The results are plotted as the reciprocal of the fraction of DNA remaining single-stranded as a function of C_0t. The maximum observed hybridization was normalized to 100 %. (Co = fraction of single-stranded DNA at time 0, Ca = fraction of single-stranded DNA at different times.) The data are taken from Fig. 1A.

The results indicate that uninfected control mice (BALB/129 and AKR) carry some sequences (up to 50 %) homologous to M-MuLV cDNA. In addition, the Moloney virus-infected mouse acquired M-MuLV-specific sequences which are not present in uninfected animals. All tissues tested contained the same amount of M-MuLV-specific DNA.

In order to examine more closely the kinetics of reassociation and to determine the number of virus copies present, the data in Fig. 1a was plotted as the reciprocal of the fraction of unhybridized probe versus the C_0t normalized to 100 % hybridization (4, 17). If all virus-specific sequences are present in the cellular DNA in equal numbers, the results would be a single straight line with a slope proportional to the number of copies of that set of sequences. On the other hand, if several sets of virus-specific sequences were present, each in different proportions, the curves would describe several slopes. It can be seen that the viral probe anneals with the DNA extracted from the M-MuLV-infected mouse as though there were two distinct sets of virus-specific sequences in the cell DNA (Fig. 2). The $C_0t_1/_2$ for each component was calculated, giving a value of 1,100 mole·sec/liter for the slow annealing component of M-MuLV-specific sequences and a value of 80–100 mole·sec/liter for the fast annealing sequences in comparison to a $C_0t_1/_2$ of 2,200 for unique cell DNA. The slopes of this slow annealing set of sequences in Fig. 2 is about 2.5 times steeper and the slope of the fast annealing set about 15

Table I: Detection of M-MuLV-specific DNA and RNA in tissues of the Moloney virus-infected mouse.

Organ	Histology: extent of infiltration with lymphoma cells	No. of M-MuLV specific sequences Fast annealing set	Slow annealing set	M-MuLV specific RNA %
lymph node	High	15–30	2–3	0.43
kidney	High	15–30	2–3	0.41
spleen	High	15–30	2–3	0.31
thymus	Medium	15–30	2–3	0.088
liver	Low	15–30	2–3	0.005
lung	None	15–30	2–3	0.19
testis	None	15–30	2–3	0.017
brain	None	(+)*	(+)*	0.005
control liver, spleen, kidney	n. t.	15–30	0	0
AKR liver	n. t.	15–30	0	n. t.

Table1: Detection of M-MuLV specific DNA and RNA in various tissues of the Moloney virus-infected mouse.

The number of M-MuLV-specific DNA copies in haploid mouse genomes was calculated from the $C_0t_{1/2}$ values of each class of virus-specific sequences relative to the $C_0t_{1/2}$ of unique DNA (Figs. 1 and 2). The concentration of M-MuLV-specific RNA was calculated from the $C_rt_{1/2}$ values in Fig. 3.

* The brain DNA was positive for M-MuLV-specific DNA but no quantitation was possible due to the small amount of brain DNA isolated.

times steeper as compared to the self-annealing of cellular DNA. These values and the $C_0t_1/_2$ values suggest about 2–3 copies per haploid genome for the slow annealing component and about 15–30 copies for the fast annealing component of sequences complementary to M-MuLV. Thus, the Moloney virus-infected animals acquired 2–3 copies of Moloney virus-specific sequences not present in control animals (Table I).

d) *Differential transcription of M-MuLV specific sequences in different organs:* M-MuLV cDNA was annealed with RNA extracted from eight organs of the M-MuLV-infected mouse and also with RNA from some organs of control mice (Fig. 3). RNA extracted from the various organs of the experimental animal

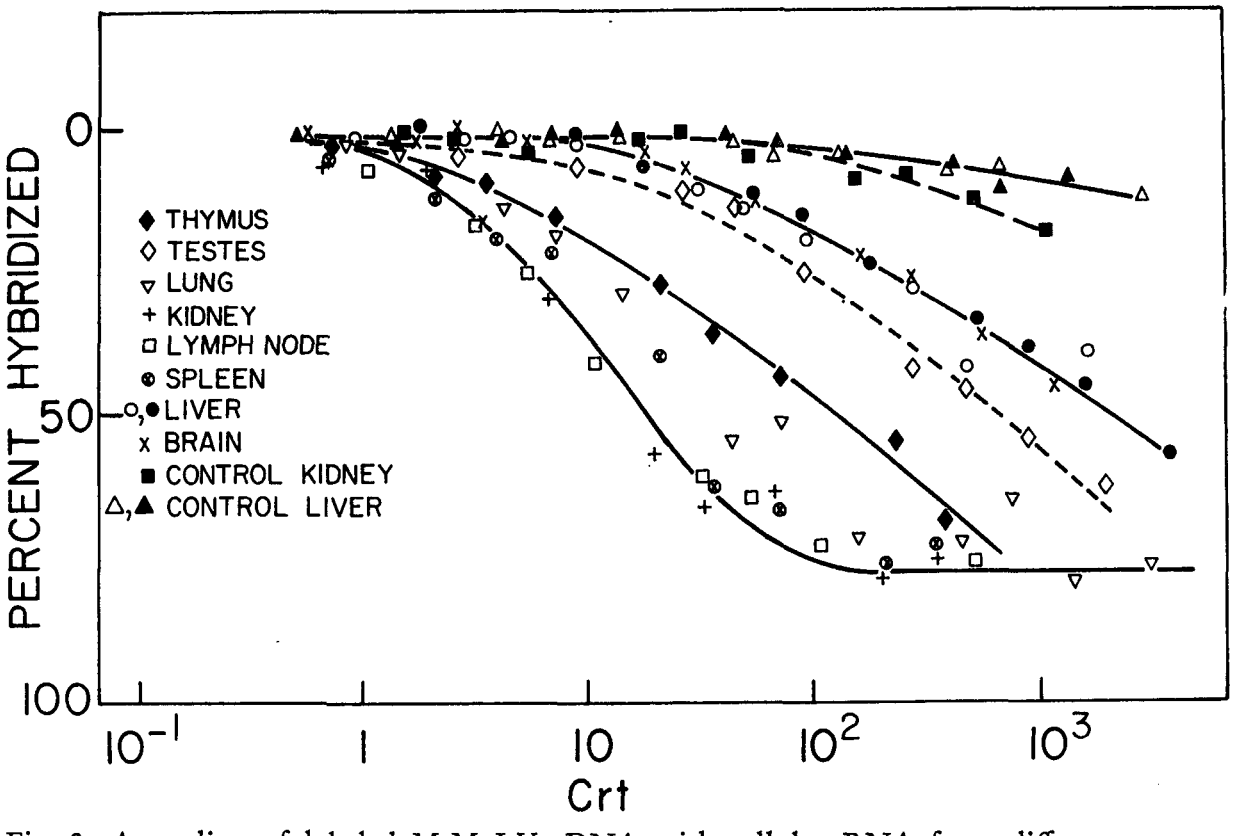

Fig. 3: Annealing of labeled M-MuLV cDNA with cellular RNA from different organs of the M-MuLV infected and control mice. Annealing in conditions of RNA excess was performed as described (12). Per cent hybrid formation is displayed as a function of C_rt, corrected to standard annealing conditions (25).

efficiently hybridized with the M-MuLV cDNA, while control RNA annealed only small amounts at extremely high C_rt values. Maximal hybridization for the M-MuLV cDNA was 75 % as determined by annealing to purified M-MuLV 60–70S RNA.

The amount of M-MuLV specific RNA present in the various organs was determined from the half-saturation values of hybridization ($C_rt_1/_2$) (12), and was found to vary over a wide range (Table 1). Between 0.3 and 0.43 % of the total RNA extracted from spleen, lymph nodes and kidney was virus-specific, whereas liver, testes and brain contained approximately 50–100-fold less M-MuLV-specific RNA. The thymus and the lung showed intermediate levels of virus-specific RNA. With the exception of the lung, the levels of M-MuLV-specific RNA appeared to

correlate roughly with the degree of infiltration of a given organ with lymphoma cells (see Table 1). These results indicate that although all tissues contained the same number of M-MuLV DNA copies, these virus genes were expressed at different levels in different organs.

II. *Genetic transmission of M-MuLV.*

The previous experiments established that infection of preimplantation mouse embryos can lead to animals carrying M-MuLV-specific DNA sequences in many, if not all, the tissues of the adult. We were interested in studying whether integration of the virus into the germ line could take place since this would allow us to map the integration site of an exogenously infecting virus and to compare this site with the known integration sites of endogenous viruses. Therefore, we investigated whether genetic transmission of M-MuLV from a viremic father to its offspring can occur.

The male used for these experiments (mouse No. 339 in Table 2) was derived from a 4–8 cell preimplantation embryo infected with M-MuLV. The animal showed moderately elevated levels of serum p30 (0.8–1.0 μg/ml) at six weeks of age and was bred with unexposed females. Of 80 progeny tested so far, seven had infectious M-MuLV in the serum at 4–5 weeks of age. This observation suggests either that approximately 10 % of the sperm of the father carried virus-specific information in its genome and transmitted it genetically to his offspring or that about 10 % of the offspring were congenitally infected from the viremic father via the mother (as opposed to genetic transmission, 18). Earlier observations seem to rule out the latter explanation since leukemic males infected *in utero* or after birth with M-MuLV do not transmit the disease to their offspring (5–7). If, on the other hand, germ line transmission had occurred, two testable predictions should be fulfilled.

1) The number of M-MuLV-specific DNA copies in the viremic N-1 animals should be constant in all organs of individual animals (in contrast to animals infected after birth, see below).

2) Viremic N-1 males mated to uninfected females should transmit the virus according to simple Mendelian expectations to the next generations.

a) *M-MuLV-specific DNA sequences in N-1 animals:* The first 25 progeny of the viremic male infected at the preimplantation stage with M-MuLV were tested for both infectious virus in their serum as well as for M-MuLV-specific DNA sequences in some of their organs. Four of these 25 animals showed infectious virus with titers between 10^2–10^3 XC PFU/ml serum. Only in DNA extracted from these four XC positive animals were M-MuLV-specific DNA sequences found, whereas in the other 21 animals, no M-MuLV-specific DNA could be detected. Figure 4 shows the annealing kinetics of ^{32}P labeled M-MuLV cDNA with DNA extracted from five different organs of one of these animals. The slopes of the annealing kinetics for non-target organs, i. e., brain, liver, kidney and muscle, are identical, whereas the spleen, a target organ of M-MuLV infection, anneals with M-MuLV cDNA four times faster. The number of virus-specific DNA copies was calculated to be one-half copy per haploid mouse genome equivalent for the non-target organs and two copies for the spleen. Hybridization experiments with the other three viremic N-1 animals gave identical results.

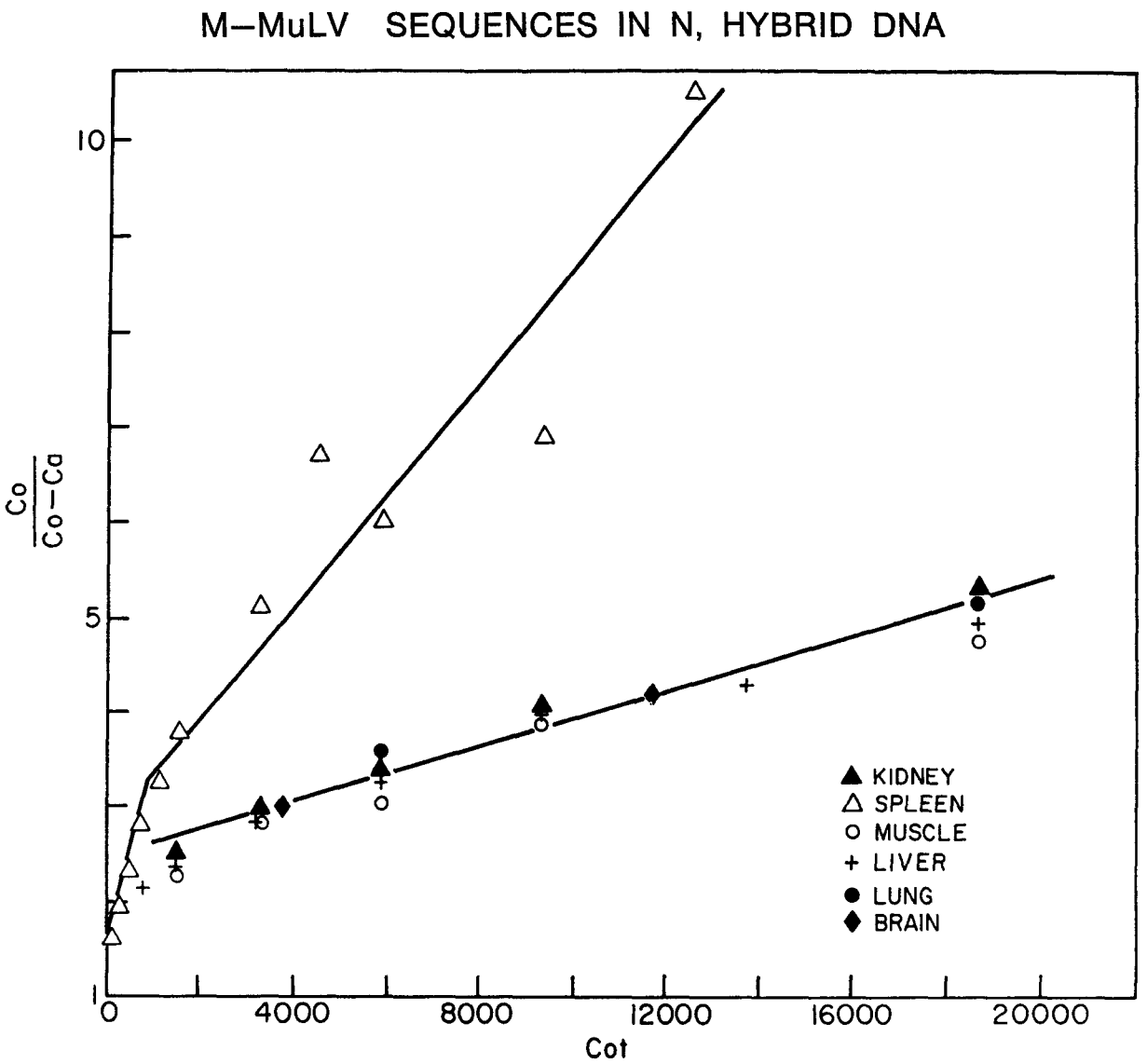

Fig. 4: Analysis of the annealing kinetics of ^{32}P-labeled M-MuLV cDNA with mouse DNA extracted from an N-1 animal derived from mating mouse No. 339 (Table 2) with an uninfected female. The reaction mixture containing 0.2 ng/ml of ^{32}P-labeled M-MuLV cDNA (1.5 x 10^8 cpm/μg) and 5 mg/ml of mouse DNA extracted from different organs was annealed as described in Fig. 1 and the data plotted as described in Fig. 2. The cDNA probe used annealed up to 92 % to M-MuLV 60–70S RNA.

These observations indicate that whenever M-MuLV DNA sequences were transmitted from the father to its offspring (in four out of 25 N-1 animals tested) the virus information was expressed as infectious virus in the serum. Secondly, the number of M-MuLV-specific DNA copies found in all non-target organs of these animals was identical and increased four-fold in the target tissues during the development of leukemia.

b) *Transmission of M-MuLV to the N-2 generation: The* hybridization results described above indicate that the viremic N-1 animals carried one-half copy of M-MuLV per haploid mouse genome equivalent in each cell, suggesting that these animals may have been heterozygotes for one integration site of M-MuLV. This would predict a 50 % transmission of M-MuLV to the N-2 generation.

One viremic N-1 male and two viremic N-2 males were used to test this hypothe-

Table II: Genetic Transmission of M-MuLV

Mouse No.	Mode of Infection with M-MuLV	Transmission of M-MuLV to Offspring when Mated with Uninfected Females	
		No. of Viremic Offspring	%
339 ♂	4–8 cell preimplantation stage	7/80	9 %
921 ♂	N-1 of No. 339	19/30	63 %
901–3 ♂		4/ 8	50 %
901–10 ♂	N-2 of No. 339	6/20	30 %
		Total 29/58	50 %
1 ♂		0/35	0 %
2 ♂	Infected as newborns with	0/29	0 %
4 ♂		0/32	0 %
9 ♂	M-MuLV	0/25	0 %
		Total 0/121	0 %

Table 2: Genetic transmission of M-MuLV.
Four–eight cell embryos were infected with M-MuLV and transplanted to foster mothers as described in the text. One viremic mouse derived from these embryos (male No. 339) was bred with uninfected BALB/c females and the resulting N-1 generation was analyzed for infectious virus in the serum. Viremic N-1 males were bred with normal BALB/c females to yield N-2 and N-3 animals. Furthermore, newborn mice were infected with M-MuLV and bred with BALB/c females after development of viremia. All the progeny were tested for infectious M-MuLV in the serum by the XC assay.
Figure Legends

sis. The N-1 male (No. 921, Table 2) was derived by mating mouse No. 339 with an uninfected BALB/c female. The N-2 males (No. 901–3 and 901–10 in Table 2) were derived from a viremic daughter of mouse No. 339 mated with an uninfected BALB/c male. Annealing kinetics of DNA extracted from various organs of this female with M-MuLV cDNA have been described in Fig. 4. These three viremic males were mated to uninfected females and the resulting N-2 and N-3 generations were tested for infectious virus in the serum. The results in Table 2 indicate that of a total of 58 N-2 and N-3 animals tested, 29 were positive for infectious M-MuLV. These data strongly favor the hypothesis that M-MuLV was integrated into the germ line of these mice and was transmitted genetically to the offspring according to simple Mendelian expectations.

Table 2 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 (5–7).

III. *Infection of newborn mice with M-MuLV.*

The experiments described above have shown that after infection of an animal at the preimplantation stage with M-MuLV, virtually all tissues of the resulting adult can carry the same amount of virus-specific sequences per cell, regardless of whether the tissue represents a "target" tissue for the virus or not. The situation

might be very different when infection takes place at a later developmental stage, i. e., after birth, when all cells of the animal are fully differentiated. To investigate this possibility, newborn mice were infected with M-MuLV and following development of viremia and leukemia, the DNA from different organs was analyzed for the presence of virus-specific sequences. The results obtained with one of these animals are described below.

When sacrificed at four months of age, this animal appeared terminally ill with extensive tumor cell infiltration in many organs. Radioimmunoassay revealed 2 µg/ml serum of murine p30. Histological examination demonstrated almost 100 % lymphoma cell infiltration in the highly enlarged thymus and spleen, approximately 30–50 % infiltration in the liver and less than 10 % infiltration in the kidneys. Brain and muscle had little if any signs of lymphoma cell infiltration.

The DNA was extracted from all these tissues and annealed with ^{32}P-labeled M-MuLV cDNA (Fig. 5). The slopes of the DNA annealing kinetics revealed two sets of virus-complementary sequences comparable to the results described in Fig. 2. But, in contrast to the results obtained with the animal infected at the preimplantation stage (Figs. 1, 2) or in animals with genetically transmitted virus (Fig. 4), the curves indicate that different amounts of the slow annealing set of

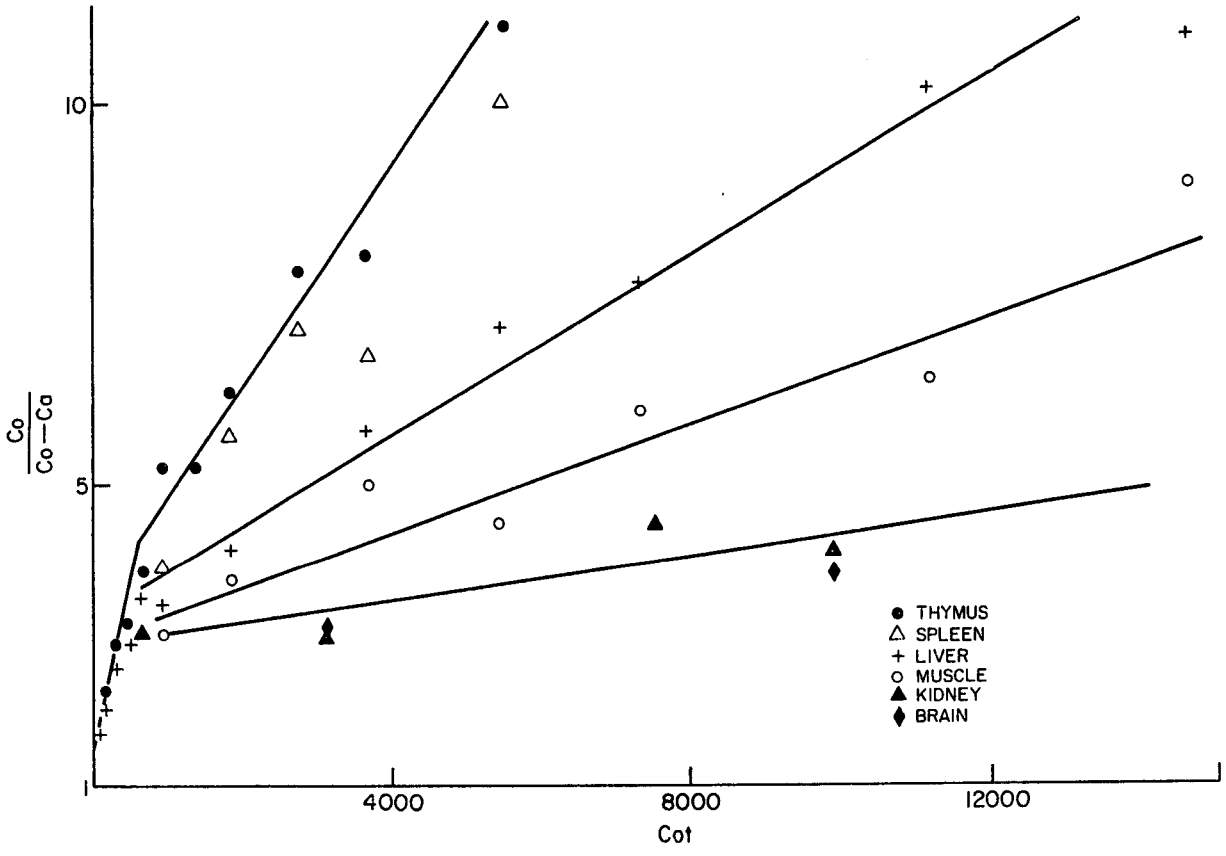

Fig. 5: Analysis of the association kinetics of ^{32}P-labeled M-MuLV DNA with mouse DNA extracted from an animal infected after birth with M-MuLV. The reaction mixture containing 0.2 ng/ml of ^{32}P-labeled M-MuLV cDNA (8×10^7 cpm/µg) and 6 mg/ml of mouse DNA extracted from different organs was annealed as described in Fig. 1, and the data are plotted as described in Fig. 2. The cDNA probe used annealed up to 90 % to M-MuLV 60–70S RNA.

M-MuLV-specific sequences were present in the different organs. Spleen and thymus showed the highest concentration of virus-specific DNA, comparable to the concentration found in each of the organs of the animal described in Figs. 1 and 2. On the other hand, the slow annealing M-MuLV cDNA sequences annealed more slowly with the DNA extracted from the other organs. From the slopes of these curves, it can be calculated that in comparison to the thymus and spleen, the concentration of M-MuLV-specific DNA present in the liver was only about 0.4 times as much, in the muscle about 0.25 and in brain and kidneys approximately 0.05–0.1 times as much. With the exception of the muscle, these values roughly correlate with the extent of histological detected lymphoma cell infiltration. This suggests that the virus-specific DNA sequences detected in the DNA of the different organs was derived primarily from the infiltrating tumor cells and not from the parenchymal cells. This correlation does not hold for the muscle and may be due either to extensive tumor cell infiltration of the skeletal muscles undetected in the limited number of histological sections examined, or to muscle cells being susceptible to virus infection (in contrast to other parenchymal cells).

Discussion

Murine leukemia induced by exogenously infecting M-MuLV or by the endogenous Gross virus develops as a typically thymus-derived disease. Other RNA tumor viruses, such as Friend virus or murine mammary tumor virus, are characterized by a different organ-tropism, i. e., they transform spleen erythroblasts or mammary gland cells, respectively, but not the thymus derived lymphoblasts. One of the aims of this investigation was to study the possible basis of the organ-tropism of a given tumor virus.

We, therefore, compared the type of tissues that can be infected by M-MuLV at different stages of development. We have chosen two extreme stages of development for infection, the preimplantation 4–8 cell embryo and the newborn mouse. At the 4–8 cell stage of development, no differentiation or commitment of the single blastomers can be recognized (19), whereas the newborn mouse can be considered as an essentially fully differentiated organism. By testing for the presence of M-MuLV-specific DNA sequences in different tissues of the adult leukemic mouse, we were able to demonstrate that the developmental stage of an animal at which the infection takes place indeed determines which tissues can be infected and which tissues are not susceptible to infection. Furthermore, we studied the expression of M-MuLV genetic information present in target and non-target organs.

I. *Tissue distribution of M-MuLV specific sequences.*

The leukemic animal described in this paper infected at the 4–8 cell stage with M-MuLV (Figs. 1 and 2, Table 1) was derived from a BALB/c 129 cross, both of which are low leukemia incidence strains. The animal had developed the leukemia at two months of age and the pathology of this disease was typical for a Moloney virus-induced lymphatic leukemia and distinctly different from the pathology of a spontaneous leukemia in these mouse strains. This, together with the isolation of N-B tropic virus from the serum, tends to exclude the possibility that the animal developed a spontaneous leukemia but rather indicates that this leukemia

was induced by the infecting M-MuLV. This conclusion is further supported by the hybridization studies.

The DNA-DNA hybridization experiments revealed one class of M-MuLV complementary sequences in uninfected animals and two classes of sequences in the Moloney virus-infected animal (Figs. 1 and 2). The more abundant class of M-MuLV complementary sequences was present at approximately 15–30 copies per haploid genome in infected and uninfected animals. A second less frequent set of Moloney virus-specific sequences was found only in the experimental animal at a frequency of 2–3 copies per haploid mouse genome.

Multiple copies of the endogenous C-type viruses have been detected in a variety of species (1, 4, 20, 21) and the number of copies of endogenous viruses varies considerably, depending on the system studied and the viral probe used. The class of M-MuLV complementary sequences we have detected in uninfected animals may represent sequences of endogenous viruses that are homologous to part of the M-MuLV cDNA.

Eight different tissues derived from all three germ layers of the Moloney virus-infected animal carried the same number of M-MuLV specific DNA copies, regardless of whether the respective organ was infiltrated with lymphoma cells or not. This observation suggests that the virus DNA was integrated into the host genome, possibly at specific sites, rather than existing as an independently replicating plasmid. That chromosomal integration of RNA tumor viruses occurs following exogenous infection has been demonstrated recently (22, 23).

The tissue distribution of viral DNA sequences following infection at the preimplantation stage is in sharp contrast to the situation found after infection of newborn animals with leukemia virus. In chicks infected with avian myeloblastoma virus at one day of age, virus-specific DNA sequences occurred only in cells from tumors or tumor cell infiltrated organs, whereas tumor cell free "non-target" tissues did not contain viral specific sequences (8). The experiments described in Fig. 5 showed similar results. When newborn mice were infected with Moloney virus, virus-specific sequences were found primarily in leukemia target tissues such as thymus and spleen. The amount of virus-specific sequences found in other organs was correlated to the extent of infiltration with lymphoma cells of the respective organ, suggesting that the parenchymal cells did not become infected with virus, although muscle tissue was a possible exception. These observations suggest that susceptibility of the different cells of an animal to virus infection may be determined by the developmental stage of the animal at the time of infection. Once the animal has developed to birth, i. e., to a fully differentiated stage, only certain target tissues are susceptible or accessible to leukemia virus infection. In contrast, when the animal is infected at the 4–8 cell stage, i. e., prior to any detectable cell differentiation, the organ-tropism of the virus does not determine which cells become infected and consequently virus infection can result in animals carrying the virus information in possibly all the differentiated cells of the adult.

II. *Genetic transmission of M-MuLV.*

The experiments discussed above suggest that infection of newborn animals should not lead to germ line integration of the virus. Indeed, in experiments done previously germ line transmission of leukemia virus introduced into mice by

transplacental infection or by infection after birth was never observed (5–7, Table 2). The experiments described in Fig. 4 and Table 2 indicate that infection of preimplantation embryos, on the other hand, can lead to virus integration into the germ line and to genetic transmission of the exogenously infecting virus. The data in Fig. 4 suggest that only one copy of M-MuLV DNA was transmitted vertically, thus resulting in an N-1 animal carrying only one-half copy of M-MuLV-specific DNA per haploid mouse genome. It is likely that the male used for these studies (mouse No. 339 in Table 2) carried the virus integrated at only one site in approximately 10 % of his sperm, suggesting that this animal is characterized by germ line mosaicism analogous to allophenic mice (24). The 50 % transmission of M-MuLV from the N-1 to the N-2 and N-3 generations indicates that the integrated virus behaved essentially like a Mendelian dominant gene. Experiments are presently being conducted to map the integration site.

The observation that non-target organs of viremic N-1 animals carried one-half copy of M-MuLV DNA per haploid mouse genome but target organs like the spleen and the thymus carried two copies per haploid mouse genome (Fig. 4) indicates that during the development of leukemia an amplification of the germ line transmitted virus DNA had taken place. It is not known if this amplification is due to reintegration of the virus into the mouse genome or if the observed amplification is the result of selective chromosomal duplication in the transformed cells. In any event, these observations and the results described in Figs. 1 and 2 and Table 1 suggest that the maximum number of virus copies that can integrate is 2–3 copies per haploid mouse genome. The viremic animals derived from mouse No. 339 (Table 2) might therefore carry M-MuLV integrated into their germ line at only one out of two or three possible integration sites.

III. *Expression of M-MuLV.*

The animal described in Figs. 1 and 2 carried equal amounts of M-MuLV-specific DNA in all tissues tested. This enabled us to compare the expression of the virus information in target and non-target organs of M-MuLV infection.

The virus information present in the cells was expressed to very different extents in the various tissues. For most tissues, the amount of virus-specific RNA found roughly correlated with the degree of infiltration with lymphoma cells in the respective organs. RNA from brain, liver and testes, which histologically showed little or no infiltration contained 50–100 times fewer viral sequences than RNA from the highly infiltrated spleen, lymph nodes and kidneys (Table 1). The lung was the only clear exception in this correlation with no tumor cell infiltration observed in the histological specimen but nevertheless with a relatively high concentration of viral-specific RNA expressed. The contribution in these measurements of viral-specific RNA from virus particles present in the serum has not yet been determined.

Several interrelated aspects of known gene expression and ontogeny may explain these results. One possibility is that viral DNA was transcribed into RNA in lymphoma cells only. The amount of virus-specific RNA found in different tissues would then reflect the extent of infiltration. In any case, it is evident that M-MuLV transcription is one manifestation of differential gene expression in different tissues. All tissues contained the same amount of M-MuLV DNA, so it

is likely that the M-MuLV virus gene came under control of host regulatory elements such as those involved in normal tissue differentiation.

These experiments suggest a second control mechanism involved in the organ-tropism of RNA tumor viruses. If, as in these experiments, an M-MuLV gene is introduced into the DNA of all cells in an animal, its expression still appears to be repressed in all cells except the target cells. Therefore, in addition to organ-tropism at the level of adsorption, penetration or integration of the virus (as shown by the data on M-MuLV infected newborn animals), a second level of organ-tropism appears to be operating at the level of the expression of the integrated virus genome.

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