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Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation

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ABSTRACT **We show that an electric treatment in the form of high-frequency, low-voltage electric pulses can increase more than 100-fold the production and secretion of a recombinant protein from mouse skeletal muscle. Therapeutical erythopoietin (EPO) levels were achieved in mice with a single injection of as little as 1 μg of plasmid DNA, and the increase in hematocrit after EPO production was stable and long-lasting. Pharmacological regulation through a tetracycline-inducible promoter allowed regulation of serum EPO and hematocrit levels. Tissue damage after stimulation was transient. The method described thus provides a potentially safe and low-cost treatment for serum protein deficiencies.**

Genes can be transferred into skeletal muscle cells of rodents and primates by intramuscular injection of plasmid DNA, and the resulting gene expression has been reported to last as long as several months (1, 2). Similarly, various viral vectors such as adenoviral, retroviral, and AAV-based vectors (3), have been used to transduce myofibers *in vivo*. The i.m. injection of plasmid DNA, however, has several advantages over viral vectors. First, plasmid DNA vectors are easier to construct and can be prepared as pharmaceutical-grade solutions (4) without the risk of contamination with wild-type infectious particles. Second, previous infection by wild-type adenovirus or AAV may induce a neutralizing antibody response that could preclude administration of the recombinant virus. In contrast, anti-DNA antibodies have never been detected in experiments of muscle DNA injection (2), therefore it is possible to readminister plasmid DNA by i.m. injection if repeated therapy or escalation is required.

Despite the promise of i.m. injection of plasmid vectors for treating serum protein deficiencies, several important issues remain to be addressed before this approach becomes feasible for human gene therapy. The potential clinical usefulness of direct gene transfer to muscle of plasmid DNA is in fact limited by the low and highly variable level of gene expression (1, 2, 5, 6). Therefore, although DNA injection is potentially very powerful as a vaccination method because a low level of gene expression is sufficient to trigger immunoresponses, it is necessary to increase the efficiency of DNA uptake after i.m. injection of plasmid vectors before using this technique as a standard gene correction procedure.

One of the most efficient methods implemented to achieve gene transfer and expression in mammalian cells is based on electric pulses (7). Electroporation has been used to introduce foreign DNA in different cell types (7), but it has also recently met with some success in *in vivo* applications. Gene transfer by electrical permeabilization has been obtained in skin (8, 9), corneal endothelium (10), melanoma (11), brain (12), liver, (13) and muscle (14) of experimental animals.

We have shown previously that electropermeabilization can increase severalfold the uptake by rat muscle of a plasmid encoding the *Escherichia coli* lacZ gene (15). In this study, we show that exposure of skeletal muscle to a pulsed electric field increases more than 100-fold the expression of a transgene injected i.m. in mice. Clinically effective quantities of circulating EPO were obtained for at least 6 months after a single injection of minimal amounts of plasmid DNA. Moreover, the protein in circulation and hematocrit levels were regulated by coadministering a plasmid carrying the mouse EPO (mEPO) cDNA under the control of a tetracycline-inducible promoter and a second construct carrying the reverse tetracycline-dependent transactivator protein (rtTAnls) (16). Muscle electropermeabilization can therefore provide a safe and cost-effective treatment for a variety of diseases by producing therapeutic proteins for systemic effects.

MATERIALS AND METHODS

DNA Constructs. The complete mEPO coding region, including 40 bp of the 5′ untranslated region (17), was assembled from synthetic oligonucleotides as described (18).

Plasmid pCMV/mEPO was constructed by inserting the mEPO coding sequence as an *Eco*RI–*Bam*HI 0.6-kb fragment into pViJnsB (19), which contains the cytomegalovirus (CMV) immediate/early region promoter and enhancer with intron A followed by the BGH polyadenylation signal.

pUHD 10.3/mEPO was constructed by inserting the mEPO coding sequence as an *Eco*RI–*Bam*HI fragment in pUHD 10.3 (20), which contains human CMV minimal promoter with heptamerized tet-operators and the SV40 polyadenylation signal. The entire cassette containing CMV minimal promoter, mEPO coding sequence, and SV40 poly(A) was excised as an *Xho*I–*Hin*dIII 1.5-kb fragment and cloned blunt in *Msc*I–*Bst*XI of pViJnsB (19). The resulting plasmid was called pOr/EPO.

The rtTAnls gene consisting of the tetR^r–VP16 fusion preceded by Kozak sequence and nuclear localization sequence from adenovirus E1A protein was excised as an *Eco*RI–*Bam*HI 1.0-kb fragment from pUHD172–1neo and cloned into *Eco*RV of pV6D-A (D. Montgomery, unpublished data). The resulting plasmid was called pMCK/rtTAnls. The pV6D-A plasmid contains the complete muscle creatine kinase (MCK) 3.3-kb promoter region (21) with a BGH poly(A) signal in pVijnsB. Plasmid DNA was prepared by standard double CsCl gradient purification and resuspended in sterile saline solution.

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Abbreviations: EPO, erythropoietin; mEPO, mouse EPO; CMV, cytomegalovirus; rtTAnls, reverse tetracycline-dependent transactivator protein; MCK, muscle creatine kinase.

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Animals and Treatments. Six-week-old female mice were used in all experiments; BALB/c, C57BL/6, DBA/2J, C3H, and CD1 mice were purchased from Charles River Breeding Laboratories. Mice were maintained in standard conditions under a 12-hour light/dark cycle, provided irradiated food (4RF21, Mucedola), and chlorinated water ad libitum. All animal procedures were conducted in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana no. 40, February 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication No. 85–23, 1985). Where indicated, doxycycline·HCl (Sigma) was dissolved in the drinking water to a final concentration of 400 μg/ml in 5% sucrose, pH 6.0.

Electrostimulation. Mouse quadriceps muscles were surgically exposed and injected with a predetermined amount of plasmid DNA. Steel electrodes in the form of parallel 0.2-mm wires about 3 cm long and 5 mm apart were brought into contact with the muscle in parallel orientation with respect to the muscle fibers. The electric field was applied in a pulsed form through a Pulsar 6bp-a/s bipolar stimulator (FHC, Bowdoinham, ME), and each cycle of stimulation comprised a 1-second pulse train of square bipolar pulses delivered every other second. Each train consisted of 10^3 pulses of 200-μsec length and 45-V amplitude. Pulses were monitored by using a digital oscilloscope (Fluke 96 Scopometer).

Hematocrit and EPO Measurement. Hematocrits were measured by centrifugation of blood obtained from mouse retroorbital cavity. Serum levels of mEPO were measured by using a commercially available assay (R & D Systems) for human EPO cross-reactive with mEPO and known amounts of rmEPO (Boehringer Mannheim) as a standard reference. Results were analyzed by using ANOVA or χ^2 analysis (STATVIEW, Abacus Concept, Berkeley, CA). A P value <0.05 was considered significant.

Histology. Tissues were harvested, fixed overnight in buffered 4% paraformaldehyde, and imbedded in paraffin blocks following standard procedures. Sections 3–5 μm thick were stained by hematoxylin and eosin.

RESULTS

Long-Term EPO Production by Plasmid DNA Injection and Muscle Electroporation. We have previously shown that muscle electroporation in rats can increase the uptake of a plasmid encoding the *E. coli* lacZ gene (15). To test the efficacy of muscle electroporation in delivering clinically significant levels of a therapeutic protein and to analyze the persistence of transgene expression, we chose to use EPO as a reporter gene. For this purpose, plasmid pCMV/mEPO was constructed carrying the mEPO cDNA under the control of the CMV immediate early promoter/enhancer.

C57BL/6 mice were injected in the quadriceps muscle with 50 μg of pCMV/EPO, and one group of mice was electrostimulated immediately after injection. The electrical conditions used in this experiment consisted of 10 trains of 10^3 pulses with an amplitude of 45 V, a length of 200 μsec per phase, and a current of 50 mA. Hematocrit levels determined at selected time points increased exclusively in electrically stimulated mice (Fig. 1), reaching a peak level of 82% 84 days after injection, and remained elevated for at least 6 months after treatment. No significant change in hematocrit was detected in the unstimulated mice or in electrically stimulated mice injected with saline (Fig. 1). These data indicate that electrostimulation of DNA-injected muscle greatly enhances the transduction efficiency.

Dose–Response Correlation. To determine the minimal effective dose of pCMV/EPO, BALB/c mice were injected

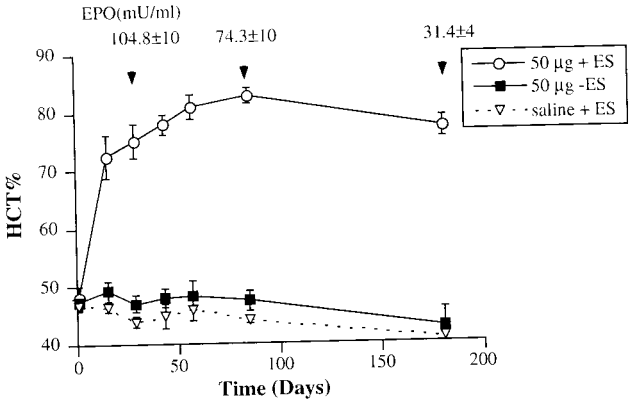

FIG. 1. Hematocrit levels in C57BL/6 mice injected with 50 μg of pCMV/mEPO or with saline with (+ES) or without (−ES) electrical stimulation. Data are the mean ± SD of hematocrit and serum EPO measured in five animals. Serum EPO levels at different time points are indicated.

with different amounts of DNA; serum EPO levels and hematocrit were measured over time (Fig. 2 *A* and *B*). Serum EPO measured 7, 28, 56, and 84 days posttreatment correlated with the amount of DNA injected, and ranged from undetectable levels (<5 milliunits/ml) with ≤0.5 μg of DNA (not shown) to a maximal level of 563 milliunits/ml (1 mU = 10pg)

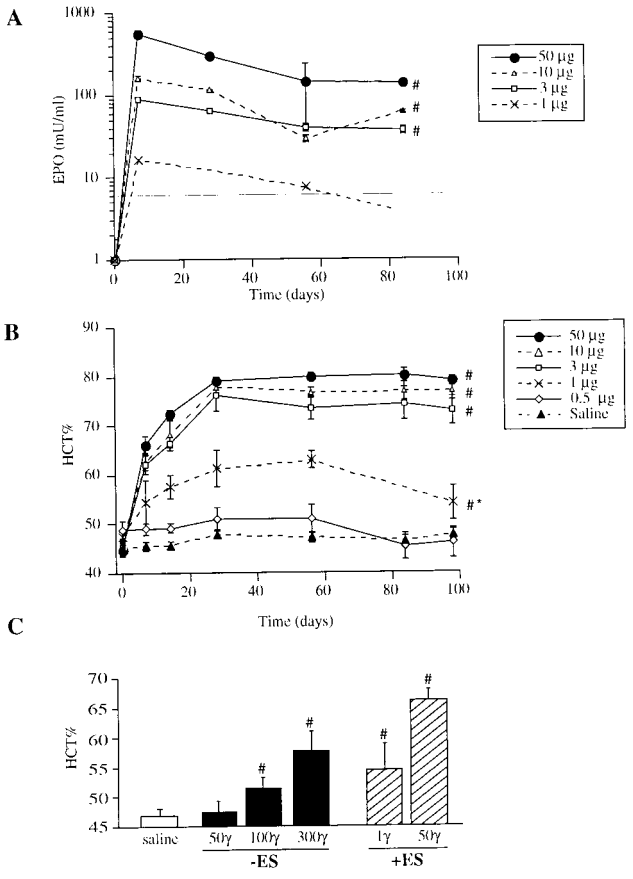

FIG. 2. (*A*) Serum EPO levels in BALB/c mice injected with different amounts of pCMV/EPO. The horizontal line indicates the limit of detection of the assay. (*B*) Hematocrit levels. (*C*) Comparison of hematocrit levels in mice injected with different DNA doses with (+ES) or without (−ES) electrostimulation. Data are the mean ± SD of hematocrit and serum EPO measured in five animals. #, Significantly different from the saline control; ∗, significantly different from 50 μg.

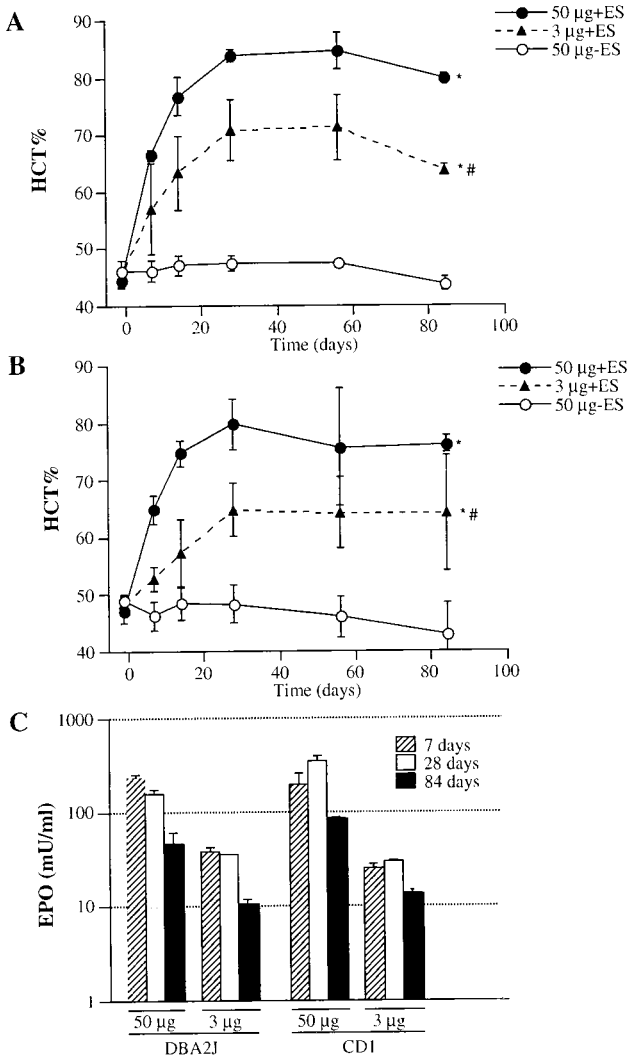

FIG. 3. Hematocrit levels in DBA/2J (*A*) and CD1 (*B*) mice injected with 3 or 50 μg of pCMV/EPO with (+ES) or without (−ES) electrical stimulation. Data are the mean ± SD of hematocrit measured in four animals. (*C*) Serum EPO levels. *, Significantly different from 50 μg −ES; #, significantly different from 50 μg +ES.

at day 7 in mice injected with 50 μg of DNA. The circulating protein reached a peak level 7 days after treatment, decreased variably in the different groups from 1/2 to 1/3 of the initial value after 56 days, and remained constant thereafter. EPO was undetectable in mice injected with 50 μg of pCMV/EPO but not electrically stimulated (data not shown). Additionally, hematocrit increased significantly in mice injected with ≥1 μg of the EPO plasmid (Fig. 2*B*). The level of hematocrit was significantly higher in all of the mice injected with ≥1 μg of DNA than in saline-treated controls for at least 3 months.

It has been shown that long-term expression of EPO in mice can be achieved by i.m. injection of plasmid DNA (22). To determine the minimal DNA dose required for an efficient muscle transduction without electrostimulation, mice were

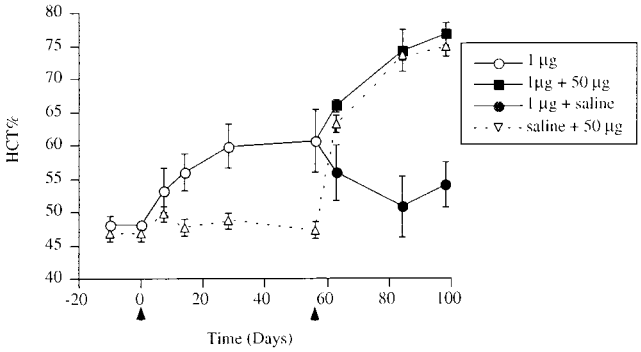

FIG. 4. Readministration of pCMV/EPO. The right quadriceps of a group of eight mice was injected at day 0 (arrow) with 1 μg of plasmid (○). At day 56 (arrow), in half of the mice, the left quadriceps was injected with 50 μg of the same plasmid (■) or with saline (●). A group of four mice (△) was injected with saline at day 0 and with 50 μg of pCMV/EPO at day 56. Each injection was followed by electrical stimulation. Data are mean ± SD.

injected i.m. with 50, 100, or 300 μg of pCMV/EPO. One week after injection, a significant increase in hematocrit was observed in mice injected with ≥100 μg of plasmid (Fig. 2*C*), and the extent of increase was comparable to that obtained in mice injected with only 1 μg followed by electrostimulation (Fig. 2*C*). Similar results were obtained in two other strains of mice: the inbred strain DBA2/J (Fig. 3*A*) and the outbred CD1 (Fig. 3*B*). Serum EPO levels in injected mice are reported in Fig. 3*C*; the decrease in circulating EPO with time is comparable to that observe in BALB/C mice, and at 84 days after injection, EPO levels are reduced between 2 and 3.5 fold with respect to the peak. Thus, electrostimulation enhances >100-fold the uptake and expression of plasmid DNA after i.m. injection in different mice strains.

Transgene Readministration. Readministration of the plasmid DNA is an important issue in diseases where repeated therapy or escalation is required, therefore we wondered whether our electrical treatment activated an immune reaction against the injected plasmid and consequently hampered readministration.

BALB/c mice were injected with 1 μg of pCMV/EPO, and a second group was injected with saline followed by electrical stimulation. Fifty-six days after treatment, mice injected with pCMV/EPO were divided into two groups with comparable mean hematocrit levels. Animals of the first group were left untreated, while those of the second group and mice injected with saline were reinjected with 50 μg of pCMV/EPO. Circulating EPO and hematocrit levels, determined 1, 4, and 6 weeks after the second treatment, increased comparably in the mice pretreated with saline or with plasmid DNA (Table 1 and Fig. 4), whereas they decreased in mice that received a single treatment. In agreement with these results, no significant levels of anti-DNA antibodies were detected in sera of mice injected with 50 μg of pCMV/EPO (data not shown).

Transcriptional Regulation of EPO Expression. To regulate EPO expression, we exploited the tetracycline-inducible system described by Gossen *et al.* (16) and inserted the mouse EPO cDNA downstream of a minimal CMV promoter flanked with seven repeats of the tetracycline operator (23). rtTAnls,

Table 1. Serum EPO levels in mice treated with pCMV/EPO

Treatment, μg	Days postinjection					
	7	28	56	63	84	98
1	16.2 ± 0.8	7.4 ± 1.0	<5	<5	<5	<5
1 + 50	16.2 ± 0.8	7.4 ± 1.0	<5	277 ± 100	144 ± 16	119 ± 3
Saline + 50	<5	<5	<5	286 ± 10	122 ± 11	78 ± 6

Values given are serum EPO levels in milliunits/ml.

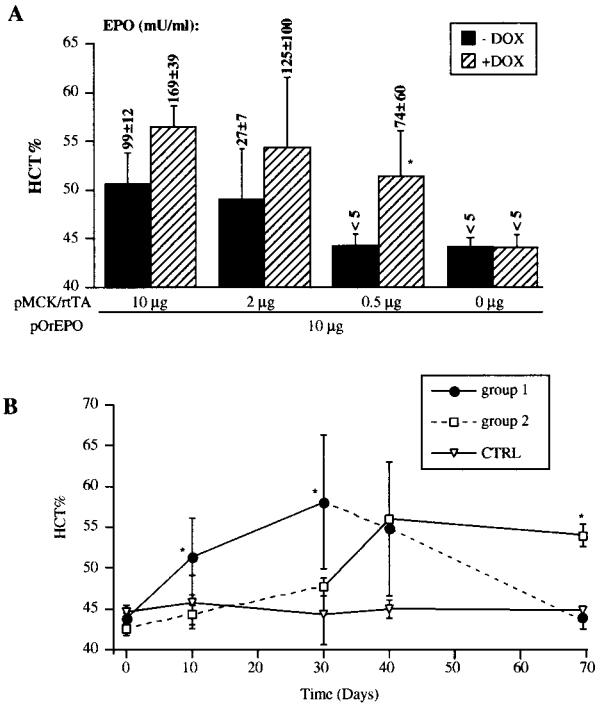

FIG. 5. (*A*) Hematocrit levels 10 days after injection in mice treated with 10 μg of pOr/EPO and different amounts of pMCK/rtTAnls as indicated. Serum EPO levels are indicated at the top of each column. (*B*) Hematocrit levels over time in mice injected with 10 μg of pOr/EPO and 0.5 μg of pMCK/rtTAnls. Solid lines indicate the presence of doxyxycline in the drinking water, dashed lines the absence. Group 1 mice were treated with DOX from day 0 to day 30; Group 2, from day 30 to day 70. Data are the mean ± SD of hematocrit as measured in four animals. *, Significantly different from mice not treated with DOX.

which activates transcription in the presence of doxycycline, was cloned under the control of the MCK promoter (21).

In a preliminary experiment, different amounts of pOr/EPO were injected to determine the basal level of expression in the absence of the transactivator; the maximal dose of plasmid that could be injected without significant hematocrit increase was 10 μg. This dose was chosen for subsequent experiments; C3H mice were injected with 10 μg of pOr/EPO and different doses (10, 2, and 0.5 μg) of pMCK/rtTAnls and stimulated immediately after injection by using standard electrical parameters. Additionally, doxycycline was given to half of the injected animals by adding it to the drinking water.

Ten days after injection, hematocrit and serum EPO increased significantly in the absence of doxyxcycline induction in mice injected with 10 or 2 μg of pMCK/rtTAnls (Fig. 5*A*), and the extent of induction in the presence of doxycycline was low (Fig. 5*A*). In contrast, in mice injected with 0.5 μg of pMCK/rtTAnls, hematocrit increased only in mice treated with doxycycline and remained at basal levels in the absence of induction (Fig. 5*A*). The induction of EPO expression was further characterized in this group of mice (Table 2).

Hematocrit increased from 43.9 ± 0.6 before injection to 58.1 ± 8.2% 30 days after treatment in the presence of induction (Fig. 5*B*, group 1) whereas it remained at 47.7 ± 1.1% in the absence of doxycycline (Fig. 5*B*, group 2). After 30 days, the doxycycline treatment was started in mice of group 2 and stopped in mice of group 1. As a consequence of induction, hematocrit increased in group 2 mice, reaching 55.5 ± 4.9% 10 days after initiation of the doxycycline treatment. The slow decline of the hematocrit levels in mice of group 1 on withdrawal of doxycycline was expected, given the 24-day half-life of mouse erythrocytes. Tetracycline-inducible promoters can therefore be exploited for efficient transcriptional regulation following plasmid DNA injection and muscle electroporation.

Table 2. Serum EPO levels in mice treated with 10 μg of pOr/EPO and 0.5 μg of pMCK/rtTAnls

	Days postinjection				
	0	10	30	40	70
Group 1	<5	75 ± 69	48 ± 42	<5	<5
Group 2	<5	<5	<5	34 ± 26	23 ± 4
Control	<5	<5	<5	<5	<5

Group 1 mice were treated with DOX from day 0 to day 30; Group 2, from day 30 to day 70. Control mice were treated with DOX from day 0 to day 70. Values given are serum EPO levels in milliunits/ml.

Analysis of Tissue Damage. Extensive and irreversible damage of skeletal muscle after electrical trauma can be ascribed to rhabdomyolysis and secondary release of myoglobin because of increased skeletal muscle cell membrane permeabilization (24). It was therefore important to test whether the electrical conditions used for muscle electropermeabilization caused muscle damage.

Histological analysis of electrostimulated muscles was performed 24 hours, 7 days, and 1 month after stimulation. No tissue alteration was detected 24 hours after electrical treatment (data not shown). One week postinjection, the presence of areas with an increased number of fibers with central nuclei was the most prominent change in the electrostimulated muscles (Fig. 6*A* and *B*). These areas were of variable size in different samples and never exceeded 10–20% of the total muscle (Fig. 6*A*). In normal muscle, the number of fibers with central nuclei was never >5% of the total. An increase in this percentage is considered a very unspecific sign of disturbance of muscle function (25). The distribution of the altered muscle fibers along a linear pathway (Fig. 6*A*) suggests that damage occurs in the regions of higher current density, i.e., just below the needle electrodes.

Small necrotic areas with lymphocyte infiltrations were occasionally detected in the central region of the muscle (Fig. 6 *C* and *D*) probably corresponding to the injection site. The damage was transient, and 1 month after treatment, muscles appeared normal (data not shown), and the altered fibers were no longer detected.

DISCUSSION

In this study, we have demonstrated that muscle DNA injection followed by electrostimulation yields high levels of EPO expression in the treated animals. The 100-fold improvement in muscle transduction over naked DNA injection alone makes muscle electroporation one of the most efficient methods of nonviral gene delivery described so far. Induction of muscle regeneration by cardiotoxin or bupivacaine can increase transduction efficiency up to 80-fold (26); however, this method is very unlikely to be routinely applicable to humans given the high toxicity of these drugs. Enhancement of gene expression has also been observed with polyvinyl-based delivery systems such as poly(vinyl alcohol) or polyvinylpyrrolidone; however, the extent of improvement was variable and only 10- to 15-fold higher than DNA formulated in isotonic saline (27, 28).

While this work was in progress, Aihara and Miyazaki (14) reported that muscle electropermeabilization can be achieved by an electrical treatment with different features from those described in this study, i.e., six monopolar pulses of 50 msec with an electric field of 200 V/cm. This treatment improves the uptake of a plasmid encoding mIL-5 with an up to 100-fold increase in the level of the circulating cytokine compared with DNA injection alone. A significant improvement in muscle

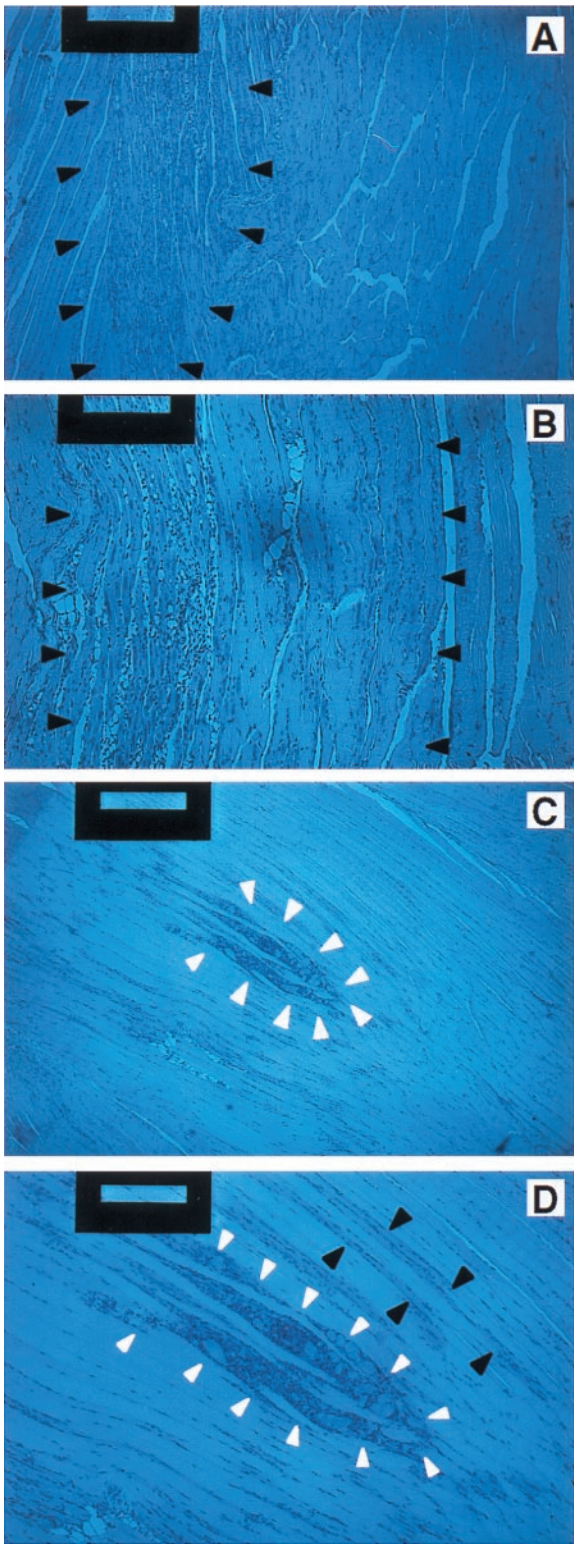

FIG. 6. Histological analysis of electrostimulated muscles 1 week after injection; black arrowheads indicates the fibers with central nuclei. White arrowheads indicate the necrotic region. [Bar = 105 μm (*A* and *C*) and 45 μm (*B* and *D*).]

transduction can be therefore obtained with different types of electrical stimuli.

The reason for the slow decline in EPO expression over time (Fig. 1 and 2) is presently unclear, and two possible mechanisms could be invoked to explain this finding: plasmid DNA degradation or promoter attenuation. Indeed, it has been shown that the cytokines interferon-γ and tumor necrosis factor-α inhibit transgene expression from several viral promoters, including human CMV, delivered by adenoviral, retroviral, or plasmid vectors *in vivo* (29). Alternatively, an immune response directed against the injected transgene could also account for the observed decline in expression. This latter possibility, however, seems to be less likely in view of published studies where the species-specific EPO gene was administered through adenoviral (30–32) or adeno-associated viral (33) vectors and no anti-EPO antibodies were detected.

The electrical parameters for muscle electrostimulation are peculiar if compared with electroporation of cells in culture in that low-voltage high-frequency pulses are used *in vivo* vs. single or a few high-voltage (kV/cm) discharges used in conventional cell electroporation. Although the basic phenomenology of electroporation remains uncertain, the strength of the electric field required to permeabilize the cell membrane depends on the cell size. The larger the cell, the lower is the minimal effective electric field (34). In comparison to other cell types, skeletal muscle cells are quite large and should be more susceptible to electropermeabilization. Indeed, it has been previously reported that exposing rat skeletal muscle to an electric field exceeding 60 V/cm results in a decrease in muscle impedance (24).

A relevant issue in gene therapy approaches is the possibility of regulating the levels of the therapeutic protein according to the need of each patient. The good correlation between the amount of plasmid injected and EPO levels in circulation on muscle electroporation allows the DNA dose to be injected to be precisely calculated to obtain the desired therapeutic effect. Thus, subsequent readministrations could allow the fine tuning of protein production. Recombinant human EPO has been shown to stimulate erythropoiesis in anemic patients with chronic renal failure; however, this therapy requires frequent treatments (35). We have seen that the minimal effective dose of the EPO-encoding plasmid is 1 μg per mouse and therefore on the order of 50 μg/kg of body weight. The increase in hematocrit with this amount of DNA lasts for at least 3 months; it is thus reasonable to speculate that only one or a few treatments with a low amount of DNA should be sufficient to stably ameliorate the anemia associated with chronic renal failure in humans. It should be noted, however, that the therapeutic levels of EPO are on the order of hundreds of pg per ml of serum; the adaptation of muscle electroporation to other diseases such as hemophilia, requiring higher levels of the therapeutic protein in circulation, will probably require higher amounts of injected DNA. Moreover, given the actual electrode configuration—a pair of wire electrodes placed 4 mm apart—the amount of DNA that can be electroporated per site is limited; therefore, the application of the electric field methodology to larger animals will require multiple injection/stimulations. Alternatively, a different electrode configuration, i.e., stimulating electrodes placed at a larger distance, should allow electroporation of a larger number of muscle fibers. Thus, these and other issues potentially affecting electroporation efficiency, such as volume of injection and type of muscle to be transduced, must be thoroughly addressed for this methodology to be implemented in clinical applications.

We show that transcriptional regulation can be achieved by using a tetracycline-inducible system. The strength of the promoter driving rtTAnls expression and hence the ratio between the induced plasmid and the transactivator are crucial for achieving optimal results. Major differences between rtTA and rtTAnls have been observed in experiments of transient transfection, whereby rtTAnls yielded a higher basal activity in the absence of doxycycline. So far, the reason for this high basal activity has not been investigated and might be attributed to the elevated accumulation of the protein in the nucleus on transient overexpression or to a somehow altered affinity of the protein to its cognate binding sequence (H. Bujard, personal comunication). The construction of a single plasmid

carrying both the rtTAnls or rtTA and the EPO genes with suitable regulation elements would be critical to making progress toward the application of this approach in humans. Additionally, given the bacterial origin of the rtTAnls, immune rejection could follow on expression in muscle cells. Although an immune reaction against the transactivator has not been observed in mice (36–38), it cannot be excluded that the foreign nature of the rtTAnls could hamper its long-term expression in other species and limit its utility in controlling gene expression.

In conclusion, the efficacy and safety of this treatment, together with the possibility of modulating the foreign gene expression, make muscle electroporation an attractive strategy for gene therapy applications.

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