Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector
(postimplantation embryo/microinjection of virus-producing cells/Eco gpt gene expression)

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ABSTRACT The potential use of retrovirus vectors to transduce foreign genetic information into cells of different tissues of an animal was explored by introducing a recombinant genome carrying the Eco gpt gene into postimplantation mouse embryos. To obviate the need for preparing concentrated virus stocks, φ2-2-5 cells producing the replication-defective murine sarcoma virus (MSV)-gpt virus were microinjected directly into embryos. The φ2-2-5 cells were mixed with cells producing replication-competent Moloney murine leukemia virus (Mo-MuLV) to facilitate spread of the vector. A high percentage of the manipulated embryos continued to develop without disturbance and were analyzed either prior to birth or as adults for expression of both helper and Eco gpt virus. Microinjection of as few as 10 Mo-MuLV-producing cells resulted in viremia of >50% of the embryos or adults, 25%–30% of which produced MSV–gpt recombinant virus in a variety of organs including thymus, spleen, lung, kidney, and brain. The fraction of vector-producing cells, however, was 3 to 5 orders of magnitude lower than that of helper-virus-producing cells. Our results demonstrate that a selectable gene can be introduced by retroviral vectors into animals and can be expressed in a wide variety of different somatic tissues.

A variety of different procedures have been developed for the introduction of foreign genes into eukaryotic cells. In most studies to date, cloned genes have been introduced into mammalian cells cultured in vitro (1, 2), and the expression of the transduced genes has been monitored either transiently after gene transfer (3, 4) or after the generation of stable transformants (5, 6). An alternative approach for studies of mammalian gene expression and regulation is the introduction of genes into pluripotent cells in vivo. Such an approach provides the means for studying the expression and regulation of genes throughout a developmental pathway. To date, the introduction of genes into cells in vivo has been achieved by microinjection of cloned DNA into mouse zygotes (7–14). This procedure offers stable in the introduction of sequences into germ-line cells, yet the expression of such sequences in somatic tissues has been variable.

Retrovirus vectors have recently been used in a number of laboratories to introduce genes into cultured cells (15–23). These vectors are particularly well suited for gene transfer in that they infect a variety of cell types and introduce genes at high efficiency. Studies of the interaction of Moloney murine leukemia virus (Mo-MuLV) with cells of the developing mouse embryo (24–27) have suggested the possibility of using retroviral vectors for the transfer of genes in vivo as well. Exposure of preimplantation mouse embryos to Mo-MuLV results in the efficient integration of proviral copies in germ-line cells (24–26). However, preimplantation mouse embryos are nonpermissive for virus expression and replication (28); infection of embryos at the preimplantation stage leads to efficient de novo methylation of proviral sequences upon chromosomal integration (29). In contrast, retroviruses can replicate unrestrained in cells of most somatic tissue when introduced into embryos between day 8 and day 10 of gestation (27, 29). Upon birth of the animal, however, replication is restricted to cells of lymphatic tissue (24, 27). To investigate the potential of retroviral vectors for introducing foreign genes into cells of many somatic tissues of an animal, we microinjected mouse embryos at the postimplantation stage with virus-producing cells and analyzed late embryos or adults derived from infected embryos for the presence and expression of the recombinant provirus. A retrovirus vector carrying the Eco gpt gene, termed murine sarcoma virus (MSV)–gpt, was used for these studies, because expression of the dominant marker gene is easy to detect in tissue culture cells (6). Our results demonstrate that retrovirus vectors can indeed be used to integrate and express foreign genes in a variety of different somatic tissues in the mouse.

MATERIALS AND METHODS

Mice. BALB/c and C57BL/6 mice were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). 129/ter SV breeding pairs were from L. Stevens. All mice were bred in our mouse colony at the Heinrich-Pette-Institut.

Cells. The packaging cell line φ2-2-5 was derived by transfection of pMSV–gpt DNA into φ2 cells (unpublished data; ref. 30). The cells were free of any detectable Mo-MuLV helper virus and produced a titer of 5 × 10⁶ gpt colony-forming units per ml.

The Mo-MuLV helper-virus-producing cell line C12C was derived by transfection of NIH 3T3 cells with the highly infectious pMOV-3 clone (31) followed by 2-fold end point dilution of Mo-MuLV and subsequent cell cloning. C12C cells contain one copy of Mo-MuLV per cell and produce a helper virus titer of 5 × 10³ XC plaque-forming units per ml.

Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Selection medium for gpt colonies was as described (6).

Microinjection of Mid-Gestation Embryos. Females of the inbred mouse strains C57BL/6, BALB/c, and 129/ter SV were mated with males from the same strain, and the day of vaginal plug formation was counted as day 1 of gestation. The pregnant mice were anesthetized with 15–20 µg of Methomidat·HCl per g of weight (Janssen GmbH, Neuss, F.R.G.) and ether prior to manipulation. The developing embryos were infected between day 8.5 and 10.5 of gestation by microinjection of virus-producing cells directly through the uterus wall into the ventral third of the decidual swelling or

Abbreviations: Mo-MuLV, Moloney murine leukemia virus; MSV, murine sarcoma virus; SV40, simian virus 40; kb, kilobase(s).
into the placenta, essentially as described (27). Approximately 0.2–0.5 μl of cell suspensions containing \(5 \times 10^5\) ϕ2-2.5-C12 or 5 \(\times 10^3\) C12C cells per ml were injected into the embryo or into the placenta using a micropipette with a closed tip and a hole of \(\approx 30\) μm diameter at one side.

Radioimmunoassay. Microinjected animals were tested 4 weeks after birth for viremia by a competitive radioimmunoassay for the presence of p30 in the serum as described (32).

Assay for Presence of Defective MSV-gpt in the Blood. Blood samples (100–200 μl) were taken from viremic animals 5 weeks after birth and diluted 1:10 in phosphate-buffered saline/2% fetal calf serum. NIH 3T3 cells seeded the day before at a density of \(0.5 \times 10^6\) cells per ml in 24-well plates in the presence of 2 μg of Polybrene per ml were overlaid with 0.3 ml of diluted blood samples. Twenty-four hours later, the blood cell suspension was washed off and the NIH 3T3 cells were passaged onto Petri dishes (10-cm diameter) containing Eco gpt selection medium. Plates were fixed and stained with Giemsa (Merck) 10–14 days later.

Infectious Center Assay. Organs were removed from adult mice derived from microinjected embryos between 2 and 4 months after birth or from day 19 embryos, respectively, and single cell suspensions were obtained as described (32). Spleen cells (1 \(\times 10^6\)) from adult mice or 1 \(\times 10^6\) cells from different organs (brain, liver, kidney, lung, heart, and thymus) as well as the carcass of day 19 embryos, respectively, were seeded onto NIH 3T3 cells grown to 30%–50% confluency in medium containing 2 μg of Polybrene per ml on Petri dishes (5-cm diameter). The cells were passaged 2 days later onto Petri dishes (14-cm diameter) containing Eco gpt selective medium. Plates were fixed and stained 2 weeks later. Mycophenolic acid-resistant colonies from some plates were isolated by using cloning cylinders and grown under Eco gpt selective conditions. Supernatants of mycophenolic acid-resistant colonies were titered for Mo-MuLV helper virus by an XC plaque assay (33) as well as for MSV-gpt defective virus.

Primary Embryonal Cell Culture. Single cell suspensions from lung and part of the carcass from day 19 embryos were prepared and plated onto Petri dishes containing Dulbecco’s modified Eagle’s medium/20% fetal calf serum. When attachment of cells was apparent (normally 3–5 days after seeding), medium was changed. After reaching confluency, one-half of the primary culture was passaged onto Petri dishes containing Eco gpt selective medium, and \(\approx 3\) weeks later, mycophenolic acid-resistant colonies derived from secondary embryonal cells were transfected with simian virus 40 (SV40) to derive permanent cell lines. The other half of the primary cultures was cocultivated with NIH 3T3 cells as described above, followed by selection for mycophenolic acid resistance.

Isolation of DNA and Restriction Enzyme Analysis. High molecular weight DNA from organs of adult mice or embryos and from tissue culture cells was prepared as described (34). The DNA was digested with 5 μl 1 (3 units per μg of DNA) (Bethesda Research Laboratories) as described by the supplier. DNA fragments were separated on 0.8% agarose gels and transferred to nitrocellulose filters (35). \(^{32}\)P-labeled nick-translated probes of pSV2-gpt were prepared and hybridized to nitrocellulose filters as described (36).

RESULTS

Microinjection of Virus-Producing Cells into Mid-Gestation Mouse Embryos. Fresh cell suspensions of the two cell lines ϕ2-2.5 and C12C, which produce the replication-defective MSV-gpt and Mo-MuLV helper virus, respectively, were mixed in a ratio of 100:1. A total of \(1–3 \times 10^6\) cells was microinjected into day 8.5 to 10.5 mid-gestation mouse embryos; \(33\%\) of the injected embryos (93/277) survived the physical trauma of microinjection to adulthood, one-half of which developed viremia (45/92), as shown by the presence of p30 in their serum 4 weeks after birth. These results show that microinjection of Mo-MuLV-producing cells into mid-gestation mouse embryos results in productive infection with the same efficiency as compared with microinjection of concentrated virus stocks as described (27). They furthermore indicate that introduction of viable cells into the mid-gestation embryos does not interfere with the subsequent normal development.

Expression of MSV-gpt Virus in Cells of Embryos and Adult Mice. Expression of MSV-gpt virus was analyzed by an infectious center assay. Cells were isolated from different tissues of infected embryos at day 19 of gestation or from adult animals, cocultivated with NIH 3T3 cells, and subjected to Eco gpt selective conditions after 2 days. The number of mycophenolic acid-resistant NIH 3T3 clones was used to calculate the fraction of cells of different organs from the experimental animals that produced the defective MSV-gpt virus. To monitor the presence of Mo-MuLV helper virus, NIH 3T3 cells were cocultivated with cells from the carcass of day 19 embryos and tested for infectious Mo-MuLV virus by the XC plaque assay. The adult animals were assayed for viremia by radioimmunoassay for presence of p30 in the serum.

Table 1 shows the results obtained with cells from different tissues of day 19 embryos that had been microinjected with virus-producing cells at day 9 of gestation. The XC plaque assay indicated that 8 of 10 embryos produced Mo-MuLV helper virus, while expression of infectious MSV-gpt virus was detected in 3 embryos. Embryo 2 produced MSV-gpt virus in kidney, lung, brain, heart and thymus, and in the carcass. Embryo 7 showed evidence for MSV-gpt expression in lung and carcass and embryo 8 showed virus expression in lung, carcass, and brain. No virus expression could be detected in liver cells from any of the animals. This is consistent with previous results, which show low levels of expression after introduction of Mo-MuLV into day 8 embryos (27). On the average, between 5 and 500 mycophenolic acid-resistant NIH 3T3 colonies were induced by 10^7 embryonic cells from different organs. This indicates that, under our experimental in vitro conditions, approximately every 5 \(\times 10^3\) to 5 \(\times 10^4\) cell produced MSV-gpt recombinant virus.

To analyze whether cells from infected mid-gestation embryos would continue to express MSV-gpt virus under in vivo conditions and could be selected directly for mycophenolic acid drug resistance, primary cultures on lung and the carcass of day 19 embryos were established (see Materials and Methods). Expression of MSV-gpt-defective virus could be detected after cocultivation of secondary cultures from embryos 2, 7, and 8 when cocultivated with NIH 3T3 cells, confirming the results shown in Table 1. Furthermore, mycophenolic acid-resistant colonies could be selected directly from secondary carcass cell cultures of embryo 7 (see Table 1). To derive permanent cell lines, four drug-resistant clones were transformed with SV40, isolated, and grown into mass cultures. Twenty-five adult animals derived from microinjected mid-gestation embryos and shown to be viremic by radioimmunoassay were analyzed for MSV-gpt virus expression at 2–4 months of age. Single cell suspensions were prepared from spleen, lung, and kidney, and infectious center assays were carried out as described above. As shown in Table 2, cells from 6 of 25 spleens, 2 of 24 lungs, and 1 of 24 kidneys gave rise to mycophenolic acid-resistant colonies after cocultivation with NIH 3T3 cells. Between 1 and 200 drug-resistant NIH 3T3 colonies were induced by 10^6 cells from different organs, similar to the frequency observed with cells.
from 19 embryos (Table 1). In contrast to the low frequency of MSV-gpt virus-producing cells, 1 in 10 to 20 spleen cells produced infectious Mu-MuLV helper virus, as shown by an infectious center assay and analysis for XC plaque formation (data not shown), confirming previous results (26). When NIH 3T3 cultures were exposed to fresh blood samples of the experimental viremic animals, no mycophenolic acid-resistant colonies were observed, whereas a Mu-MuLV helper virus titer of \( 10^8 \) XC plaque-forming units per ml was found in the same blood samples.

To analyze whether infectious MSV-gpt virus can be rescued from drug-resistant NIH 3T3 clones after cocultivation with cells from different organs of infected animals, single colonies were isolated and grown into mass culture. The supernatant of these clones was titrated for Mu-MuLV and MSV-gpt virus. Table 3 shows that all mycophenolic acid-resistant NIH 3T3 clones produced both viruses at a titer that was comparable to the titer of the CI2C and \( \psi 2 \)-2-5 cell lines, respectively, which were used to infect the mid-gestation embryos.

The results summarized in Tables 1, 2, and 3 indicate that the recombinant MSV-gpt virus can be detected in different tissues of 19 embryos or adults that have been exposed to virus-producing cells at the mid-gestation stage. The fraction of cells from infected embryos producing the recombinant Eco gpt virus was 3 to 5 orders of magnitude lower than the fraction of cells producing Mu-MuLV helper virus.

### Table 1. Expression of Mo-MuLV and MSV-gpt in 19 embryos derived from microinjected embryos

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Expression of Mo-MuLV</th>
<th>Expression of MSV-gpt in</th>
<th>Kidney</th>
<th>Liver</th>
<th>Brain</th>
<th>Lung</th>
<th>Carcass</th>
<th>Heart and thymus</th>
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8/10 1/8 0/8 2/8 3/8 3/8 1/4

Day 19 embryos derived from microinjected mid-gestation embryos were assayed for expression of Mo-MuLV helper virus and MSV-gpt-defective virus by an infectious center assay. Fraction of cells expressing MSV-gpt in different organs was 1-10 per \( 10^7 \) (++), 10-100 per \( 10^7 \) (++), or <1 per \( 10^7 \) (–), respectively. The titer of Mo-MuLV was not calculated.

### Table 2. Expression of MSV-gpt virus in adult mice derived from microinjected embryos

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Blood from 25 viremic 5-week-old animals was analyzed for MSV-gpt expression. Spleen, lung, and kidney cells from 2- to 4-month-old animals were analyzed for MSV-gpt expression in an infectious center assay. Fraction of cells expressing MSV-gpt was 1-10 per \( 10^7 \) (++), 10-100 per \( 10^7 \) (+++), or <1 per \( 10^7 \) (–), respectively.

### DNA Analysis for gpt Sequences

To show the presence of MSV-gpt sequences in DNA from in vitro selected, mycophenolic acid-resistant clones (see above), high molecular weight DNA was prepared. The DNA was digested with Sst I, a restriction enzyme that cuts once in the 5' as well as the 3' terminal repeat of the defective MSV-gpt genome, generating an internal fragment of 3.1 kilobases (kb). After separation on agarose gels and transfer to nitrocellulose filters, the DNA was probed with a \( 32P \)-labeled nick-translated pSV2-gpt DNA probe (6).

Fig. 1A shows that all different drug-resistant NIH 3T3 clones derived by cocultivation with spleen, lung, or kidney cells of infected animals contained a single gpt-specific Sst I fragment corresponding in size to the internal 3.1-kb Sst I fragment of MSV-gpt present in the DNA from \( \psi 2 \)-2-5 cells, which were used for infection of the embryos (compare Fig. 1A, lanes b–f to lane a). In addition, DNA from \( \psi 2 \)-2-5 cells contained gpt-specific fragments of higher molecular weight, most probably because of rearrangement of the MSV-gpt genome during DNA transfection (lane a).

When DNA from four SV40-transformed clones derived directly from the carcass of embryo 7 (see above) was analyzed, a gpt-specific Sst I fragment \( 3.1 \) kb long was found (Fig. 1B, lanes b–e). The pattern of Sst I fragments of higher molecular weight was identical to that of the drug-resistant NIH 3T3 clones.

### Table 3. Titration of MSV-gpt-defective virus and Mo-MuLV helper virus from mycophenolic acid-resistant NIH 3T3 clones derived by cocultivation with spleen, lung, and kidney cells from viremic animals

<table>
<thead>
<tr>
<th>NIH 3T3 clone</th>
<th>MSV-gpt titer, gpt cfu/ml</th>
<th>Mo-MuLV titer, XC pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3/spleen (20)</td>
<td>( 1.6 \times 10^4 )</td>
<td>( 2.5 \times 10^5 )</td>
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<tr>
<td>3T3/spleen (4, 10, 12, 13, 18)</td>
<td>+ (NT)</td>
<td>+ (NT)</td>
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<tr>
<td>3T3/lung (18)</td>
<td>( 1.1 \times 10^4 )</td>
<td>( 2.0 \times 10^5 )</td>
</tr>
<tr>
<td>3T3/kidney (16)</td>
<td>( 2.5 \times 10^4 )</td>
<td>( 7.8 \times 10^4 )</td>
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<td>Controls</td>
<td>( \psi 2 )-2-5</td>
<td>( 5 \times 10^4 )</td>
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</table>

Mycophenolic acid-resistant NIH 3T3 clones derived after cocultivation with cells from spleen, lung, and kidney cells from 2- to 4-month-old viremic animals were titrated for MSV-gpt-defective virus on NIH 3T3 cells, followed by Eco gpt selection, and for Mo-MuLV helper virus by the XC plaque assay (33). NT, not titrated; cfu, colony-forming units; pfu, plaque-forming units. Numbers in parentheses represent animal number.
molecular weight was identical for all four clones but different from the ϕ2-2-5 cell line (compare Fig. 1B, lanes b-e to lane a), suggesting that only one independent transformant was derived. These additional fragments may represent integrated copies of SV40 DNA, because the probe used for these studies, 32P-labeled pSV2-gpt, contains SV40 sequences. These results suggest that the mycophenolic acid-resistant clones isolated directly from secondary embryonal cell culture are derived from infected embryonal cells rather than from ϕ2-2-5 cells that might have survived in the embryo.

When DNA from kidney, heart, lung, and brain of viremic 2- to 4-month-old mice and from the carcass as well as placenta from infected day 19 embryos was analyzed, no MSV-gpt sequences (at least <0.1 copy per cell) could be detected in any case (data not shown).

**DISCUSSION**

In this paper, we show the potential of retroviral vectors to transduce foreign genes into somatic cells of the postimplantation mouse embryo and to express these genes in many tissues of the resulting late embryo or adult animal. The bacterial Eco gpt gene carried by a replication-defective viral vector was chosen as a marker gene because in vitro selection for drug resistance is highly sensitive and allows detection of a small percentage of cells that express the transduced gene. In contrast to previous work in which concentrated virus stocks were microinjected into mid-gestation mouse embryos (27), in the work presented here we used virus-producing cells for microinjection. To facilitate replication and spread of the recombinant virus in the developing embryo, cells producing the replication competent Mo-MuLV were coinjected. Our results show that, in fact, microinjection of virus-producing cells is as efficient as using concentrated virus stocks and that microinjection of as few as 10 cells producing Mo-MuLV is sufficient to establish productive virus infection in ~50% of the infected embryos. The microinjection of virus-producing cells instead of virus greatly facilitates the introduction of foreign genes into animals, because it obviates the need to concentrate and purify recombinant virus stocks from cells that may produce low titers of virus.

A total of 1–3 × 10^6 cells were microinjected into the embryo, with the recombinant MSV-gpt virus-producing ϕ2-2-5 cells being in a 100-fold excess over the Mo-MuLV helper virus-producing cells. We cannot formally exclude the possibility that the injected cells survived for extended periods of time and were competent to produce virus even in the adult. The failure to cause visible disturbance of further embryonic development, however, suggests that the cells did not replicate in the embryo for an appreciable length of time after microinjection. It therefore seems likely that infection of embryonal cells occurred soon after the injection and that the recombinant virus may have spread during the subsequent stages of development by replication and superinfection of embryo cells. Consistent with this interpretation was the finding that the integration pattern of the MSV-gpt virus in cell clones directly derived from an infected late embryo was different from the integration pattern in ϕ2-2-5 cells.

When infected animals were analyzed either at day 19 of embryogenesis or as adults, cells producing Eco gpt recombinant virus were detected in a variety of tissues including brain, kidney, lung, spleen, and thymus and not in circulating blood cells. This is consistent with previous observations that Mo-MuLV can productively infect cells of all somatic tissues when introduced at the postimplantation stage (27). The fraction of cells producing the recombinant virus as detected by an infectious center assay was, however, 3–5 orders of magnitude lower than the fraction of cells producing helper virus. This figure most likely represents a minimum estimate, because the efficiently replicating helper virus may have decreased the number of Eco gpt infectious centers by virus interference (37). Furthermore, overproduction of the Eco gpt gene in cells of the developing embryo may be toxic, thus limiting the number of mycophenolic acid-resistant infectious centers in vitro. Apparent cell toxicity due to overproduction of XGPRT has been reported in tissue-culture experiments (38). Our results suggest that Mo-MuLV replicates much more efficiently in the infected embryo thus possibly limiting the initial infection and the later spread of the recombinant virus in vivo. We do not yet know whether the presence of actively replicating helper virus may in fact decrease rather than increase the fraction of cells expressing the transduced Eco gpt gene.

Retroviral vectors carrying a variety of cellular genes have been constructed during recent years (15–23). So far, these genes have been transduced successfully by virus infection of cells cultured in vitro (15–21, 23), and more recently, of hematopoietic stem cells (22). The results presented in this paper indicate that retrovirus vector-mediated introduction and expression of foreign genes in a wide range of somatic tissues in vivo is also possible. Recently, we have used the same retroviral vector to insert the Eco gpt gene into the germ line of mice by infection of preimplantation embryos (unpublished data). Thus, the availability of retroviral vector systems greatly enhances our ability to genetically manipulate somatic and germ cells of animals and may ultimately provide the means for targeting foreign genes to specific cells and potentially to correct genetic defects in somatic tissues.

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