Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1874–1878, March 1982
Cell Biology

Differentiation and virus expression in BALB/Mo mice: Endogenous Moloney leukemia virus is not activated in hematopoietic cells

(transplantation of hematopoietic stem cells/*Mov-1* genome expression/Southern blotting technique/viremia)

WALTER FIEDLER, PETER NOBIS, DETLEV JÄHNER, AND RUDOLF JAENISCH

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

Communicated by George Klein, November 16, 1981

ABSTRACT **The endogenous Moloney leukemia virus (M-MuLV) in the BALB/Mo substrain of mice is activated during the first week after birth. Virus replication occurs in cells of the lymphatic system. Lymphoid cells therefore represent the target cells for virus replication and leukemic transformation. To investigate whether virus activation occurs during lymphoid cell differentiation, hematopoietic stem cells carrying the endogenous *Mov-1* genome were transplanted to sublethally irradiated BALB/c mice. Effective colonization of the recipients was demonstrated by Southern DNA hybridization. No activation of the endogenous *Mov-1* genome occurred during a 4-month observation period after the transplantation. Thus the first step in development of disease in BALB/Mo mice involves activation of the *Mov-1* locus in a nonhematopoietic cell followed by superinfection of lymphatic cells and subsequent virus replication and virus spread.**

Murine leukemia viruses (MuLVs) such as the AKR and Moloney strains integrate the proviral genome into lymphoid cells and transform specifically T cells by an unknown mechanism (1, 2). BALB/Mo mice transmit the exogenous Moloney MuLV (M-MuLV) as a Mendelian gene (3). The viral genome was shown to be integrated on chromosome 6, and this genetic locus was named *Mov-1* (4). Virus is activated soon after birth, and activation is followed by somatic proviral DNA amplification and virus expression in the lymphatic tissues—spleen and thymus (5, 6). Thus, cells of the lymphatic tissues represent the target cells for virus infection and for virus replication and therefore carry two types of M-MuLV copies: the endogenous, genetically transmitted copy at the *Mov-1* locus and somatically acquired "exogenous" copies. Thymus-specific leukemia develops several months later. All three events—i.e., virus expression, provirus DNA amplification, and development of leukemia—can be prevented by injecting newborn BALB/Mo mice with anti-M-MuLV antiserum (7). Thus, superinfection of lymphoid cells appears to be an essential step for the development of viremia. The available evidence suggests that activation of the endogenous M-MuLV in BALB/Mo mice as well as in other substrains developed in this laboratory occurs at specific stages of development and differentiation (8, 9). To better understand the relationship between differentiation and virus genome activation, we investigated whether activation of the *Mov-1* genome occurs in the same cell type that is the target cell for replication and transformation. For this, hematopoietic stem cells of BALB/Mo mice carrying only the endogenous *Mov-1* genome were transplanted to BALB/c recipients. Our results indicate that the viral genome is not activated during hematopoietic cell differentiation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mice. BALB/Mo mice were derived as reported (3, 10). Mice heterozygous at the *Mov-1* locus [(BALB/c × BALB/Mo)F_1] were derived by mating BALB/Mo males with BALB/c females. Timed matings were performed to obtain embryos of defined development stages.

Radioimmunoassay. The concentration of the M-MuLV internal protein p30 was determined by competitive radioimmunoassay with radioiodinated M-MuLV p30 and rabbit antiserum directed at p30 (7, 11).

Cell Transfer. Single cell suspensions were prepared by mincing organs in Dulbecco's modified Eagle's medium. The cells were filtered through a Nyltex 100-μm-pore filter to remove the stromal tissues. Bone marrow was obtained by flushing the femur with the modified Eagle's medium. The cells were washed twice by centrifugation and resuspended in the modified Eagle's medium. Viable cells were counted by use of trypan blue solution, and 10^7, 10^6, or 10^5 cells were injected into the tail vein of irradiated mice.

Irradiation of Mice. BALB/c males were irradiated with 800 to 900 rads (1 rad = 0.01 gray), using an x-ray source (12).

DNA Extraction and Restriction Enzyme Analysis. Spleen, thymus, and liver were obtained from recipient BALB/c mice 4 months after transfer and high molecular weight DNA was extracted as described (6).

The DNA was digested with *Eco*RI restriction endonuclease, separated on 0.5% agarose gels, blot transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled M-MuLV cDNA as described (6).

Antiserum Treatment. Antiserum-treated mice heterozygous at the *Mov-1* locus were obtained as described (7). BALB/c females mated with BALB/Mo homozygous males were injected with 0.3 ml of rabbit anti-M-MuLV serum at day 15 of gestation. The newborn mice were injected with antiserum at the day of birth and subsequently at weekly intervals (7).

RESULTS

Virus Is Not Activated in Transplanted BALB/Mo F_1 Cells. We have shown previously that viremic mothers transmit virus efficiently to their offspring by congenital infection (13). Therefore, to obtain donor animals that have not been exposed to maternal exogenous virus, cells were isolated from embryos or adults heterozygous at the *Mov-1* locus [(BALB/c × BALB/Mo)F_1]. These animals were derived by mating BALB/c females with homozygous BALB/Mo males and for simplicity will be referred to as BALB/Mo F_1 mice.

Abbreviations: MuLV, murine leukemia virus; M-MuLV, Moloney strain of MuLV; kb, kilobase(s).

Table 1. Survival of irradiated animals reconstituted with BALB/Mo F_1 cells

Donor	Cells transferred: Type	Cells transferred: Number	Reconstituted animals, survivors/total	Control animals, survivors/total
Embryos day 15	Liver	10^7	1/1	3/7
		10^6	5/6	
		10^5	0/2	
Embryos day 18–19	Liver	10^7	1/3	4/25
		5×10^6	4/4	
		10^6	8/8	
		10^5	2/4	
	Thymus	10^6	7/7	
Two-month-old antibody-treated adult	Bone marrow	10^7	1/1	1/7
		10^6	1/1	
		10^5	1/1	
	Thymus	10^7	1/1	
		10^6	1/1	
	Spleen	10^7	1/1	
		10^6	2/2	
		Total	36/43 (83%)	8/39 (20%)

Four- to 5-week-old BALB/c mice were irradiated with 800–900 rads and transplanted with BALB/Mo F_1 cells. Animals in the control group did not receive cells. All animals were observed for a period of 4 months.

Fetal liver or thymus cells from BALB/Mo F_1 embryos or cells from spleen, bone marrow, or thymus from adults that had been treated with anti-M-MuLV antiserum were transferred to irradiated BALB/c mice. It has been shown that no infectious virus is present in BALB/Mo F_1 embryos (9) or in anti-M-MuLV-treated BALB/Mo F_1 adults (7). Thus no "exogenous" M-MuLV copies were present in the transferred cells, and development of virus in the recipient animals would be due to activation of the endogenous M-MuLV genome.

Table 1 shows that 20% of BALB/c mice survived irradiation with 800–900 rad. Survival was increased to approximately 80% when the irradiated mice were reconstituted with liver, thymus, spleen, or bone marrow cells from BALB/Mo F_1 embryos or from adult mice. The results suggest that transplanted BALB/Mo F_1 cells colonized the irradiated recipients efficiently.

Virus activation was monitored by testing the recipients for viremia. The animals were bled every other week for a period of 4 months and the serum was tested for M-MuLV p30 by using a radioimmunoassay. Table 2 shows that none of the recipients developed viremia after being grafted with as many as 10^7 cells from BALB/Mo F_1 embryos or anti-M-MuLV-treated adults.

The following controls were performed to estimate the number of infectious units required to induce viremia in adult recipients. Unirradiated 8-week-old BALB/c mice were grafted with serial dilutions of spleen or thymus cells that were obtained from untreated, viremic adult BALB/Mo F_1 mice. Table 3*A* shows that viremia developed within 2–3 weeks even in recipients of as few as 100 cells. We have estimated previously that 10 spleen cells of an adult BALB/Mo mouse can form one infectious center (7). Furthermore, serial dilutions of a virus preparation were injected into BALB/c recipients and in parallel quantitated in an XC assay. The results in Table 3*B* demonstrate that as few as 10 infectious units as quantitated in the XC assay were able to induce viremia when injected into normal adult recipients.

Protein extracts were prepared from spleens of grafted recipients and analyzed for the presence of M-MuLV by competitive radioimmunoassay. Table 4 shows that the concentration of p30 is approximately 1 ng/mg of protein in normal BALB/c mice and about 50–300 ng/mg of protein in viremic BALB/Mo F_1 mice, confirming previous observations (7). A slight increase between 1 and 5 ng of p30 per mg of protein was found in extracts from reconstituted recipients. This increase was similar in irradiated BALB/c mice that had not received BALB/Mo F_1 cells and probably represented activation of murine endogenous viruses due to irradiation. The results in Tables 2 and 4 demonstrate that neither infectious virus nor p30 is induced in the BALB/Mo F_1 donor cells.

Table 2. p30 in serum of animals reconstituted with BALB/Mo F_1 cells (age 3–6 months)

Donor	Cells transferred: Type	Cells transferred: Number	Animals with p30/total grafted
Embryos day 15	Liver	10^7	0/1
		10^6	0/5
Embryos day 18–19	Liver	10^7	0/1
		5×10^6	0/4
		10^6	0/8
	Thymus	10^6	0/7
Two-month-old antibody-treated adult	Spleen	10^7	0/1
		10^6	0/2
	Bone marrow	10^7	0/1
		10^6	0/1
		10^5	0/1
	Thymus	10^7	0/1
		10^6	0/1
		Total	0/34

BALB/c mice were grafted with BALB/Mo F_1 cells as described in Table 1. All recipients were kept for 4 months and tested for viremia at biweekly intervals, using a radioimmunoassay (7).

Detection of BALB/Mo F_1 Cells in Recipients. Molecular hybridization was used to demonstrate the presence of BALB/Mo F_1 cells in BALB/c recipients. DNA was isolated from spleens, thymuses, and livers and was digested with *Eco*RI, separated on agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled M-MuLV cDNA as described (6). Because BALB/Mo mice carry the endogenous M-MuLV copy (*Mov-1* locus) in a 27-kilobase (kb) *Eco*RI fragment, this was used as a marker to distinguish DNA of BALB/Mo F_1 and BALB/c origin. Fig. 1*A* shows the analysis of liver, spleen, and thymus DNA of a 5-month-old BALB/c recipient that had been reconstituted with liver cells from a 15-day BALB/Mo F_1 embryo 4 months previously. In DNA from spleen and thymus, but not in liver of the same animal, a 27-kb fragment characteristic of the *Mov-1* locus in BALB/Mo mice was detected (lanes d–f). The multiple bands of lower molecular weight represent sequences of endogenous murine type C viruses cross-reacting with the probe. Similarly, the 27-kb *Mov-1* fragment was seen in DNA from thymus and spleen of BALB/c recipients that were reconstituted with cells of a liver from a 19-day-old BALB/Mo F_1 embryo (Fig. 1*B*, lanes d–f) or with bone marrow or spleen cells from an anti-M-MuLV-treated adult (Fig. 1*C*). No 27-kb fragment was seen in the liver of the same animals.

To estimate the fraction of BALB/Mo F_1 cells in the BALB/c recipients, reconstitution experiments were performed. DNA from animals homozygous or heterozygous at the *Mov-1* locus (Fig. 1 *A* and *B*, lanes a and b) and a 1:1 mixture of both heterozygous BALB/Mo DNA and BALB/c DNA (Fig. 1 *A* and *B*, lanes c) were analyzed. Because the donor cells in the grafting experiments had been embryos heterozygous at the *Mov-1* locus [(BALB/c × BALB/Mo)F_1], the intensity of the 27-kb band in DNA from heterozygous BALB/Mo mice (lanes b) corresponds to a pure population of BALB/Mo F_1 cells, whereas the intensity in lane c corresponds to the presence of 50% BALB/Mo F_1 cells.

By comparing the intensity of the 27-kb band in DNA from the experimental animals with that in DNA of the reconstruction experiments, it is estimated that the cell population in spleen and thymus of the animal in Fig. 1*A* contained 50–70% BALB/Mo F_1 cells; in the same organs from the animals in Fig. 1*B* and *C*, 20–50% were of BALB/Mo F_1 origin. Analyses of the kind shown in Fig. 1 confirmed that BALB/Mo F_1 cells had repopulated the reconstituted BALB/c recipients and that 4 months after grafting between 20% and 70% of the lymphatic cell population were of donor origin.

Table 3. Injection of normal BALB/Mo F_1 cells or of virus into unirradiated BALB/c mice

(*A*) Grafting of BALB/Mo F_1 cells

Cells transferred		Animals with p30/total grafted
Type	Number	
Spleen	10^6	1/1
	10^5	1/1
	10^4	1/1
	10^3	1/1
	10^2	1/1
Thymus	10^6	1/1
	10^5	1/1
	10^4	1/1
	10^3	1/1
	10^2	1/1
	Total	10/10

(*B*) Infection with virus

Dilution of virus	NIH 3T3 cultures inducing XC plaques/total infected	Animals with p30/total injected
10^{-1}	1/1	1/1
10^{-2}	2/2	1/1
10^{-3}	2/2	1/1
10^{-4}	4/4	2/2
10^{-5}	3/4	1/2
10^{-6}	1/4	0/2

Eight-week-old BALB/c mice were grafted with cells from adult, viremic BALB/Mo F_1 mice (*A*) or with serial dilutions of a virus preparation obtained from a tissue culture supernatant of fibroblasts chronically producing M-MuLV (*B*). To quantitate the virus, independent dishes with NIH 3T3 cells were infected with serial dilutions of the virus. The fraction of infected cultures inducing XC plaques after several passages is given in *B*. All injected animals were tested for development of viremia as described for Table 2. Grafted animals became positive for p30 within 2–3 weeks.

Table 4. p30 in spleens of animals reconstituted with BALB/Mo F_1 cells

Donor	Cells transferred		p30 content in spleen extracts, ng p30/mg protein
	Type	Number	
Embryos day 15	Liver	10^7	0.9
		10^5	1.3
Embryos day 18	Liver	10^7	9.0
		10^6*	2.8
		10^6*	5.0
Embryos day 19	Liver	10^6*	3.7
		10^6*	2.9
	Thymus	10^6	2.5
Two-month-old antibody-treated adult	Spleen	10^7	4.7
		10^6	1.8
	Bone marrow	10^7	2.6
	Thymus	10^7	1.9
		Mean	3.2
BALB/c			1
BALB/Mo			50–300
Irradiated control BALB/c			3.3

Extracts from spleens were prepared 4 months after reconstitution or from control mice and the concentration of p30 was determined by competitive radioimmunoassay (7, 11).
* Cells from different embryos were tested independently.

DISCUSSION

The endogenous M-MuLV is activated invariably in every BALB/Mo mouse during the first week after birth (3, 9), leading to a predominantly lymphotropic expression of M-MuLV (5). The age-dependent kinetics of expression of viral RNA and of appearance of infectious virus in different tissues of BALB/Mo mice has been quantitatively determined previously (5, 7, 9). Other substrains of mice developed in this laboratory activate virus at different stages during their development, leading to distinct phenotypes of tissue-specific virus expression. In the Mov-13 substrain, for example, virus is activated during embryogenesis, leading to virus expression in lymphatic as well as nonlymphatic tissue (9).

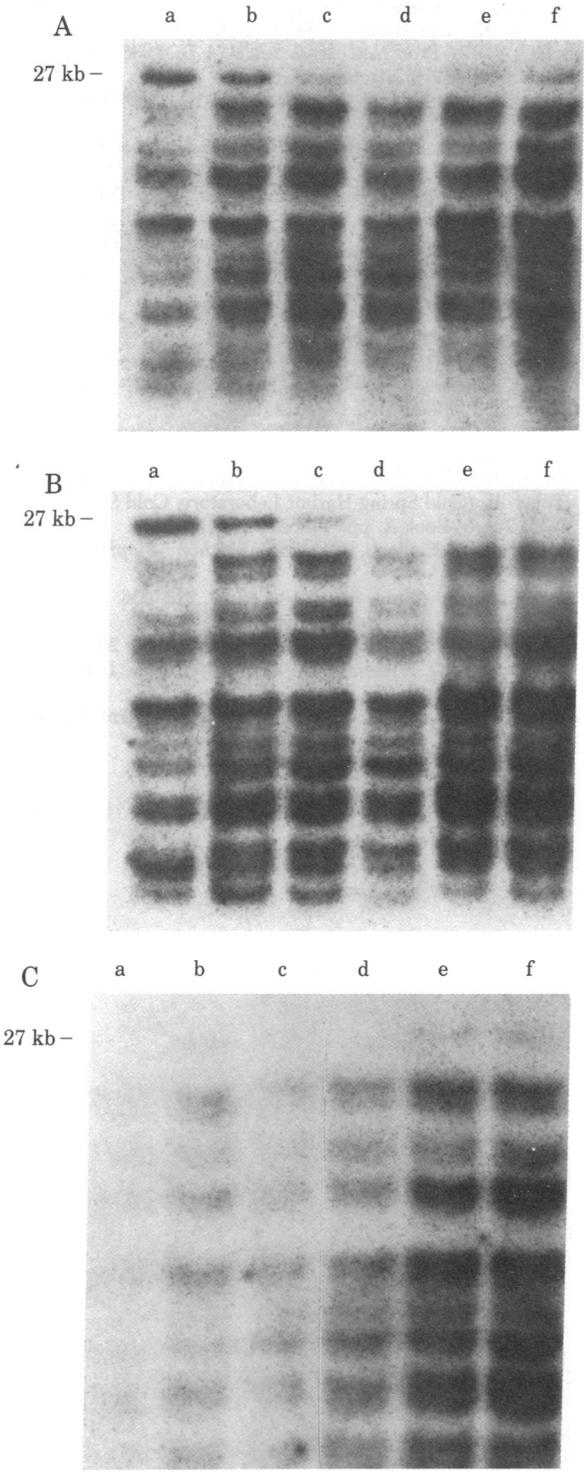

FIG. 1. Detection of M-MuLV-specific sequences in organs of reconstituted BALB/c mice. Irradiated BALB/c mice were transplanted with BALB/Mo F_1 cells as described in Table 1. DNA was extracted from different organs of the recipients 4 months later and analyzed for the presence of *Mov-1* sequences by restriction enzyme analysis with *Eco*RI (6). Controls were from mice homozygous or heterozygous at the *Mov-1* locus or from a 1:1 mixture of heterozygous BALB/Mo and BALB/c mice. (*A*) Lanes: a, homozygous BALB/Mo liver; b, heterozygous BALB/Mo liver; c, 1:1 mixture heterozygous BALB/Mo and BALB/c; d, liver from BALB/c, reconstituted with 10^7 liver cells from BALB/Mo F_1 embryo at day 15 of gestation; e, spleen, same animal as in d; f, thymus, same animal as in d. (*B*) Lanes: a, homozygous BALB/Mo; b, heterozygous BALB/Mo liver; c, 1:1 mixture of heterozygous BALB/Mo and BALB/c liver; d, liver from BALB/c, reconstituted with 10^6 liver cells from BALB/Mo F_1 embryo at day 19 of gestation; e, spleen, same animal as in d; f, thymus, same animal as in d. (*C*) Lanes: a, liver from BALB/c, reconstituted with 10^7 bone marrow cells from a 2-month-old anti-M-MuLV-treated BALB/Mo F_1 mouse; b, spleen, same animal as in a; c, thymus, same animal as in a; d, liver from BALB/c, reconstituted with 10^7 thymus cells from a 2-month-old anti-M-MuLV-treated BALB/Mo F_1 mouse; e, spleen, same animal as in d; f, thymus, same animal as in d.

Two mechanisms of virus activation can be envisaged: (*i*) Activation of the exogenous viral genome occurs first in a specific population of cells at a specific stage of development. (*ii*) Activation of virus can occur in any cell with a given probability. This probability would be higher in Mov-13 than in BALB/Mo mice. The tissue-specific pattern of virus expression in the adult then would be due to superinfection of target cell populations that are different at different stages of development (13).

Previous experiments have shown that viral RNA in BALB/Mo mice is detected first in spleens and thymuses of newborn animals. This led to the hypothesis that lymphatic cells may be the ones in which the endogenous *Mov-1* locus is activated (5, 8). However, because lymphatic cells acquire new exogenous M-MuLV copies by superinfection, it was not possible to decide whether virus replication reflected the primary transcription of the endogenous *Mov-1* gene in the lymphatic cell or, rather, was due to superinfection with virus activated in some other cells.

The present experiments were undertaken to distinguish between these possibilities. Hematopoietic and lymphopoietic cells carrying the endogenous M-MuLV genome at the *Mov-1* locus only were transplanted to BALB/c mice. The recipients were irradiated with a sublethal dose to enhance survival and colonization by the donor cells. The overall survival rate of the irradiated mice increased from 20% to 80% after reconstitution, suggesting that donor cells had colonized the recipients. This conclusion was supported by the restriction enzyme analysis of DNA from spleen and thymus (Fig. 1), showing that 20–70% of the cells in lymphatic tissues of the transplanted BALB/c mice were of BALB/Mo F_1 origin. Although we have not characterized the type of BALB/Mo F_1 cells present, it appears likely that pluripotent stem cells from fetal liver, bone marrow, and spleen give rise to clones that differentiated along lymphopoietic as well as erythropoietic pathways (14–16). Transplanted thymic cells, however, should contain committed stem cells with a restricted potential for differentiation only. This is compatible with the observation that the fraction of BALB/Mo F_1 cells in animals reconstituted with 10^7 thymus cells was lower than in animals reconstituted with 10^7 fetal liver cells (Fig. 1).

None of the recipients developed viremia or had p30 in their lymphatic tissues during a period of 4 months after transplantation. Viremia is a highly sensitive indicator for virus expression because injection of as few as 10 infectious units into unirradiated adults will lead to a general infection of the hematopoietic system followed by high virus titers in the serum (Table 3). Our results therefore indicate that the *Mov-1* locus was not activated in donor cells, which presumably had undergone all steps of lymphopoietic and erythropoietic cell differentiation (15) during a 4-month period after their transfer.

The hematopoietic system has a high cell turnover rate. It has been estimated, for example, that the total population of 2×10^8 cells in the thymus of a 6-week-old mouse is renewed every 4–5 days (17). Assuming that 500–5000 pluripotent stem cells are present in 10^7 fetal liver or adult bone marrow cells (16, 18), it could be estimated that 35–40 cell divisions were required to repopulate just the thymus of the irradiated host and to permit cell renewal for a period of 4 months after transplantation.

Our results therefore exclude the possibility that M-MuLV originates from hematopoietic cells in BALB/Mo mice, meaning that virus is activated neither during differentiation nor after a large number of cell divisions. Recent experiments analyzing the DNA methylation pattern also argued against expression of the *Mov-1* locus in lymphatic organs of BALB/Mo mice (19). That cell division *per se* does not activate the *Mov-1* locus is also supported by the failure of BALB/Mo fibroblasts to produce virus even after prolonged growth in tissue culture (4, 20). Therefore infectious virus appearing in every BALB/Mo animal during the first week after birth (5, 9) probably originates in cells of nonhematopoietic origin. Our results indicate that virus is activated at a specific stage of differentiation in a specific cell population, as has been proposed earlier (8). The mechanism of tissue-specific genome activation, however, remains to be defined.

We thank Drs. M. Breindl, C. Stewart, and F. Lehmann-Grube for constructive criticism. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk. The Heinrich-Pette-Institut is supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie und Gesundheit. This study was performed as part of the doctoral thesis of W.F. at the University of Hamburg.

1. Bishop, M. (1978) *Annu. Rev. Biochem.* **47**, 35–88.
2. Hayward, M. & Neal, B. (1980) *Curr. Top. Microbiol. Immunol.* **91**, 217–276.
3. Jaenisch, R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1260–1264.
4. Breindl, M., Doehmer, J., Willecke, K., Dausmann, J. & Jaenisch, R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1938–1942.
5. Jaenisch, R. (1979) *Virology* **93**, 80–90.
6. Jähner, D., Stuhlmann, H. & Jaenisch, R. (1980) *Virology* **101**, 111–123.
7. Nobis, P. & Jaenisch, R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3677–3681.
8. Jaenisch, R. (1980) in *The Molecular Biology of Tumor Viruses*, ed. Stephenson, J. (Academic, New York), pp. 131–162.
9. Jaenisch, R., Jähner, D., Nobis, P., Simon, J., Löhler, J., Harbers, K. & Grotkopp, D. (1981) *Cell* **24**, 519–529.
10. Jaenisch, R. (1977) *Cell* **12**, 691–696.
11. Strand, M., Lilly, F. & August, J. T. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3682–3686.
12. Storer, J. B. (1975) in *Biology of the Laboratory Mouse*, ed. Green, E. (Dover, New York), 2nd Ed., pp. 427–446.
13. Jaenisch, R. (1980) *Cell* **19**, 181–188.
14. Johnson, G. & Metcalf, D. (1978) in *Differentiation of Normal and Neoplastic Hematopoietic Cells*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Clarkson, B., Marks, P. A. & Till, J. E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 5, Book A, pp. 49–62.
15. Till, J. & McCulloch, E. (1980) *Biochim. Biophys. Acta* **605**, 431–460.
16. Suzuki, S. & Axelrad, A. (1980) *Cell* **19**, 225–236.
17. Bryant, B. J. (1972) *Eur. J. Immunol.* **2**, 38–45.
18. Cantor, H. & Weissman, I. (1976) *Prog. Allergy* **20**, 1–64.
19. Stuhlmann, H., Jähner, D. & Jaenisch, R. (1981) *Cell* **26**, 221–232.
20. Bacheler, L., Jaenisch, R. & Fan, H. (1979) *J. Virol.* **29**, 899–906.