JOURNAL OF VIROLOGY, Nov. 1989, p. 4857–4865
0022-538X/89/114857-09$02.00/0

Vol. 63, No. 11

Transfer of a Mutant Dihydrofolate Reductase Gene into Pre- and Postimplantation Mouse Embryos by a Replication-Competent Retrovirus Vector

HEIDI STUHLMANN,[1]† RUDOLF JAENISCH,[1,2] AND RICHARD C. MULLIGAN[1,2]*

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142,[1] and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[2]

Received 18 May 1989/Accepted 1 August 1989

In order to explore the potential of retrovirus vectors for efficiently transferring foreign genes into mouse embryos, a replication-competent recombinant Moloney murine leukemia virus (Mo-MLV) vector carrying a mutant dihydrofolate reductase (DHFR) cDNA insert in the U_3 region of the viral long terminal repeat was used to infect pre- and postimplantation embryos. When preimplantation mouse embryos were infected with the vector, as expected, the provirus integrated into the embryos and the germ line with the same efficiency as that observed with wild-type Mo-MLV, leading to inactivation of the recombinant virus. In contrast, when postimplantation mouse embryos were microinjected with virus-producing cells, between 90 to 100% of the surviving animals proved to be infected with the virus. The recombinant virus spread as efficiently as wild-type Mo-MLV in the infected embryos, resulting in up to three to five proviral copies per genome in heart, thymus, and brain tissues. Substantial expression of mutant DHFR*-coding viral message was found in all somatic tissues analyzed, the amounts correlating with the proviral copy number in the respective organ. These results suggest that replication-competent vectors are useful for efficient transfer and expression of foreign genes into tissues or whole animals when virus spread is needed.

Retrovirus vectors are powerful tools for the transfer of foreign genes because they have been shown to infect efficiently a variety of cells in vitro and in vivo (16, 18, 33–35). However, one potential use for the vectors that has not yet been fully exploited is to introduce and express foreign genes in mice at different stages of development. This approach would greatly facilitate the understanding of mechanisms of expression of defined genes during development as well as of the effect of foreign gene expression on development. Furthermore, retrovirus-mediated gene transfer might be used to mark cell lineages during development as has been demonstrated already in other systems (16, 18, 21, 25, 31, 33).

Most retrovirus vectors described so far are replication defective and require helper functions for packaging their RNA into infectious virus particles. Such replication-defective vectors have been successfully used for the introduction of several genes into preimplantation mouse embryos (7, 13, 24, 28, 32). However, as has been shown for wild-type (wt) virus, expression of these vectors has been shown to be suppressed when the gene is under the control of the viral long terminal repeat (LTR) (13, 28). In contrast, when replication-competent Moloney murine leukemia virus (Mo-MLV) is used to infect postimplantation mouse embryos between days 8 and 10 of embryogenesis, no inactivation of viral genes occurs and the virus can replicate unrestricted in most somatic tissues (9, 15). In an attempt to exploit that property, we previously used a replication-defective retrovirus for infection of postimplantation mouse embryos and showed that the bacterial marker gene Ecogpt can be introduced and expressed in many somatic tissues of the infected animal (29). However, the fraction of cells infected with the vector was very low, indicating the need for virus spread after infection of postimplantation embryos (29).

In order to overcome this limitation, we have constructed Mo-MLV-derived replication-competent vectors carrying in addition a selectable gene. A series of vectors carrying inserts with a mutant dihydrofolate reductase (DHFR*) cDNA (26) as a selectable marker gene in the U_3 region of the viral LTR have been constructed and analyzed in vitro for stability of the virus and expression of DHFR (30). In this report, we describe the use of such a replication-competent vector for introducing the mutant DHFR* gene at different stages of embryogenesis. We examined the efficiency of infection, the levels of DHFR* expression, and the properties of the recombinant virus in vivo. In addition, we explored the possibility of methotrexate selection of the infected embryos in vivo.

MATERIALS AND METHODS

Mice, cell lines, and mouse embryo infection. BALB/c C57BL/6J, and CBA mice were obtained from the Jackson Laboratories and bred in our mouse colony. NIH 3T3, XC cells, and DHFR*-5H-E cells (30) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum, penicillin, and streptomycin. The clone DHFR*-5H-E was derived by three passages of MLV DHFR*-5 in vitro without methotrexate selection and subsequent single-cell cloning. DHFR*-5H-E cells contain 15 to 20 proviral copies per cell and produce a titer of 10^7 XC plaques per ml and 5×10^5 Mtx^r colonies per ml, respectively.

Four- to sixteen-cell C57BL/6J preimplantation embryos were isolated and cocultivated with DHFR*-5H-E cells for 24 to 28 h, as described previously (11, 14). Embryos which had developed to the late morula-blastocyst stage were transferred into the uteri of pseudopregnant (C57 male × CBA female) foster females.

Postimplantation mouse embryos were microinjected in

* Corresponding author.
† Present address: Department of Biochemistry, Stanford University Medical Center, Stanford, CA 94305.

situ at day 9.5 of gestation (vaginal plug counted as day 1) with 10^3 to 10^4 DHFR*-5H-E cells, as described previously (9, 29). The cells were treated before microinjection with 10 μg of mitomycin C per ml for 3 h to prevent mitosis. The presence of p30 in the serum of mice was determined by a competitive radioimmunoassay as described previously (20).

DNA preparation and Southern hybridization analysis. High-molecular-weight DNA from embryo and adult tissues was prepared as described previously (10). Agarose gel electrophoresis and Southern hybridization was performed by standard procedures (19), using ZETABIND membranes (AMF/Cuno, Inc.) for DNA transfer. A 660-base-pair (bp) DHFR* cDNA fragment was nick translated by using standard procedures (19).

RNA preparation and S1 nuclease analysis. Organs from embryos or adult mice were removed and quick-frozen in liquid N_2. The tissues were homogenized in 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7)–0.5% sarcosyl–0.1 M β-mercaptoethanol–1 drop of Antifoam A (Sigma Chemical Co.), using a polytron (Brinkmann Instruments, Inc.). Total cellular RNA was prepared by the method of Chirgwin et al. (2) as described by Cone et al. (3).

S1 nuclease analysis was performed essentially as described by Hentschel et al. (5), using 1 μg of total cellular and 5 μg of *Saccharomyces cerevisiae* tRNA as carrier for each reaction. A ^{32}P-5′-end-labeled 840-bp *Ssp*I-*Bam*HI fragment and a 1,300-bp *Ssp*I-*Sca*I fragment from a pLTR subclone (22) containing a simian virus 40 (SV40) promoter-DHFR* cDNA insert were used as probes. Hybridization was performed with RNA concentrations of 0.5 μg/ml at 52°C for 18 h. The hybrids were digested with 30 U of S1 nuclease (Boehringer Mannheim Biochemicals) for 1 h at 37°C. Protected DNA fragments were analyzed by electrophoresis through 7% polyacrylamide-urea gels.

Infectious center assays. To analyze virus production in embryonal tissues, single-cell suspensions were prepared by a limited digestion of tissue (30 to 60 min at 37°C) in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.1) containing 1% heat-inactivated chicken serum (GIBCO Laboratories)–0.1 mg of trypsin per ml–0.1 mg of collagenase per ml–0.01 mg of DNase I (Worthington Diagnostics) per ml. Cells were counted, and serial dilutions from 10^{-1} to 10^{-5} of the single-cell suspension were plated in DMEM-F12 (1:1) medium supplemented with 15% fetal calf serum–10 mM HEPES (pH 7.3)–2 mM L-glutamine–penicillin–streptomycin–fungizone–4 μg of Polybrene per ml along with NIH 3T3 cells seeded 1 day earlier in 24-well petri dishes either at a density of 5×10^4 cells per well (for a methotrexate assay) or 0.5×10^4 cells per well (for an XC assay) (23). Cells were passaged 2 days later into methotrexate selection medium (DMEM supplemented with 10% dialyzed calf serum–0.3 mM methotrexate [Lederle Laboratories]). To analyze for infectious virus, the cells were subjected directly to an XC assay (23) at 5 to 6 days after cocultivation.

To analyze for MLV DHFR*-5 in the blood of infected animals, 200 μl of blood was collected retroorbitally and diluted 1:2 in HEPES buffer (pH 7.1) containing 10 U of heparin per ml–2% calf serum. NIH 3T3 cells in 24-well dishes were overlaid with 0.2 ml of diluted blood samples and analyzed for Mtx^r and XC-positive virus as described above.

Methotrexate treatment of embryos. Postimplantation embryos were injected either with DHFR*-5H-E cells or DMEM medium, respectively. Pregnant females were injected intraperitoneally with 0.2 to 0.6 mg of methotrexate (Lederle) in a single or double dose between days 11.5 and 18.5 of pregnancy. Surviving animals were tested for viremia at 4 to 6 weeks of age by a competitive radioimmunoassay as described previously (20).

RESULTS

Characteristics of MLV DHFR*-5 producer cell line used for embryo infection. Since the construction of the replication-competent vector pMLV DHFR*-5, and the generation of virus-producing cell lines has been described elsewhere (30), only the main characteristics of the virus are summarized here. pMLV DHFR*-5 is a replication-competent Mo-MLV-derived vector carrying an insert of about 1 kilobases (kb) in length in the U_3 region of the viral LTR upstream from the viral promoter and enhancer sequences. The insert encompasses a 660-bp mutant DHFR* (26) cDNA and upstream SV40 early region promoter sequences (Fig. 1 bottom). The virus-producing cell line DHFR*-5H-E was derived by three virus passages in vitro without drug selection and subsequent single-cell cloning. DHFR*-5H-E cells contain about 15 proviral copies per cell and produce a titer of 10^7 XC plaques per ml and 5×10^5 Mtx^r colonies per ml, respectively. The proviruses carry a 40- to 65-bp deletion in the SV40 sequences encompassing the promoter and part of the 21-bp repeats (30), suggesting that DHFR* expression is under the transcriptional control of the virus. DHFR*-5H-E cells were used for infection of pre- and postimplantation mouse embryos. To distinguish between MLV DHFR*5 virus and mouse strains carrying integrated MLV DHFR*-5 sequences, we refer to MLV DHFR*5 virus as MLV DHFR* virus throughout the article. Mouse strains are numbered (e.g., MLV DHFR*-1).

Infection of preimplantation mouse embryos. Preimplantation mouse embryos were infected in vitro at the 4- to 16-cell stage by cocultivation with MLV DHFR*-producing cells and transplanted to pseudopregnant foster females as described previously (11, 14). Of 29 animals derived from the infected embryos, 13 were found to carry MLV DHFR* sequences in their tail DNA, thus indicating a similar efficiency of infection as that found with wt Mo-MLV or Mo-MLV sup, containing a bacterial *supF* gene in the LTR (11, 27). Figure 1 (top) shows a *Pvu*II digest of tail DNAs from the 13 infected animals, probed with a ^{32}P-labeled 660-bp DHFR* fragment. Each tail DNA gave rise to a 3′ internal *Pvu*II fragment of about 1.10 kb in length, indicating that the proviral copies in the infected animal contained the same 50- to 100-bp deletion in the SV40 sequences present in the MLV DHFR provirus from the producer cell line (compare lanes P and C) (30). Furthermore, all mice were mosaics with respect to proviral integration, as indicated by 5′ flanking *Pvu*II fragments of submolar intensities. The infected animals harbored between one to nine MLV DHFR*-5 integration sites (Fig. 1 top).

To determine whether MLV DHFR* proviruses would be genetically transmitted, six mosaic males were mated with normal C57BL/6J females. The results are shown in Table 1. When a total of 214 F_1 offspring were analyzed, 14 different mice carrying genetically transmitted proviruses (DHFR*-1 to DHFR*-14) could be identified. Whereas some founder animals transmitted proviral sequences with very low efficiency through the germ line (e.g., ♂F), nearly 100% of the F_1 offspring from ♂L were positive for one or more provirus integrations, each offspring carrying between two to five MLV DHFR* copies (data not shown). These results indicate that a large fraction of germ cells in mosaic animal ♂L was infected with multiple copies of MLV DHFR*.

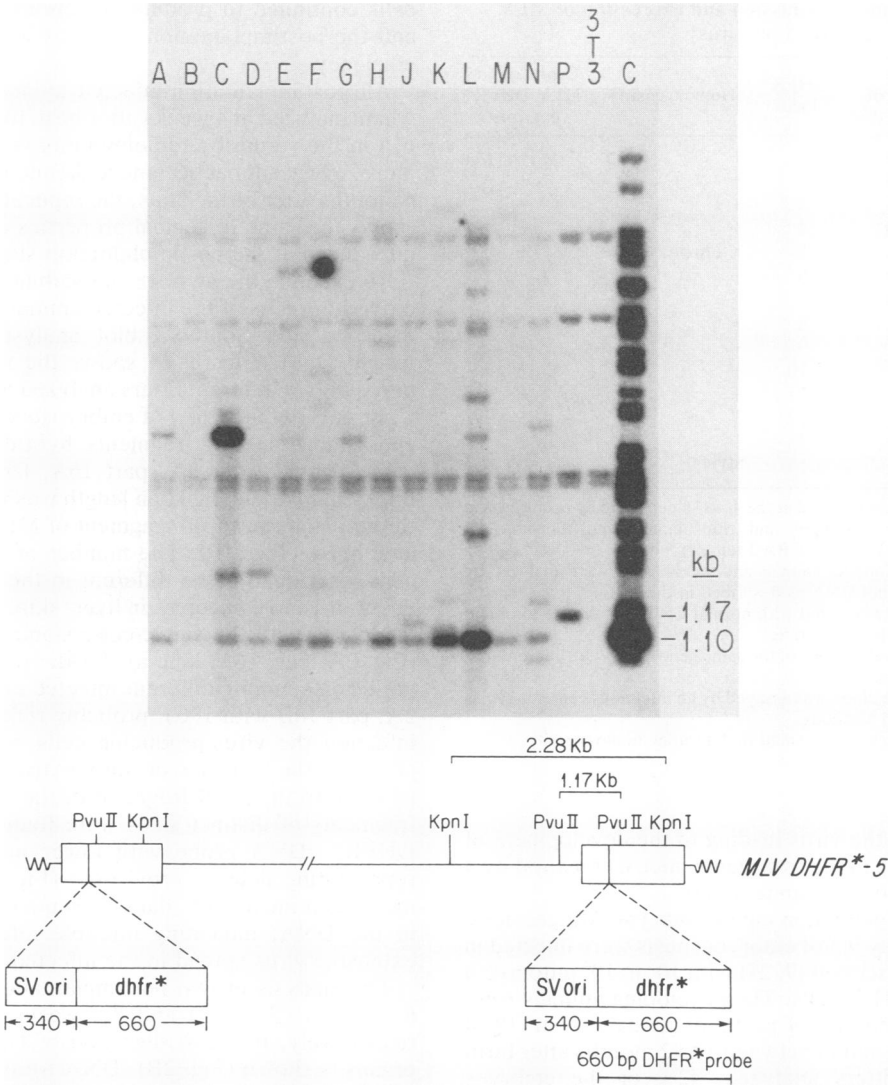

FIG. 1. Southern hybridization analysis of tail DNA from 13 mice infected as preimplantation embryos with MLV DHFR*-5 recombinant virus. (Top) High-molecular-weight DNA (10 μg) from the tail was digested to completion with *Pvu*II. The filter was hybridized with 2×10^7 cpm of ^{32}P-labeled 660-bp DHFR* cDNA fragment. Lanes: A to N, tail DNA from founder animals A to N; P, 10 μg of NIH 3T3 DNA mixed with 15 pg of pMLV DHFR*-5, *Pvu*II digested; 3T3, 10 μg of NIH 3T3 DNA, *Pvu*II digested; C, 10 μg of DNA from clone DHFR*-5H-E, *Pvu*II digested. The bands in the uninfected NIH 3T3 control (lane 3T3) represent hybridization of the DHFR* probe with endogenous DHFR fragments. The size markers indicate the positions of the internal *Pvu*II fragment in pMLV DHFR*-5 (1.17 kb) and in the proviral copies present in the tail DNAs (1.10 kb), respectively. The intensely hybridizing fragment in lane C is probably due to a contamination in the restriction enzyme digest. (Bottom) Schematic diagram of integrated proviral DNA derived from MLV DHFR*-5 infection, the restriction enzymes, and the probe used for Southern hybridization analysis. Solid lines indicate proviral sequences; waved lines indicate host DNA sequences.

Further breeding analysis of the F_1 offspring showed that of 13 mice carrying different MLV DHFR* integration sites, 10 could be obtained homozygous so far (see Table 1). Analysis of the DHFR*-5 locus indicated proviral integration on the X chromosome (data not shown).

The germ line-transmitted proviral MLV DHFR* copies were highly methylated as shown by digestion of the DNA from F_1 offspring with the methylation-sensitive enzyme *Sma*I (data not shown). When analyzed for expression of infectious virus, none of the mosaic founder animals nor their offspring carrying MLV DHFR* proviral copies contained detectable amounts of viral p30 in the serum at 4 weeks or 2 or 4 months after birth, using a radioimmunoassay (20). However, by using a very sensitive infectious center assay from blood, infectious MLV DHFR* virus could be detected in most strains at levels 2 to 3 orders of magnitude lower compared with fully viremic Mov 14 control mice (see Table 1). Furthermore, none of the mosaic mice nor offspring transmitting the MLV DHFR* provirus was found to develop leukemia up to 6 to 12 months after birth. However, after treatment of primary embryo fibroblast cultures or whole animals with 5-azacytidine (data not shown) (12), the proviruses could be activated and the animals were found to develop viremia as with wt Mo-MLV. These results indicate that the MLV DHFR* provirus transmitted through the germ line can give rise to infectious virus.

TABLE 1. Germ line transmission and expression of MLV DHFR*-5 provirus[a]

Founder animal[b]	Germ line transmission of proviral copies (%)[c]	DHFR* locus	Homozygosity[d]	Expression of MLV DHFR* provirus[e]
♂A	4/25 (16)	1	+	+
♂K	10/38 (26)	2	+	+
		3	+	+
♂M	21/61 (34)	4	+	+
		5	X chromosome	+
♂F	4/41 (10)	6	+	−
♂L	15/16 (93)	7	+	+
		8	+	+
		9	+	+
		10	+	+
		11	+	+
		12	?[f]	+
		13	?	+
♂B	1/33 (3)	14	ND[g]	ND

[a] C57BL/6J embryos were infected at the 4- to 16-cell stage by cocultivation with DHFR*-5H-E cells overnight and transplanted into the uteri of pseudopregnant (C57BL/6J male × CBA/J female) females.

[b] The genotypes of the founder animals and the offspring were analyzed by Southern blot analysis of tail DNA as described in the text.

[c] Male founder animals were bred with normal C57BL/6J female to obtain germ line transmission of proviral copies.

[d] Heterozygous males and females of the same genotypes were intercrossed to generate homozygous strains.

[e] Virus expression in the blood was assayed by an infectious center assay as described in Materials and Methods.

[f] ?, Number of offspring was too small to determine homozygosity.

[g] ND, Not determined.

However, spread of the virus leading to the development of viremia and leukemia in the transgenic mice is impaired by a mechanism that is not well understood.

Infection of postimplantation mouse embryos. Midgestation mouse embryos at day 9.5 of embryogenesis were injected in situ as previously described (9, 29) with 10^3 to 10^4 mitomycin C-treated DHFR*-5H-E cells. The developing animals were analyzed either at the end of gestation at days 17 to 19 of embryogenesis or as adults between 1 to 2 months after birth (Table 2). Of 13 litters analyzed, 42% of the embryos survived the physical trauma of manipulation at the time of examination. About 97% of the surviving animals proved to be infected with the recombinant virus when analyzed for the presence of proviral copies in their DNAs as well as for virus production in an infectious center assay when examined before birth (see below) or for viremia by a p30 radioimmunoassay as adults, respectively. These results show that after treatment with mitomycin C, the injected cells continued to produce infectious virus in the embryos and the postimplantation embryos were infected with high efficiency.

TABLE 2. Infection of midgestation mouse embryos[a]

Time of analysis	No. of litters injected	No. of survivors/ no. of embryos injected (%)	No. of infected animals/no. of animals tested (%)
17–19 days of embryogenesis	3	15/18 (83)	14/15 (93)
1–2 mo after birth	10	23/72 (32)	21/21 (100)
Total (avg %)		38/90 (42)	35/36 (97)

[a] Mouse embryos at day 9.5 of gestation were microinjected in situ with 10^3 to 10^4 mitomycin C-treated DHFR*-5H-E cells as described in Materials and Methods. The developing animals were analyzed either before birth between days 17 and 19 of gestation or as adults at 1 to 2 months of age for infection with the recombinant virus as described in the text.

All postimplantation-infected animals developed viremia when analyzed at 4 weeks after birth for the presence of viral p30 in the serum by radioimmunoassay (Table 2). Furthermore, every animal became leukemic and died between 4 to 6 months after birth. Thus, the replication-competent vector shows the same biological properties as wt Mo-MLV when introduced at the postimplantation stage (9).

To examine the number and stability of proviral copies in different organs of the infected animals, we performed *Kpn*I digestion and Southern blot analysis of high-molecular-weight DNA. Figure 2A shows the results from four embryos of two different litters analyzed at day 18 (part 18A, B, C) or day 19 (part 16A) of embryogenesis. In addition to the endogenous DHFR fragments hybridizing to the DHFR* cDNA probe (Fig. 2A, part 16A, lane c), a predominant fragment of about 2.2 kb in length was visible, corresponding to the 3′ internal *Kpn*I fragment of MLV DHFR* (part 16A, lane b; see Fig. 1B). The number of proviral genomes per cells was found to be different in the various organs, with about three to five copies in liver, skin, and limb DNAs when compared with the single-copy standard of pMLV DHFR* (Fig. 2A, part 16A, lane a). Furthermore, the copy number varied also among different infected embryos (compare Fig. 2A, part 18B with 18C), probably reflecting whether during infection the virus-producing cells were delivered into or close to the embryo or into extraembryonic tissues. In addition to the *Kpn*I fragment of the expected size, smaller fragments of distinct sizes were found to hybridize to the DHFR* cDNA probe with lower intensities, presumably representing deleted proviruses (Fig. 2A). No *Kpn*I fragments that included 5′ flanking sequences could be detected in the DNA, indicating multiple integration sites due to extensive virus spread in the infected embryos.

The analysis of two postimplantation-infected animals at 6.5 weeks (♂ 37-27) and 8.5 weeks (♀ 27-9) after birth, respectively, by *Kpn*I digestion of DNA from 10 different organs is shown (Fig. 2B). DNA from every somatic tissue analyzed as well as from ovary and testis tissues contained up to three to five proviral copies per cell as estimated by the intensity of the 3′ internal *Kpn*I fragment. As for the analysis of infected embryos at days 18 to 19 of gestation, distinct smaller *Kpn*I fragments of lower intensities were visible. Similar results were obtained when three other mice infected as postimplantation embryos were analyzed at 1 to 2 months of age (data not shown). Although none of these animals had developed leukemia at the time of examination, additional submolar *Kpn*I fragments indicative of specific integration were detected in thymus DNA from some animals, suggesting the onset of clonal tumor growth in the thymus (Fig. 2B, ♂ 37-27, lane e). The highest number of MLV DHFR* proviruses were invariably found in spleen and heart tissues, whereas liver, kidney and testes or ovary tissues contained significantly lower copy numbers.

The data in Fig. 2 show that infection of postimplantation embryos with MLV DHFR* resulted in efficient insertion of proviral genomes in the DNA of all somatic tissues analyzed. The data also indicate that extensive virus spread occurred in the infected embryos, during which the majority of the proviruses apparently did not accumulate deletions. Although proviral copies were found in the DNA from testes and ovary tissues (Fig. 2B), no germ line transmission of MLV DHFR* could be detected when 71 offspring of vire-

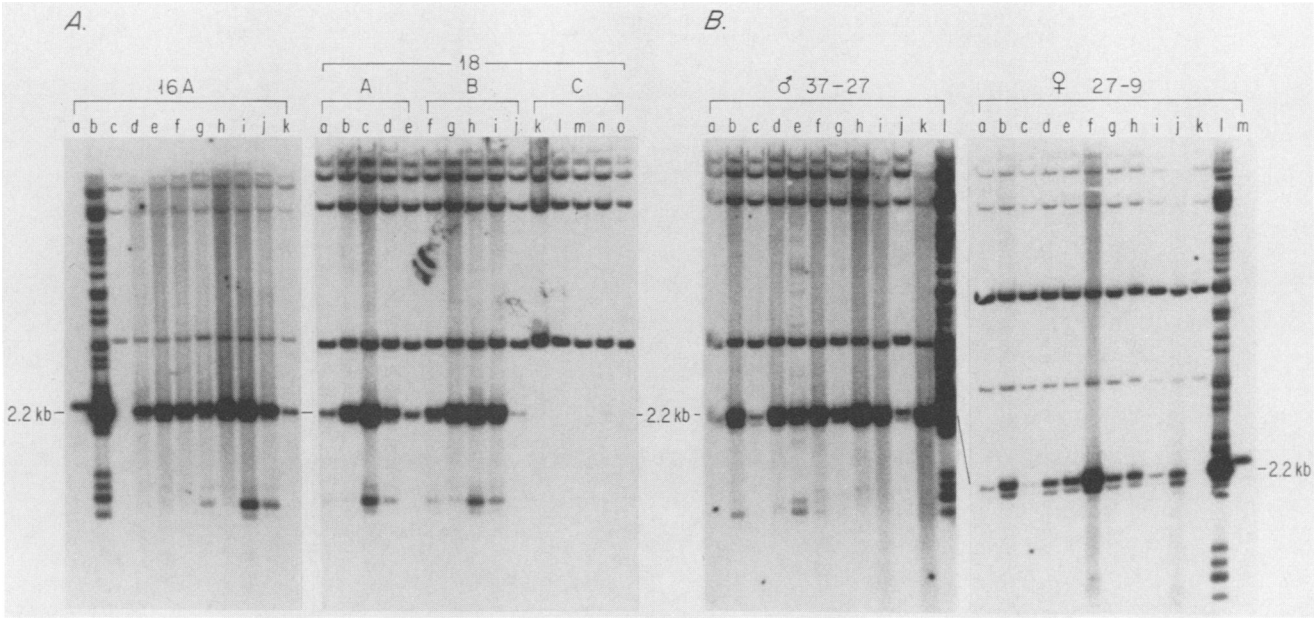

FIG. 2. Southern hybridization analysis of organ DNA from animals microinjected with virus-producing cells at day 9.5 of embryogenesis. High-molecular-weight DNA (10 μg) was digested to completion with *Kpn*I. The filters were hybridized with 2×10^7 cpm of ^{32}P-labeled 660-bp DHFR* fragment. (A) Analysis of four embryos from two litters at day 18 (18A, B, C) or day 19 (16A) of embryogenesis. (16A) Lanes: a, 15 pg of pMLV DHFR*-5 DNA; b, 10 μg of DHFR*-5H-E DNA, *Kpn*I digested; c, 10 μg of NIH 3T3 DNA, *Kpn*I digested; d to k, 10 μg of DNA from thymus (lane d), kidney (lane e), lung (lane f), brain (lane g), liver (lane h), skin (lane i), limb (lane j), and placenta (lane k). (18A, B, C) Ten micrograms of DNA from brain (lanes a, f, and k), liver (lanes b, g, and l), skin (lanes c, h, and m), limb (lanes d, i, and n), and placenta (lanes e, j, and o). (B) Analysis of two adult animals at 6.5 (♂ 27-37) and 8.5 (♀ 27-9) weeks after birth. Lanes: Ten micrograms of DNA from liver (lane a), spleen (lane b), kidney (lane c), intestine (lane d), thymus (lane e), heart (lane f), lung (lane g), brain (lane h), ovary (♀ 27-9, lane i), muscle (♂ 37-27, lane i), testes (♂ 37-27, lane j), tail (♀ 27-9, lane j; ♂ 37-27, lane k), NIH 3T3 control (♀ 27-9, lane k), DHFR*-5-E control (lane l), 15 pg of pMLV DHFR*-5 (lane m). The size marker indicates the position of the 2.2-kb internal *Kpn*I fragment in the MLV DHFR*-5 DNA.

mic, postimplantation-infected males were analyzed for the presence of virus-specific copies (data not shown).

Expression of MLV DHFR* in postimplantation infected animals. To determine the fraction of MLV DHFR*-5 virus-producing cells in a given organ from postimplantation-infected animals, we employed an infectious center assay. Single-cell suspensions from different organs of day 18 embryos (Fig. 2A, part 18A, B, C) were prepared, and serial dilutions were plated onto NIH 3T3 cells. The fibroblast cells were tested for the production of infectious virus by an XC plaque assay (23) as well as for expression of the mutant DHFR* by methotrexate selection (see Materials and Methods). The results, summarized in Table 3, show a rough correlation between the frequency of infectious centers and the number of proviral copies in the DNA of the respective tissues (Fig. 2A). In embryos 18A and 18B, which were infected with about one to five proviral copies per cell in various organs (see Fig. 2A), infectious MLV DHFR* virus was expressed in cells from every organ analyzed in vitro. In thymus, brain, and spleen tissues, we found the highest viral titer, with up to 1 of 20 cells yielding an XC plaque and 1 of 40 cells yielding Mtx^r colonies in infectious center assays. As found with MLV DHFR* virus fibroblast producer cells (30), the XC titers were consistently higher compared with the Mtx^r colony titers, probably because of the higher DHFR* expression levels necessary to confer resistance to methotrexate. These frequencies are in the same order of magnitude as found for viremic Mov animals (11). It should be emphasized that the infectious center assay may underestimate the fraction of infected cells in vivo in the animal (see

TABLE 3. Frequency of infectious centers per 1,000 cells in organs from day 9.5 microinjected embryos[a]

Embryo	Organ	No. of proviral copies	No. of infectious centers/10^3 cells	
			Mtx^r colonies	XC plaques
18A	Brain	1	0.8	10
	Thymus		25	50
	Spleen		0.3	3
	Liver	3–5	0.2	7.6
	Lung		1.3	13
	Kidney		0.04	1.2
18B	Brain	2	25	130
	Thymus		0.25	25
	Spleen		0.2	20
	Liver	3–5	2.5	1.5
	Lung		1.7	3
	Kidney		0.7	2.7
18C	Brain	<0.1	0.15	0.2
	Thymus		<0.04	0.01
	Spleen		0.025	0.025
	Liver	<0.1	<0.001	0.001
	Lung		<0.002	<0.01
	Kidney		<0.002	<0.01

[a] Mouse embryos were infected at day 9.5 of gestation with MLV DHFR*-5 virus as described in Materials and Methods. Three embryos from one litter were dissected at day 18 of embryogenesis. Single-cell suspensions were prepared, and serial dilutions were cocultivated with NIH 3T3 cells in the presence of 4 μg of Polybrene per ml (see Materials and Methods). The NIH 3T3 cells were assayed for virus infection by a methotrexate assay as well as an XC plaque assay (23). The number of proviral copies in brain and liver DNAs were estimated from the intensities of *Kpn*I fragments by Southern analysis (Fig. 2A).

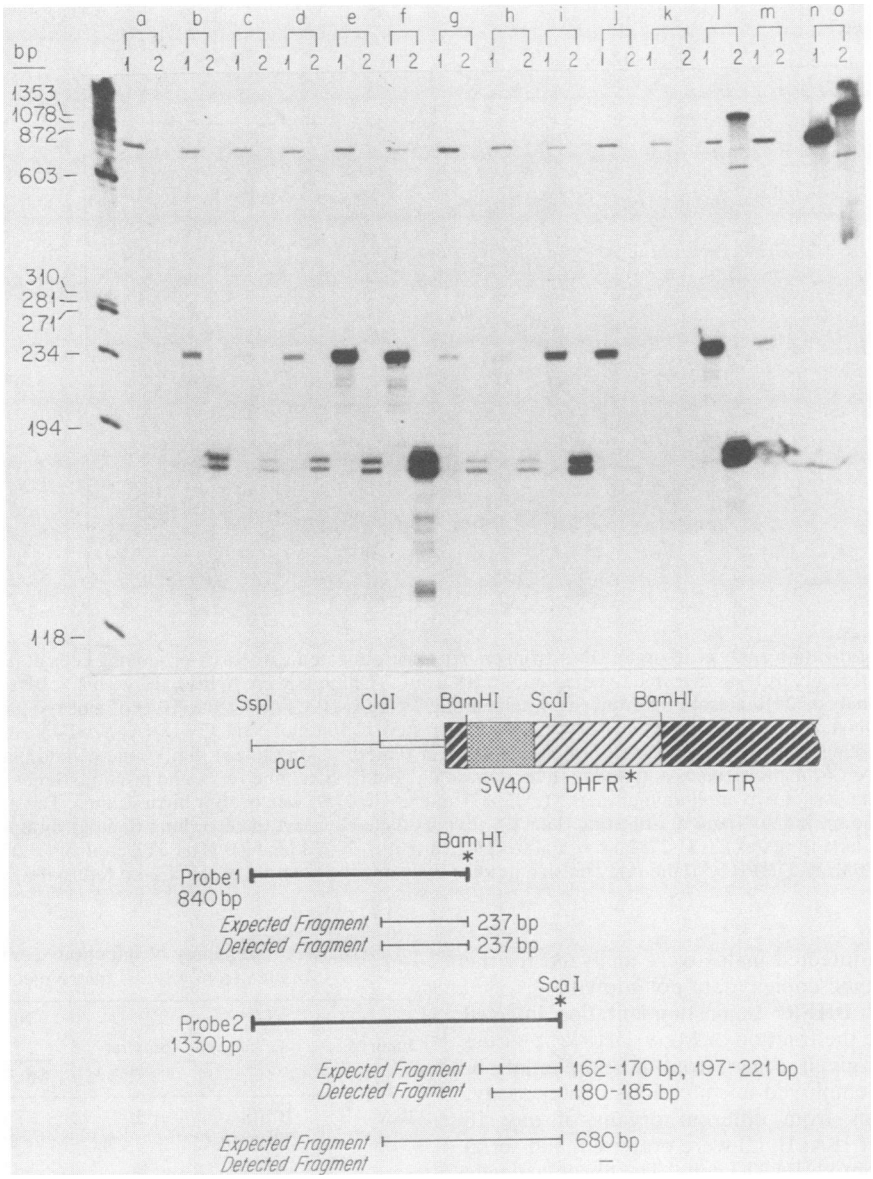

FIG. 3. S1 hybridization analysis of Mo-MLV- and DHFR*-specific transcripts in tissues from postimplantation-infected animal ♀ 27-9. One microgram of total cellular RNA mixed with 5 μg of *S. cerevisiae* tRNA was hybridized with 5×10^3 cpm of a ^{32}P-5′-end-labeled 840-bp *Ssp*I-*Bam*HI fragment (probe 1) or a 1,300-bp *Ssp*I-*Sca*I fragment (probe 2). The protected S1 fragments were separated on a 7% polyacrylamide urea gel. Lanes: RNA from liver (lane a), spleen (lane b), kidney (lane c), intestine (lane d), thymus (lane e), heart (lane f), lung (lane g), brain (lane h), muscle (lane i), ovary (lane j), NIH 3T3 control (lane k), DHFR*-5H-E cells (lane l), wt Mo-MLV-producing clone (lane m), and probe 1 (lane n) and probe 2 (lane o) alone. On the left, the sizes of the ϕX fragments, cut with *Hae*III, are indicated. The structure and sizes of pLTR DHFR*-5 DNA, the DNA probes, and the protected fragments are indicated at the bottom.

Materials and Methods) since only a fraction of the infected cells may give rise to an infectious center.

To examine the expression of the integrated MLV DHFR*-5 genomes, total RNA was isolated from different organs of adult mice infected at the postimplantation stage and analyzed by S1 hybridization. Figure 3 shows the results using RNA from animal ♀ 27-9 (Fig. 2B). Hybridization of genomic or spliced *env* MLV DHFR* RNA to probe 1 gave rise to a protected 237-bp S1 fragment. The same fragment was also obtained by using RNA from NIH 3T3 clones producing either MLV DHFR* virus or wt Mo-MLV virus, respectively (Fig. 3, lanes l and m). Using probe 2, which was labeled 104 bp inside the DHFR* cDNA at the *Sca*I site, two protected S1 fragments of 180 and 185 bp in length were found in all tissues, identical to those found in the producer cell clone DHFR*-5H-E (Fig. 3, lane l). No fragments indicating a transcriptional start site in the SV40 early promoter (1) could be detected. This result shows that the virus replicating in the postimplantation-infected animals had identical 3′ boundaries of the deletion in the SV40 promoter sequences as those of the virus used for infection. When the 5′ deletion boundaries were analyzed by S1 mapping, a rather heterogeneous population of protected fragments, identical to those using DHFR*-5H-E RNA, was found (data not shown). Thus, as suggested by Southern analysis of the proviral DNA, the MLV DHFR* retrovirus

vector, carrying a 50- to 70-bp deletion in the SV40 promoter and 21-bp repeat region, was stable upon replication in infected midgestation embryos and did not give rise to further deletions.

The levels of RNA from ♀ 27-9, as determined by S1 analysis, were consistent with the number of proviral copies present in the respective organs (Fig. 2B), indicating no restriction to virus expression in the postimplantation-infected animals. Virus-specific RNA from heart tissue was about as abundant as that in the DHFR*-5H-E producer clone (Fig. 3, compare lane f with lane l). In contrast, liver RNA contained barely visible amounts of viral RNA (Fig. 3, lane a). These results were confirmed by Northern blot analysis of RNA (data not shown).

Methotrexate treatment of postimplantation-infected animals. The results presented so far showed that postimplantation mouse embryos could be infected with high efficiency by using a replication-competent MLV DHFR* virus and that expression of the inserted genome could be detected at substantial levels before and after birth in different organs of the infected mice. We therefore addressed the question whether resistance to methotrexate could be detected in vivo in the infected animals. For this, we attempted to design a scheme of drug treatment either during late stages of gestation or after birth, respectively. This scheme should preferentially allow infected animals to survive while uninfected controls would die. In a series of methotrexate treatments of 23 litters during embryonal development, animals in the same litter at day 9.5 of gestation were either injected with MLV-DHFR*-producing cells or with DMEM, respectively. Of 224 total embryos, 161 (72%) were infected with virus. Between 0.2 and 0.6 mg of methotrexate was administered intraperitoneally to the pregnant mother in a single or double dose at later stages of gestation (days 11.5 to 18.5). Of 37 surviving animals, 21 (57%) tested positive for viremia at 4 to 6 weeks of age by using a p30 radioimmunoassay (20). Thus, although we could define conditions which were lethal to the embryos but not to the pregnant mother, the methotrexate treatment exhibited an equally toxic effect on both infected and control animals. A similar result was found when postimplantation-infected animals were treated with methotrexate as adults (data not shown).

DISCUSSION

In this study, we explored the possibility of using replication-competent retrovirus vectors for gene transfer into whole mice in vivo at different stages of development. For this, we used an Mo-MLV-derived vector which carries a mutant DHFR* cDNA as well as SV40 regulatory sequences inserted in the U_3 region of the viral LTR. The resulting virus was shown to stably transmit a methotrexate-resistant phenotype during multiple rounds of replication in vitro and was produced at high titers from fibroblast cells.

Infection of mouse embryos at the preimplantation stage resulted in efficient infection of embryos and insertion of the recombinant virus into the germ line. However, similar to wt Mo-MLV, the viral sequences became inactivated in most tissues and highly methylated. When initiating these experiments, we were interested in analyzing whether expression of DHFR* could be facilitated from the internal SV40 promoter in the viral LTR. However, the germ line-transmitted provirus proved to contain a nonfunctional SV40 promoter because of a deletion event which had occurred during initial virus passages in vitro (30), and no DHFR* transcript could be detected in the mice. In future experiments, we are planning to construct retrovirus vectors which contain additional nonviral transcriptional signals for expression of heterologous genes. Most of the strains containing germ line-integrated MLV DHFR* proviral sequences activated infectious virus at very low levels, which could be detected in the serum as well as primary fibroblast cultures by cocultivation experiments (unpublished results). Spread of virus was obviously impaired in the animals, the mechanism for which remains obscure.

Injection of mouse embryos at the postimplantation stage with virus-producing cells resulted in nearly 100% efficiency of infection. Furthermore, analysis of infected animals at later stages of development indicated that the replication-competent MLV DHFR* vector could spread without restriction in all somatic tissues in the infected embryo, resulting in up to one to five proviral copies per cell in some organs. In contrast, infection of postimplantation embryos with replication-defective vectors, either alone or in combination with helper Mo-MLV, resulted in only a very low fraction of cells infected with the recombinant vector (29; unpublished results). Although the MLV DHFR* virus had to replicate extensively while spreading throughout the embryo, the majority of proviral copies appeared not to have accumulated further deletions. The low level of deleted provirus observed is presumably a consequence of the large number of rounds of replication in the developing embryo. Similar results were obtained from studies with Mo-MLV recombinant virus containing a bacterial suppressor tRNA in the LTR (22). We can exclude the presence of surviving virus-producing fibroblasts used for infection of the embryos, since these cells had been treated with mitomycin C before injection.

The biological properties of MLV DHFR* virus were similar to those of wt Mo-MLV when introduced into postimplantation embryos; upon infection, the virus was expressed and replicated to give rise to viremia and subsequently to thymus-derived leukemia (9, 15). The infection was limited, however, to somatic cells in infected postimplantation embryos, since no germ line transmission of MLV DHFR* could be detected. This result is in agreement with those of earlier studies, showing a very low frequency of germ line integration (11). Analysis of postimplantation infected embryos revealed that cells from every organ were able to express infectious virus and that substantial levels of viral RNA containing the DHFR* cDNA were expressed in some organs like heart, thymus, spleen, and brain. In general, the analysis showed a correlation between the number of proviral copies and the amount of RNA expressed in a given organ.

The ability of MLV DHFR* to confer methotrexate resistance upon infected cells in vitro raised the possibility that similar selective schemes might be used in vivo. Unfortunately, although expression of the DHFR* gene could be demonstrated in vivo, preliminary attempts at developing a selection scheme in vivo were not successful. Besides the possibility that not every somatic cell was infected and that the expression of the DHFR* gene in vivo was insufficient for methotrexate selection, several other possibilities exist. First, the timing and precise dosage of methotrexate administration necessary for effective selection may be very critical and is not yet defined. Whereas the conditions of selection used in the current study did lead to the death of uninfected embryos while sparing the mother, the death of embryos carrying the MLV DHFR provirus may have been due to effects on maternal-derived cells supporting embryological development. Also, it is possible that normal folate

metabolism may be critical for early stages of development and that altered levels of DHFR expression may be toxic to the developing embryo. We are currently examining these possibilities. Recently, systemic resistance to methotrexate in transgenic mice carrying a mutant DHFR* gene has been reported (8). These mice contained between one to several copies of the DHFR* gene in every cell, yet expression of DHFR* was very low (4). In view of the results presented in this report, the prolonged survival of the transgenic mice is surprising.

In conclusion, the results presented in this report suggest that infection of postimplantation mouse embryos with replication-competent vectors is an important route to introduce foreign genes efficiently into somatic tissues. In the avian system, Rous sarcoma virus-derived replication-competent vectors have been used recently to transfer and express foreign genes under the transcriptional control of the viral LTR (6, 17). In future experiments, we plan to introduce genes which are potentially important for development. Furthermore, we are interested in the development of vectors which will target expression of the recombinant genome to specific cell types both in the embryo and in the adult, thus enabling us to study the function of specific genes in distinct cell types at defined stages of development.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA38497 and HD00635 from the National Institutes of Health to R.C.M.

We thank Doris Grotkopp for excellent technical assistance.

LITERATURE CITED

1. **Baty, D., H. A. Barrera-Saldana, R. D. Everett, M. Vigneron, and P. Chambon.** 1984. Mutational dissection of the 21 bp repeat region of the SV40 early promoter reveals that it contains overlapping elements of the early-early and late-early promoters. Nucleic Acids Res. **12**:915–931.
2. **Chirgwin, J. M., A. E. Pzybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
3. **Cone, R. D., A. Weber-Benarous, D. Baorto, and R. C. Mulligan.** 1987. Regulated expression of a complete human β-globin gene encoded by a transmissible retrovirus vector. Mol. Cell Biol. **7**:887–897.
4. **Gordon, J. W.** 1986. A foreign dihydrofolate reductase gene in transgenic mice acts as a dominant mutation. Mol. Cell. Biol. **6**:2158–2167.
5. **Hentschel, C. C., J. C. Irminger, P. Bucher, and M. Birnstiel.** 1980. Sea urchin histone mRNA termini are located in gene regions downstream of putative regulatory sequences. Nature (London) **285**:147–151.
6. **Hunt, L. A., D. W. Brown, H. L. Robinson, C. W. Naeve, and R. G. Webster.** 1988. Retrovirus-expressed hemagglutinin protects against lethal influenza virus infections. J. Virol. **62**: 3014–3019.
7. **Huszar, D., R. Balling, R. Kothary, M. C. Magli, N. Hozumi, J. Rossant, and A. Bernstein.** 1985. Insertion of a bacterial gene into the mouse germ line using an infectious retrovirus vector. Proc. Natl. Acad. Sci. USA **82**:8587–8591.
8. **Isola, L. M., and J. W. Gordon.** 1986. Systemic resistance to methotrexate in transgenic mice carrying a mutant dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA **83**:9621–9625.
9. **Jaenisch, R.** 1980. Retroviruses and embryogenesis: microinjection of Moloney leukemia virus into midgestation mouse embryos. Cell **19**:181–188.
10. **Jaenisch, R., K. Harbers, A. Schnieke, J. Lohler, I. Chumakov, D. Jahner, D. Grotkopp, and E. Hoffmann.** 1983. Germ line integration of Moloney murine leukemia virus at the Mov13 locus leads to recessive lethal mutation and early embryonal death. Cell **32**:209–216.
11. **Jaenisch, R., D. Jahner, P. Nobis, I. Simon, J. Lohler, K. Harbers, and D. Grotkopp.** 1981. Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. Cell **24**:519–529.
12. **Jaenisch, R., A. Schnieke, and K. Harbers.** 1985. Treatment of mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues. Proc. Natl. Acad. USA **82**:1451–1455.
13. **Jahner, D., K. Haase, R. Mulligan, and R. Jaenisch.** 1985. Insertion of the bacterial *gpt* gene into the germ line of mice by retroviral infection. Proc. Natl. Acad. Sci. USA **82**:6927–6931.
14. **Jahner, D., and R. Jaenisch.** 1980. Integration of Moloney leukemia virus into the germ line of mice: correlation between genotype and virus activation. Nature (London) **287**:456–458.
15. **Jahner, D., H. Stuhlmann, C. L. Stewart, K. Harbers, J. Lohler, I. Simon, and R. Jaenisch.** 1982. *De novo* methylation and expression of retroviral genomes during mouse embryogenesis. Nature (London) **298**:623–628.
16. **Keller, G., C. Paige, E. Gilboa, and E. F. Wagner.** 1985. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature (London) **318**:149–154.
17. **Kornbluth, S., F. R. Cross, M. Harbison, and H. Hanafusa.** 1986. Transformation of chicken embryo fibroblasts and tumor induction by the middle T antigen of polyomavirus carried in an avian retroviral vector. Mol. Cell. Biol. **6**:1545–1551.
18. **Lemischka, I. R., D. H. Raulet, and R. C. Mulligan.** 1986. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell **45**:917–927.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Nobis, P., and R. Jaenisch.** 1980. Passive immunotherapy prevents expression of endogenous Moloney virus and amplification of proviral DNA in BALB/Mo mice. Proc. Natl. Acad. Sci. USA **77**:3677–3681.
21. **Price, J., D. Turner, and C. Cepko.** 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc. Natl. Acad. Sci. USA **84**:156–160.
22. **Reik, W., H. Weiher, and R. Jaenisch.** 1985. Replication-competent Moloney murine leukemia virus carrying a bacterial suppressor tRNA gene: selective cloning of proviral and flanking host sequences. Proc. Natl. Acad. Sci. USA **82**:1141–1145.
23. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
24. **Rubenstein, J. L. R., J.-F. Nicolas, and F. Jacob.** 1986. Introduction of genes into preimplantation mouse embryos by use of a defective recombinant retrovirus. Proc. Natl. Acad. Sci. USA **83**:366–368.
25. **Sanes, J. R., J. L. R. Rubenstein, and J.-F. Nicolas.** 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. **5**:3133–3142.
26. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80**:2495–2499.
27. **Soriano, P., and R. Jaenisch.** 1986. Retroviruses as probes for mammalian development: allocation of cells to the somatic and germ line lineages. Cell **46**:19–29.
28. **Stewart, C. L., S. Schuetze, M. Vanek, and R. F. Wagner.** 1987. Expression of retroviral vectors in transgenic mice obtained by embryo infection. EMBO J. **6**:383–388.
29. **Stuhlmann, H., R. Cone, R. C. Mulligan, and R. Janeisch.** 1984. Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. Proc. Natl. Acad. Sci. USA **81**:7151–7155.
30. **Stuhlmann, H., R. Jaenisch, and R. C. Mulligan.** 1989. Construction and properties of replication-competent murine retroviral vectors encoding methotrexate resistance. Mol. Cell. Biol. **9**:100–108.
31. **Turner, D. L., and C. L. Cepko.** 1987. A common progenitor for

neurons and glia persists in rat retina late in development. Nature (London) **328**:131–136.

32. **van der Putten, H., F. M. Botteri, A. D. Miller, M. G. Rosenfeld, H. Fan, R. M. Evans, and I. M. Verma.** 1985. Efficient insertion of genes into the mouse germ line via retroviral vectors. Proc. Natl. Acad. Sci. USA **82**:6148–6152.
33. **Williams, D. A., I. R. Lemischka, D. G. Nathan, and R. C. Mulligan.** 1984. Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature (London) **310**:476–480.
34. **Wilson, J. M., D. M. Jefferson, J. R. Chowdhury, P. M. Novikoff, D. E. Johnston, and R. C. Mulligan.** 1988. Retrovirus-mediated transduction of adult hepatocytes. Proc. Natl. Acad. Sci. USA **85**:3014–3018.
35. **Wolff, J. A., J.-K. Yee, H. F. Skelly, J. C. Moores, J. G. Respess, T. Friedmann, and H. Leffert.** 1987. Expression of retrovirally transduced genes in primary cultures of adult rat hepatocytes. Proc. Natl. Acad. Sci. USA **84**:3344–3348.