Introduction of genes into preimplantation mouse embryos by use of a defective recombinant retrovirus
(transgenic mice/murine leukemia virus)

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ABSTRACT Two-cell and four-cell preimplantation mouse embryos were cocultured in vitro with fibroblasts producing the recombinant retrovirus M-MuLV-neo. No wild-type helper virus was detected in these cultures. Of the embryos that survived the in vitro cultivation and the reimplantation into foster mothers, 2 of 15 that were tested contained the proviral genome. The provirus integrated as a single copy at a unique site. We estimate that ~20% of the cells in each of the two transgenic fetuses contained the provirus.

For investigation of a number of problems, it is important to be able to introduce DNA into embryonic cells. Presently, genes are introduced into mammalian embryos by microinjection of DNA into one of the pronuclei of the fertilized egg (1). The resulting integrated copies of the gene are usually found as long tandem repeats, a result that complicates studies requiring a single well-defined gene. This problem can be circumvented if the injected DNA is a transposable element, as exemplified by the P elements of Drosophila. These molecules, when injected into a fly embryo of the appropriate genotype, efficiently insert the exogenous DNA as a unique copy with a well-defined structure (2).

Mammalian retroviruses share many of the characteristics of transposons (3). They integrate into the host chromosome by a specific mechanism, thereby producing a provirus of a predictable structure (3). Also, one can easily introduce a single proviral copy into the host genome. These facts, coupled with the rapid development of recombinant retroviruses as vectors to introduce new genes into cells (4–12), make this technology an attractive alternative to microinjection. Using recombinant retroviruses, several groups have succeeded in introducing genes into embryonal carcinoma cells (8–12). Further, it has been shown that wild-type retroviruses can infect preimplantation mouse embryos and stably integrate their proviral genome in germ-line cells (13, 14). Injection of a mixture of wild-type and recombinant retrovirions into postimplantation mouse embryos has led to the successful introduction of the Escherichia coli gpt gene into the somatic tissues of mice (15). However, this approach introduces the recombinant viral genome into only a very small fraction of the embryo’s cells.

In this paper, we report the infection of preimplantation embryos with a recombinant retrovirus that carries the bacterial neomycin-resistance gene (neo). This gene was thus stably introduced into the mouse genome by means of viral stocks that did not contain leukemogenic wild-type virions.

MATERIALS AND METHODS

Embryos. Embryos were isolated from 3- to 4-week-old female mice that had been induced to superovulate (16, 17).

Following the injection of chorionic gonadotropin, the female mice were mated. The females were killed by cervical dislocation 44 or 54 hr later, and the embryos (at two- and four-cell stages, respectively) were isolated. The embryos were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (4.5 mg/ml), pyruvic acid (0.1 mg/ml), and NaHCO3 (3.7 mg/ml) and supplemented with 10% fetal bovine serum (Biochrom, Berlin), penicillin (5 x 105 units/ml), and streptomycin (0.1 mg/ml). The zona pellucida was removed by placing the embryos in a solution of acidic Tyrode’s solution (18), and the embryos were then cocultured for 24 hr with the virus-producing cell line at 37°C in air containing 12% CO2. No more than four embryos were placed in the same culture well in order to obviate extensive aggregation of the blastomeres.

Virus and Infection. In these experiments, we have used a cell line that produces a defective recombinant retrovirus called M-MuLV-neo (8, 9). This virus is a derivative of the Moloney murine leukemia virus (M-MuLV) in which the neo gene (19) has replaced the viral structural genes. The neo gene, which encodes an enzyme that phosphorlates amingoglycosides, is under the transcriptional control of the M-MuLV promoter. The M-MuLV-neo provirus has been introduced into ϕ2 cells (4); the ϕ2 cell line produces defective recombinant retroviruses free of wild-type virions. Periodically, our viral stocks were assayed for the presence of wild-type virions by the S+L− focus-forming test (3) and were found to contain less than 1 focus-forming unit/ml. Twenty-four hours before the embryos were isolated, 1 ml of medium containing 105 cells was transferred to each well of 48-well tissue culture plates (16-mm well diameter, Costar, Cambridge, MA). One day later, the cultures were semiconfuent and the medium contained about 106 neo-transducing virions per milliliter. Polybrene (Sigma) was added (4 μg/ml) to these cultures just before addition of the embryos (but see Table 1, footnote §).

After the 24-hr cocultivation, the embryos were transferred to a 60-mm glass Petri dish (siliconized with a solution of 2% dimethyl dichlororosilane in 1:1,1-trichloroethane, BDH) containing the complete DMEM medium. They were incubated for an additional 48 hr, after which the expanded blastocysts were washed in phosphate-buffered saline (138 mM NaCl/3 mM KCl/8 mM Na2HPO4/1.5 mM KH2PO4, pH 7.4) containing bovine serum albumin (4 mg/ml) and then were reimplanted in pseudopregnant foster mothers (20).

Analysis of Fetal DNA. Four days after reimplantation, the pregnant foster mothers were killed, and the embryos were isolated and separated from their extraplacental membranes. They were frozen, minced with a scalpel, and then digested for 90 min at 37°C with proteinase K (1 mg/ml, Merck) in 10 mM Tris-HCl, pH 8.0/10 mM EDTA, pH 8.0/150 mM NaCl/0.2% Na2SO4. This solution then was extracted three times with equal volumes of phenol/chloro-

Abbreviations: neo, neomycin-resistance gene; M-MuLV, Moloney murine leukemia virus; LTR, long terminal repeat.
RESULTS

In Vitro Retroviral Infection of Preimplantation Mouse Embryos. Two- and four-cell-stage mouse embryos were obtained from parents of a variety of genotypes (see Table 1). After their zona pellucidae were removed, the embryos were placed in culture wells containing fibroblasts constitutively producing a recombinant retrovirus. This defective retrovirus, M-MuLV-neo, from which the gag, pol, and env genes have been deleted, contains the bacterial neo gene under the transcriptional control of the M-MuLV long terminal repeat (LTR) (8, 9). These fibroblasts do not produce wild-type M-MuLV virions (4). The embryos were grown in these wells until they reached the compacted-morula stage and then were transferred to a siliconized Petri dish containing virus-free medium and incubated until they reached the expanded-blastocyst stage. Then they were reimplanted into the uterus of pseudopregnant foster mothers.

In the initial experiments, the embryos were cocultivated with the fibroblasts until they reached the blastocyst stage. This technique was successful at producing blastocysts and, eventually, one transgenic fetus (Table 1). However, the embryos at the stage of compaction have a strong tendency to adhere to the fibroblasts. To circumvent this difficulty, the embryos were transferred to a siliconized Petri dish when they reached the compacted-morula stage.

With this procedure, 50–80% of the two- and four-cell-stage embryos developed into blastocysts in culture (data not shown). Although the two-cell stage embryos grew well in vitro, only 7 of 310 developed into fetuses. On the other hand, of the reimplanted blastocysts that were derived from embryos cultured in vitro from the four-cell stage, ~30% developed into 17-day-old fetuses (Table 1).

Analysis of Fetal DNA. If a virus infects only one of an embryo’s blastomeres, the resulting mouse has a high probability of being a mosaic with respect to the tissue distribution of the provirus. For this reason, we began our studies by sacrificing the fetuses at 17 days of gestation and analyzing DNA extracted from the total fetus.

By Southern blot analysis, the presence of the M-MuLV-neo provirus was detected in 2 of the 15 fetuses analyzed (Fig. 1). Two types of analysis were performed; in each one, a probe homologous to the neo gene was used. First, to determine whether the provirus had suffered a rearrangement, the DNA was digested with Xba I, an enzyme that cuts the provirus twice (once in each LTR). The Xba I fragment derived from each fetus was the same size as the control Xba I fragment derived by digestion of the plasmid pM-MuLV-neo with Xba I (Fig. 1), thereby demonstrating that no major rearrangement had taken place. The second type of analysis involved determining the number of proviral integration sites. This was achieved by digesting the DNA with BamHI, which cuts the provirus once, just 3’ of the neo gene. Each

![Fig. 1](image-url)

**FIG. 1.** (Upper) Southern blot analysis of fetal DNA, demonstrating the presence of an integrated copy of the M-MuLV-neo provirus. F1 and F2 correspond to fetuses derived from embryos placed in culture at the two- and the four-cell stage, respectively. Each DNA sample was digested separately with the restriction enzymes BamHI and Xba I. Note that the Xba I digestion of the F1 sample is incomplete. The size, in kilobases (kb), of the fragments is shown at left. (Lower) A schematic representation of the proviral structure, in which the size, in base pairs (bp), and the positions of the LTRs, the neo gene, and the restriction enzyme recognition sites are indicated.

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**Table 1. Efficiency of obtaining transgenic mice**

<table>
<thead>
<tr>
<th>Stage of embryo*</th>
<th>No. of blastocysts reimplanted1</th>
<th>No. of fetuses formed</th>
<th>Fetuses containing M-MuLV-neo, no./no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-cell</td>
<td>305</td>
<td>7 (2%)</td>
<td>1/7†</td>
</tr>
<tr>
<td>Four-cell</td>
<td>278</td>
<td>76 (27%)</td>
<td>1/8‡</td>
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* A number of different genotypes were used; no reproducible difference in their ability to be cultured in vitro was detected. The crosses included (C57BL/6 × CBA/J)F1 × SJL/J, (C57BL/6 × CBA/J)F1 × (C57BL/6 × DBA/2)F1, (C57BL/6 × SJL/J)F1 × (C57BL/6 × SJL/J)F1, and (CBA/J × SJL/J)F1 × (C57BL/6 × DBA/2)F1. The female genotype precedes the male genotype.

1 Foster mothers were (C57BL/6 × CBA/J)F1 or (C57BL/6 × BALB/c)F1.

2 The positive fetus was derived from the cross (C57BL/6 × SJL/J)F1 × SJL/J.

3 The positive fetus was derived from the cross (CBA/J × SJL/J)F1 × (C57BL/6 × DBA/2)F1. Note that Polybrene was not used in this experiment. This embryo was cultured with the virus-producing cells until the blastocyst stage.
integation site will thus give rise to a fragment of a defined length determined by the distance to the next BamHI site in the flanking genomic DNA. In each case, only one fragment was detected (Fig. 1), showing that there was only one integration event in each embryo. We have performed additional analyses using EcoRI (which does not cut the proviral DNA) and have obtained similar results (data not shown). Finally, in these two fetuses, the provirus was found to be present at \( \approx 0.2 \) genome equivalent (data not shown).

**DISCUSSION**

This paper describes the introduction of a bacterial gene into the mouse chromosome by infection of preimplantation embryos with defective retrovirions, free of wild-type helper virus. These experiments are an extension of the studies of Jaenisch and coworkers (13, 14), who demonstrated that wild-type retroviruses can introduce their genome into preimplantation mouse embryos. Jaenisch and other colleagues subsequently reported that injection of a mixture of recombinant and wild-type retrovirions into postimplantation embryos (8.5–10.5 days of gestation) leads to the introduction of the recombinant viral genome into the embryo’s cells (15). Although this approach will be useful for a number of studies; it uses a leukemogenic wild-type virus and results in a low percentage of cells that are infected with the recombinant virus. These low efficiency seriously decreases the probability of introducing the recombinant genome in the germ cells.

As an alternative approach, we have infected preimplantation embryos with helper-free recombinant retrovirions carrying the bacterial neo gene. Of the fetuses that survived, 15 were tested and 2 were found to contain the M-MuLV-neo provirus in a significant fraction of their cells (10–30%, 0.2 genome equivalent, see Results). In each case, only one major site of proviral integration was detected, which suggests that, initially, only one blastomere was infected and that its descendants gave rise to all of the proviral-containing tissues. This procedure should therefore prove helpful to the study of cell lineages during mouse development.

In these studies, we used a recombinant retrovirus in which the LTR functions as the promoter for the neo gene. Several groups have suggested that the LTR is inactive in embryonic cells (9–11, 22–24). Further, when a retrovirus infects an embryonic cell, the expression of its genes is repressed throughout most of development and only becomes activated at a low frequency as the animal matures (14, 25). Hence, we suspect that the M-MuLV-neo provirus will rest in a nontranscribed state and therefore will not be useful for expressing newly introduced genes.

In one modified version of the M-MuLV-neo virus, M-MuLV-SV\(k\)-neo, the expression of the neo gene is under the transcriptional control of the SV\(k\) promoter (a composite simian virus 40 early promoter/herpes simplex virus type I thymidine kinase gene promoter). We have shown that M-MuLV-SV\(k\)-neo is much more efficient at expressing the neo gene in embryonic cells (8, 9) and expect that the internal SV\(k\) promoter should help gene expression throughout development.

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