Muscle electrotransfer as a tool for studying muscle fiber-specific and nerve-dependent activity of promoters

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Muscle electrotransfer has recently become a promising tool for efficient delivery of plasmids and transgene expression in skeletal muscle. This technology has been mainly applied to use of muscle as a bioreactor for production of therapeutic proteins. However, it remains to be determined whether muscle electrotransfer may also be accurately used as an alternative tool to transgenesis for studying aspects of muscle-specific gene control that must be explored in fully mature muscle fibers in vivo, such as fiber specificity and nerve dependence. It was also not known to what extent the initial electrical stimulations alter muscle physiology and gene expression. Therefore, optimized conditions of skeletal muscle electroperoration were first tested for their effects on muscles of transgenic mice harboring a pM310-CAT transgene in which the CAT reporter gene was under control of the fast IIB fiber-specific and nerve-dependent aldolase A pM promoter. Surprisingly, electrostimulation led to a drastic but transient shutdown of pM310-CAT transgene expression concomitant with very transient activation of MyoD and, mostly, with activation of myogenin, suggesting profound alterations in transcriptional status of the electroporated muscle. Return to a normal transcriptional state was observed 7–10 days after electroporation. Therefore, we investigated whether a reporter construct placed under control of pM could exhibit fiber-specific expression 10 days after electrotransfer in either fast tibialis anterior or slow soleus muscle. We show that not only fiber specificity, but also nerve dependence, of a pM-driven construct can be reproduced. However, after electrotransfer, pM displayed a less tight control than previously observed for the same promoter when integrated in a chromatin context.

fast or slow fibers; aldolase A; MyoD; myogenin

MATURE ADULT MUSCLE is a highly specialized and heterogeneous tissue composed of different types of fibers expressing predominantly slow (type I)- or fast (classified as types IIA, IIX, and IIB according to the isoform of myosin heavy chain (MyHC) that they express)-twitch contractile protein isoforms and different sets of glycolytic or oxidative metabolic enzymes. The establishment and maintenance of muscle fiber diversity rely on a complex interplay between intrinsic controls linked to muscle cell precursor heterogeneity and both neural and hormonal influences (6, 38, 56). Because of this complexity, suitable muscle cell culture models reproducing adult-specific muscle fast/glycolytic or slow/oxidative phenotypes are lacking. Hence, the delineation of cis-acting DNA elements involved in gene regulation operating in mature muscle, as fiber specificity and nerve influence, must involve in vivo approaches. In this context, transgenic mice models proved to be particularly appropriate because transgenes are subjected to all the intrinsic and extrinsic factors likely to influence their specific transcription. However, as this approach is particularly time consuming, an attractive alternative is the in vivo transfection of skeletal muscle, which is a faster and easier method for analyzing gene regulation in a fully mature and innervated myofiber context. To transfect muscles, direct injection of naked DNA was first used (9, 11, 26, 57). In most cases, this gene transfer technique proved to be valuable in achieving appropriate fiber type (8, 21, 24, 52, 55, 58) or nerve-dependent expression (2, 15) of plasmids placed under control of muscle-specific promoters. However, this approach suffers from low transfer efficiency and a high interindividual variability of gene expression. It was recently shown that electrical stimulations after injection significantly increase the number of transfected fibers and reduce gene expression variability (Refs. 1, 13, 28, 32; reviewed in Refs. 33, 43, 48). In vivo electroporation of skeletal muscle thus became a preferred way for gene delivery. Because of the persistence of DNA in an epimodal state for months, and its relative safety compared with viral methods, this technology rapidly appeared as particularly attractive for using skeletal muscle as a bioreactor for secretion of therapeutic proteins within muscles or into the circulation (3, 12,
With this perspective, most studies aiming at the improvement of this technology focused on the best electroporation parameters to obtain high levels of transfected fibers and persistent expression of transgenes (16, 18, 29, 31, 35, 42, 50). However, although electroporation-mediated transfection yields higher expression, electrical pulses may induce some transient tissue trauma, which in turn may affect transcriptional activity of transfected promoters. Moreover, many muscle properties such as fiber-specific expression are altered by repeated electrical stimulation (36). Hence, we do not know to what extent the electrical pulses used for electroporation may alter muscle physiology and the transcriptional status of fast and slow fibers. This may be particularly important when expression vectors include a promoter that is highly sensitive to physiological status of muscle, as is the case for the muscle-specific aldolase A promoter (called pM) that was previously characterized in transgenic mice models (40, 41, 45–47). Therefore, in the present study, we used either an integrated transgene (pM310-CAT 98) or an electrotransferred plasmid construct harboring the same fast muscle-specific and nerve-dependent pM promoter to investigate whether some muscle physiology parameters are altered after electrical pulse delivery and to what extent fiber-specific and nerve-dependent transcription can be accurately studied by using muscle electroporation.

MATERIALS AND METHODS

Mice. pM310-CAT 98 transgenic mice, back-crossed for at least 10 generations on a C57BL/6 genetic background, were generated and characterized as previously described (41). C57BL/6 mice were purchased from Charles River Laboratories. Adult pM310-CAT 98 mice of either sex and 7- to 10-wk-old C57BL/6 females were used for muscle electroporation or for muscle denervation. Mice were anesthetized with a mixture of ketamine and xylazine (80 mg/kg and 4 mg/kg, respectively). All experiments using animals were conducted in accordance with the European guideline for the care and use of laboratory animals.

Plasmid constructs. Different expression vectors were used: pCMV-LacZ carries β-galactosidase reporter gene flanked by the cytomegalovirus (CMV) immediate-early promoter-enhancer plus intron A and bovine growth hormone transcription termination. pM310-Luci was derived by cloning proximal regulatory sequences (bp –310 to +45 relative to the transcription start site) of the human aldolase A muscle-specific promoter (identical to those used to derive the pM310-CAT transgene; Ref. 41) into pGL3 firefly luciferase reporter vector (Promega). pRSV-Renilla contained the Rous sarcoma virus promoter-enhancer inserted upstream of the coding sequence of the Renilla luciferase (Promega). pM-PLAP was obtained by subcloning, in pSP72 vector (Promega), both proximal regulatory sequences from the pM (sequences 1792–2250 of the published sequence; Ref. 25) and a part of the intron preceding the first coding exon (sequences 4098–4616) upstream of human placental alkaline phosphatase (PLAP) cDNA (10). After amplification in DH-5α bacterial cells, supercoiled plasmids were purified by two cycles of ethidium bromide-CsCl equilibrium density gradient ultracentrifugation and dissolved in 0.9% NaCl isotonic solution.

Muscle electroporation. For each experiment at least five mice were used and anesthetized, and before DNA injection leg hair was shaved. Depending on the experiment, different plasmid mixtures were injected in 50 μl of 0.9% NaCl in the tibialis anterior, the gastrocnemius, or the soleus muscle with a 29-gauge needle. For optimization of electroporation conditions, only 2 μg of pCMV-LacZ was injected in tibialis anterior, whereas for investigation of fiber specificity, a mixture containing 13 μg of pM-PLAP and 2 μg of pRSV-Renilla was injected in both tibialis anterior and soleus. In these experiments, PLAP was preferred over CAT as reporter gene because PLAP activity is easier to detect histochemically. For studying nerve dependence a mixture containing 13 μg of pM310-Luci and 2 μg of pRSV-Renilla was injected in the denervated tibialis anterior. The electrical field was applied with caliper rule electrodes coated with ultrasound transmission gel (Aquasonic 100, Parker) and placed on each side of the leg. Six square-wave 130 V/cm pulses, lasting 60 ms each with a 100-ms interval, were then applied with a BTX electroporator. Mice were killed by cervical dislocation, and muscles were collected at the indicated times after electroporation. For experiments aimed at monitoring muscle alterations, mice received intraperitoneal injection of Evans blue dye [EBD; 100 μl of 2% EBD in phosphate-buffered saline (PBS)] 12 h before they were killed.

Denervation of mice. Mice were anesthetized, and the right sciatic nerve was then exposed and surgically cut as previously described (46). For experiments combining denervation and electroporation, DNA solution was injected 3 days after denervation, before clear signs of muscle atrophy could be observed.

Measurement of luciferase activity. Protein extracts were made from dissected muscles and then assayed for firefly and Renilla luciferase activities according to Promega’s protocols with the Dual Luciferase Assay. Luciferase and Renilla activities were measured from the same sample in an automated Berthold Lumat LB 9507 luminometer.

Histochemical analyses. Muscles were dissected, embedded in Cryomatrix, frozen in isopentane at liquid nitrogen temperature, and cross-sectioned in a microtome cryostat. Seven- to ten-micrometer-thick serial sections were further processed for different staining and detection. For assessment of tissue damage and morphology, sections were stained with hematoxylin-eosin and examined under a light microscope. For EBD staining, sections were fixed in acetone, cooled at –20°C for 10 min, rinsed in PBS, and mounted with Vectashield medium (Vector Laboratories). EBD staining was monitored under a fluorescence microscope.

For β-galactosidase and PLAP staining, muscle sections were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C and then rinsed three times in PBS. Sections were stained for β-galactosidase activity at 37°C overnight in X-Gal buffer (1 × PBS, 0.5 M X-Gal, 2 mM ferricyanide, 2 mM ferrocyanide, 2 mM MgCl₂). After PBS washes, sections were dehydrated and mounted with Eukitt. For PLAP staining, sections were further processed for different staining and detection. For assessment of tissue damage and morphology, sections were stained with myeloperoxidase and examined under a light microscope. For EBD staining, sections were fixed in acetone, cooled at –20°C for 10 min, rinsed in PBS, and mounted with Vectashield medium (Vector Laboratories). EBD staining was monitored under a fluorescence microscope.

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D5, SC-71, BF-F3, or BF-35 mouse monoclonal antibodies (as described in Ref. 44). BA-D5 is specific for MyHC-I, SC-71 for MyHC-IIA, BF-F3 for MyHC-IIIB, and BF-35 for all MyHC types except MyHC-IIX.

Northern blot analysis. Right tibialis or gastrocnemius of pM310-CAT 98 transgenic mice was injected with 50 μl of physiological serum and electroporated as described above. Corresponding left muscles were not electroporated and served as controls. Both electroporated and control muscles were collected at different times after electroporation (1, 2, 6, 24, or 48 h and 5, 7, or 10 days after electroporation). Electroporated and control muscles were taken, frozen in liquid nitrogen, and stored at −80°C until RNA extraction was performed by the guanidinium thiocyanate single-step procedure (7). Northern blot analysis was carried out as previously described (46). Membranes were successively hybridized with CAT, MyoD, myogenin, skeletal actin, and R45 probes (described in Ref. 46) labeled with [32P]dCTP by using the Megaprime DNA labeling system (Amersham). The R45 probe that hybridizes with 18S ribosomal RNA was used for sample normalization. Relative amounts of specific mRNAs were quantified by measuring on the blots the radioactivity associated with the different bands with a STORM 850 Phosphorimager and Image-Quant 5.0.

Statistics. Statistical analysis was performed with StatView software. Values are means ± SE. Differences between relative CAT mRNA level mean values in control and electroporated muscles were analyzed by Mann-Whitney test, whereas differences between relative Luci-to-Renilla expression ratios in control and denervated tibialis were analyzed by Fisher’s test. In both cases, \( P < 0.05 \) was accepted as the level of statistical significance.

RESULTS

Optimization of muscle electroporation conditions. Consensus electroporation conditions that seem to give a satisfactory gene delivery with the most limited muscle trauma use several pulses of short duration (several tens of milliseconds) at a low voltage. Because optimal conditions may differ according to electrode design, different parameters, mainly inspired by those used in References 32 and 13, were checked for electroporating mouse leg tibialis anterior and gastrocnemius. Muscle transfer efficiency was assessed by injecting 2 μg of a β-galactosidase gene-encoding plasmid (pCMV-LacZ) and quantification 5 days later of β-galactosidase-positive fibers on electroporated muscle sections. In our hands, electroporation parameters that reproducibly gave a good transfer efficiency without obvious muscle lesions were six pulses of 60-ms duration, with 100-ms intervals, applied at 130 V/cm. Under these conditions, up to 42% of β-galactosidase-expressing fibers could be reproducibly detected in the electroporated tibialis anterior (Fig. 1). β-Galactosidase-positive fibers were mainly located in muscle periphery, close to the sites where electrodes were applied. On these sections, no obvious damaged areas were detected on muscle surface.

To identify potential muscle pathological changes associated with electric pulses, hematoxylin-eosin staining of electroporated tibialis was combined with β-galactosidase activity detection on serial sections of muscles sampled 12 h, 5 days, and 10 days after electroporation (Fig. 2). Whereas control muscle fibers exhibited polygonal joined cells with peripheral nuclei (Fig. 2A), 12 h after electrostimulation some muscle alterations were evidenced by the presence of numerous (10–20%) fibers with centrally located nuclei and round-shaped and less joined fibers (Fig. 2C). At this stage, no β-galactosidase-positive fibers could be detected (Fig. 2D) and in some respects electroporated muscle resembled freshly denervated muscle (Fig. 2B). Only very few central nuclei were still observed 5 days after electroporation, whereas the situation returned to normal after 10 days. However, local inflammation appeared around fibers then expressing the β-galactosidase protein (Fig. 2, E–H). This macrophage infiltration is likely caused by the known antigenicity of β-galactosidase protein. No significant increase or decrease in β-galactosidase-positive fibers could be observed when muscles were sampled 10 days instead of 5 days after electroporation (not shown).

To detect more subtle damage to muscle fibers, EBD was injected intraperitoneally into electroporated mice 12 h before they were killed for muscle sampling. EBD is a small molecule that stains fibers permeabilized by membrane destabilization. When mice were killed 12 h after electroporation (EBD injection being performed just after electroporation), EBD uptake could be observed in numerous fibers (up to 40%), mainly located at muscle periphery of the electroporated muscle (Fig. 3A). Membrane permeabilization linked to electroporation was transient, because only a few EBD-stained fibers were still observed 5 days after electroporation (Fig. 3B) and no more could be detected 10 days after (not shown). Five days after electroporation, among EBD stained fibers none expressed the β-galactosidase protein (data not shown). This suggests that after a few days, fibers that have not recovered from electrical permeabilization are degenerative.

Fig. 1. X-Gal staining on an electroporated tibialis anterior (TA) section. Two micrograms of pCMV-LacZ plasmid were injected in TA of C57BL/6 mice before electrotransfer, and β-galactosidase activity was revealed on muscle sections 5 days later.
Fig. 2. Comparison between untreated TA (A), denervated TA (sampled 3 days after surgical cutting of the sciatic nerve; B), and TA submitted to pCMV-LacZ (2 μg) electrotransfer (C–H) after treating cryosections to hematoxylin-eosin (A–C, E, G) or X-Gal (D, F, H) staining. Electroporated muscles were removed for analysis 12 h (C, D), 5 days (E, F), or 10 days (G, H) after injection and electrical pulse delivery. Arrows indicate regenerating fibers with central nuclei. Numbered fibers are the same fibers in E and F.

Fig. 3. Evans blue dye (EBD) staining on electroporated TA sections. TA were taken 12 h (A) or 5 days (B) after pCMV-LacZ (2 μg) electrotransfer.
Altogether, these data show that electrical pulses allowing an efficient plasmid delivery induced only very transient phenotypic and morphological alterations of muscle fibers. An optimal expression of pCMV-LacZ, and an almost complete muscle morphological recovery, could already be detected 5 days after electroporation.

**Molecular alterations in electroporated muscles.** To further analyze at the molecular level potential alterations of muscle physiology linked to electrical pulses, we checked the effects of the electroporation process on the expression of a muscle-specific transgene (pM310-CAT) previously shown to be very sensitive to muscle physiological status. In this transgene, proximal regulatory sequences of the fast muscle-specific Pm promoter from the glycolytic aldolase A gene (bp –310 to +45 relative to the transcription start site) are linked to a CAT reporter gene. These sequences were shown to be sufficient to mimic the fast glycolytic IIB fiber specificity of endogenous Pm promoter in a transgenic mice model (44, 45). These Pm promoter sequences were also shown to be highly sensitive to both denervation and muscle hyperactivity, as observed in the context of myotonic ADR mice, whose muscles display chronic electrical after-activity (46). pM310-CAT transgene activity could thus be used as an indicator of muscle status after electroporation. Mice from a previously described pM310-CAT 98 transgenic line were thus submitted to a sham electroporation experiment in which only physiological serum was injected into one of their tibialis anterior and gastrocnemius muscles before electrical pulse delivery. Electroporated tibialis anterior and gastrocnemius were sampled with control nonelectroporated contralateral muscles at different times. pM310-CAT transgene expression was estimated by Northern blot experiments, allowing observation of immediate effects at the transcriptional level. Because of the high stability of the CAT protein, CAT assays could mask such effects. Surprisingly, as illustrated in a representative Northern blot (Fig. 4A) and quantified in Fig. 4B, pM310-CAT mRNA was found to be significantly diminished in electroporated muscles sampled 5 days after the experiment. This downregulation of pM310-CAT transgene expression was reminiscent of what we previously observed (46) after muscle denervation and is also illustrated in Fig. 4A for tibialis anterior and gastrocnemius sampled 3 days after the sciatic nerve was cut. The decrease of pM310-CAT expression linked to electroporation was more drastic in tibialis anterior (23% of residual activity) than in gastrocnemius (45% of residual activity). This difference could be related to the fact that the tibialis anterior is smaller and reproducibly more efficiently electroporated than the gastrocnemius (data not shown). However, pM310-CAT transcriptional downregulation observed after electroporation was only transient, because 7–10 days after electrical pulse delivery pM310-CAT mRNA levels were no more significantly different from those found in nonelectroporated control contralateral muscles (Fig. 4, A and B). The transient alteration of muscle transcriptional status induced by electroporation was also reflected by reexpression of MyoD and myogenin mRNAs (Fig. 4, A and C). These myogenic factors (4), although barely detectable in adult control muscles (Fig. 4, A and C), are
reexpressed in regenerating fibers (30) and after denervation (Refs. 14, 39, 53, 54; see also Fig. 4A). MyoD and myogenin reexpression was first detectable 6 h after electroporation (Fig. 4C). MyoD was strongly activated, but only very transiently, because within 2 days its mRNA level returned to that of control muscles. In contrast, myogenin reexpression was still observed 5 days after muscle electrical stimulation and a delay of 7–10 days was required for a return to control levels. No change in the transcription of a mature adult skeletal gene such as α-skeletal actin (Fig. 4C) could be observed.

Altogether, these results show that, even in electroporation conditions giving no morphological muscle alterations, the process leads to profound but transient molecular alterations that may in turn alter transcription of muscle-specific genes. Seven to ten days after electroporation seem to be necessary for the muscle fibers to recover a normal physiological state.

Fiber specificity and nerve dependence of electroporated aldolase A pM promoter. We then investigated whether our electroporation conditions, with a delay of 10 days before muscle sampling, could allow reproduction, and thus study, of both fiber specificity and nerve dependence of the pM promoter via an in vivo injected DNA plasmid construct. To analyze pM promoter fiber specificity, we coinjected in the mouse tibialis anterior and soleus muscles a pM-PLAP construct in which the same regulatory sequences as those used in the pM310-CAT transgene were linked to human PLAP. Tibialis anterior is a mainly fast mixed muscle in which all fiber types are represented (43% fast/glycolytic IIB, 26% fast/glycolytic-oxidative IIX, 24% fast/oxidative IIA, and 7% slow/oxidative type I). Soleus is a slow muscle mainly composed of type I fibers (~55%) in addition to IIA fibers (40%) and a small amount of IIX fibers (5%). Fiber type repartition of both PLAP and β-galactosidase activity was assessed 10 days after electroporation by histochemical analysis of cross sections of injected muscles. Detection of alkaline phosphatase and β-galactosidase activity was combined with fiber typing with antibodies against the different MyHC isoforms on serial muscle sections. In the tibialis anterior, 114 β-galactosidase- and/or PLAP-labeled fibers were thus analyzed. We found that ~50% of LacZ-positive fibers were of the IIB phenotype as illustrated in Fig. 5 (fibers 8 and 9) or mixed IIB/IIX (as fiber 6), 40% of type IIX (as fibers 1, 2, and 4), and the remaining transfected fibers were in majority IIA (as fiber 7, which is a mixed IIA/IIX) with few type I. By contrast, among PLAP-positive fibers, 97% were IIB

Fig. 5. Fiber-specific expression of an electroporated pM-placenta alkaline phosphatase (PLAP) construct in TA. As indicated in each panel, serial sections of TA from C57BL/6 mice electroporated with a mixture of pM-PLAP (13 μg) and pCMV-LacZ (2 μg) were histochemically stained for LacZ, PLAP (AlcP), and immunohistochemically stained with a set of myosin heavy chain (MyHC)-specific monoclonal antibodies for identification of fast type IIB, IIA, and IIX (this antibody stains all fibers except those expressing MyHC IIX) and slow type I fibers. Numbered fibers are the same fibers in all panels.
fibers (Fig. 5, fibers 5, 8, and 9) and 3% corresponded to mixed IIB/IIX fibers (as fiber 6), showing that electroporated pM-PLAP construct is preferentially expressed in IIB fibers. In tibialis anterior, few PLAP-positive fibers were found negative or very faint for LacZ (as illustrated for fiber 5 in Fig. 5). This phenomenon could be due to the fact that in these experiments the coelectroporated pCMV-LacZ plasmid is sixfold less abundant than pM-PLAP construct. However, in the electroporated soleus, in which 54% of fibers were analyzed, although 80% of LacZ-positive fibers were found negative for PLAP, 20% nevertheless expressed PLAP, which were either of type IIA or type I (illustrated in Fig. 6). These results show that although IIB fiber specificity of the pM promoter is well reproduced in electroporated tibialis anterior, it is much less tightly controlled when electroporated in soleus than what was observed in the pM310-CAT or pM-PLAP transgenic mouse models, in which no transgene expression could be observed in the soleus (Ref. 44; J.-P. Concordet, unpublished data).

Expression of pM310-CAT transgene was shown previously to be highly sensitive to muscle denervation (Ref. 46; see also Fig. 4A), and the cis-acting DNA regulatory elements involved in response to innervation were identified within the proximal pM promoter region present in this transgene. To find out whether pM nerve dependence could be reproduced and studied in electroporated muscle, the same pM regulatory sequences were cloned upstream of the luciferase reporter gene to obtain the pM310-Luci construct. pM310-Luci was coinjected and electroporated with a RSV- Renilla plasmid, used as control for electroporation efficiency, either in control innervated or denervated tibialis anterior. As illustrated in Fig. 7, when analyzed 10 days after the muscle electrottransfer, pM310-Luci activity was found significantly reduced in absence of the nerve, with a 2.7-fold difference between innervated and denervated muscle. Although statistically significant, the magnitude of the decrease remains far from the dramatic falling off of transgene transcription that was observed in the transgenic mouse model after denervation. These results show that the nerve dependence of pM activity may be reproduced in the electroporated muscle but that some sharp controls are partly lost.

**DISCUSSION**

In vivo electroporation has recently become a very promising approach by which gene expression, regulation, and function can be studied in different vertebrate developmental systems including mouse, bird, zebra fish (49), and Xenopus (review in Ref. 48). In the adult, skeletal muscle is probably the most accessible and studied tissue for gene electrotransfer, thus providing a potentially powerful alternative method to transgenesis for studying muscle-specific gene control. Indeed, DNA constructs can be analyzed rapidly in the context of fully differentiated fibers, with much better transfection efficiency than that obtained by simple injection of naked DNA. However, several important issues concerning conditions that are required to reproduce accurate gene control remain to be addressed before this approach can be used routinely to delineate cis-regulatory elements involved in muscle-specific gene regulation, such as fiber specificity and nerve dependence.

![Fig. 6. Expression of an electroporated pM-PLAP construct in soleus fibers. As indicated in each panel, serial sections of soleus from C57BL/6 mice electroporated with a mixture of pM-PLAP (13 μg) and pCMV-LacZ (2 μg) were histochemically stained for LacZ, PLAP (Alc P), and immunohistochemically stained with MyHC-specific monoclonal antibodies for identification of fast type IIA and slow type I fibers. Numbered fibers are the same fibers in all panels.](http://ajpcell.physiology.org/)

![Fig. 7. Nerve-dependent expression of an electroporated pM310-Luci construct. A mixture containing 13 μg of pM310-Luci and 2 μg of pRSV-Renilla was electrottransferred in both control contralateral left TA and right denervated TA from C57BL/6 mice the right sciatic nerve of which was surgically cut 3 days before. Relative Luciferase/Renilla expression ratios in control and denervated muscles are averages from 12 mice, and error bars represent SE. The difference was statistically significant (P < 0.05).](http://ajpcell.physiology.org/)
Most studies aiming at the best parameter calibration for transient or durable expression of transfected plasmids use viral ubiquitous promoters, and maximal plasmid expression is generally reached 5 days after injection (1, 29). In the present study we obtained similar results with a pCMV-LacZ plasmid when using electrotransfer parameters that, in our hands, gave no visible burning lesions at the surface of the injected muscles, only transient morphological alterations in fiber integrity, and reproducibly high DNA transfection efficiency. Electrical pulses partially increase gene transfer, with cell membrane permeabilization allowing DNA entry (32). To follow both fiber status and β-galactosidase staining at different times after pCMV-LacZ electrotransfer, we used EBD staining as an in vivo membrane destabilization tracer. Twelve hours after electroporation a large proportion of fibers were found to be EBD positive, whereas β-galactosidase expression was still undetectable. However, the EBD-stained fiber proportion (40–50%) and peripheral localization coincide well with those of LacZ-positive fibers detected 5 days after electrotransfer, suggesting that EBD-stained fibers are those that have also taken up plasmid DNA. At this early stage, the presence of some central nuclei and fibers with round morphology, which are signs of regeneration, was also observed and electroporated muscles resembled freshly denervated muscles. In contrast, 5 days after electrotransfer, although β-galactosidase was found strongly expressed in a high number of fibers, only a few fibers were still EBD stained, none of them being LacZ positive, and almost no more centrally located nuclei could still be observed. These results suggest that only the few fibers that have been irreversibly damaged and can no longer express protein still exhibit EBD staining at this stage.

Interestingly, although, by these criteria, injected muscles seemed to have already recovered 5 days after electroporation, the strong downregulation of the pM310-CAT transgene observed when the process was applied to transgenic muscles indicated that, at this stage, muscle transcription was still profoundly altered. Such alterations were also reflected by the reexpression of myogenic factors. Indeed, we observed that a few hours after muscle electroporation, MyoD and myogenin mRNAs were both upregulated. These myogenic regulatory factors (MRFs) are activated early during myogenesis (4) but downregulated in adult muscle, where MyoD and myogenin remain preferentially expressed at low levels in fast-twitch and slow-twitch skeletal muscles, respectively (20, 54). MyoD and myogenin were also reported to be reexpressed during muscle regeneration (30) and after denervation (5, 14, 39, 53). In our experiments, MyoD was only very transiently induced a few hours after electrotransfer whereas myogenin reexpression was more sustained. At these early stages, the time course of MyoD reactivation followed by myogenin upregulation probably reflects satellite cell activation that fits with morphological detection of some regenerating fibers. However, 5 days after electrotransfer, when no more signs of fiber regeneration could be evidenced, although MyoD expression was returned to that found in the control a very strong myogenin expression could still be detected. This myogenin upregulation was concomitant with transgenic pM transcriptional shutdown. An even more dramatic and sustained increase in expression of myogenin than that observed for MyoD was also observed after muscle denervation (5, 39, 53). This, together with the decrease in pM310-CAT transgene expression similar to that observed after denervation, argues in favor of an early onset of myogenic factors first coinciding with signs of muscle regeneration, followed by a “denervation-like” phenomenon leading mainly to an upregulation of myogenin and concomitant downregulation of pM promoter. Interestingly, myogenin and pM310-CAT mRNAs returned simultaneously to normal levels between 7 and 10 days after electrotransfer. These results support our previous hypothesis (46) of a direct link between myogenin upregulation and reduction of aldolase A pM promoter activity that could be explained by competition of myogenin with upstream stimulatory factors (USFs) for binding to the E-box present in this promoter. Indeed, in adult muscles myogenin remains mostly expressed in oxidative slow-twitch muscles, and its overexpression in muscles of transgenic mice was shown to lead to a switch toward oxidative metabolism, with a concomitant decrease of expression of glycolytic enzymes (19). The upregulation of myogenin linked to electroporation of fast muscles such as tibialis anterior and gastrocnemius could similarly induce a transient switch toward a more oxidative phenotype and concomitant inhibition of the glycolytic pM promoter. The denervation-like phenomenon could result either from transient and reversible alteration of neuromuscular junctions or from transient phenotypic changes in fibers, linked to repeated electrical pulses used for electrotransfer. Indeed, electrical stimulation has long been known to induce phenotypic changes in muscle. Notably, increasing electrical activity stimulates muscle-specific gene expression associated with a slow/oxidative muscle phenotype (36), and it has been postulated that myogenic factors provide a link between electrical activity and gene regulation (19). Therefore, our results emphasize that we must not ignore potential muscle physiological alterations, when electrical stimulations are used to get a better gene transfection. According to our data, 7–10 days seem to be necessary for a correct muscle molecular recovery.

Under these conditions, a pM-PLAP construct electroporated in the tibialis anterior was shown to reproduce the fast IIB fiber specificity of the promoter. Although an almost equivalent amount of IIB and IIX fibers was found to be transfected, only IIB or some hybrid IIB/IIX fibers could express PLAP. Despite the presence of ~24% of IIA fibers in tibialis anterior, a lesser proportion were found to be transfected. This result could probably be explained by the more peripheral localization of IIB and IIX fibers, thus more prone to DNA uptake. Indeed, in soleus we could efficiently transfect both type IIA and type I fibers. The majority (80%) of transfected type IIA and type I fibers in the
soleus were not able to activate pM, showing that specificity is globally maintained. However, compared with the relatively strict IIB specificity observed in tibialis anterior, the finding that in soleus up to 20% of transfected type IIA and type I fibers could express pM was somewhat unexpected. The pM-PLAP construct used in this study was also used to derive several transgenic lines: as previously observed for CAT activity in pM310-CAT transgenic mice, PLAP transgene activity was found restricted to IIB fibers, with a fainter expression only in a small number of IIX fibers. In particular, no PLAP activity could be detected in the soleus of pM-PLAP lines (Concordet J-P, unpublished data; and Grifone R, Laclef C, Spitz F, Demignon J, Guidotti JE, Kawakami K, Xu PX, Kelly R, Daegelen D, Concordet J-P, and Maire P, unpublished observations). A regeneration-associated derepression of fast troponin I (TnI) fiber type specificity after direct gene transfer in soleus without electrical stimulation was recently reported (17). In contrast to the fast TnI promoter, which is expressed in both regenerating fast and slow muscles, pM is only active in fully differentiated postnatal muscle fibers, so that it is difficult to imagine that only regeneration could account for the more leaky expression of pM-PLAP in the soleus. It is well known that the denervated regenerating soleus undergoes transition to a faster phenotype and that such effects of short-term denervation are highly dependent on muscle phenotype (54). Given our observation that electroporation produces a transient phenomenon looking like denervation, it is tempting to propose that the seemingly less tight control of pM fiber specificity in soleus is in fact due to transient switching of some electroporated type IIA and type I fibers toward a more glycolytic phenotype. Indeed, the metabolic status of fibers is known to be more rapidly prone to fiber plasticity than expression of sarcomeric protein isoforms (36), and overexpression of myogenin in transgenic muscles leads to a switch of metabolism, without any alteration of MyHC isoform expression (19). In the same way, transient deregulation linked to electroporation in soleus could lead to a burst of pM expression in some fibers that, because of PLAP protein stability, would remain stained without any change in their MyHC phenotype. Such side effects linked to electrical pulses used for electroporation would thus have different consequences in tibialis anterior (transient switch to a more ‘oxidative’ phenotype and inhibition of pM transcription) and soleus (deregression of pM transcription). Although transient inhibitory effect in fast muscles would not alter specificity, pM transcription being only delayed, transient deregression of pM in soleus would be sufficient to allow persistent expression of the pM-driven reporter gene in some slow fibers. Transient permissiveness of some oxidative fibers for pM transcription could also be favored by the high copy number of the electroporated pM-PLAP, in addition to the fact that the construct was not inserted in a chromatin environment as is a transgene. The fact that, after electrotransfer of an empty plasmid vector, soleus from pM-PLAP transgenic mice still failed to express PLAP argues in favor of such an hypothesis (Grifone et al., unpublished observations). Together, these differences could also explain why, by using this approach, the nerve-dependent control of pM can be reproduced but not as tightly as that observed in the transgenic mice model. Indeed, when a pM-CAT construct was used in electrotransfer conditions as defined above for a study aimed at further characterizing pM DNA elements involved in fiber type-specific transcriptional activation, global CAT activity was found about fivefold more active in the tibialis anterior than in the soleus (Grifone et al., unpublished observations). This difference was ~100-fold for an integrated pM310-CAT transgene (40, 41).

In summary, by using aldolase A pM muscle-specific promoter, whose transcription is very sensitive to muscle physiology (46), we show that injection followed by electrical stimulation delivery leads to a transitory but profound alteration of muscle transcriptional status. This alteration is probably consecutive to a denervation-like process that may differ with muscle phenotype. However, 7–10 days after electrotransfer, treated muscles have sufficiently recovered to reproduce both fiber-specific and nerve-dependent transcription, thus allowing further dissection in an adequate in vivo context of DNA elements involved in such regulations.

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DISCLOSURES

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