Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3357–3361, April 1992
Medical Sciences

Expression of human factor IX in mice after injection of genetically modified myoblasts

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Communicated by Oscar D. Ratnoff, January 9, 1992 (received for review October 24, 1991)

ABSTRACT **Hemophilia B is an X chromosome-linked recessive bleeding disorder. To develop a somatic gene therapy for this disease, we have examined whether mouse skeletal myoblasts can serve as efficient vehicles for systemic delivery of recombinant factor IX. When mouse myoblasts (C2C12) transduced with a Moloney murine leukemia virus-based vector containing the bacterial β-galactosidase gene were injected into mouse skeletal muscles, they fused with the existing and regenerating myofibers and continued to express β-galactosidase. C2C12 myoblasts that were infected with recombinant retroviruses containing a human factor IX cDNA secreted biologically active human factor IX into the culture medium at a rate of 2.6 μg per 10^6 cells per day. Myotubes derived from these cells in culture continued to express human factor IX (0.68 μg/day from myotubes derived from 10^6 C2C12 cells). After injection of the transduced C2C12 myoblasts into skeletal muscles of mice, the systemic level of recombinant human factor IX was found to be as high as ≈1 μg/ml of serum. These results provide the rationale for using skeletal myoblasts as an efficient gene delivery vehicle in the somatic gene therapy for hemophilia B.**

Factor IX is a plasma glycoprotein that plays a pivotal role in the middle phase of the blood coagulation cascade (1). It is normally synthesized in liver and secreted into the circulation. A deficiency of biologically active factor IX in circulation results in an X chromosome-linked recessive bleeding disorder, hemophilia B (Christmas disease). The current treatment for this disease by plasma protein replacement therapy is effective but complicated by serious side effects, such as possible exposure of patients to blood-borne pathogenic viruses, including hepatitis and human immunodeficiency viruses. Somatic cell gene therapy may provide an alternative safe treatment for this disorder (2). In such an approach, the normal factor IX gene is transferred into target somatic cells that can stably produce active factor IX and transport it into the circulation. The somatic cells used must be able to efficiently carry out various post-translational modifications, such as γ-carboxylation, required for the biological activity of factor IX (1). Genetically modified skin fibroblasts implanted in mice as *in dermis* or subcutaneous implants can produce and secrete recombinant factor IX into the circulation (3, 4). This approach, however, has suffered from poor stability of expression (5) and inefficient transportation of recombinant proteins into the circulation (4).

Skeletal myoblasts have several unique properties that make them attractive for use in somatic gene therapy. Proliferating myoblasts are readily isolated and cultured in large numbers (6, 7). More importantly, these cells can fuse with existing muscle fibers when injected into muscle tissues (8). In this report, we demonstrate the expression of the recombinant genes for human factor IX and β-galactosidase by injecting genetically modified myoblasts into mouse skeletal muscles. This *ex vivo* approach in mice has resulted in the systemic delivery of a high level of recombinant factor IX.

MATERIALS AND METHODS

Recombinant Retroviruses. Moloney murine leukemia virus-derived retrovirus vectors (LIXSN and BAG) have been described (9, 10). LIXSN contains a 1.4-kilobase human factor IX cDNA under the direct control of the 5′ long terminal repeat (LTR) and a neomycin-resistance gene with a simian virus 40 promoter. BAG contains a bacterial β-galactosidase gene controlled by the LTR promoter and an expression unit of the simian virus 40–neomycin-resistance gene (10). Amphotropic retroviruses of these vectors with titers of 10^5–10^6 colony-forming units/ml were prepared with ψ-crip as described (9, 11).

Cell Culture and Retrovirus Infection of Cells. C2C12 cells are clonally derived myoblasts from leg skeletal muscles of an adult C3H mouse and have all of the characteristics of myoblasts (6, 7). This cell line can differentiate into contracting myotubes that express various muscle proteins and is, therefore, used as a model for skeletal myoblasts in the present study. C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% (vol/vol) fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Approximately 5×10^5 cells were seeded in a 100-mm plate 1 day before exposure to 10 ml of retrovirus stock medium containing Polybrene at 8 μg/ml for 20 h, as described (9). The retrovirus-infected cells were cultured in regular medium for 1 day, and then neomycin-resistant cells were selected in medium containing G418 (GIBCO, 1 mg/ml, 50% active) for 1 week. All resistant cells (≈10^5 independent colonies) were pooled and expanded for further studies. Differentiation of C2C12 myoblasts into multinucleated myotubes was carried out by exposing the confluent myoblast cultures to DMEM with 2% (vol/vol) horse serum for 3 days.

Analysis of Human Factor IX in Culture Medium and Mouse Serum. The amount of human factor IX and its activity in culture medium were quantitated by an ELISA using a mouse anti-human factor IX monoclonal antibody (HFIXα40) and the one-stage clotting assay, as described (9). To determine the amount of human factor IX in blood, mouse serum (0.1–0.2 ml) was collected by tail bleeding and assayed by an ELISA. The monoclonal antibody (HFIXα40) used does not cross-react with mouse, rat, rabbit, or bovine factor IX (unpublished data).

Myoblast Injection. Pathogen-free male C3H mice of 6 weeks old were anesthetized by intraperitoneal injections of 0.3 ml of 2.4% (wt/vol) Avertin (2,2,2-tribromoethyl alcohol) in phosphate-buffered saline (PBS; 4.3 mM Na_2HPO_4/1.4 mM $KHPO_4$/2.7 mM KCl/113 mM NaCl, pH 7.4). The posterior sides of four limbs and the back were shaved and cleaned with 75% alcohol. Transduced C2C12 myoblasts were resuspended in 0.5–0.75 ml of serum-free DMEM at 4

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Abbreviation: LTR, long terminal repeat.

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$\times 10^7$ cells per ml and loaded into a 1-ml tuberculin syringe. Muscle groups on the posterior sides of four limbs and on both sides of the spine were surgically exposed, and myoblasts were injected at 20–50 sites through a 30-gauge needle. Each injection released 10–20 μl of cell suspension. The incisions were closed with stainless steel autoclips (MikRon Precision). To prevent any possible immune rejection of transplanted cells, immunosuppressive agent cyclosporine [Sandoz, 5 mg/kg (body weight)] was given by intraperitoneal or intramuscular injection 1 day before myoblast transplantation and every day thereafter. On various days, mouse serum (0.1–0.2 ml) was collected by tail bleeding and was frozen at −70°C until further analysis.

Immunostaining Analysis of Cells. Immunostaining of myoblasts and myotubes was carried out using a Histostain-SP kit (Zymed Laboratories) as described (9). Briefly, cells were grown on gelatin-coated glass cover slides and fixed with 4% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature. The cells were then incubated with mouse anti-human factor IX monoclonal antibody (HFIXα40), biotinylated goat anti-mouse antibody, and then horseradish peroxidase-conjugated streptavidin and 3-amino-9-ethylcarbazole. Slides were counterstained with hematoxylin to show cell structure and photographed with a Nikon microscope.

Histological Staining for β-Galactosidase Activity. Cultured cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Muscle tissues were treated with the same fixing solution at 4°C overnight. Fixed tissues or cells were rinsed two or three times in PBS and incubated in staining solution [5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (1 mg/ml)/1 mM $MgCl_2$/0.5 mM potassium ferriferrocyanide/PBS] at 37°C overnight. For tissue sections, fixed and stained muscles were embedded in glycol methacrylate and sectioned at 4 μm. These sections were counterstained with hematoxylin and eosin and were visualized with a Nikon microscope.

Protein Blot Analysis. The molecular size of the recombinant human factor IX was analyzed on a protein blot (9). A sample (5 ml) of the 2-day culture medium was harvested from a dish containing transduced myoblasts or myotubes, precipitated with barium citrate, and then immunoprecipitated. The precipitated protein fraction was subjected to protein blot analysis, as described (9).

Detection of Antibody Against Human Factor IX. Samples of purified human factor IX (Calbiochem, 80 ng per lane) were electrophoresed on a SDS/10% polyacrylamide gel and then electroblotted onto an Immobilon-P membrane (Milipore). The blot was cut into strips containing one lane and separately incubated in a serum sample diluted 1:10 in 1% bovine serum albumin/0.05% Tween 20/Tris-buffered saline (50 mM Tris·HCl, pH 7.4/0.15 M NaCl) at 37°C for 3 h. The specific mouse antibody bound to human factor IX protein on the filter was detected with goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad) at a 1:500 dilution and diaminobenzidine substrate (Sigma) at 0.5 μg/ml in PBS/0.01% H_2O_2.

RESULTS AND DISCUSSION

By using a C2C12 cell line as a model, we have examined skeletal myoblasts for their ability to produce recombinant factor IX and their potential as a vehicle for efficient *ex vivo* gene transfer. In this approach, β-galactosidase gene was also expressed to optimize the procedures.

Expression of the β-Galactosidase Gene *in Vitro* and *in Vivo*. C2C12 cells were infected with BAG viruses and selected for G418 resistance. Southern blot analysis indicated that the BAG vector was integrated into genomes of cells without any rearrangements (data not shown). These cells produced β-galactosidase activity as shown by histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Fig. 1 *A* and *C*). The myotubes differentiated from these C2C12 cells continued expressing β-galactosidase (Fig. 1 *B* and *D*), indicating that the LTR promoter-controlled expression of β-galactosidase gene is not turned off during myogenic differentiation. C2C12 myoblasts transduced with BAG viruses were then injected into limb muscles of five mice. Mice were individually sacrificed at various time points (7, 18, 22, 60, and 122 days after the cell injection), and their limb muscle tissues were stained for β-galactosidase. β-Galactosidase activity was observed in muscle tissues of all mice injected with the cells. Fig. 2 *A–D* shows typical results of β-galactosidase staining of muscle tissues from the mice sacrificed 22 and 60 days after the cell injection. Fusion of the injected myoblasts with the existing and regenerating myofibers resulted in the formation of mosaic myofibers that were easily identified by the β-galactosidase staining on microscopic sections of the tissues (Fig. 2 *E* and *F*). Approximately 50–90% of myofibers within some injection areas were stained blue, indicating a high efficiency of fusion between the injected myoblasts and the host myofibers. Longitudinal staining of β-galactosidase within some individual myofibers was at least 2 mm in length. Cross-sections of muscle cells frequently showed β-galactosidase staining localized to one side of the cells, indicating that cytoplasmic diffusion of the β-galactosidase produced may not be efficient. In the fused

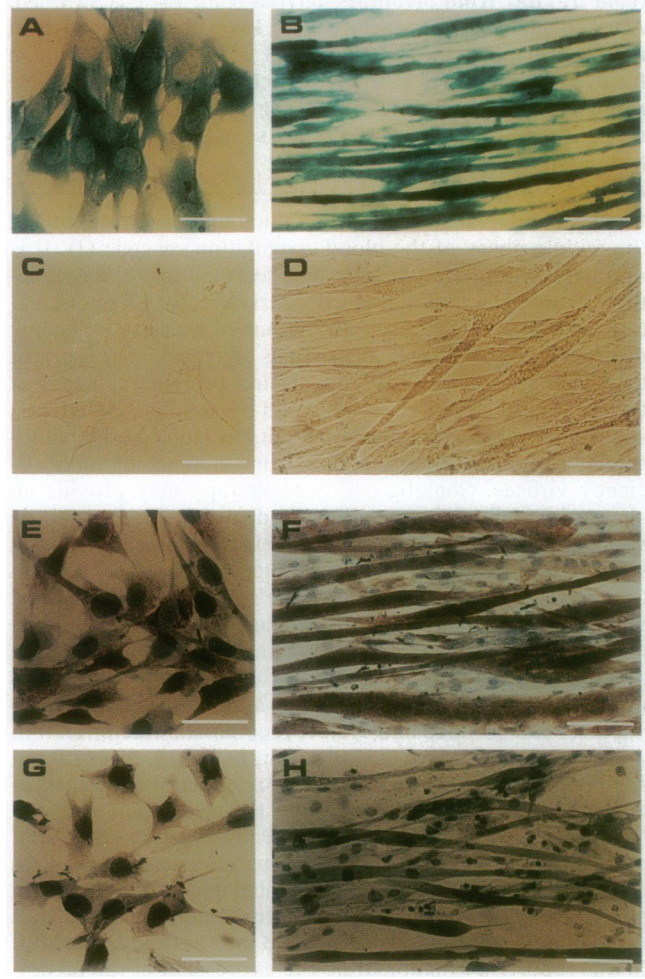

FIG. 1. Production of β-galactosidase and human factor IX in transduced C2C12 myoblasts (*A*, *C*, *E*, and *G*) and in their differentiated myotubes (*B*, *D*, *F*, and *H*). (*A*, *B*, *G*, and *H*) Transduced with BAG. (*C–F*) Transduced with LIXSN. (*A–D*) β-Galactosidase staining (blue color). (*E–H*) Immunostaining of human factor IX (reddish brown). (Bars: *A*, *C*, *E*, and *G*, 50 μm; *B*, *D*, *F*, and *H*, 100 μm.)

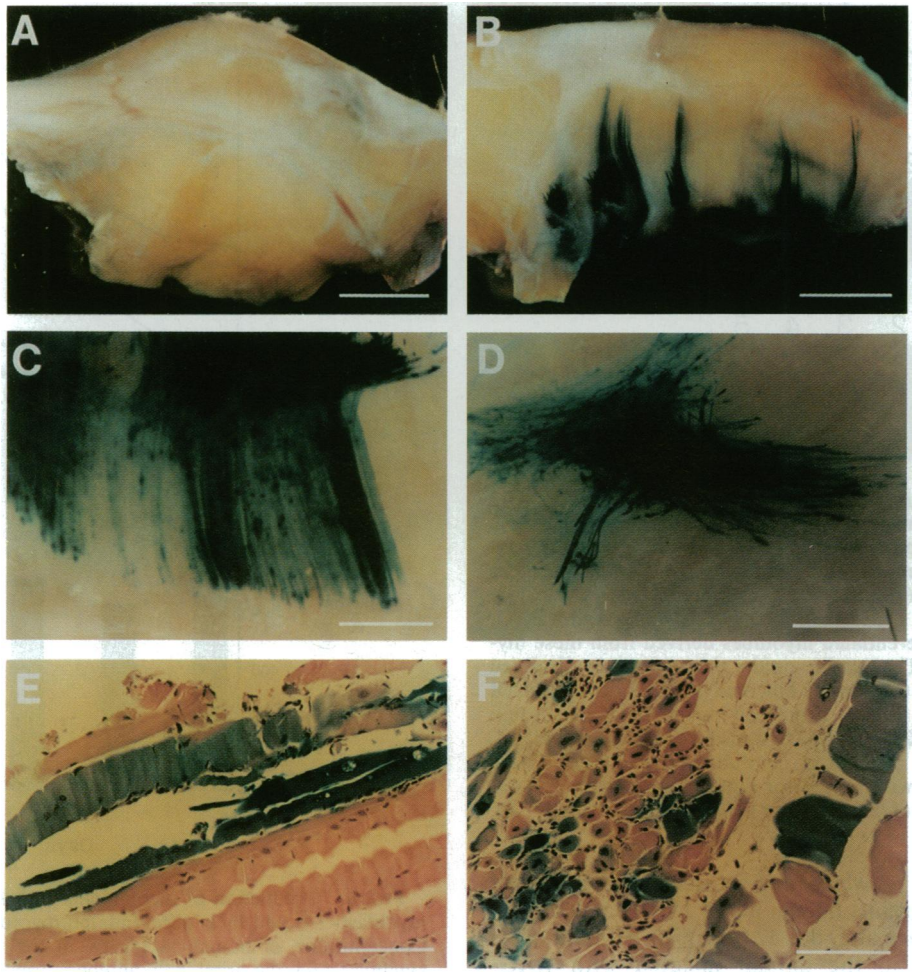

FIG. 2. Expression of β-galactosidase in mouse skeletal muscles injected with genetically modified C2C12 myoblasts. (*A*) Muscles in a hind limb removed 22 days after injection of 5×10^6 myoblasts transduced with LIXSN. (*B* and *C*) Muscles in a hind limb removed 22 days and 2 months, respectively, after injection of 5×10^6 myoblasts transduced with BAG. (*D*) Same as *C*, but showing the formation of disorganized myofibers on the surface of a limb muscle. (*E* and *F*) Longitudinal sections and cross-sections of the muscle stained for β-galactosidase activity as shown in *B*. Photographs were taken through a stereo microscope (Olympus) (*A–D*) or a Nikon microscope (*E* and *F*). β-Galactosidase activity is shown as dark blue. (Bars: *A* and *B*, 4 mm; *C* and *D*, 1 mm; *E* and *F*, 100 μm.)

mosaic cells, central nuclei were often observed. Myoblasts occasionally placed on the surface of muscles fused among themselves, forming a myofiber-like structure in a disorganized manner (Fig. 2*D*). These results showed that a recombinant gene can be efficiently transferred into adult mouse muscles by injecting genetically modified myoblasts resulting in its prolonged expression *in vivo*.

Expression of Human Factor IX *in Vitro* and *in Vivo*. Expression of the recombinant human factor IX by skeletal myoblasts was tested using C2C12 cells transduced with LIXSN. Southern blot analysis showed that these cells have LIXSN integrated into their genomes without any detectable rearrangement. Presence of the recombinant human factor IX in the cytoplasma of cells was easily detected by *in situ* immunostaining of the transduced cells with a mouse anti-human factor IX monoclonal antibody (Fig. 1 *E* and *G*). RNA blot analysis (9) showed a 4.4-kilobase transcript as expected for myoblasts infected with the LIXSN retrovirus (Fig. 3*B*). These cells secreted human factor IX into the culture medium at a rate of ≈2.6 μg per 10^6 cells per day (Fig. 4*A*). The recombinant human factor IX produced was indistinguishable from the plasma factor IX in its size (Fig. 3*C*) and also showed a high specific activity (81–89% of the normal human plasma factor IX), which is comparable to activities produced by other cell types including skin fibroblasts (3, 4), hepatocytes (12), and endothelial cells (9). This clearly indicates that the muscle cells have efficient mechanisms of post-translational modification, such as γ-carboxylation, required for factor IX activity.

Human factor IX was continuously expressed in multinucleated myotubes derived from the transduced myoblasts as monitored by *in situ* immunostaining (Fig. 1 *F* and *H*). The level of factor IX mRNA in the myotubes was as high as that of myoblasts (Fig. 3*B*). The transduced myotubes secreted an approximately constant amount of recombinant human factor IX into the culture medium every day (≈0.68 μg/day from myotubes derived from 10^6 myoblasts) for at least 4 days (Fig. 4*B*). This indicates that factor IX proteins were synthesized *de novo* by the myotubes. The clotting assay confirmed that human factor IX secreted by these myotubes is as active as that produced by the myoblasts.

When the C2C12 myoblasts transduced with LIXSN were injected into skeletal muscles of four mice, a significant amount of recombinant human factor IX was detected in the sera of all mice as early as the second day after myoblast injection. The expression of factor IX in two representative mice that received 2 or 3×10^7 transduced myoblasts was followed for 4 weeks (Fig. 5*A*). The level of recombinant human factor IX in the serum of a mouse injected with 3×10^7 cells gradually increased over a period of ≈2 weeks after injection, reaching a peak of ≈1 μg/ml on day 12. The level of human factor IX in mouse serum then gradually declined

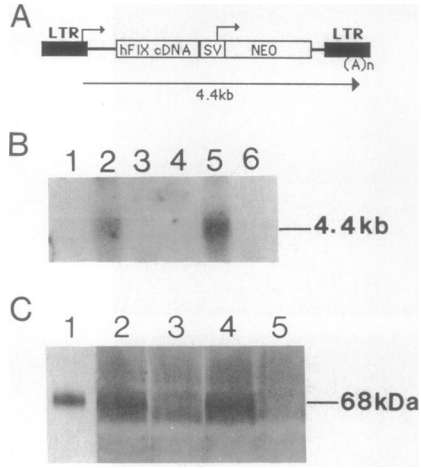

FIG. 3. RNA and protein blot analyses of the transduced C2C12 cells and their differentiated myotubes. (*A*) Structure of LIXSN vector with the expected size of factor IX transcripts (large arrow). Small arrows indicate transcription start sites. (*B*) RNA blot analysis of total cellular RNA (10 μg) from C2C12 myoblasts. Lanes: 1–3, nontransduced cells or cells transduced with LIXSN or with BAG, respectively; 4–6, differentiated myotubes derived from these myoblasts, respectively. The number on the right shows the observed size of the transcripts. (*C*) Protein blot analysis of human factor IX in the culture medium. Lanes: 1, purified human factor IX (25 ng); 2 and 3, medium of LIXSN- and BAG-transduced C2C12 myoblasts, respectively; 4 and 5, medium of myotubes derived from C2C12 myoblasts transduced with LIXSN or BAG, respectively. The observed molecular mass of human factor IX is shown on the right. kb, Kilobases.

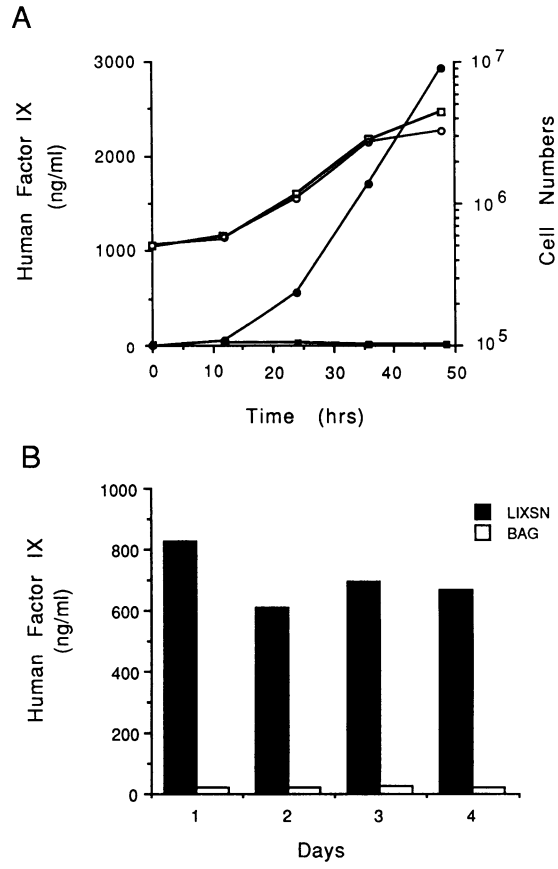

FIG. 4. Production of recombinant human factor IX by C2C12 myoblasts (*A*) and their differentiated myotubes (*B*) in culture. (*A*) Concentration of human factor IX in the culture medium (solid symbols) and the total cell numbers (open symbols) of C2C12 transduced with LIXSN (circles) or BAG (squares) were plotted against the culture time. Transduced and selected C2C12 cells (5×10^5 cells) were seeded in 60-mm dishes in 5 ml of DMEM supplemented with 20% (vol/vol) factor IX-depleted fetal bovine serum and vitamin K_1 (10 μg/ml). The culture medium was harvested from two dishes of each cell line at each time point and assayed in duplicate for factor IX antigen by ELISA (9). Cells were collected by treating with 0.25% trypsin and cell numbers were counted with a hemocytometer. (*B*) Daily production of recombinant human factor IX by myotubes derived from C2C12 myoblasts transduced with LIXSN or BAG. About 5×10^6 myoblasts were induced to differentiate into myotubes. After differentiation, the medium was replaced every 24 h with 5 ml of DMEM containing 20% factor IX-depleted fetal bovine serum and vitamin K_1 (10 μg/ml). The human factor IX antigen in the culture medium was assayed by ELISA. Each point represents the mean of four duplicated assays.

to the basal level in ≈4 weeks and an increasing amount of specific antibodies against human factor IX was detected in the serum of these mice after day 12 (Fig. 5*B*). The antibodies were not detected in the animal injected with BAG-transduced myoblasts (Fig. 5*B*). The transduced factor IX cDNA sequence was clearly detected by polymerase chain reactions in the muscle tissues 2 months after the myoblast injection (data not shown). These results indicate that the decline of factor IX is primarily due to an immune response against the human factor IX that is secreted into circulation. As expected, cyclosporine, which was administered daily to these animals to prevent possible cell rejection, did not completely suppress humoral immune response against human factor IX (13).

In the present study, retroviral LTR promoters were used for the expression of both human factor IX and β-galactosidase. It has been reported that the retroviral LTR promoter is gradually inactivated *in vivo* in transplanted skin fibroblasts (5). This does not appear to occur in muscle cells because the intracellular expression of β-galactosidase was maintained for at least 4 months (data not shown).

The distribution and elimination of factor IX infused into the circulatory system are best explained by a two-compartment model (14, 15), which can be expressed by the equation: $C_{ss} = K_0 T_{1/2}/0.693 V_d$, where C_{ss}, K_0, $t_{1/2}$, and V_d are steady-state concentration, constant production (or infusion) rate, half-life, and body distribution volume, respectively (16). If the $t_{1/2}$ and V_d for factor IX in mouse are the same as in dog and human (23 h and 0.25 liter/kg, respectively) (14, 15), then the peak factor IX level (1020 ng/ml of serum) observed in the circulation of the mouse injected with 3×10^7 cells was calculated to be 22.6% of the potential level estimated from the *in vitro* production rate (679 ng/day by myotubes derived from 10^6 transduced myoblasts). This systemic recovery of the recombinant factor IX in the circulation is at least 5–10 times higher than that observed for the skin fibroblast implants (4). Several factors might contribute to the higher efficiency in this system, including the excellent survival of the injected cells, the high-level expression, and the efficient transportation of the gene products into the circulation.

Direct injection of DNA into muscles can result in the expression of recombinant genes (17, 18). To test the potential application of this approach for producing factor IX, we injected three mice with 100–200 μg of LIXSN DNA or other factor IX expression constructs into the skeletal muscles. None of the animals produced any detectable human factor IX for a period of 1 month after injection. We speculate that this is due to a low efficiency of DNA transfection with this approach as observed by others (17, 18). The present approach, which employs the direct injection of genetically modified myoblasts, may have several advantages over the reported direct DNA injection method. Myoblast cell fusion is an efficient natural process that inherently occurs in myogenesis and muscle regeneration. Myoblasts, which can

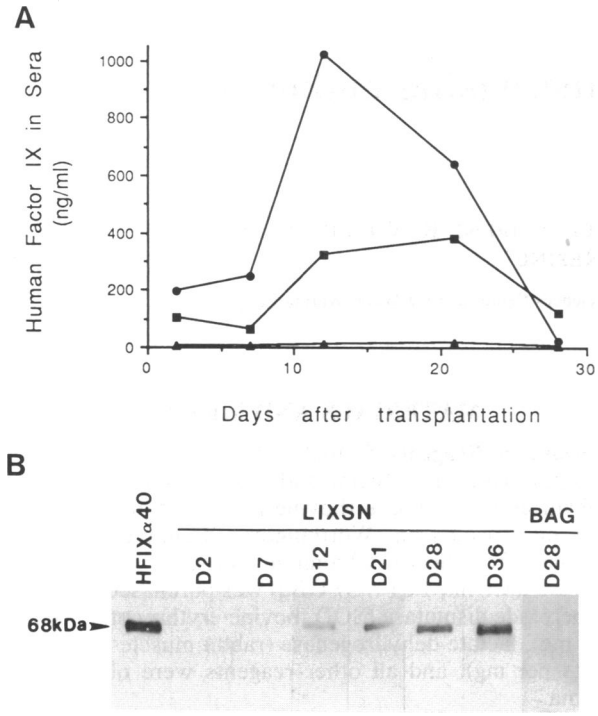

FIG. 5. (*A*) Recombinant human factor IX detected in the serum of mice implanted with myoblasts. ▲, Mouse implanted with 2×10^7 C2C12 cells transduced with BAG; ■, mouse implanted with 2×10^7 C2C12 cells transduced with LIXSN; ●, mouse implanted with 3×10^7 C2C12 cells transduced with LIXSN. Data are the mean of the two duplicated assays for human factor IX antigen. (*B*) Detection of mouse anti-human factor IX antibodies in the sera of transplanted mice. Serum samples obtained on various days from a mouse (circles in *A*) injected with 3×10^7 C2C12 myoblasts transduced with LIXSN or a mouse (triangles in *A*) injected with 2×10^7 myoblasts transduced with BAG were analyzed by protein blot assay. The size of purified human plasma factor IX is shown on the left. The mouse anti-human factor IX monoclonal antibody HFIXα40 was used as a positive control.

be obtained from a small biopsy of muscle tissue of patients, can be efficiently transduced with retrovirus vectors (19) and well characterized *in vitro* before being injected into the muscle tissues. This *ex vivo* approach, therefore, provides a method of efficiently introducing into adult muscles a recombinant gene that can be stably integrated into the genome of muscle cells and expressed for a long time.

Injection of normal myoblasts into skeletal muscles is now under clinical trial to treat hereditary Duchenne muscular dystrophy (20–24). However, histocompatible normal myoblasts are not available for dystrophic patients. The patients transplanted with histoincompatible normal myoblasts must rely on the prolonged administration of immunosuppressive agents such as cyclosporine to prevent immunologic rejection (23, 24). By genetically modifying patients' own myoblasts with the normal dystrophin gene using a method similar to the one described here, an effective gene therapy may be developed for this disorder. In addition, this *ex vivo* approach may also be applicable to many other diseases requiring the efficient transportation of gene products into circulation.

The approach described in this report needs to be further refined for testing the long-term stable expression of recombinant factor IX *in vivo*, such as by employing autologous myoblasts instead of C2C12 cells and the factor IX cDNA sequence obtained from the same animal species. This optimized approach can then be further tested in dogs with hemophilia B for its efficacy.

We thank K. Brabec, L. Dudus, and Dr. K. Christensen in the cell morphology core of this campus for their help in histological techniques. This work was supported in part by research grants from the National Institutes of Health (HL38644 and PO1-DK42718) and the Hemophilia Foundation of Michigan. We thank the Michigan Gastrointestinal Research Center (NIDDK34933) and the Clinical Research Center (NIH MO1-RR00042) of the University of Michigan for their help.

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