*Proc. Nat. Acad. Sci. USA*
Vol. 72, No. 10, pp. 4008–4012, October 1975
Cell Biology

# Infection of preimplantation mouse embryos and of newborn mice with leukemia virus: Tissue distribution of viral DNA and RNA and leukemogenesis in the adult animal

(*in vitro* infection of 4–8 cell embryos with Moloney murine leukemia virus/development/leukemia/DNA and RNA annealing kinetics)

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*Communicated by David Baltimore, June 16, 1975*

ABSTRACT **Explanted mouse embryos derived from low leukemia incidence strains were infected with Moloney murine leukemia virus (M-MuLV) at the 4–8 cell stage of development. After cultivation *in vitro* to the blastocyst stage, the embryos were surgically transferred to the uteri of pseudopregnant surrogate mothers. Of 15 animals born, one developed a leukemia at 8 weeks of age. When autopsied, this leukemia was found to be of the lymphatic type, as is typical for the M-MuLV-induced disease. In addition, infectious M-MuLV virus was isolated from the serum.**

**Molecular hybridization tests for the presence of M-MuLV-specific sequences were conducted on DNA and RNA extracted from eight different organs. The DNA·DNA reannealing experiments revealed the presence of two classes of M-MuLV-specific sequences in equal concentrations in all tissues tested. The less abundant class of M-MuLV-specific sequences was not detected in tissues from uninfected animals or in non-target tissues of leukemic animals infected at birth. The results are consistent with the working hypothesis that the virus was integrated in all cells of the animal, possibly including the germ line.**

**Fifty to 100 times more M-MuLV-specific RNA was detected in tumor tissues than was found in non-target organs such as liver, brain, and testes. Since all organs contained the same amount of virus-specific DNA, these results indicate that the M-MuLV-specific DNA can be differentially expressed in different tissues.**

All mouse strains that have been studied carry genetic information of C-type viruses integrated in their genome. Oncogenic C-type virus can be readily isolated from strains such as AKR mice with a high incidence of leukemia, and it has been shown that these endogenous viruses are transmitted vertically (1–3). Virus production in AKR mice is controlled by two dominant loci, one of which has been mapped on linkage group I (4). So far it has not been possible to map the integration site of an exogenously infecting virus, since infection of newborn animals leads to virus integration into a few target tissues only. Most other tissues, notably the germ line, do not become infected (5, 6).

One way to obtain an animal carrying exogenous virus genes in every cell including the germ line would be to infect the animal 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 simian virus 40 (SV 40) DNA carried SV40-specific sequences in some of their organs (7, 8).

We report here the successful infection and development of Moloney murine leukemia virus (M-MuLV)-infected embryos into mature adult mice and the experimental recovery of M-MuLV-specific DNA and RNA sequences from the tissues of one of these animals.

Abbreviations: M-MuLV, Moloney murine leukemia virus; cDNA, DNA complementary to M-MuLV RNA; $C_0t$ and $C_rt$, product of initial DNA and RNA concentration, respectively, and incubation time.

## MATERIALS AND METHODS

**Virus.** M-MuLV clone no. 1 was grown and purified as described (9, 10). Virus stocks were titered by endpoint dilution using the XC plaque test (11).

**Complementary DNA (cDNA).** Virus-specific DNA probes were prepared from purified M-MuLV stock in the presence of 100 μg/ml of actinomycin D; probe synthesis was catalyzed by endogenous RNA-dependent DNA polymerase (9, 10). $^{32}$P-Labeled dCTP and/or dTTP at a specific activity of 100 Ci/mmol (New England Nuclear) was used as radioactive precursor. The DNA synthesized had a specific radioactivity of 60 to 120 × $10^6$ cpm/μg and sedimented between 5.5 and 6 S in alkaline sucrose gradients. It annealed up to 90% to M-MuLV 60–70S RNA.

**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 (Sigma) (12). The embryos were infected with $10^8$ PFU/ml of M-MuLV in medium containing 2 μg/ml of Polybrene (Aldrich) for 5 hr and subsequently cultured *in vitro* for 24 hr. At this time, they were surgically transplanted to the uterine horns of pseudopregnant ICR foster mothers (12).

**Extraction of Nucleic Acids.** Mouse tissues were removed and extracted as described (7, 8). The sodium dodecyl sulfate lysed cells were digested with 50 μg/ml of Pronase and extracted with phenol–chloroform (7, 8). DNA was purified by digestion with RNase (7, 8) and banded in ethidium bromide–CsCl gradients. After sonication (7, 8), the DNA sedimented at approximately 6 S in alkaline sucrose gradients. RNA was purified as described previously (9, 10).

**Molecular Hybridization** DNA·DNA and DNA·RNA hybridizations were carried out as described previously (7–10).

The cell DNA was in a 2 to 10 × $10^6$-fold excess over the $^{32}$P-labeled M-MuLV cDNA. Input radioactivity was 400–800 cpm per experimental point.

## RESULTS

### Infection of 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 6 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.

### Induction of leukemia and isolation of virus

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 2 months of age, the animals were bled and the serum was tested for the presence of murine p30 protein by radioimmune assay (11). Whereas, 14 animals were negative in this test (less than 0.03 μg/ml of serum), one mouse showed a high level of 1.9 μg/ml of serum. Two weeks later this animal was sacrificed. Autopsy and histological examination revealed a typical lymphatic leukemia and massive infiltration of lymph nodes, spleen and kidneys with lymphoma cells, intermediate infiltration of the thymus, and little infiltration of the liver. The lung, brain, and testes did not show any tumor cell infiltration (Table 1).

Infectious N-B tropic virus was recovered from the serum at a titer of 2.5 × $10^4$ PFU/ml (11).

### 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.

Table 1. Detection of M-MuLV-specific DNA and RNA in various tissues of the Moloney-virus-infected mouse

| Organ | Histology: extent of infiltration with lymphoma cells | No. of M-MuLV-specific sequences: Fast-annealing set | No. of M-MuLV-specific sequences: 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. |

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. n.t. = not tested.

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

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 of Fig. 1A were plotted as the reciprocal of the fraction of unhybridized probe versus the $C_0t$ (product of initial DNA concentration and incubation time) normalized to 100% hybridization (3, 13). 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 1100 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 2200 for unique cell DNA. The slopes of this slow-annealing set of sequences in Fig. 2 are about 2.5 times steeper and the slope of the fast-annealing set about 15 times steeper as compared to the self-annealing of cellular DNA. These values and the $C_0t_{1/2}$ values suggest 2–3 copies per haploid genome for the slow-annealing component and about 15–30 copies for the fast-annealing component of M-MuLV-specific sequences. Thus, the Moloney-virus-infected animal acquired 2–3 copies of Moloney-virus-specific sequences not present in control animals (Table 1).

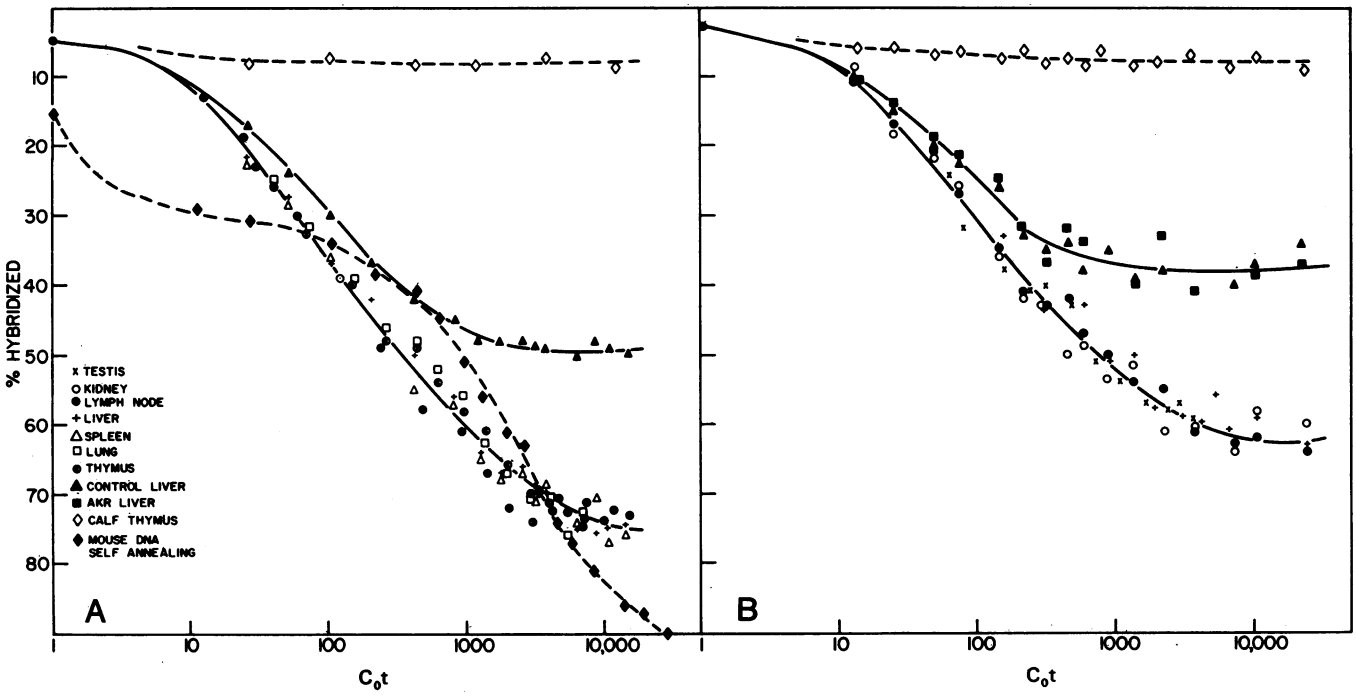


FIG. 1. Association kinetics of $^{32}$P-labeled M-MuLV cDNA with mouse cellular DNA derived from various organs of the mouse infected at the 4–8 cell stage with M-MuLV, from control mice (BALB/c 129 and AKR), or from calf thymus. The reaction mixture containing 0.5 ng/ml of $^{32}$P-labeled M-MuLV cDNA ($1.2 \times 10^8$ cpm/$\mu$g), 4 mg/ml of mouse or calf thymus DNA in 0.01 M Tris·HCl, pH 7.0, 1 mM EDTA, 1 M NaCl, was heat denatured and incubated at 68°. Percent hybridization is plotted as a function of $C_0t$, corrected to standard annealing conditions (3). In replicate experiments, the two different cDNA probes used annealed to 80% (A) or 70% (B) to M-MuLV 60–70S RNA.

## 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 efficiently hybridized with the M-MuLV cDNA, while control RNA annealed only small amounts at extremely high $C_rt$ values ($C_rt$ is product of RNA concentration and incubation time).

The amount of M-MuLV-specific RNA present in the various organs was determined from the half-saturation values of hybridization ($C_rt_{1/2}$) (9, 10), 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 to 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 level 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.

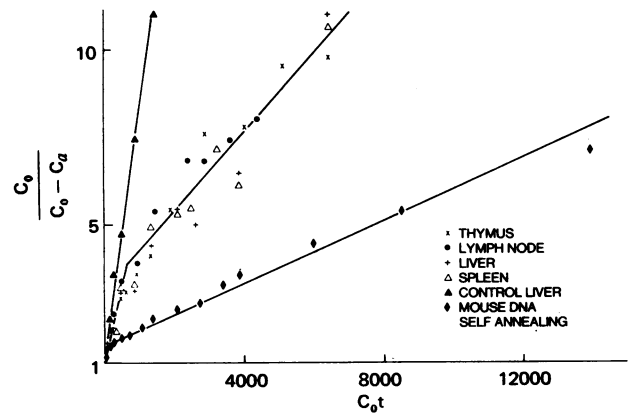


FIG. 2. Analysis of the association kinetics of $^{32}$P-labeled M-MuLV cDNA with mouse DNA by the method of Wetmur and Davidson (13). The results are plotted as the reciprocal of the fraction of DNA remaining single-stranded as a function of $C_0t$. The maximum observed hybridization was normalized to 100%. ($C_0$ = fraction of single-stranded DNA at time 0, $C_a$ = fraction of single-stranded DNA at different times.) The data are taken from Fig. 1A.

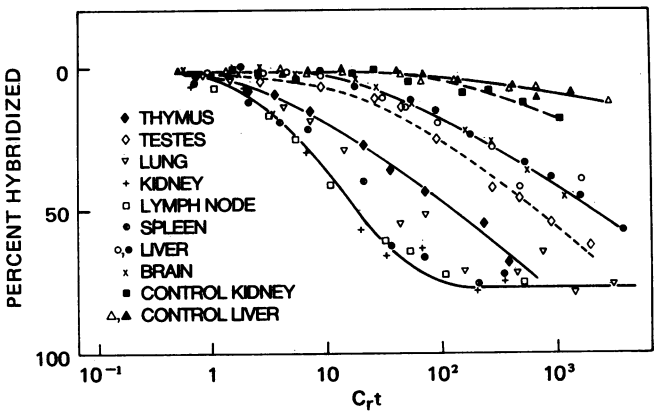


FIG. 3. Annealing of labeled M-MuLV cDNA with cellular RNA from different organs of the M-MuLV-infected and control mice. Annealing in conditions of RNA excess was performed as described (9, 10). Percent hybrid formation is displayed as a function of $C_rt$, corrected to standard annealing conditions (9, 10).

### 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, 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 4 months of age, this animal appeared terminally ill with extensive tumor cell infiltration in many organs. Radioimmunoassay revealed 2 $\mu$g/ml of 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. 4). The slopes of the DNA annealing kinetics revealed two sets of virus-specific 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 and 2), the curves indicate that different amounts of the slow-annealing set of 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 histologically 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).

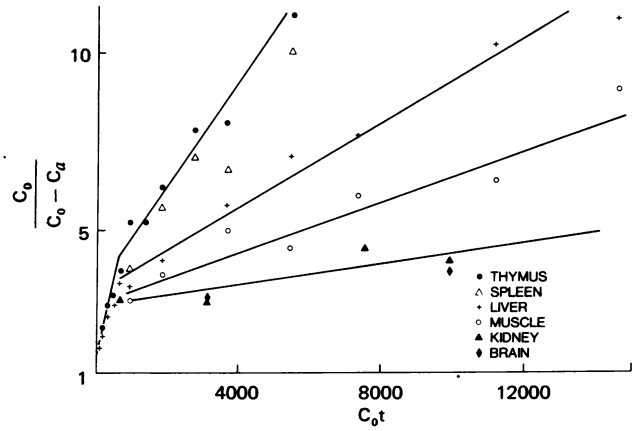


FIG. 4. Analysis of the association kinetics of $^{32}$P-labeled M-MuLV cDNA with mouse DNA extracted from an animal infected after birth with M-MuLV. The reaction mixture containing 0.2 ng/ml of $^{32}$P-labeled M-MuLV cDNA ($8 \times 10^7$ cpm/$\mu$g) and 6 mg/ml of mouse DNA extracted from different 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.

## DISCUSSION

The leukemic animal described in this paper infected at the 4–8 cell stage with M-MuLV was derived from a BALB/c × 129 cross, both strains of which are low leukemia incidence. The animal had developed the leukemia at 2 months of age and the pathology of this disease was typical for a Moloney-virus-induced lymphatic leukemia. This, together with the isolation of N-B tropic virus from the serum, indicates that the 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-specific sequences in uninfected animals and two classes of sequences in the Moloney-virus-infected animal. The more abundant class of M-MuLV specific 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 DNA copies of endogenous C-type viruses have been detected in a variety of species (1–3, 14, 15) and the number of copies of endogenous viruses varied considerably, depending on the system studied and the viral probe used. The class of M-MuLV-specific 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 (16, 17).

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. Chicks infected with avian myeloblastosis virus (AMV) at one day of age carried virus-specific DNA sequences only in cells from tumors or tumor-cell-infiltrated organs, whereas tumor-cell-free "non-target" tissues did not contain viral specific sequences (5). The experiments described in Fig. 4 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 dif-

ferent 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 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.

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.

These results suggest 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. Since all tissues contained the same amount of M-MuLV DNA, this would postulate that the virus gene came under control of host regulatory elements, possibly those involved in normal tissue differentiation.

A larger series of experiments has been started, to repeat the observations described in this paper. So far, from 44 mice born after infection with Moloney leukemia virus at the preimplantation stage, three have developed elevated gs (group-specific) antigen titers at four weeks of age. Preliminary evidence suggests that M-MuLV can be integrated into the germ line and transmitted vertically to the offspring.

We thank Dr. F. Dixon for his support. The excellent technical assistance of V. Cox and J. Dausman is gratefully acknowledged. This work was supported in part by a Basil O'Connor Starter Grant from the National Foundation, by National Institutes of Health Grant nos. CA-15561-02 and CA-15747-02. B.C. received a postdoctoral fellowship, J-241, from the American Cancer Society.

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