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Genetics

Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector

(gene therapy/anemia/skeletal muscle/serum protein)

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ABSTRACT **Erythropoietin (Epo)-responsive anemia is a common and debilitating complication of chronic renal failure and human immunodeficiency virus infection. Current therapy for this condition involves repeated intravenous or subcutaneous injections of recombinant Epo. In this report, we describe the development of a novel muscle-based gene transfer approach that produces long-term expression of physiologically significant levels of Epo in the systemic circulation of mice. We have constructed a plasmid expression vector, pVRmEpo, that contains the murine Epo cDNA under the transcriptional control of the cytomegalovirus immediate early (CMV-IE) promoter, the CMV-IE 5′ untranslated region, and intron A. A single intramuscular (i.m.) injection of as little as 10 μg of this plasmid into immunocompetent adult mice produced physiologically significant elevations in serum Epo levels and increased hematocrits from preinjection levels of 48 ± 0.4% to levels of 64 ± 3.3% 45 days after injection. Hematocrits in these animals remained elevated at greater than 60% for at least 90 days after a single i.m. injection of 10 μg of pVRmEpo. We observed a dose–response relationship between the amount of plasmid DNA injected and subsequent elevations in hematocrits. Mice injected once with 300 μg of pVRmEpo displayed 5-fold increased serum Epo levels and elevated hematocrits of 79 ± 3.3% at 45 days after injection. The i.m. injected plasmid DNA remained localized to the site of injection as assayed by the PCR. We conclude that i.m. injection of plasmid DNA represents a viable nonviral gene transfer method for the treatment of acquired and inherited serum protein deficiencies.**

A large number of inherited and acquired serum protein deficiencies including hemophilia A, diabetes mellitus, and the erythropoietin (Epo)-responsive anemias are currently treated by repeated intravenous or subcutaneous injections of purified or recombinant proteins. Although largely effective, such therapies are both expensive and inconvenient. Moreover, in diseases such as hemophilia A, there is not sufficient recombinant protein available to allow a comprehensive program of prophylactic therapy. Given these problems, there has been considerable interest in developing novel gene-based therapies for such serum protein deficiencies. An initial series of studies demonstrated that skeletal myoblasts genetically modified *in vitro* could be reimplanted by intramuscular (i.m.) injection and would subsequently produce stable physiological levels of recombinant proteins in the systemic circulation of adult immunocompetent mice (1–7). Subsequently, several groups have demonstrated the stable production of recombinant serum proteins after a single i.m. injection of replication-defective adenovirus (RDAd) vectors (8–13). Despite these initial successes, both myoblast transplantation and i.m. injection of RDAd vectors have thus far been associated with problems that may preclude their widespread clinical application (14–20).

Previous studies by Wolff and coworkers (21, 22) demonstrated that plasmid DNA injected directly into muscle can be taken up by skeletal myocytes adjacent to the site of injection and expressed for at least 19 months. The prospect of using i.m. injection of plasmid DNA to treat serum protein deficiencies has distinct advantages compared with either myoblast transplantation or the i.m. injection of recombinant viral vectors (21–23). (*i*) Plasmid vectors are relatively simple to construct and homogeneous preparations of plasmid vectors can be produced easily and economically. (*ii*) Plasmid DNA injection is an *in vivo* gene transfer approach that could be readily used to treat large numbers of patients. (*iii*) Such an approach would obviate the need for an infectious vector, eliminating the risk of insertional mutagenesis associated with the use of retroviral vectors and also possibly reducing immune responses that have been associated with the use of RDAd vectors in immunocompetent hosts. Despite these advantages, the use of plasmid DNA injection to treat serum protein deficiencies has been precluded by the finding that plasmid DNA is inefficiently taken up by muscle cells and manifests relatively low-level expression compared with viral vectors.

In this report, we describe the construction and characterization of a novel plasmid vector that produces high-level expression and secretion of Epo after i.m. injection into immunocompetent adult mice. A single i.m. injection of as little as 10 μg of this plasmid produced physiologically significant levels of murine (m) Epo in the systemic circulation of adult immunocompetent mice and resulted in significant elevations in hematocrits that were stable for at least 90 days. The injected plasmid DNA remained localized at the site of injection and the amount of Epo production (as reflected by the elevated hematocrits) was proportional to the dose of plasmid DNA injected. Thus, i.m. injection of plasmid DNA represents a feasible approach to the treatment of serum protein deficiencies.

MATERIALS AND METHODS

Plasmid Vectors. pVRhEpo contains an 840-bp *Not*I human (h) Epo cDNA fragment from pAdEF1hEpo (8) cloned into

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Abbreviations: Epo, erythropoietin; mEpo, murine Epo; hEpo, human Epo; i.m., intramuscular; RDad, replication-defective adenovirus; mU, milliunit(s); SCID, severe combined immunodeficiency; CMV-IE, cytomegalovirus immediate early.

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the *Not*I site of the plasmid vector pVR1012 (Fig. 1*A*) (24). pVRmEpo contains a 620-bp *Sal*I–*Bgl*II murine Epo cDNA fragment obtained by PCR of pAdEF1 mEpo (25) with sense and antisense primers (5′-GGGGTCGACGGCGGGGAGATGGGGGTGCCCG and 5′-GGGAGATCTAGTTCACCTGTCCCCTCTCCTGC, respectively) and cloned into the *Sal*I and *Bgl*II sites of pVR1012. pVRβgal contains the bacterial *lacZ* gene cloned into the multiple cloning site (MCS) of pVR1012 and pVR1902 contains the canine factor IX cDNA cloned into the MCS of pVR1012.

Transfections. C2C12 myoblasts (10^6 cells in a 10-cm tissue culture dish) were transfected with 15 μg of pVRmEpo using the LipofectAmine reagent (GIBCO/BRL). Approximately 16 hours later, the transfected cells were placed in fusion medium (DMEM/2% horse serum/1% penicillin/streptomycin) and the cells were allowed to fuse into myotubes overnight. Medium was harvested at the times indicated after fusion and assayed for mEpo using a radioimmunoassay (RIA) (26).

Intramuscular Injection of Plasmid DNA. Purified plasmid DNA was resuspended in sterile PBS −/− (GIBCO/BRL) at a concentration of 3 mg/ml. Mice were injected i.m. into the tibialis anterior or rectus femoris muscles with 50–100 μl of DNA solution (10–100 μg of DNA) per site. Hematocrits were measured as described (8) on blood collected by tail vein or orbital venipuncture. All animal experimentation was performed in accordance with National Institutes of Health guidelines in the A. J. Carlson Animal Research Facility of the University of Chicago or at Vical, Inc.

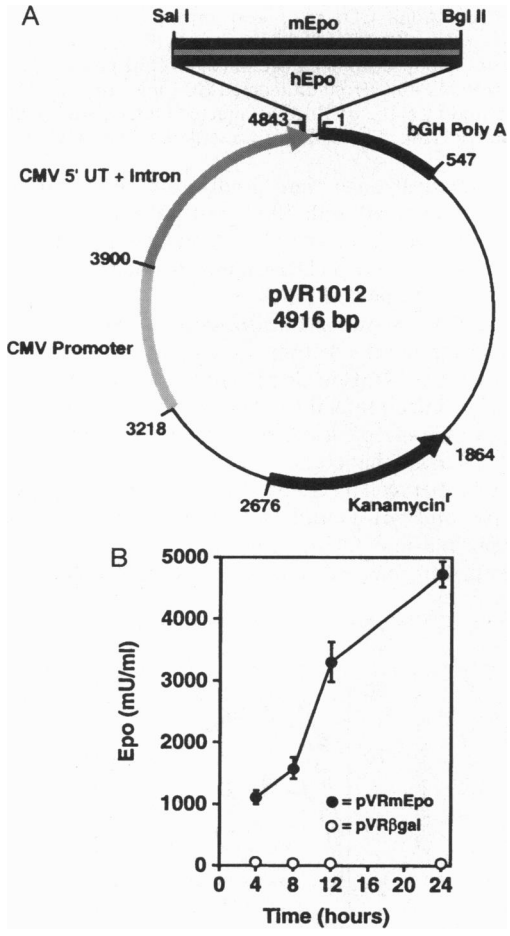

FIG. 1. *In vitro* analysis of pVRmEpo. (*A*) Schematic illustration of pVRmEpo and pVRhEpo. The hEpo or mEpo cDNAs were cloned into pVR1012 (24) 3′ of the CMV-IE promoter, CMV-IE 5′ untranslated (UT) region, and intron A. The bovine growth hormone polyadenylylation signal (bGH Poly A) is located immediately 3′ of the Epo cDNAs. (*B*) Time course of mEpo secretion after transfection of 10^6 C2C12 cells with 15 μg of pVRmEpo (●) or pVRβgal (○). The data are shown as the concentration of Epo in mU/ml. Each data point represents the mean ± SEM of three experiments. In some cases error bars are too small to be visualized.

Epo Assays. Tissues were harvested from mice 90 days after injection with plasmid DNA, homogenized in approximately 1 ml of Epo specimen diluent buffer (R and D Systems), and centrifuged at 10,000 × *g* for 10 min, and supernatants were collected. mEpo levels were measured in cell culture supernatants, serum, and tissue lysates using a RIA that is specific for mEpo (26).

PCR–Southern Assays. Total cellular DNA was isolated from mouse tissues as described (8). For SCID (severe combined immunodeficient) mice, approximately 1 μg of total cellular DNA from each tissue was subject to the PCR using primers corresponding to sequences within the hEpo cDNA (5′-CCAGACCCCGAAGCATGG) and the pVR1012 plasmid (5′-GGAAGACTTAAGGCAGCG). For CD-1 and BALB/c mice, approximately 100 ng of cellular DNA from each tissue was subjected to the PCR using primers corresponding to sequences within the mEpo cDNA (5′-GAAGTCAGGCTACGTAGACCACTG) and the pVR1012 plasmid (5′-GTCTGAGCAGTACTCGTTGC). The resulting PCR products were fractionated on a 1% agarose gel and examined by Southern blot analysis using a radiolabeled *Bam*HI–*Pvu*II fragment of the hEpo cDNA or a radiolabeled *Bgl*II–*Sac*I fragment of the mEpo cDNA as probes. All cellular DNA samples were also subjected to the PCR using primers specific for the cardiac troponin C (cTnC) gene, and the products were visualized in ethidium bromide-stained agarose gels as described (8). PCR conditions were 35 cycles (94°C for 1 min, 72°C for 1 min) followed by a 10-min extension at 72°C.

RESULTS

***In Vitro* Analysis of pVRmEpo.** To construct a plasmid expression vector that could program high-level production and secretion of recombinant proteins from skeletal myofibers *in vivo*, we cloned the hEpo and mEpo cDNAs into pVR1012 (24), a plasmid vector that contains a eukaryotic expression cassette controlled by the cytomegalovirus immediate early (CMV-IE) promoter and the CMV-IE 5′ untranslated and intron A sequences followed by the bovine growth hormone polyadenylylation signal (Fig. 1*A*) (24). pVR1012 was used in these experiments because this plasmid backbone has been shown to program high-level luciferase expression after i.m. injection into immunocompetent mice (24).

In initial experiments, C2C12 skeletal myoblasts were transfected with pVRmEpo and allowed to fuse into multinucleated myotubes. Culture supernatants from these transfected myotubes were assayed for mEpo at various times after transfection (Fig. 1*B*). C2C12 cells transiently transfected with pVRmEpo produced mEpo at approximately 2000 milliunits (mU) per hour per 10^6 cells (4800 mU/ml, 10 ml/24 hours). Supernatants from control (pVRβgal-transfected) C2C12 cells did not contain detectable mEpo levels (Fig. 1*B*). Thus, pVRmEpo programmed high-level mEpo expression and secretion after transfection into cultured skeletal myocytes. Similar high-level hEpo expression and secretion was observed after transfection of C2C12 cells with pVRhEpo (data not shown).

Intramuscular Injection of Epo Expression Vector DNA Produces Physiologically Significant Levels of Epo in the Systemic Circulation of Mice. To determine whether i.m. injection of a plasmid Epo expression vector could produce physiologically significant levels of Epo in the systemic circulation of adult mice, adult SCID mice were injected i.m. with 300 μg of pVRhEpo or the control plasmid pVR1012, which does not contain a cDNA insert. Hematocrits of the pVRhEpo-injected mice rose from preinjection values of 48 ± 1.2% to values of 68 ± 2.4% within 14 days of injection and

remained elevated at this level for the 90-day time course of the experiment. These elevated hematocrits were significantly different from those of control mice injected with identical amounts of pVR1012 ($P < 0.006$) (Fig. 2*A*). Injection of adult immunocompetent CD1 mice with 300 μg of pVRmEpo produced similar elevations in hematocrits (74 ± 2.4% in the pVRmEpo-injected animals versus 47 ± 1% in control injected animals; $P < 0.01$) that were also sustained over the 90-day time course of the experiment (Fig. 2*B*).

Elevated Serum and Muscle Epo Levels in the pVRmEpo-Injected Mice. To demonstrate directly that the increased hematocrits observed in the pVRmEpo-injected mice reflected persistently elevated serum mEpo levels in these animals, we assayed serum mEpo levels 90 days after injection by using a RIA that can detect mEpo (26). Serum mEpo levels in the pVRmEpo-injected animals were significantly elevated compared with mEpo levels in serum from control mice (52 mU/ml in the pVRmEpo-injected mice versus 8 mU/ml in the control mice; $P < 0.03$) (Fig. 3). To determine the site of mEpo production in the pVRmEpo-injected mice, tissue lysates prepared from liver, kidney, and muscle at the site of i.m. injection (IM) were assayed for mEpo by RIA. Murine Epo levels in lysates from the pVRmEpo-injected muscle were significantly elevated compared with levels in control uninjected muscle lysate (130 mU/ml in the pVRmEpo-injected muscle versus 2 mU/ml in the uninjected muscle; $P < 0.0001$) (Fig. 3). There were no significant differences in the mEpo levels detected in the other tissue lysates tested from the pVRmEpo-injected or uninjected animals. Thus, the elevated hematocrits observed in the pVRmEpo-injected animals reflected persistent production and secretion of recombinant mEpo from the pVRmEpo-injected muscle.

A Dose–Response Relationship Between the Amount of DNA Injected and the Subsequent Elevation in Hematocrit. To determine directly if the level of polycythemia observed after i.m. injection of pVRmEpo was proportional to the amount of DNA injected, BALB/c mice were injected i.m. with 10, 100, or 300 μg of pVRmEpo, and hematocrits were measured during the 90 days after injection. i.m. injection of as little as 10 μg of pVRmEpo resulted in stable elevations in hematocrits from preinjection values of 48 ± 0.4% to postinjection levels of 64 ± 3.3% at 45 days after injection (Fig. 4). Injections of 100 or 300 μg of DNA caused further increases in hematocrits to levels of 79 ± 3.3% at 45 days after injection that declined to 67 ± 4.7% at 90 days after injection. The hematocrits observed in each treatment group were significantly elevated at each time point from those observed in control mice injected with 300 μg of pVR1012 ($P < 0.004$). Thus, the observed levels of polycythemia were proportional to the amount of pVRmEpo DNA injected at least over the range of 10–100 μg of injected DNA.

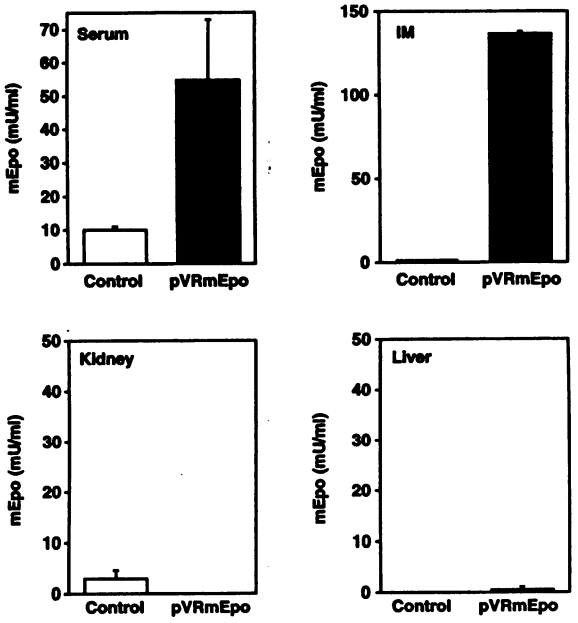

FIG. 3. mEpo levels in serum and tissue lysates after i.m. injection of pVRmEpo. Adult CD1 mice were injected i.m. with 300 μg of pVRmEpo. mEpo levels were determined by RIA (26) in serum and tissue lysates 90 days after i.m. injection of pVRmEpo ($n = 3$). Control samples were obtained from uninjected CD1 mice ($n = 3$). IM lysates were prepared from the pVRmEpo-injected muscle. All samples were assayed in triplicate. The data are presented as the mean ± SEM.

Plasmid DNA Remains Localized at the Site of i.m. Injection. To determine the distribution of plasmid DNA after i.m. injection with pVRmEpo or pVRhEpo, mice were sacrificed 90 days after injection and total cellular DNA from a number of tissues was assayed for the presence of pVRmEpo DNA by using a PCR assay that could detect as little as 0.00001 copy of the plasmid per cell (Fig. 5). In SCID mice injected with pVRhEpo and CD1 and BALB/c mice injected with pVRmEpo, plasmid DNA could be detected in lysates prepared from the injected muscle. In some animals, a barely

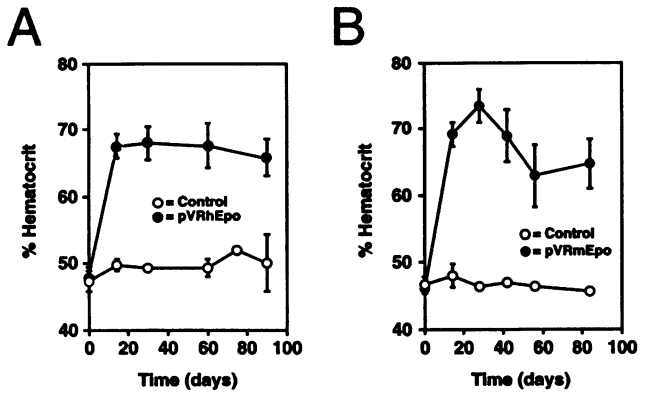

FIG. 2. Hematocrits after i.m. injection of pVRhEpo or pVRmEpo. (*A*) SCID mice were injected i.m. with 300 μg of pVRhEpo (●) ($n = 5$). Control mice received an identical injection of 300 μg of pVR1012 (○)($n = 3$). Hematocrits were measured at the times indicated from tail vein blood. (*B*) Adult CD1 mice were injected with 300 μg of pVRmEpo (●) ($n = 10$) or pVR1012 (○) ($n = 5$). Each data point represents the mean ± SEM. In some cases error bars are too small to be visualized.

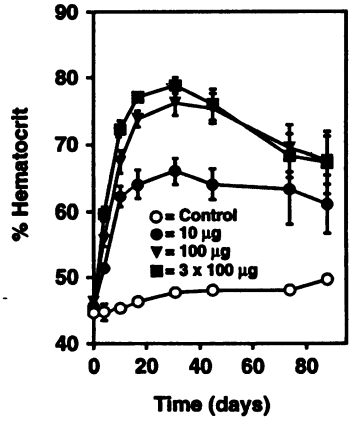

FIG. 4. Dose-dependent elevations in hematocrits after i.m. injection of pVRmEpo. Adult BALB/c mice were injected i.m. with 10 μg (●) ($n = 5$), 100 μg (▼) ($n = 5$), or 300 μg (■) ($n = 5$) of pVRmEpo. Control mice (○) were injected with 300 μg of pVR1902 ($n = 10$). At the times indicated, hematocrits were measured from ophthalmic venous blood samples. The data are presented as mean ± SEM.

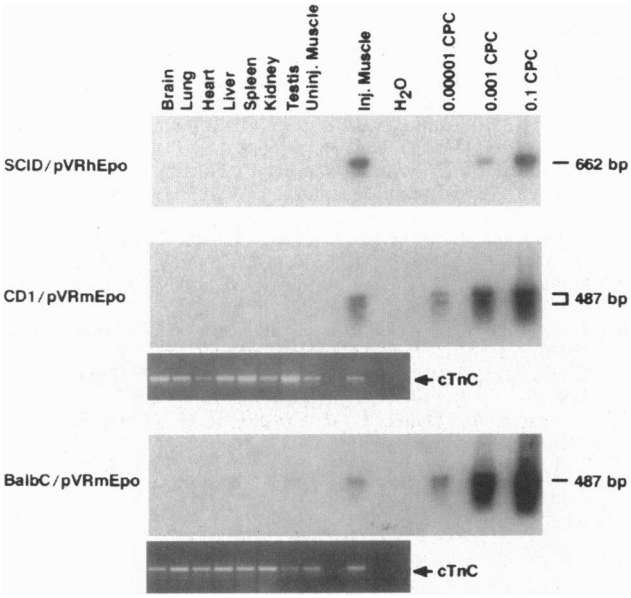

FIG. 5. Localization of plasmid DNA after i.m. injection of pVRmEpo. Adult SCID mice were injected i.m. with 300 μg of pVRhEpo. Adult CD1 or BALB/c mice were injected i.m. with 300 μg of pVRmEpo. Ninety days after injection, total cellular DNA from different tissues was assayed for the presence of pVRhEpo or pVRmEpo DNAs by the PCR. All DNA samples were also assayed by PCR for the cellular cardiac troponin C (cTnC) gene to ensure the reproducibility of the PCRs. Uninj Muscle, uninjected muscle; Inj Muscle, injected muscle; H_20, a negative control PCR lacking cellular DNA. Each experiment included mixtures of 1 μg of control cellular DNA plus 0.00001–0.1 copy per cell of purified pVRhEpo or pVRmEpo plasmid DNAs. The sizes of the expected PCR products in bp are shown to the right of each autoradiogram. The pVRmEpo PCR product often appears as a doublet due to alternative priming sites within the plasmid.

detectable signal was also observed in the liver. Thus, preponderance of plasmid DNA remained localized at the site of i.m. injection.

DISCUSSION

In the studies described in this report, we have constructed a novel plasmid expression vector, pVRmEpo, that directs high-level production and secretion of mEpo from skeletal myocytes *in vitro*. The i.m. injection of as little as 10 μg of pVRmEpo into adult immunocompetent mice resulted in dose-dependent elevations in hematocrits that remained stable for at least 90 days. The increased hematocrits observed in the pVRmEpo-injected mice reflected persistent production of mEpo from the injected muscle and secretion of this protein into the systemic circulation. Finally, the injected DNA remained predominantly localized to muscle at the site of injection. Although several previous reports have demonstrated persistent low-level reporter gene expression after i.m. injection of plasmid expression vectors in rodents (21–23), to our knowledge, this report represents the first demonstration of the delivery of physiologically significant levels of recombinant protein to the systemic circulation after the i.m. injection of a plasmid DNA expression vector. Our positive results likely reflect both the increased transcriptional efficiency of the pVR1012 vector (24) and the potency of Epo as a cytokine.

Previous studies have demonstrated that i.m. injection of RDAd vectors can also be used to deliver physiologic levels of Epo to the systemic circulation of mice (8, 27). However, the i.m. injection of plasmid DNA has a number of distinct advantages compared with the use of RDAd vectors. First, plasmid DNA vectors are easier to construct, can accept large cDNA inserts, and can be prepared as pure chemical solutions without the risk of contamination with wild-type infectious particles. In addition, i.m. injection of adult immunocompetent animals with RDAd has been associated with immune responses that eliminate virus-infected cells in 14–28 days, thereby producing only transient recombinant gene expression *in vivo* (16–20). Of equal importance, previous infection with wild-type adenovirus results in a neutralizing antibody response that may preclude administration of an RDAd vector (16, 19). In contrast, our results demonstrate long-term Epo expression after a single i.m. injection of plasmid DNA even in adult immunocompetent animals. Moreover, we were unable to detect antibodies against mEpo in the sera of mice 90 days after injection with pVRmEpo (data not shown). Therefore, it should be possible to readminister plasmid DNA by i.m. injection if repeated therapy or dose escalation is required.

Despite the promise of i.m. injection of plasmid vectors for the treatment of serum protein deficiencies, several important issues remain to be addressed before this approach is feasible for human therapy. First, the observed dose–response curve in mice suggests that it would be necessary to administer approximately 28 mg of plasmid DNA to produce significant elevations in hematocrits in patients using pVRmEpo (or pVRhEpo) (assuming a linear relationship between plasmid dose and mEpo production, a requirement for 10 μg of plasmid DNA for a mouse, and a ratio of 2800:1 for the body weight of a human versus a mouse). The adaptation of this approach to diseases such as hemophilia that require higher levels of circulating protein would require even larger amounts of DNA. Therefore, it would be desirable to increase the efficiency of plasmid DNA uptake or recombinant gene expression (or both) after i.m. injection of plasmid vectors prior to their use in human gene therapy. Alternatively, it may be possible to increase the half-life of the recombinant proteins in the systemic circulation. Finally, it will be important to develop systems for regulating recombinant gene expression in muscle *in vivo* after i.m. injection of plasmid vectors, particularly for diseases such as diabetes mellitus and the Epo-responsive anemias. Despite these hurdles, our results suggest that i.m. injection of improved plasmid DNA vectors may be useful for the treatment of a number of inherited and acquired human serum protein deficiencies.

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