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RESEARCH ARTICLE

Electrotransfer of naked DNA in the skeletal muscles of animal models of muscular dystrophies

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The electrotransfer of naked DNA has recently been adapted to the transduction of skeletal muscle fibers. We investigated the short- and long-term efficacy of this methodology in wild-type animals and in mouse models of congenital muscular dystrophy (dy/dy, dy^{2J}/dy^{2J}), or Duchenne muscular dystrophy (mdx/mdx). Using a reporter construct, the short-term efficacy of fiber transduction reached 40% and was similar in wild-type, dy/dy and dy^{2J}/dy^{2J} animals, indicating that ongoing muscle fibrosis was not a major obstacle to the electrotransfer-mediated gene transfer. Although the complete rejection of transduced fibers was observed within 3 weeks in the absence of immunosuppression, the persistency was prolonged over 10 weeks when transient or continuous immunosuppressive regimens were used. Using therapeutic plasmids, we demonstrated that electrotransfer also allowed the transduction of large constructs encoding the laminin $\alpha 2$ chain in dy/dy mouse, or a chimeric dystrophin-EGFP protein in mdx/mdx mouse. The correct sarcolemmal localization of these structural proteins demonstrated the functional relevance of their expression in vivo, with a diffusion domain estimated to be 300 to 500 μm. However, degeneration–regeneration events hampered the long-term stability of transduced fibers. Given its efficacy for naked DNA transfer in these models of muscular dystrophies, and despite some limitations, gene electrotransfer methodology should be further explored as a potential avenue for treatment of muscular dystrophies. Gene Therapy (2001) **8,** 1097–1107.

Keywords: *dy/dy mouse; mdx/mdx mouse; dystrophin; laminin $\alpha 2$ chain; naked DNA; electrotransfer*

Introduction

Muscular dystrophies are devastating genetic diseases frequently leading to premature death of affected children or young adults. Spontaneous animal models have been described for the most frequent form, Duchenne muscular dystrophy (DMD),[1–3] and also for the occidental form of congenital muscular dystrophy (CMD).[4–11] These models have helped the investigations of potential therapeutic avenues, including the recent approaches based on gene transfer. In skeletal muscle, transduction efficacy is largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver a gene to target cells with minimal toxicity.

Nonviral vectors are particularly suitable due to their simplicity of production and use, absence of size restriction, and lack of immune response. The simplest approach to nonviral delivery systems is direct gene transfer with naked plasmid DNA,[12] which is smaller than other vectors, and may be produced easily on a large scale with standardized quality. However, the use of naked DNA is hampered by the poor efficacy of transduction *in vivo*. Although transduction yields are sufficient to trigger immune reactions in certain situations,[13–16] they would not reach the threshold required for the development of a functional benefit. The electrotransfer methodology has been recently adapted to skeletal muscle transduction *in vivo* with high efficacy and reproducibility, the expression of reporter or secreted proteins being increased by a factor of one to many hundreds.[17–23] This physical approach improves naked DNA transfer efficacy by a two-component process consisting of membrane permeabilization and DNA movement enhanced by electrophoresis.[19,24] The *in vivo* application of electric pulses across muscle tissue instantaneously and temporarily compromises the barrier function of the cell membrane, allowing foreign genetic material to enter.

Naked DNA electrotransfer studies performed up to now were aimed at improving the level and reliability of gene expression after plasmid DNA injection in healthy muscle, but this methodology could be perfectly well-adapted for gene transfer in skeletal muscles of animal models of muscular dystrophies. In the present study, we investigated the efficacy of this methodology in the *mdx/mdx* and *dystrophia muscularis* mice, which are animal models of DMD and CMD, respectively.

DMD is an X-linked disease due to the alteration of the gene coding for a large subsarcolemmal protein termed dystrophin.[1] The most widely studied animal model for

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DMD, the *mdx*/*mdx* (*mdx*) mouse,[1,2] lacks subsarcolemmal dystrophin because of a mutation in position 3185 of the *dystrophin* gene.[3] The animals are characterized by raising of the level of plasma pyruvate kinase and muscular isoform of creatine phosphokinase, histological abnormalities of muscular tissue,[2] but adult *mdx* mice show few clinical signs up to 18 months. Depending on the age of the mouse and the muscle considered, 30 to 90% of the fibers are centronucleated, which is a characteristic of adult regenerated fibers. Except the diaphragm, which is one of the most constantly solicited skeletal muscles, they do not show extensive fibrosis.[25] Depending on their age and strain, the mice present 0.1 to 1% of revertant fibers, which express a truncated form of dystrophin.[26] The lack of dystrophin makes the *mdx* mouse a good model for gene complementation. Indeed, the transfer of a dystrophin minigene into the gastrocnemius muscle of young *mdx* was reported to protect partially the transduced fibers against detrimental consequences of eccentric exercise.[27]

The occidental form of CMD is an autosomal disease due to the defect of the gene coding for the α2 chain of laminin.[11] The association of the large α2 chain with β1 and γ1 chains constitutes the laminin-2 protein, which is an extracellular protein providing a bridge between the sarcolemmal membrane and the extracellular matrix in muscle fibers.[28,29] The *dystrophia muscularis dy*/*dy* and dy^{2J}/dy^{2J} mice are animal models for the occidental form of CMD.[4–10] Both murine and human diseases share common clinical, histological and genetic features, among which are the generalized and progressive alteration of the muscular tissue. The number of fibers is decreased and their diameter is greatly variable. Strikingly, a widespread proliferation of the endomysial tissue leads to the progressive fibrosis of the muscle.[4,5,30] The fibrosis process takes place rapidly in *dy*/*dy* animals and more slowly in dy^{2J}/dy^{2J}. In the latter, the process is first segmental but becomes generalized by the age of 3 months.[5,31,32] In the absence of the laminin α2 chain, muscle regeneration is abortive.[33,34] The *dy*/*dy* animals develop a devastating disease reducing the life expectancy to 6 months, whereas dy^{2J}/dy^{2J} animals develop a milder pathology which allows them to survive up to 24 months. The difference between these models may be related to the expression of a truncated, but partially functional protein in the dy^{2J}/dy^{2J} mouse.[9,10].Using these animal models, we addressed some issues specific to dystrophic muscle: is transduction of skeletal muscle fibers efficient in animal models of muscular dystrophies using reporter plasmids, and does muscle dystrophy hamper efficacy? Does the transfer of therapeutic genes induce the expression of functional proteins in these suffering muscles *in vivo*? Does electrotransfer induce immunological responses, and does the expression of the transgene persist in these models? We show that this methodology allows efficient transduction of dystrophic muscle fibers, and that the persistency of the transgene product is dependent upon the immune system and the muscle fibers turn-over.

Results

Short-term transduction efficacy of the β-gal reporter gene in wild-type and dystrophic mice

In wild-type mice, less than 1% ($n = 4$) of the muscle fibers was found to express the reporter protein β-gal 6 days after gene transfer in the absence of electrotransfer. Conversely, the electrotransfer conditions used in this study allowed transduction of 37 ± 14% ($n = 19$) of muscle fibers when correlated to the surface containing the reporter protein, and of 47 ± 13% ($n = 8$) of muscle fibers when correlated to the number of transduced fibers (Figure 1A and B). We observed a good correlation between the results obtained using the two methodologies for measuring expression, ie computer-assisted surface measurement and manual counting of the fibers, the difference between these methodologies for measurement being not significant ($P > 0.05$). Indeed, the amount of fibrotic tissue is negligible in non-dystrophic animals.

In the *dy*/*dy* and dy^{2J}/dy^{2J} mice, the transduction efficacy rarely reached 1% (Figure 1E, G) in the absence of electrotransfer. However, the electrotransfer methodology increased the transduction of muscle fibers in these models dramatically (Figure 1F, H). Based on surface measurements, the efficacy of β-gal gene transfer was estimated to be 14.3 ± 6.3% ($n = 9$) in *dy*/*dy* animals and 23.3 ± 8.1% ($n = 9$) in dy^{2J}/dy^{2J} mice. This discrepancy, which is statistically significant ($P < 0.05$), is due to the presence of fibrotic tissue which, in the surface measurement method, is recognized as non-transduced tissue hence leading to an underestimation of the efficacy of transduction. Alternatively, the direct counting of the transduced fibers gave an efficacy of 32 ± 9.7% ($n = 5$) in *dy*/*dy* mice and 35.7 ± 14% ($n = 4$) in dy^{2J}/dy^{2J} mice, the difference being not significant, thus indicating that the plasmids were able to diffuse within the tissues and penetrate the muscle fibers with the same efficacy. These results suggest that fibrosis was not an obstacle to electrotransfer in these animal models.

In wild-type as well as in dystrophic animals, the fibers expressing the reporter protein β-gal were not homogeneously distributed over the whole muscle, suggesting that transduction occurred mainly at the multiple injection sites (Figure 1B, F, H). The length of expression of the β-gal, however, extended longitudinally over hundreds of microns (not shown). Variations in staining intensity were observed.

No damage was induced in the muscular tissue by the electrotransfer methodology in wild-type animals. No classical figure of ongoing degeneration–regeneration[25,35] was observed in the muscular tissue at the time (6 days) of analysis in wild-type mouse muscles (Figure 1C, D). The transduced fibers, which were identified by β-gal expression, were not centronucleated (Figure 1C, D). We did not observe the massive cellular infiltration that is present following delivery of cellular or viral vectors.[36,37] Given the fibrotic nature of the dystrophic muscles, it is difficult to judge about the potential damage triggered by electrotransfer in the models of muscular dystrophies.

Short-term transduction efficacy of the laminin α2 chain gene in dystrophia muscularis mice

The reporter and therapeutic constructs were mixed and injected together in these short-term cotransduction experiments. This is the first report showing that the transduction of the therapeutic laminin α2 chain cDNA led to the expression of a functional laminin α2 chain in *dy*/*dy* mice, which is correctly expressed around the muscle fiber membrane (Figure 2A, B). As for β-gal expression, staining intensity varied from fiber to fiber, some cells even showing cytoplasmic expression and fur-

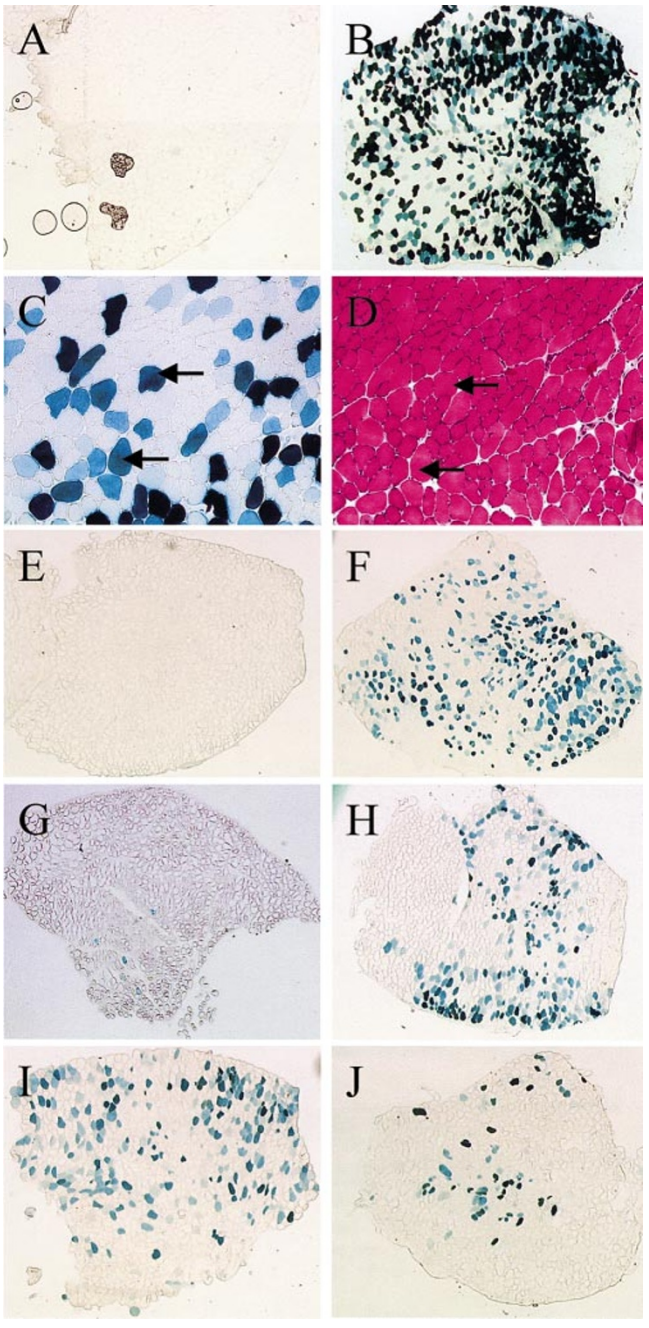

Figure 1 *Short-term expression of β-gal reporter transgene in wild-type, dy/dy and dy^{2J}/dy^{2J} animals. The pCOR β-gal plasmid was injected in the tibialis anterior of wild-type (A–D), dy^{2J}/dy^{2J} (E, F, I) and dy/dy (G, H, J) animals. The expression of the β-gal reporter protein was analyzed by X-gal histochemistry 6 days (A–H) or 1 month (I, J) after gene transfer in the absence (A, E, G) or presence (B–D, F, H–J) of electrotransfer. C and D are serial sections. In C, X-gal histochemistry localizes the fibers expressing β-gal. In D, hematoxylin-eosin staining indicates the absence of muscle degeneration, necrosis, infiltration or centronucleation in the areas of transduced fibers. Arrows localize identical fibers on serial sections. Animals used in 1-month experiments were immunosuppressed. Original magnification, ×30 (A, B, E–J), ×120 (C, D).*

thermore, transduction was not restricted to the large fibers (Figure 2A, B). However, we observed that the number of fibers expressing β-gal was higher than those expressing the laminin α2 chain since 6 to 16% ($n = 3$) of the fibers were positive for α2 chain staining, whereas up to 33% were positive for β-gal staining. As in the case of wild-type animals, the transduced fibers were not homogeneously distributed. However, the use of serial sections allowed us to estimate the domain of expression of the α2 chain to be close to 500 μm (Figure 2C–F).

Requirement of immunosuppression for long-term transgene expression

In the absence of immunosuppression, the percentage of muscle fibers expressing the foreign protein β-gal dropped dramatically 3 weeks after electrotransfer in both wild-type ($n = 4$, Figure 3B) and dystrophic animals (not shown), as compared with the percentage obtained 6 days after electrotransfer (Figure 3A). Conversely, the number of centronucleated fibers rose, suggesting an event of degeneration–regeneration (not shown). No prominent cellular infiltration was observed at the time of death, ie 3 weeks after gene transfer.

FK506 is a powerful immunosuppressant which has been used successfully in various animal models to prevent the immune rejection triggered by the expression of new antigens. For instance, FK506 blocked the cellular and humoral rejection of transgene products in cell- or virus-mediated gene transfer in mice.[38–44] Therefore, in the present study, FK506 was used to prevent the rejection of transduced muscle fibers by the mature, adult immune system of the recipient mice. As expected, the daily immunosuppression of animals following transduction led to the persistency of a high percentage of labeled fibers for several weeks in wild-type mice. The continuous immunosuppression over 3 weeks allowed the level of expression to remain high over 3 weeks (surface measurement, 40.2 ± 8.5%, $n = 8$, Figure 3D). The difference between immunosuppressed and non-immunosuppressed groups is significant ($P < 0.05$). Moreover, the discontinuation of FK506 treatment after 3 weeks still allowed a high expression level 4 weeks later, the difference with the group of mice killed after 3 weeks not being significant (surface measurement, 41.5 ± 13.9%, $n = 6$, Figure 3E). The effect was partially sustained over time since, even 10 weeks after cessation of FK506, some fibers were still expressing β-gal (Figure 3F). However, a precocious cessation of FK506 immunosuppression was not efficient at blocking cellular rejection, since a 1 week-long immunosuppression was not sufficient to allow a 3 week-long persistency of transduced fibers ($n = 4$, Figure 3C).

Long-term expression of β-gal and laminin α2 chain genes in dystrophia muscularis mice

For these long-term cotransduction experiments, all animals were immunosuppressed. One month after electrotransfer, the fibers expressing the β-gal reporter protein were still numerous (surface measurements, 8 ± 2.8% ($n = 2$) in *dy/dy* animals and 17.7 ± 5.4% ($n = 6$) in dy^{2J}/dy^{2J} animals, Figure 1I, J).

Long-term transduction efficacy of β-gal and dystrophin genes in mdx mice

All animals were immunosuppressed for these long-term cotransduction experiments. As in the CMD animal models, electrotransfer greatly increased gene transfer efficacy in this DMD animal model (Figure 4C). It should be noted that 1 month after transduction in these *mdx* mice, the number of labeled fibers has decreased, as compared with wild-type animals. The difference between the

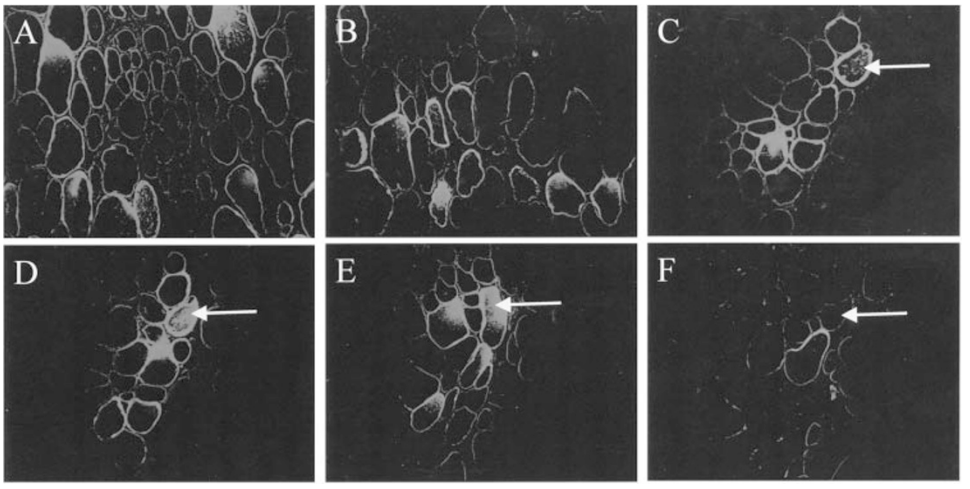

Figure 2 *Expression of the α2 chain transgene in dy/dy mice. The expression of laminin α2 chain was explored by immunohistofluorescence 6 days after electrotransfer-mediated transfer of the pMER plasmid in dy/dy muscles. The protein surrounded muscle fibers of different diameters in continuous or patchy patterns (A, B), and was also present in the cytoplasmic compartment of some fibers. The staining was restricted to portions of the muscle probably representing the injection sites. (B–F). Figures C to F: serial images of consecutive sections interspaced by 160 μm. The arrow identifies the same muscle fibers and indicates that the domain of expression of the α2 chain is restricted to about 500 μm. Original magnification: ×250.*

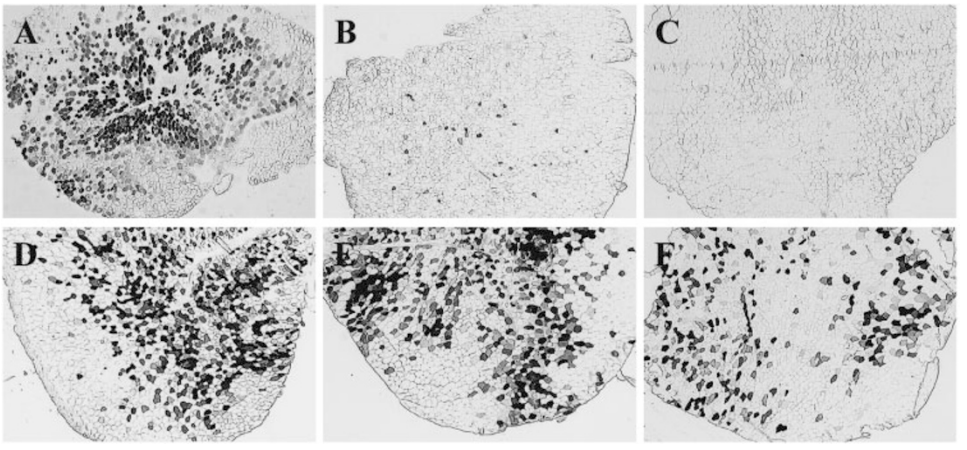

Figure 3 *Evidence of immune reactions against the fibers expressing the β-gal reporter transgene. The expression of the β-gal reporter protein was observed at various times after electrotransfer of the pCOR β-gal plasmid in adult, immunocompetent non-dystrophic mice. (A) Death at 6 days after gene transfer; (B) death at 3 weeks after gene transfer in the absence of immunosuppression; (C) death at 3 weeks after gene transfer following a 1-week long transient FK506 immunosuppression; (D) death at 3 weeks after gene transfer following a 3-week FK506 immunosuppression; (E) death at 7 weeks after gene transfer, following a 3 week-long transient FK506 immunosuppression; (F) death at 13 weeks after gene transfer, following a 3 week-long transient FK506 immunosuppression. Original magnification, ×25.*

efficacy in wild-type animals (40.2 ± 8.5%, $n = 8$) and in *mdx* animals (22.0 ± 8.9%, $n = 7$) is significant ($P < 0.03$). As observed in the other models, the distribution of the fibers expressing β-gal was not homogenous.

The transduction of the EGFP cDNA construct resulted in strong but diffuse expression of the EGFP protein in the cytoplasm of the muscle fibers (not shown). The transduction of either the minidystrophin-EGFP construct (Figure 4A–F) or the full-length dystrophin-EGFP construct (Figure 4G, I) resulted in the strong expression of EGFP protein restricted to the sarcolemmal membrane. As with previous models, staining intensity varied from one fiber to the other.

Given the nature of the constructs used in these experiments, four combinations of fiber labeling were expected following transduction, since the fibers expressing EGFP always express dystrophin: (1) β-Gal+/EGFP+/dystrophin+ fibers; (2) β-Gal+/EGFP–/dystrophin– fibers; (3) β-Gal–/EGFP+/dystrophin+ fibers; (4) β-Gal–/EGFP–/dystrophin– fibers. As the so-called revertant fibers expressing a truncated form of dystrophin are present in *mdx* muscles,[26] two other combinations were also expected: (5) β-Gal+/EGFP–/dystrophin+ fibers; (6) β-Gal–/EGFP–/dystrophin+ fibers. The six combinations were indeed identified among neighboring fibers and five out of six are shown in Figure 4F. It is worth noting that any false positive results between β-gal, EGFP and dystrophin is ruled out by the fact that some fibers expressing the β-gal did not express the EGFP, nor dystrophin, and conversely some fibers expressing EGFP or dystrophin did not express β-gal.

The analysis of serial muscle sections allowed the calculation of the nuclear domains of the reporter proteins, as illustrated in Figure 5. The expression of

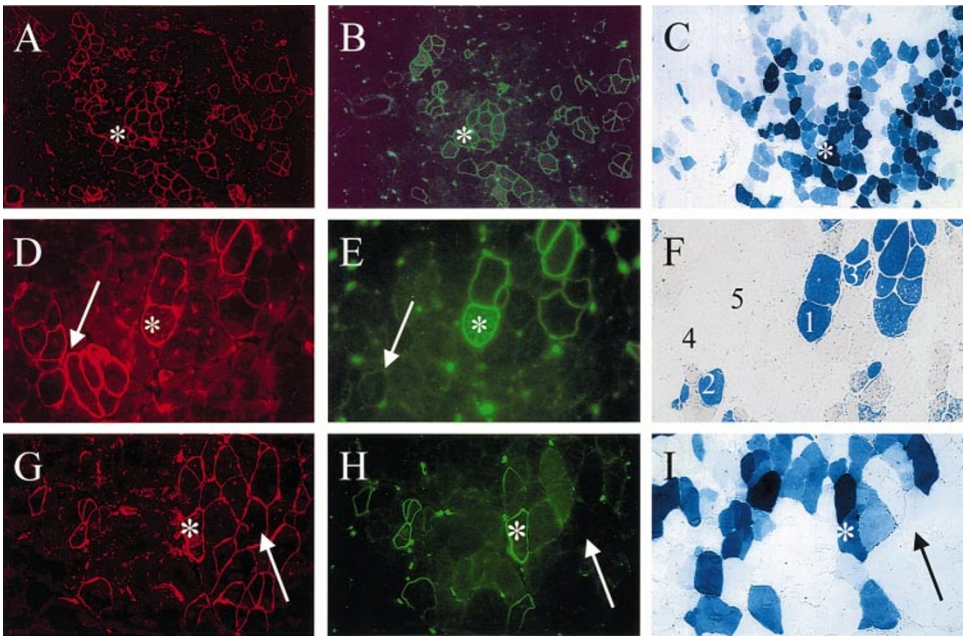

Figure 4 *Expression of β-gal, EGFP and dystrophin transgenes in mdx mice. Electrotransfer was performed in immunosuppressed mdx animals following the injection of pCOR β-gal plasmid associated to the pEGFP-Minidystrophin (A–F) construct or to the pEGFP-full length dystrophin (G–I) construct and the animals were killed 3 weeks later. The expression of mini-dystrophin (A, D), of full-length dystrophin (G), or of revertant dystrophin (A, D, G) was assessed by immunohistofluorescence. The expression of EGFP (B, E, H) was observed by direct histofluorescence. Dystrophin and EGFP were observed on the same section. The expression of β-gal was assessed by X-gal histochemistry (C, F, I) on an adjacent section. (A–C) Asterisks localize the same fibers; the fibers expressing EGFP also expressed minidystrophin, but some fibers expressing a revertant dystrophin, but no EGFP were observed. The fibers expressing the EGFP and β-gal were localized in the same clusters, although the β-gal-positive fibers were more numerous. (D–F) At higher magnification, asterisks localize the same fiber, and arrows identify a cluster of fibers expressing a revertant form of dystrophin. In F, the numbers identify five labeling combinations among neighboring fibers (see text). (G–I) The fibers expressing EGFP also expressed full length dystrophin, but some fibers also expressing a revertant dystrophin but no EGFP were observed (arrows). Asterisks localize the same fiber. Original magnification: × 90 (A–C), × 230 (D–I).*

EGFP/dystrophin was generally restricted between 32 and 40 consecutive sections (ie about 300 μm). As expected, the expression of dystrophin alone in the revertant fibers not expressing EGFP extended over the length of the skeletal muscle (Figure 5 shows a view limited to 40 consecutive sections). Strikingly, the fibers transduced with the β-gal plasmid were expressing the protein over more than 360 consecutive sections, ie 2500 μm (Figure 6).

Persistence of transgene expression in a model of acute degeneration–regeneration

Notexin is a snake venom which triggers extensive degeneration and regeneration cycles in animal models.[45,46] In small muscles, as in our experimental conditions, degeneration is rapid and complete and regeneration is achieved by 3 weeks. Centronucleation is a hallmark of regeneration. The presence of fibers of smaller diameter or irregular shape is also frequently observed in regenerated muscles.[25,35,47,48] In order to mimic an accelerated degeneration of muscle tissue, we injected notexin intramuscularly in one of the two transduced legs of immunosuppressed wild-type animals 4 weeks after the electrotransfer. Immunosuppression was continued for 1 month at which time animals were killed.

As expected (Figure 7C), notexin has induced an acute process of degeneration–regeneration, as illustrated by the very high number of centronucleated fibers in the injected muscle to be compared with the very small number of centronucleated fibers in the contralateral muscle. Indeed, the ratio of centronucleated fibers *versus* non-centronucleated fibers was over 90% in the notexin-injected muscle and less than 10% in the contra-lateral muscle (Figure 7B, D). Moreover, histological examination of the notexin-injected muscle revealed the presence of numerous small caliber fibers of irregular shape. β-Gal expression almost completely disappeared only from the notexin-injected, degenerated muscle (Figure 7A, C), since very few fibers expressed β-gal (surface measurement 1 ± 1%, $n = 7$, Figure 7A). On the other hand, a high number of muscle fibers were expressing the reporter gene in the muscle which was not damaged by notexin injection (surface measurement 35.5 ± 3.7%, $n = 4$, Figures 7A, C). The difference between these two groups is significant ($P < 0.05$). Therefore we concluded that the muscle degeneration is the cause of loss of β-gal expression after gene transfer, despite a very efficient initial level of transduction.

Discussion

Our results show that naked gene electrotransfer *in vivo* is highly efficient in both dystrophic and non-dystrophic mice. Indeed, a high number of muscle fibers is successfully transduced by using the transfer conditions described in our previous work.[18,19] The transduction was as efficient in the dystrophic fibrotic tissue as in non-dystrophic muscle. Moreover, dystrophic fibers of variable diameters were transduced. This strongly suggests that the presence of fibrotic tissue does not hamper the trans-

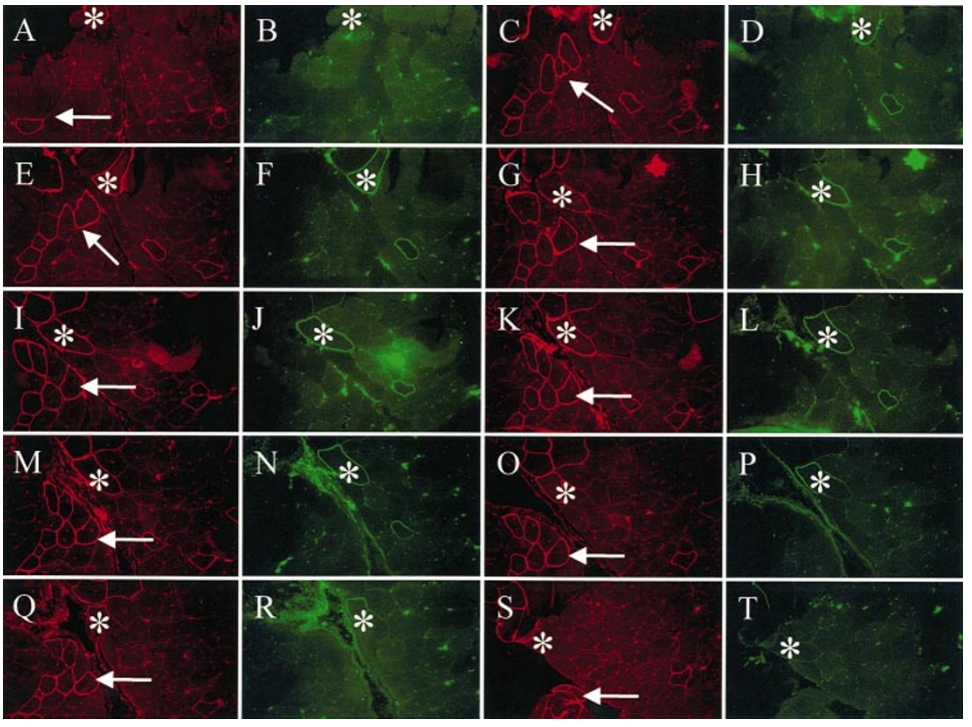

Figure 5 *Expression domains of EGFP and dystrophin in mdx muscle. Electrotransfer was performed in immunosuppressed mdx animals following the injection of the pEGFP-full length dystrophin construct and the mice were killed 3 weeks later. The expression of full-length dystrophin or of revertant dystrophin (first and third columns) was assessed by immunohistofluorescence. The expression of EGFP (second and fourth columns) was observed by direct histofluorescence. Dystrophin and EGFP were colocalized on the same section. The pairs of sections (dystrophin, EGFP) were interspaced by 32 μm. Asterisks localize fibers expressing both EGFP and dystrophin, and arrows localize a cluster of fibers expressing a revertant form of dystrophin. Note that EGFP and full-length dystrophin are expressed simultaneously over eight sets of sections, whereas the expression domain of the revertant dystrophin extends over the complete set of sections, thus pointing out a difference in the length of the domains of the two dystrophin proteins. Original magnification, ×90.*

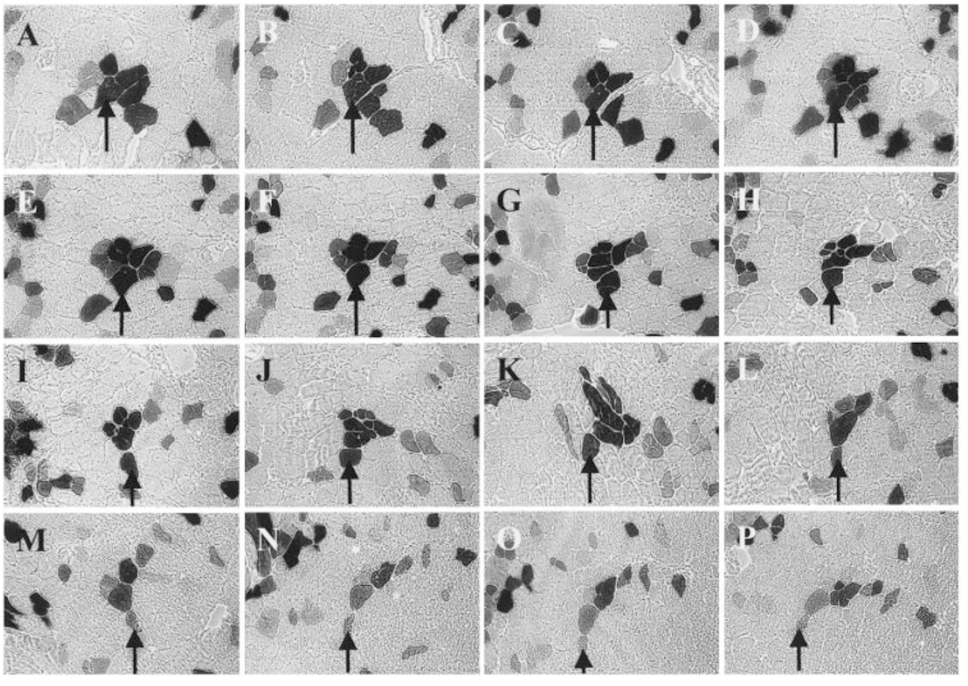

Figure 6 *Expression domain of β-gal in mdx muscle. Electrotransfer was performed in immunosuppressed mdx animals following the injection of pCOR β-gal plasmid and the mice were killed 3 weeks later. The expression of β-gal was assessed by X-gal histochemistry. The sections presented are interspaced by 200 μm, excepted for J, K and L which are separated by 100 μm. The arrows localize the same muscle fiber through the whole length of the muscle fiber. β-gal staining extends over the whole set of pictures. Note that the shape and orientation of the fibers progressively change due to the structure and orientation of the muscle. Splitting is frequently observed in the mdx muscle. Original magnification, ×110.*

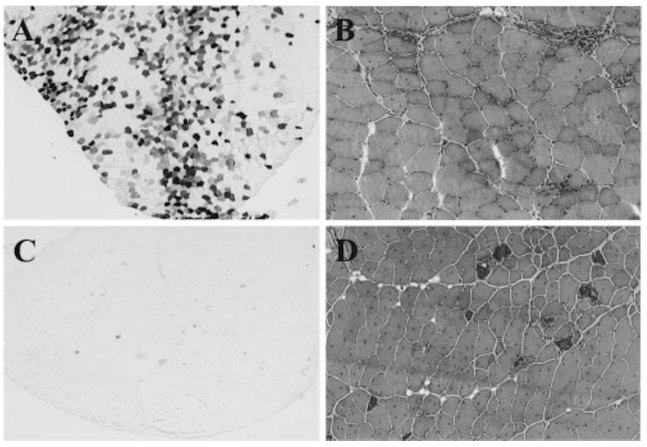

Figure 7 *Lack of persistence of transduced fibers in an acute model of degeneration–regeneration. Electrotransfer was performed in both tibialis anterior of wild-type animals following the injection of pCOR β-gal plasmid. Three weeks later, one tibialis was injected with notexin (C, D) whereas the contralateral was not (A, B). The animals were killed 4 weeks after notexin injection, ie 7 weeks after gene transfer. The expression of β-gal was assessed by X-gal histochemistry (A, C). Serial sections were stained with hematoxylin-eosin (B, D). Most fibers were centronucleated in the muscle injected with notexin (D), whereas most fibers were not in the contralateral muscle (B). Original magnification, ×35 (A, C), ×140 (B, D).*

duction of fibrotic muscle. However, the distribution of the transduced fibers was not homogenous, suggesting that naked DNA diffusion inside the muscle tissue from the injection site should still be improved. Recent studies documented the administration of various vectors through the vascular route following transient ischemia, chemically induced vasodilatation,[49] or under high physical pressure.[50] The focused ultrasound technology should also be considered.[51] Therefore, naked DNA transfer could be improved by the coupling of one of these approaches to the electrotransfer methodology.

We have chosen to quantify reporter gene expression by manual counting of transduced fibers and by computer-assisted measurement of the surface of transduced muscle, because these measurements indicated the topography and extent of transduction, the distribution of the vectors, and the possible relevance to a physiological functional benefit.

The electrotransfer parameters that were used in the present work did not trigger extended muscle damage in non-dystrophic animals as shown by the low number of centronucleated fibers and the absence of cellular infiltration either 6 days or 3 weeks (immunosuppressed animals) after electrotransfer. However, the original nature of dystrophic muscles makes difficult the evaluation of potential additional damage raised by the electrotransfer procedure in dystrophic animals. For example, the *mdx* skeletal muscle is known to be especially sensitive to eccentric contractions,[27,52,53] and one may not exclude that the electrotransfer protocol may be more detrimental in certain muscular pathologies than in others.

Our results indicate that, in immunocompetent animals, the expression of the β-gal transgene is lost within a couple of weeks. In some models, the expression of a transgene triggers a specific immune rejection directed against the transduced fibers, that results in their elimination. However, such a phenomenon is dependent on the transgene: it has not been observed for the luciferase reporter gene over a period of 1 year in C57Bl6J immunocompetent mice,[18,19] but has been observed for secreted proteins such as alkaline phosphatase or human factor IX[23] or cytoplasmic proteins such as hepatitis B envelope protein.[16]

To evaluate whether the loss of β-gal expression was due to an immune response of the host, and not to muscle instability or to promoter shut-off in muscular tissue, the animals were immunosuppressed using FK506. This agent is a blocker of interleukin-mediated T cell activation and inhibits cellular- and humoral-specific immune responses directed against neo-antigens.[54–58] FK506 has been used previously to prevent the rejection of transgene products brought in recipient animals by cellular or viral vectors.[37–44] Our present results indicate that the specific immune reactions are blocked by the permanent or discontinuous administration of the FK506 immunosuppressant. The protection, however, fades away and the number of β-gal-positive fibers decreases with time after termination of FK506. We observed that a 3-week transient immunosuppression led to a delayed persistency of the β-gal expression over 7 to 13 weeks. FK506 did not induce long-term tolerance of the neo-antigen β-gal, but may slow the rejection reaction, as observed in other experimental situations.[37,41] Our results thus confirm that β-gal antigens may trigger immune reactions, even in the absence of a cellular or viral vector.[37]

The present results were obtained in the C57BL6J mouse strain, using a transgene of prokaryotic origin. It should be underlined that the triggering and extent of immune rejection may vary according to the nature of the transgene product and the animal species or strain. The extent of immune reactions triggered by the introduction of species-specific transgenes and corresponding recombinant protein is not yet elucidated, but the present electrotransfer methodology will provide a valuable tool for the investigation of such reactions.

This study is the first report of the restoration of laminin-2 *in vivo* by direct gene transfer. Indeed, the introduction of laminin α2 chain cDNA led to the expression of a α2 chain located around the sarcolemmal membrane, and the introduction of mini-dystrophin or of full-length dystrophin cDNAs linked to the EGFP cDNA led to the production of fusion proteins which were addressed to the subsarcolemmal membrane. Taken together, these findings indicate that a functional restoration of the missing proteins is possible at the cellular level in dystrophic animals when an electrotransfer-mediated process is combined to the delivery of plasmid DNA.

Among dystrophin-positive fibers, two categories were clearly identified by the presence or absence of co-expression of EGFP. Hence, the utilisation of EGFP-dystrophin constructs expressing fusion proteins allows a clear distinction between revertant fibers expressing a truncated form of dystrophin,[26] but no EGFP, and the truly transduced fibers expressing both EGFP and dystrophin. Thus, as they allow easy discrimination between transduced and revertant fibers, the EGFP-dystrophin constructs presented here are useful tools for the design of future gene transfer protocols *in vivo*, as suggested in a previous report.[59]

From the colocalization of EGFP and dystrophin, the

domain of expression of these molecules following gene transfer can be calculated. Our studies confirm some previous findings obtained in other models, ie over 1500 μm for β-gal[60,61] and about 300 μm for dystrophin.[60,62] Along the same line, we show here that the domain of expression of the laminin-2 molecule is approximately 500 μm. These results underline one present limitation of direct gene transfer into skeletal muscles, which is the restricted segmental diffusion of the transgenic proteins inside the transduced fibers. This may be considered an advantage in situations where the local delivery of a protein is required. However, it may also be considered an impediment in situations where the long-range restoration of a structural protein is required, such as in muscular dystrophies.

The numbers of muscle fibers expressing the therapeutic proteins were lower than that observed using a β-gal reporter cDNA (see Figure 5), and the length of stained individual fibers was shorter. These observations raise several hypotheses. First, the therapeutic constructs are bigger than the reporter construct (from 12.5 to 18 kb for dystrophin constructs *versus* 5 kb for β-gal construct). Because the total amounts of DNA material injected into the muscles were similar, the number of copies were different. Moreover, in the present range of 5 to 18 kb, the influence of the physical size of constructs on transduction efficacy is likely to play an important role, since smaller plasmids have been shown to lead to higher transduction either with or without associated electrotransfer[63,64] (D Scherman, personal communication). Second, the reporter and therapeutic proteins are different in nature. While β-gal is cytoplasmic, dystrophin and laminin α2 chain have defined cellular localizations and are membrane-bound. The β-gal protein is small, whereas α2 chain and dystrophin are large and heavy, and the diffusion coefficients of these proteins may differ. Such differences were already observed in cell transplantation experiments.[60,61] Third, the plasmid constructions and preparations differ. The β-gal plasmid has a pCOR backbone and, due to its size and construction, may be present in a supercoiled form at a very high percentage (>99%). Such a situation has been shown previously to influence favorably *in vivo* transfer efficacy,[63,65] but may not be obtained with all plasmid constructs. Finally, the methods used to stain the products were different, ie the β-gal was detected by histochemistry, whereas the α2 chain and dystrophin were revealed by immunohistofluorescence.

Although expression of the reporter gene was stable over time in wild-type animals, a slow decrease with time in the percentage of labelled fibers was generally observed in dystrophic animals. A possible explanation could be that murine dystrophic muscles undergo acute or chronic cycles of degeneration–regeneration, as shown by the centronucleation of regenerated fibers.[1,2,4,5,25 30] To test this hypothesis, we triggered an acute cycle of degeneration–regeneration using notexin administration in wild-type mice in which the reporter gene was previously transduced by electrotransfer. Some snake venoms, such as notexin, trigger muscle degeneration upon intramuscular injection. This myonecrosis is limited to the mature muscle fibers and spares the blood, the nerve supplies, and the myogenic progenitors.[45,46,48] Following degeneration, small mouse muscle regenerate completely within 3 weeks[35] through the activation, proliferation and fusion of satellite cells and further maturation of the new or hybrid muscle fibers.[35,47,66]

Upon notexin administration in wild-type mice, we observed that β-gal expression was cleared from the degenerated muscle, while it persisted in the non-degenerated muscle. Therefore we concluded that the muscle degeneration is the cause of loss of β-gal after gene transfer, and that the sustained expression of the reporter gene is not compatible with acute muscle degeneration.

Since muscle regeneration proceeds by activation, proliferation and fusion of satellite cells,[35,45,47,66] our results imply that these cells have not been substantially transduced by electrotransfer *in vivo*. This is in agreement with the principle of electrotransfer, which claims a relation between cell diameter, voltage intensity, and transduction efficacy.[19,24]

Our demonstration raises a major problem for long-term expression of a therapeutic gene in the context of degenerating muscle. A number of human neuromuscular disorders are characterized by various degrees of acute or chronic degeneration–regeneration cycles and our results would suggest that in these disorders it will be important to target both muscle fibers and the satellite cells. However, if gene transfer is very efficient and leads to sufficient levels for muscle stabilization, then turnover may be slowed and the need for transduced satellite cells would become less important. Therefore, harmony should be found between the choice of the vector and transduction methodology, and the pathophysiology of the neuromuscular disorder. In this perspective, the overall functional efficacy of the electrotransfer methodology requires further investigation to define the long-term relevance of gene transfer in muscle tissue. Other groups have shown that the conversion of an important proportion of muscle fibers from dystrophin-negative to -positive was a prerequisite for a physiological functional benefit in the *mdx* model (30 to 40%).[27] The transduction efficacy presented in this study is close to these values, at least regarding the expression of β-gal, and would therefore be compatible with the development of a functional benefit.

Taken together, our results indicate that electrotransfer-mediated muscle transduction is efficient in animal models of muscular dystrophies, and that the expression of therapeutic proteins has functional relevance. Hence, the DNA electrotransfer *in vivo* is one of the strategies to be considered as a potential avenue for treatment of muscular dystrophies.

Materials and methods

Animals

Breeding pairs of *dy*/*dy* and dy^{2J}/dy^{2J} mice were purchased at Jackson Laboratories (Bar Harbor, ME, USA). From these pairs we developed colonies in our facility by heterozygous brother-sister mating. For *dy*/*dy* animals, the mice were segregated phenotypically as normal or dystrophic. For dy^{2J}/dy^{2J}, genotyping was performed as previously described.[67] Control mice were normal, genotyped littermates of dy^{2J}/dy^{2J} animals. Given the difficulties related to the production of the dystrophic animals, all mice could not be age-matched for the experiments, but they were in the range of 6 to 15 weeks old.

In both mutants, this age coincides with the time at which the muscle can still be influenced by external stimuli such as drug treatments. The *mdx* animals were from Jackson Laboratories and bred in our facility. We used 8-month-old animals. At this stage, revertant fibers are numerous. All animals were allowed free access to food and water. Studies were conducted following the recommendation of the European Convention for the Protection of Vertebrate Animals used for Experimentation.

Plasmids

The pCOR plasmid pXL3227 (pCMV-β-gal), containing the cytomegalovirus (CMV) promoter inserted upstream of the *Escherichia coli LacZ* gene coding for the cytoplasmic β-gal, was prepared as previously described.[65] The plasmid preparation contained a high percentage of supercoiled DNA (99.9%), and no RNA was detectable by gel electrophoresis. The plasmid size was 5 kb.

The pMER plasmid p941 (pCMV-α2chain) was prepared as previously described and kindly provided by Drs Ulla Wewer and Steven Loechel (Institute of Molecular Pathology, University of Copenhagen, Copenhagen, Denmark).[33] The human α2 chain cDNA was driven by the CMV promoter. The plasmid size was 12.5 kb. The pEGFP, pEGFP-minidystrophin (pEGFPMD) and pEGFP-full length dystrophin (pEGFPFLD) plasmids were prepared as previously described.[59] The human truncated or full-length dystrophin cDNA were driven by the CMV promoter and validated *in vitro* for the production of minidystrophin or full-length dystrophin. The plasmid sizes were 6 kb, 12 kb and 17 kb respectively. pMER, pEGFP, pEGFPMD, and pEGFPFLD plasmids were prepared using the Qiagen (Courtaboeuf, France) Megaprep endotoxin-free columns.

DNA injection and electric-pulse delivery

Animals were anesthetized during the whole procedure by subcutaneous injection of 30 mg/kg and 15 mg/kg of body weight of ketamine and xylazine, respectively. The skin above the tibial cranial muscle was shaved before injection. A 5-mm incision was made in the skin to visualize the underlying muscle. Twenty μg of each plasmid DNA in 20 μl of 0.9% NaCl was injected into the tibialis anterior muscle with a glass capillary in 10 different sites. The capillary thinness allowed injection into all orientations of the muscle in both depth and surface, in sites separated by 1 to 1.5 mm.[37,61] After injection, the skin was closed with resorbable sutures. Five minutes after naked DNA administration, transcutaneous electric pulses were applied through two stainless steel plate electrodes placed on either side of the hindlimb as previously described.[18,19] Briefly, square wave electric pulses were generated by an electropulsator ECM-830 (BTX, San Diego, CA, USA) with output voltage of 200 V/cm, pulse length of 20 ms, number of pulses of eight and frequency of pulse delivery of 2 Hz. Electric-field strengths (in V/cm) are reported in terms of the ratio of the applied voltage to the distance between the electrodes.

Immunosuppression

Immunosuppression was started on the day of plasmid injection using 2.5 mg/kg/day of FK506 as previously described (a kind gift from the Fujisawa Co, Osaka, Japan).[37,38] Depending on the experimental group, FK506 was maintained for 1, 3, 4 or 7 weeks.

Induction of acute degeneration–regeneration

An acute cycle of muscle degeneration–regeneration was triggered by the injection of the snake venom notexin according to the following schedule. At day 0, wild-type animals underwent electrotransfer using the β-gal reporter gene in both right and left tibialis anterior muscles. FK506 immunosuppression was started and given daily for 50 days. At day 28, the left tibialis anterior received 50 μl of notexin (Sigma, St Louis, MO, USA; 5 μg/ml). At day 50, the animals were killed and muscles were harvested and analyzed.

Preparation of muscle biopsies

The animals were killed 3 weeks after transplantation. The muscles were frozen by immersion in isopentane cooled in liquid nitrogen. The 8 μm sections were performed with a cryostat.

β-Galactosidase histochemistry

Following glutaraldehyde fixation (0.25%) and two rinses in PBS, the cryostat sections were incubated overnight in the presence of X-gal reagent (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer Mannheim, Mannheim, Germany) at room temperature as previously described.[37]

Quantification of pCOR β-gal transduction efficacy

Quantification by surface measurement: The muscle section containing the highest level of β-gal-positive fibers was selected by light microscopy. This section was digitized using a Leica microscope (Rueil-Malmaison, France) connected to a Sony 3CCD (Rueil-Malmaison) color video camera and the Thunder 1.2.0.6 software. The image was computerized at full size to allow global measurement of the surface. Following delineation of the muscle, the total surface of the muscle, the surface containing the blue precipitate, and the ratio between surfaces were calculated automatically by the NIH Image 1.62 software.

Quantification by manual counting: The same section as above was selected, digitized and color-printed on to A4 format paper using a HP Deskjet 1600 CM printer. This magnification allowed the visual counting of the muscle fibers.

Each methodology was performed by an independent observer. The analysis of correlation between the results was done using the Mann–Whitney non-parametric test run under Statview 5.0 software and indicated no significant difference when muscles of non-dystrophic animals were analyzed. However, the surface measurement systematically gave lower results when muscles of *dy*/*dy* and dy^{2J}/dy^{2J} animals were analyzed.

When indicated, statistical analysis between groups was analyzed by ANOVA paired *t*-tests run under Statview 5.0 software. The differences were considered significant when $P < 0.05$.

Laminin-2 and dystrophin immunohistochemistry

The mouse laminin α2 chain was detected with the rabbit polyclonal anti-mouse laminin α2 chain kindly provided by Dr E Engvall (Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA, USA) as previously described[61] using a Cy3-labeled second antibody. Dystro-

phin was detected with a rabbit polyclonal antibody kindly provided by Dr A Cartaud (Institut J Monod, Université Paris VII, Paris, France) as previously described,[68] using a Cy3-labeled second antibody.

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