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Genetics

Long-term *in vivo* expression of retrovirus-mediated gene transfer in mouse fibroblast implants

(vectors/factor IX/β-galactosidase/promoters/collagen implants)

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ABSTRACT **Toward the goal of gene therapy, we have been attempting to establish model somatic cell systems with the potential of sustained expression of the foreign gene. We report here that long-term expression of foreign genes in mouse embryo fibroblast implants can be achieved if a housekeeping gene promoter is used to drive transcription. Specifically, we have shown that in implants containing a β-galactosidase gene linked to either an immediate early promoter of cytomegalovirus or a dihydrofolate reductase (DHFR) gene promoter, only the DHFR promoter allows long-term expression. We propose that choice of the promoter manifests significant influence on the long-term expression of genes introduced in fibroblast implants by retroviral vectors.**

Current models for gene therapy involve the retrovirus-mediated transfer of genetic material into cells derived from a variety of somatic host tissues, including cells of the hematopoietic system, fibroblasts, hepatocytes, endothelial cells, and myoblasts (1, 2). We have previously described an approach for gene product delivery by retroviral infection of mouse skin fibroblasts (3). The transduced genes used in our previous studies in fibroblasts were the human and dog factor IX cDNAs (3, 4). Although high levels of sustained expression could be achieved in tissue culture, when transplanted as allografts in rodents, these fibroblasts produced substantial amounts of factor IX for only a short period of time (3, 5). This short duration of expression *in vivo* could theoretically be attributed to different factors: (*i*) an immune response of the host against the exogenous factor IX; (*ii*) destruction of the foreign cells after transplantation; and (*iii*) a specific decrease in transcription of the transferred gene once the transduced cells are grafted to the animal. It has been shown (3, 5) that antibodies to human factor IX are present after implantation of modified fibroblasts, which could explain, at least in part, the short period of detection of factor IX.

In this work, using different promoters to control the expression of β-galactosidase, we demonstrate that, whereas in tissue culture long-term expression can be easily obtained, the type of promoter directing transcription of the gene of interest can be one of the key factors determining long-term expression *in vivo*.

MATERIALS AND METHODS

Animal and Cell Culture Conditions. Adult male C57BL/6J mice (6–8 weeks old) and *Nu/Nu* athymic mice were obtained from The Jackson Laboratory. The retroviral packaging cell lines ψCRE and ψCRIP (6) and the cell lines NIH 3T3 and rat 208 F were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Primary fibroblasts were obtained from day 17 embryos of C57BL/6J mice and were grown in DMEM supplemented with 10% fetal calf serum. Infected cells were selected in medium containing 400 μg of G418 per ml.

Vector Construction. Retroviral vector LNCdF9L, which transduces canine factor IX, has been described (4). The vectors shown in Fig. 1 were generated by inserting a 3.1-kilobase-pair (kbp) *Bam*HI fragment containing the entire coding sequence of the β-galactosidase gene into the *Bgl* II site of plasmid LNL-SLX to generate the vector LNL-SLXβgal. The LNL-SLX vector is a derivative of LNL-XHC (7) and contains a polylinker to increase the number of cloning sites. A 350-bp *Hin*dIII fragment of the mouse dihydrofolate reductase (DHFR) promoter was cloned in the unique *Hin*dIII site of LNL-SLXβgal. A *Bam*HI/*Hin*dIII fragment containing the human intermediate early cytomegalovirus (CMV-IE) promoter [−522 to +55; a gift of J. A. Nelson (8)] was cloned in the *Bam*HI/*Hin*dIII site of LNL-SLXβgal.

Virus Production. Ten micrograms of plasmid DNA was transfected into the ecotropic packaging cell line ψCRE by the calcium phosphate coprecipitation method. The medium was changed 24 hr later, and 48 hr after transfection the culture medium was harvested and used to infect the amphotropic packaging cell line ψCRIP in the presence of Polybrene (8 μg/ml). Single colonies of infected ψCRIP were isolated by selection in the presence of G418-containing medium and expanded. Recombinant retroviruses were harvested from confluent culture dishes, filtered, and used to infect NIH 3T3 cells in the presence of Polybrene to determine the viral titers. Twenty-four hours after infection, the medium was changed to G418-containing medium and colonies were stained and counted after 12–14 days. The presence of helper virus was assayed by the marker residue method (9). Briefly, the medium from the infected cells was used to infect naive NIH 3T3 cells. The presence of β-galactosidase-positive cells was determined after 72 hr and the presence of G418-resistant colonies was quantified after 14 days. Assays for production of factor IX were carried out as described (4). The transduced fibroblasts produced ≈400 ng of canine factor IX per 10^6 cells per day.

Implantation of Infected Mouse Embryo Fibroblasts in Mice. Infected mouse embryo fibroblasts were embedded in a collagen matrix as described (3). The collagen matrix containing 2×10^6 infected fibroblasts was then grafted into the connective tissue of the dermis in the mid-back of recipient mice. To ensure rapid vascularization of the grafted tissue, a 2-mm^2 piece of Gelfoam (Upjohn) containing 2 μg of basic fibroblast growth factor was inserted into the connective tissue along with each graft as described (3). At different time

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Abbreviations: DHFR, dihydrofolate reductase; CMV, cytomegalovirus; CMV-IE, CMV immediate early region; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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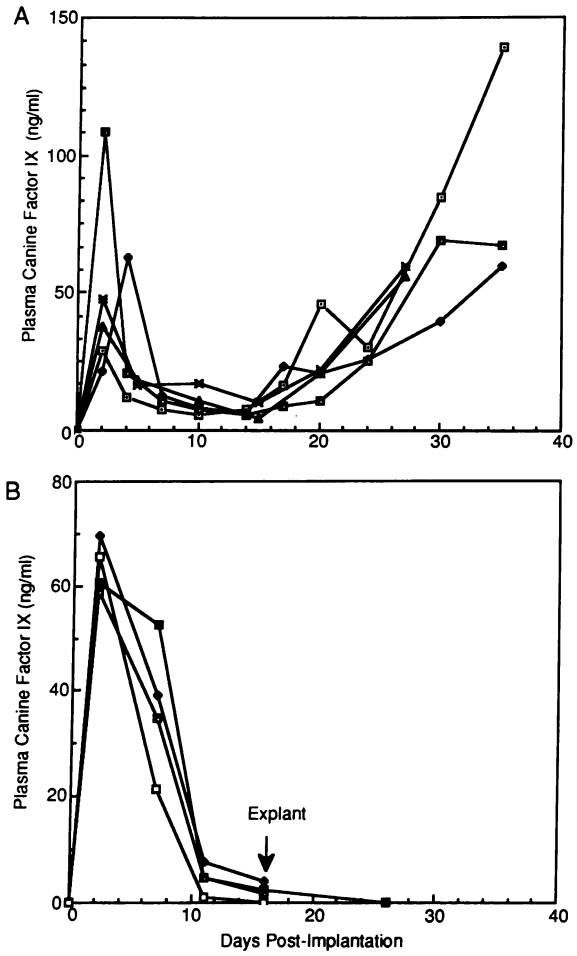

FIG. 1. Canine factor IX in mouse plasma. Levels of canine factor IX were analyzed in the plasma of mice that received two collagen implants, each containing $\approx 10^7$ cells transduced with the LNCdF9L canine factor IX virus. The collagen implants contained either transformed mouse fibroblasts (*A*) or primary skin fibroblasts (*B*) derived from adult *nu/nu* mice. Each symbol represents a different mouse. Canine factor IX levels were determined by ELISA (4).

intervals, the implanted artificial collagen matrix was removed and stained for β-galactosidase activity.

Analysis of β-Galactosidase Activity. β-Galactosidase histochemistry was assayed according to Sanes *et al.* (10) with minor modifications. Briefly, cultured cells were rinsed with phosphate-buffered saline solution (PBS) (pH 7.4) and then fixed for 5 min on ice in 2% formaldehyde/0.2% glutaraldehyde in PBS. The cells were then rinsed two times with PBS and overlaid with a solution containing 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS (pH 7.4). Incubation was at 37°C for 2–24 hr. To analyze β-galactosidase activity in the artificial collagen matrix, the fixation was prolonged for 30 min on ice.

RESULTS

Expression of Factor IX from Fibroblast Implants in Nude Mice. To partially alleviate the problem of immune response against foreign proteins, we implanted the canine factor IX infected syngeneic skin fibroblasts in nude mice. Fig. 1*A* shows that if transformed mouse fibroblasts producing canine factor IX are used as an implant, the levels of factor IX detected in mouse serum are highest at about day 5 postimplantation, and by day 10 they decline to near basal levels. However, by day 15 the levels of secreted factor IX begin to increase and continue to increase thereafter (Fig. 1*A*). Because the implant contained tumorigenic cells, which lead to the formation of palpable tumors, the increase in production of factor IX reflects cell growth. However, the experiment clearly shows that the reduction of factor IX levels by day 10 is not due to the antibodies against canine factor IX as biologically active factor IX can be detected even after 30–35 days.

In contrast, when primary *Nu/Nu* mouse fibroblasts were infected with canine factor IX retrovirus, expression of factor IX in plasma could not be detected 10–11 days postimplantation (Fig. 1*B*). After 10 days, the cells were explanted from the collagen matrices and grown in culture medium containing G418 to remove cells that may have invaded the encapsulated matrix. Approximately 10–20% of the cells in the graft were G418 resistant. Analysis of factor IX from the explanted cells showed the level of secretion to be similar to preimplanted cells, although they had matured considerably and had a reduced rate of division. These experiments suggested that the inability of the implants to produce and secrete factor IX is not due to either the immune response or rejection of the implant. We therefore argued that in the implanted cells, due to unavailability of perhaps specific growth factors, the CMV promoter is unable to drive transcription of factor IX cDNA. However, other epifactors, such as cell mass, etc., may also account for the increased expression of factor IX. Results obtained by PCR analysis also suggested that the levels of factor IX RNA in the implant had precipitously declined and were undetectable in day 16 implants (J.H.A., unpublished results).

Use of Housekeeping Gene Promoters. We next asked whether sustained expression in the implants may not be a function of the type of promoter used to initiate the transcription of the foreign gene. Since CMV-1E is an inducible promoter and may require actively growing cells, we decided to use a housekeeping promoter to maintain the constitutive levels of expression. Therefore, we constructed retroviral vectors containing murine DHFR gene promoter and the bacterial β-galactosidase gene as a reporter (Fig. 2). Clones producing high-titer amphotropic recombinant viruses were selected by infecting NIH 3T3 cells and analyzed for β-galactosidase activity and the presence of helper viruses. Fig. 2 shows that only a few clones producing $>5 \times 10^4$ neomycin-resistant (neo^R) colonies per ml could be identified, but the resultant recombinant viruses were stably propagated. The initial levels of β-galactosidase activity were higher in cells infected with LNL-SLX CMV β-galactosidase virus (visible after 2 hr of incubation) as compared to LNL-SLX DHFR β-galactosidase virus (visible after 6–8 hr of incubation). However, in a population of NIH 3T3 cells infected with LNL-SLX DHFR β-galactosidase virus, nearly all the G418-resistant cells were positive for β-galactosidase activity. In comparison, infection with LNL-SLX CMV β-galactosidase virus showed that only 50% of the G418-resistant cells were β-galactosidase positive. No replication-competent virus could be detected by marker rescue in any of the clones tested.

To further characterize the recombinant viruses containing CMV and DHFR promoters, we analyzed the RNA transcripts from cells infected with LNL-SLX CMV β-galactosidase and LNL-SLX DHFR β-galactosidase viruses. Fig. 3 shows that the transcripts of expected size (6.6, 6.1, and 3.6 kb) can be detected in virus-producing CRIP cells or mouse embryo fibroblasts. The 3.6-kb mRNA represents transcripts initiated from the CMV or DHFR promoter. No detectable levels of β-galactosidase RNA were detected in uninfected cells.

β-Galactosidase Expression in Mice. To test whether the sustained expression of β-galactosidase can be attained *in*

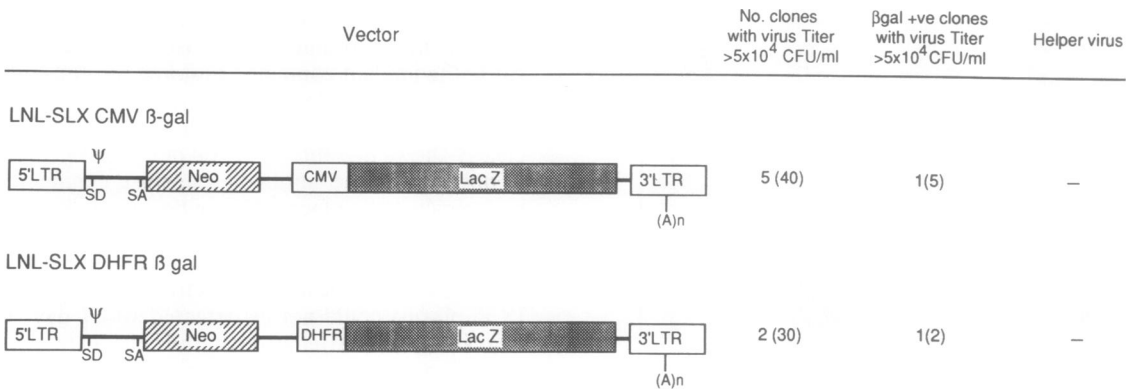

FIG. 2. Structure of retroviral vectors containing the β-galactosidase gene, titers of the recombinant retroviruses, and expression of β-galactosidase activity. The numbers of Neo^R colonies examined are in parentheses. Expression of β-galactosidase activity was determined by X-Gal staining. LTR, long terminal repeat; CFU, colony-forming units; SD, splice donor; SA, splice acceptor; (A)n, poly (A) signal.

vivo, mouse embryo fibroblasts were infected with either LNL-SLX CMV β-galactosidase or LNL-SLX DHFR β-galactosidase viruses. The infected cells were then embedded in a collagen matrix and grafted in mice. After different time intervals, the grafts were explanted and analyzed for the presence of β-galactosidase-positive cells. A minimum of two or three grafts were explanted at each time point. Fig. 4 shows that β-galactosidase-positive cells (stained blue with X-Gal) could be detected in 10-day implants with both promoters tested (Fig. 4 *C* and *D*). However, expression of β-galactosidase was detected in animals for up to 60 days (Fig. 4 *H* and *J*) only when infected with LNL-SLX DHFR β-galactosidase virus. We thus conclude that a housekeeping gene promoter like DHFR can provide sustained expression in the implants.

DISCUSSION

In this work, we have used β-galactosidase as a reporter gene to study some of the factors involved in the long-term expression of foreign genes transduced in mouse skin fibroblasts after retroviral infection. Our results demonstrate that once grafted into the animal, mouse embryo fibroblasts infected with a recombinant retrovirus can survive and express the transduced gene *in vivo* for at least 3 months (the longest time point tested so far). One of the critical points for long-term expression *in vivo* is related to the type of promoter directing transcription of the reporter gene. Indeed, long-term expression *in vivo* could not be obtained by using the CMV-IE promoter to drive the β-galactosidase gene, whereas expression persists for at least 3 months (data for 2 months are shown in Fig. 4; unpublished data) when the DHFR promoter is used.

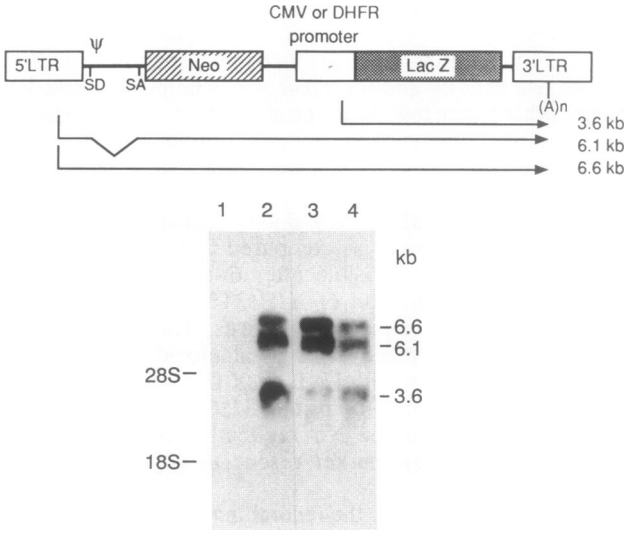

FIG. 3. Analysis of LNL-SLX CMV β-galactosidase and LNL-SLX DHFR β-galactosidase RNA. Total cytoplasmic RNA isolated from uninfected and infected primary cells and cell lines was subjected to Northern blot analysis. RNAs were fractionated on formaldehyde/agarose gel, transferred onto a nylon membrane, and hybridized to a 3.0-kb β-galactosidase probe labeled by random priming. RNAs were from primary fibroblasts (lane 1), ψCRIP-infected LNL-SLX CMV β-galactosidase (lane 2), ψCRIP-infected LNL-SLX DHFR β-galactosidase (lane 3), and primary fibroblast-infected LNL-SLX DHFR β-galactosidase (lane 4). LTR, long terminal repeat.

The choice of the bacterial enzyme β-galactosidase as a reporter gene for retrovirus-mediated gene transfer has been discussed (10–15). In well-defined conditions (10), the activity from the bacterial enzyme can be detected in the infected cells by simple histochemical staining without any background due to endogenous β-galactosidase activity. Moreover, the resulting β-galactosidase activity permits histochemical detection of individual cells expressing the integrated vector. As the recombinant retroviruses used here were helper-free and no detectable activity was present in uninfected cells, the β-galactosidase-positive cells present in the explanted grafts represent the cell population that was initially infected.

Many researchers have used recombinant retroviruses to introduce genes into primary cells. The bone marrow represents one tissue often used because of the presence of pluripotent cells capable of proliferation and differentiation during the entire life-span of the animal (16–18). However, upon reconstitution of the bone marrow with the infected cells, long-term expression of the foreign gene is generally very poor (1). We have recently described an alternative model for gene therapy by using retrovirus-infected primary fibroblasts (3). Once grafted to the animal, the exogenous gene was expressed by the infected cells but only for a short period of time (3). The relatively short duration of expression was due at least in part to an immune response, but other factors were also responsible for this short-term expression. Indeed in another study in which a similar experimental system was used (5), only short-term expression was detected in nude mice in which no immune response was detected. It is important to note that, in both cases, the promoters used to drive the gene of interest were either the CMV-IE or the retroviral 5′ long terminal repeat.

In a previous report, Li Xu *et al.* (19) reported that, in tissue culture, the choice of the promoter is not crucial for long-term expression, which is in agreement with our previous results. The role of the promoter directing transcription of the foreign gene after retrovirus-mediated gene transfer

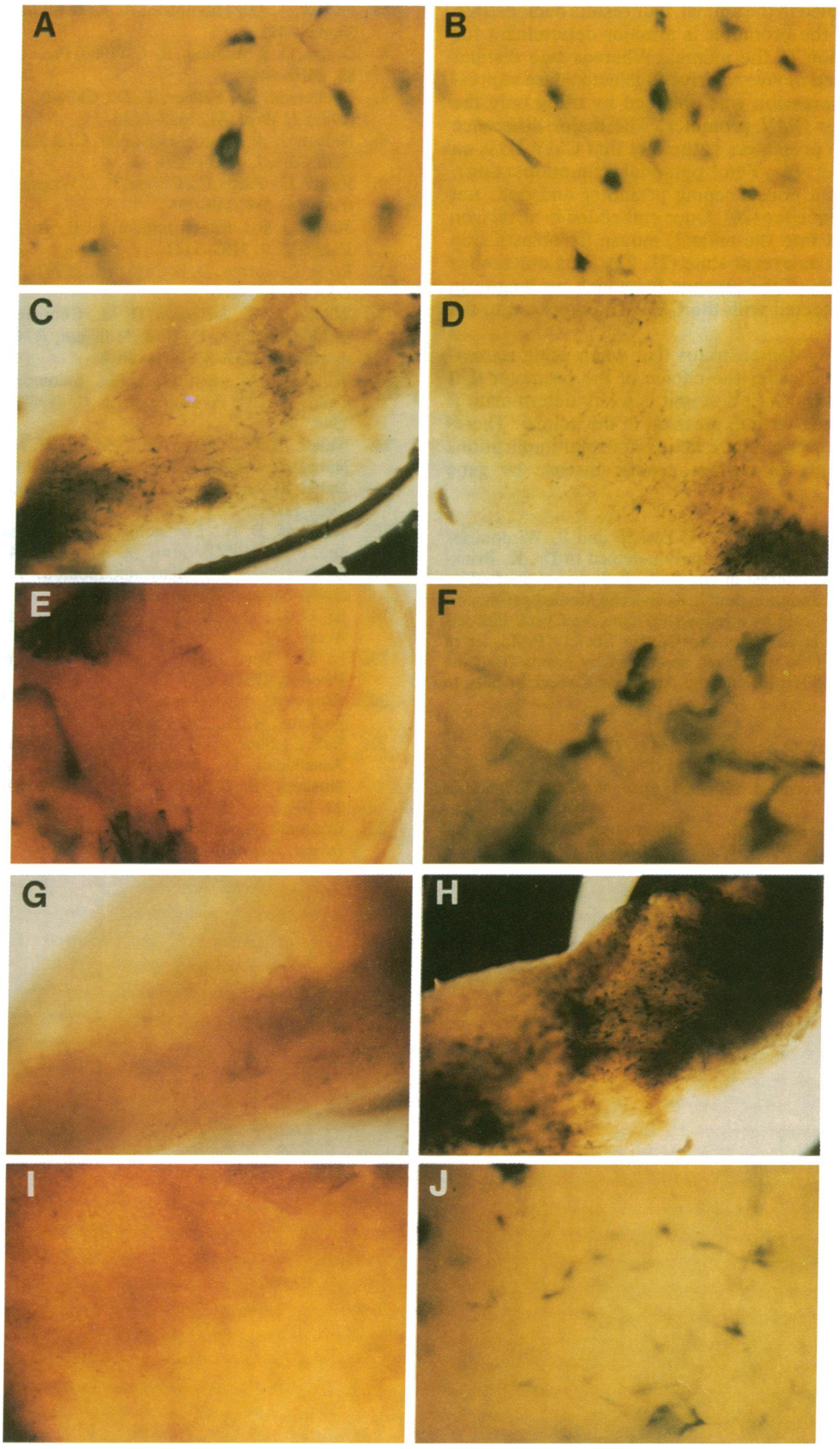

FIG. 4. Histochemical expression of β-galactosidase activity in infected fibroblasts *in vitro* and *in vivo*. Fibroblasts were infected with either SLX-CMV β-galactosidase (*A*, *C*, *E*, and *G*) or SLX-DHFR β-galactosidase (*B*, *D*, *F*, *H*, and *J*) and were embedded in a collagen matrix and stained for β-galactosidase activity. (*A* and *B*) Virus-infected cells embedded in the matrix and stained before implantation. (*C–J*) The matrices were grafted to mice and removed after different intervals in the animal. (*C* and *D*) Matrices were removed after 10 days. (*E* and *F*) Matrices were removed after 1 month. (*G*, *H*, and *J*) Matrices were removed after 2 months. (*I*) Matrix containing uninfected cells was removed after 2 months in the animal.

has never been studied in the animal. Our results demonstrate that the choice of the promoter is a major determinant for long-term expression in the animal. Whereas two distinct promoters were used *in vivo* to drive β-galactosidase expression, long-term expression was obtained by using only the DHFR and not the CMV promoter. The major difference between these two promoters is the fact that CMV-IE is an inducible promoter (J. Yen, personal communication), whereas DHFR is a housekeeping promoter and does not contain a TATA sequence (20). Once embedded in a collagen matrix and grafted into the animal, mouse fibroblasts stop growing to reach a quiescent state (21, 22). This quiescence could represent one of the reasons for which no long-term expression was detected with the CMV-IE compared to the DHFR promoter.

Finally, this study demonstrates that when using retrovirus-mediated gene transfer the choice of the promoter that drives the internal transcription unit is a key determinant if the major aim is long-term expression in the animal. Therefore, the results presented here have important implications for current attempts to correct genetic disease by gene therapy.

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