doi:10.1006/mthe.2001.0465, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Slow Accumulation of Plasmid in Muscle Cells: Supporting Evidence for a Mechanism of DNA Uptake by Receptor-Mediated Endocytosis

Saulius Šatkauskas,[1,4] Michel F. Bureau,[2] Abderrahim Mahfoudi,[3] and Lluis M. Mir[4,*]

[1]*Vytautas Magnus University, Department of Biology, LT-3000 Kaunas, Lithuania*

[2]*UMR 7001 CNRS/ENSCP/Aventis, F-94403 ,Vitry sur Seine, France*

[3]*Aventis, F- 94403, Vitry sur Seine, France*

[4]*LPPMB, UMR 8532 CNRS, Institute Gustave-Roussy, F-94805, Villejuif, France*

[*]*To whom correspondence and reprint requests should be addressed. Fax: + 33 1 42 11 52 76. E-mail: luismir@igr.fr.*

Intramuscular plasmid DNA injection results in long-term but low and variable expression of the injected genes. Optimization is difficult because the mechanism of naked DNA uptake by the cells *in vivo* is not yet determined. Here we used injections of plasmid DNA encoding luciferase to further characterize this mechanism. We analyzed the kinetics of naked DNA uptake by means of DNase I or heparin injections, using the level of luciferase expression as the indicator of DNA uptake. We demonstrated that *in vivo* heparin inhibits DNA uptake without affecting the expression of DNA internalized by means of electric pulses. Inhibition by heparin is dose dependent and compatible with the competition for the binding to a receptor. As shown also with DNase I, DNA uptake by muscle cells is slow: a progressive accumulation of the DNA in the myofibers can be found for at least 4 hours after naked DNA injection. Physical presence of DNA molecules during the uptake period, but not later, was confirmed by the facilitation of DNA uptake with appropriate electric pulses. Therefore, uptake proceeds for the entire time during which intact DNA is present in the extracellular compartment. Our results support evidence for a DNA uptake mechanism based on receptor-mediated endocytosis.

Key Words: gene therapy, DNA electrotransfer, electrogene therapy, muscle, naked DNA, plasmid DNA, kinetics, *in vivo*, heparin, receptor-mediated endocytosis

INTRODUCTION

Direct intramuscular plasmid DNA injection in mice results in the long-term expression of the injected plasmid [1,2]. However, foreign gene expression after plasmid DNA injection is relatively low and highly variable. This method still requires improvements to achieve a consistent and reproducible level of gene product. For example, gene expression was increased [3–5] and expression variability was reduced [6] if appropriate electric pulses were delivered to the tissue after injection of the naked DNA [7,8]. Long-term expression was achieved under the same conditions [9,10]. These results show that the injection of naked DNA alone could be a quite efficient procedure, providing that DNA could actually enter the cells in a reproducible and efficient way. In fact, this is what the electric pulses do: they induce the transient permeabilization of the cells and facilitate DNA uptake by means of the electrophoretic forces [6,11]. Therefore, because the crucial step in the development of gene therapy by means of naked DNA injections is the translocation of DNA across the plasma membrane, it is important to determine the actual mechanism of naked DNA uptake by cells in animal tissues.

Several mechanisms have been proposed to account for DNA transfer across the plasma membrane: mechanically induced large membrane disruption; existence of small transient membrane pores; active processes implying either the caveolae or the T-tubule system of the muscle fibers; or receptor-mediated endocytosis [12–14]. It has been suggested that the needle used to inject the DNA into the tissues may induce large membrane disruption. Then plasmid could enter the cells through the induced pathways. Similarly, small membrane pores, resulting from high-velocity injection and increased hydrostatic pressure in the tissue, may contribute to DNA uptake. Nevertheless, both hypotheses are not supported by histological data [14,15]. Systemic or direct injection of naked plasmid also

1525-0016/01 $35.00

doi:10.1006/mthe.2001.0465, available online at http://www.idealibrary.com on IDEAL

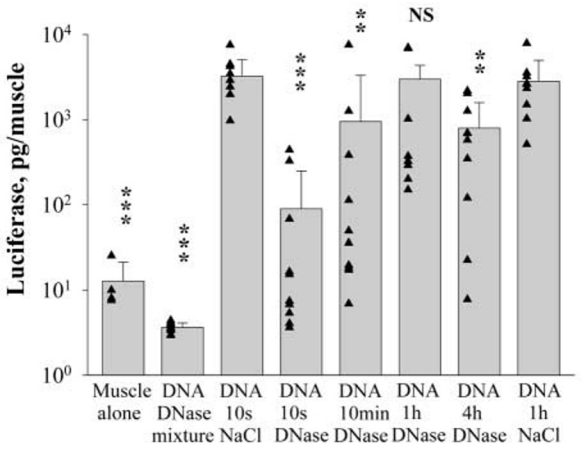

FIG. 1. Luciferase gene expression after intramuscular injection of plasmid DNA followed by injection of either physiological saline or DNase I. We injected 3 μg of DNA (in 10 μl of 0.9% NaCl) into the muscle. Then, at various times, we administered 20 μl of either NaCl (0.9%) or DNase I (6 mg/ml) through the same injection path. Filled triangles, individual experimental values; histogram, mean value ± S.D. All the DNA + DNaseI experimental conditions (except column at 1 h; NS, not significant) are statistically different (***$P < 0.001$, **$P < 0.01$) with respect to the DNA + NaCl experimental conditions.

results in efficient DNA uptake by the hepatocytes. This fact is not consistent with the implicated role of the muscular T-tubule system and suggests the existence of a more general DNA uptake mechanism. The origin of the idea that receptor-mediated endocytosis could account for DNA uptake lies in the comparison with oligonucleotide uptake. Indeed, *in vitro* studies revealed the existence of specific membrane proteins that bind oligonucleotides and polynucleotides. Because oligonucleotide uptake can be saturated and is temperature dependant, it suggests that the uptake might be mediated by receptors [16,17]. This concept was reinforced by *in vivo* studies showing that luciferase expression after naked DNA injection was inhibited by an excess of noncoding DNA or dextran sulfate, showing that DNA uptake mechanisms could be saturated as well [18].

To further characterize the actual mechanism of DNA uptake, we decided to determine the kinetics of naked DNA uptake in the skeletal muscle. We estimated the uptake of DNA by its main and most important consequence, the expression of the reporter gene encoded by the injected plasmid. We analyzed the kinetics of DNA uptake by either interrupting DNA transfer into the cells or facilitating the transfer of DNA molecules across the plasma membrane. DNA degradation outside the myofibers, mediated by injections of DNase I, showed that DNA uptake by the muscle cells is slow and that progressive accumulation of DNA in the myofibers can be detected for at least 4 hours after naked DNA injection in the muscle. We obtained similar results by means of heparin injections that suppress DNA expression by inhibiting its uptake. At 4 hours after injection, intact DNA is still present outside the myofibers in amounts sufficient to lead to the maximum level of expression measured in our experiments. These data reinforce the assumption that the mechanism of plasmid DNA uptake is consistent with receptor-mediated endocytosis.

RESULTS

Kinetics of Plasmid DNA Uptake Determined by Means of DNase I Injections

Intramuscular injection of 3 μg of luciferase plasmid in 30 μl of physiological saline resulted in the expression of about 3 ng of the protein at 48 hours after the injection. When we co-injected 3 μg of luciferase plasmid and DNase I (6 mg/ml) into mice muscles, gene expression was completely inhibited and was similar to the reaction background found in the control muscles (Fig. 1). When we injected 20 μl of physiological saline containing the same amount of DNase I immediately (that is, 10 ± 5 seconds) after the DNA (3 μg in 10 μl) injection in the treated muscle, gene expression was almost completely inhibited (Fig. 1), demonstrating that the DNA was still accessible to digestion by the enzyme.

Inside the cells plasmid DNA is protected from the enzyme, so it seemed possible to inject DNase I at various times after DNA injection and to measure the plasmid expression that corresponds to the net DNA uptake achieved before the DNase I injection. The kinetics of DNA uptake obtained by this method revealed that DNA internalization starts very rapidly after the injection and that, afterwards, DNA slowly and progressively accumulates in the muscle fibers. Four hours after plasmid injection, DNase I was still able to slightly affect luciferase expression, showing that DNA uptake was not completed even at 4 hours after injection (Fig. 1). The two-injection procedure did not affect, by itself, luciferase expression. Indeed, injection of the DNA in 10 ml followed, 1 hour later, by the injection of 20 μl of normal saline resulted in almost the same luciferase activity as a single injection of plasmid DNA in 30 μl (Fig. 1).

DNA uptake by muscle fibers at 1 hour after the DNA injection could be considered as almost completed because no statistically significant difference was found with respect to control groups ($P > 0.05$; Fig. 1). However, a few individual luciferase expression measurements are high and very different from most of the values measured within the experimental group. The existence of these few discordant determinations may be explained by the nature of the experimental procedure. Indeed, in such experiments, the second injection must be done precisely through the needle track made by the first injection because DNase I must be administered into the same site as the plasmid. Moreover, the specific anatomical structure of the muscle, which is composed of bundles of muscle fibers, can also contribute to the difficulty of the

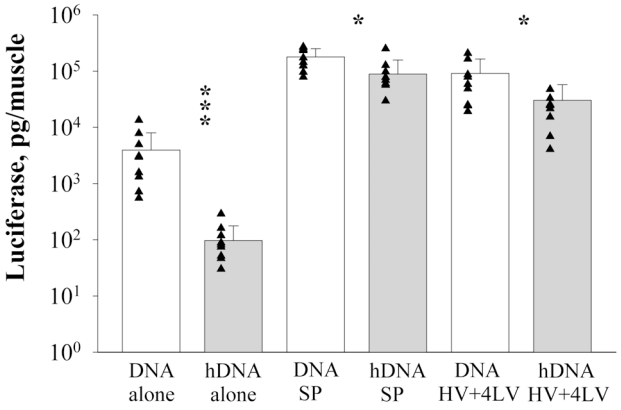

FIG. 2. Influence of heparin on luciferase uptake and expression in mice muscles. We injected 3 μg of DNA (in 30 μl of 0.9% NaCl) without (DNA) or with heparin (hDNA: DNA + 3.6 I.U. heparin) into the muscle. Then we submitted (or not) the muscles to either simple electric pulses (SP) or a HV + 4LV sequence of pulses. Filled triangles, individual experimental values; histogram, mean value ± S.D. In all cases, the DNA + heparin results are statistically different (***$P < 0.001$; *$P < 0.05$) with respect to DNA alone under the same experimental conditions.

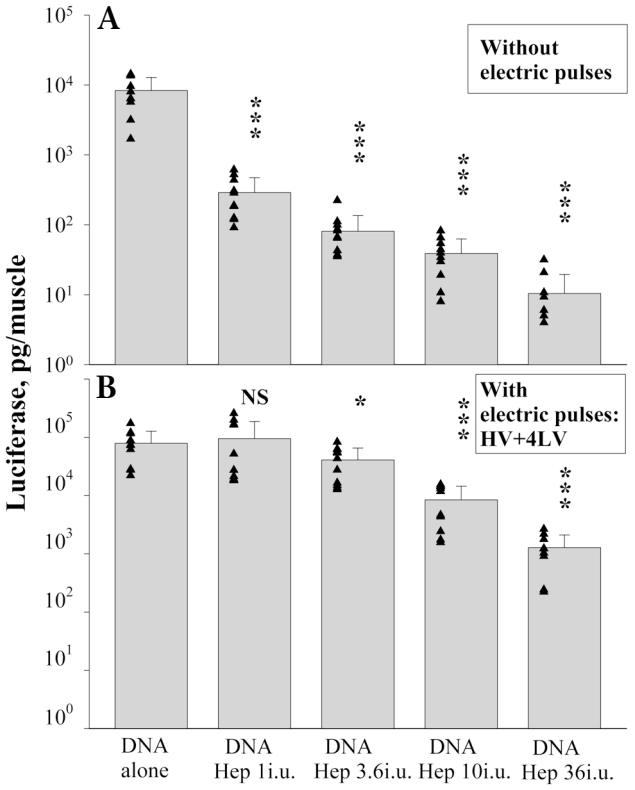

FIG. 3. Influence of heparin concentration on luciferase expression in mice muscles. We injected 3 μg of DNA (in 30 μl of NaCl) with or without heparin (1, 3.6, 10, or 36 I.U.) into the muscle and no electric pulse was applied (A) or we delivered a sequence of HV + 4LV electric pulses to the treated muscle approximately 30 s later (B). Filled triangles, individual experimental values; histogram, mean value ± S.D. (A) All DNA + heparin conditions are statistically different (***$P < 0.001$) with respect to DNA alone. (B) All DNA + heparin conditions (except 1 I.U. of heparin; NS, not significant) are statistically different (***$P < 0.001$, *$P < 0.05$) with respect to DNA alone.

experiment. If the second injection is not performed with enough precision, the injected solution can spread along the muscle without interacting with most of the injected plasmid (in fact, technical difficulties were already evident during the experiments in a few cases corresponding to these values). In the case of a 10-minute time period, presence of the discordant value does not impair the significance of the reduction in luciferase expression, and, in the case of the 1-hour time point, removal would lead to a set of data showing that DNA uptake was not yet completed at 1 hour (statistical significance with respect to control: $P < 0.001$), confirming the data obtained at 4 hours.

In Vivo Inhibition of DNA Expression by Heparin

To further analyze the kinetics of DNA uptake by muscle cells, we used another chemical compound that would inhibit DNA expression in another way than the simple physical destruction of the injected DNA. For this purpose we used the polyanionic compound heparin, which can inhibit oligonucleotide and plasmid DNA uptake by the cells *in vitro* [19,20]. When we co-injected heparin with DNA in mice muscles, we found that it strongly inhibited DNA expression (Figs. 2 and 3). Indeed, the injection of 30 μl of a mixture containing 3 μg of DNA and 3.6 I.U. of heparin resulted in a large reduction ($P < 0.001$; Fig. 2) of luciferase expression in the muscle, to about 1% of the expression obtained by the injection of 3 μg of DNA alone (in 30 μl). When we delivered appropriate electric pulses (either simple electric pulses [6,10] or the combination of HV + 4LV pulses [11]) to the muscles to increase DNA transfer into the fibers, we detected a very small (about 2 times) reduction in the levels of luciferase when heparin was co-injected with the DNA (Fig. 2). Because we found this large difference in the heparin-induced reduction in gene expression whether or not electric pulses were delivered to the muscles after DNA injection, we decided to analyze the effects of other amounts of heparin.

In all cases, we found that, in the absence of electric pulse delivery, heparin significantly inhibited DNA expression at all concentrations tested (Fig. 3A). However, at 1.0 I.U. of heparin units per muscle, there was no reduction at all in luciferase gene expression if electric pulses were delivered, whereas in the absence of the electric pulses, we already detected a large reduction of luciferase expression, to about 2% of the expression obtained in the controls without heparin ($P < 0.001$; Fig. 3). This difference in the inhibition caused by heparin in the presence or absence of the electric pulses suggests that gene expression by heparin was altered mostly due to the inhibition of DNA

doi:10.1006/mthe.2001.0465, available online at http://www.idealibrary.com on IDEAL

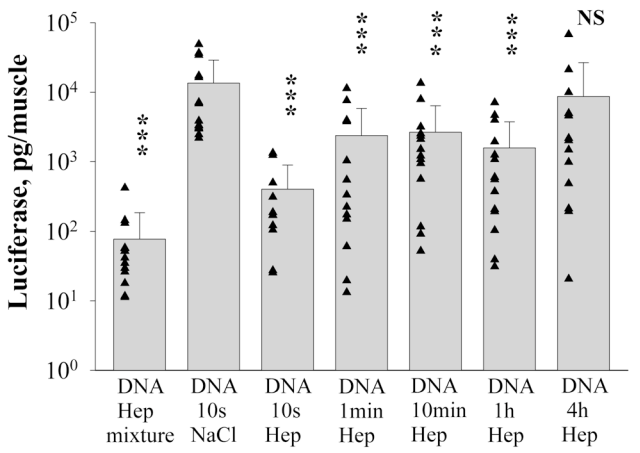

FIG. 4. Luciferase gene expression after intramuscular injection of plasmid DNA followed by the injection of either physiological saline or heparin. We injected 3 μg of DNA (in 10 μl of NaCl) into the muscle. Then, at various times, we administered 20 μl of either NaCl (0.9%) or heparin (3.6 I.U. per muscle) through the same injection path. In the first lane we injected plasmid DNA and heparin together. Filled triangles, individual experimental values; histogram, mean value ± S.D. For all DNA + heparin conditions (except for the injection of heparin 4 h after DNA injection) results are statistically different (***$P < 0.001$) with respect to those of the DNA + NaCl conditions.

uptake into the muscle cells and that heparin could be used to analyze DNA uptake kinetics.

We also found that inhibition increased with increasing amounts of heparin in the heparin-DNA mixtures. Because heparin is an anticoagulant compound, it provoked some bleeding in the treated muscle when we used 10 and 36 I.U. (we never detected such bleeding for the lower (1 and 3.6 I.U.) heparin doses). For these high heparin doses, in addition to luciferase expression being almost abolished in the absence of the electric pulses, DNA expression began to be significantly lower than that obtained after the electrotransfer of DNA in the absence of heparin (Fig. 3B). This reduction may be a result of either the existence of the bleeding (that is, the heparin toxicity due to its anticoagulant activity) or some other intrinsic toxicity of the heparin (because electric pulses can facilitate its penetration into muscle cells as well).

Kinetics of Plasmid DNA Uptake Determined by Means of Consecutive Heparin Injections

In light of these observations, even though 3.6 I.U. of heparin per muscle slightly reduced luciferase gene expression after delivery of electric pulses, we chose this dose for the analysis of DNA uptake kinetics because it leads to the highest difference in gene expression with and without electric pulse delivery (Fig. 3).

The addition of heparin 10 seconds after DNA injection is somehow less efficient in inhibiting DNA transfer than the same amount of heparin mixed with DNA before the injection (Fig. 4). Nevertheless, inhibition is large enough to follow the kinetics of DNA uptake. After 1 hour or even 1 minute, a small fraction of the DNA is either already transferred or at least in a situation in which heparin can no longer inhibit its transfer (Fig. 4). Only after 4 hours could heparin no longer significantly inhibit DNA uptake. However, analysis of the individual measurements showed that, even at this time, heparin was able to affect plasmid expression in some of the muscles. On the whole, the kinetics pattern was similar to that obtained with DNase I (Figs. 1 and 4). We concluded from these results that the DNA uptake process is slow, continuous, and almost completed 4 hours after plasmid injection.

Persistence of the Injected Plasmid DNA in the Muscle

Because DNA can leave the muscle and endogenous endonucleases can affect the injected DNA [21], the pattern of DNA uptake kinetics could be consistent with the DNA half-life in the muscle. To test this hypothesis, it was necessary to estimate how long the DNA remained in an intact and active form in the tibialis cranialis muscle. For this purpose, at various times after DNA injection, we facilitated DNA uptake by means of electric pulses. We obtained the same levels of electrotransferred DNA expression even though electric pulses were applied up to 4 hours after the injection. Moreover, these levels were more than 10 times higher than those achieved after the simple injection of naked DNA (Fig. 5). However, we lost the facilitation of DNA uptake by electric pulses when the electric pulses were delivered 6 or 8 hours after the injection. At these times, luciferase activity was similar to that obtained after the simple injection of the naked DNA. This result shows that, 6 hours after intramuscular injection of plasmid, the DNA that has not yet been internalized in the muscle cells has been either removed from the muscle or inactivated.

DISCUSSION

Here we have analyzed the kinetics of plasmid DNA uptake by the skeletal myofibers. After DNA injection in the muscle, we perturbed its spontaneous uptake by muscle cells and at various times measured luciferase expression. We used several approaches to perturb DNA uptake and to follow its kinetics. They included the injections into the treated muscle of either DNase I, to achieve the physical digestion of the plasmid, or heparin, to inhibit its uptake. In spite of differences in the two approaches, the DNA uptake kinetics patterns were similar (Figs. 1 and 4). DNA uptake starts rapidly after its administration into the muscle and, afterwards, DNA slowly and progressively accumulates into the myofibers. This takes at least several hours and is nearly completed 4 hours after DNA injection (Figs. 1 and 4). Slow DNA uptake kinetics in muscle agree with

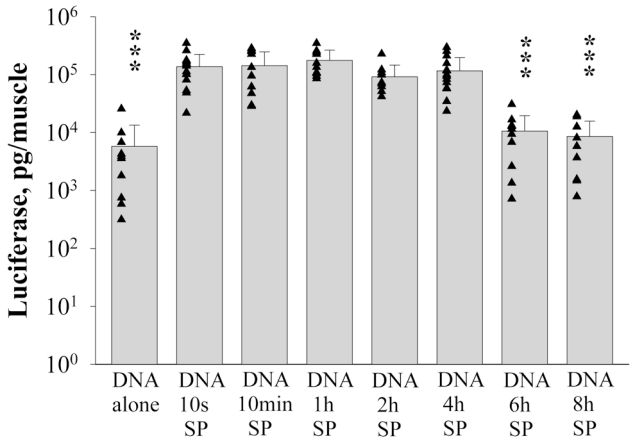

FIG. 5. Luciferase gene expression after intramuscular injection of plasmid DNA followed by the electrotransfer of the DNA still present in the muscle. We injected 3 μg of DNA (in 30 μl of NaCl) into the muscle. Then we pulsed the muscles at various times using simple electric pulses (SP). Filled triangles, individual experimental values; histogram, mean value ± S.D. For all the experimental conditions labeled with three asterisks, results are statistically different (***$P < 0.001$; one-way ANOVA test) with respect to unlabeled conditions, whereas no statistical difference exists between the labeled conditions or the between the unlabeled conditions.

the observation that plasmid uptake by hepatocytes was not immediate but required at least 1 hour [14]. In view of the slow, progressive DNA accumulation within muscle cells, it is possible to discuss the different mechanisms that could account for plasmid DNA uptake *in vivo*.

It has been assumed that DNA can simply diffuse through membrane disruptions induced by direct intramuscular injections. Several groups have already intensively analyzed this hypothesis. However, no strong evidence supporting such a route for DNA entry was obtained [2,14,15,22]. Contrary to this, it was found that the myofibers damaged by the injection procedure almost never expressed the *lacZ* gene [22]. Moreover, it has been demonstrated that, even when hydrostatic pressure is applied to liver to enhance naked DNA transfer, the hepatocytes that take up the DNA are not permeabilized [14]. It is now possible to add that such a mechanism would not be compatible with the slow and progressive DNA uptake shown here because such disruptions cannot be long-lived without impairing the survival of muscle fibers. Finally, our findings that heparin suppresses DNA expression (Figs. 2 and 3), and that this is consistent with heparin activity outside of the cell, indicate that most likely neither large membrane disruptions nor spontaneous pores are responsible for DNA uptake.

Alternatively, it has been suggested that DNA entry into the cells across the plasma membrane may be mediated by the T-tubular systems found in myofibers. However, the ability of other organs like rat liver [23], thyroid [24], lung [25], spleen, heart, and kidney [14] to uptake and express naked DNA is due to a more general mechanism of plasmid uptake.

Another mechanism proposed to explain DNA uptake was receptor-mediated endocytosis [14]. We have previously dealt with receptor-mediated endocytosis in the uptake of exogenous molecules by intact cells *in vitro* and *in vivo*. We have shown that bleomycin, a hydrophilic molecule currently used as an anticancer drug, does not diffuse freely across the plasma membrane. The main target of bleomycin is nuclear DNA, therefore, bleomycin must cross the plasma membrane to exert its cytotoxic activity. Naked DNA must do the same kind of translocation into the cell to reach the compartments where, through its transcription and translation, it can express its genes. In the case of bleomycin, the cells take up bleomycin by means of receptor-mediated endocytosis [26]. Indeed, a membrane protein able to bind the bleomycin molecules was found [27]. The direct link between the number of these membrane proteins exposed at the cell surface and the sensitivity of the cells to bleomycin were also demonstrated [28]. Finally, the turnover rate of the constitutive endocytosis was also found to have a role in the number of bleomycin molecules internalized and thus in the toxicity of this molecule [26]. Bleomycin can use a mechanism like receptor-mediated endocytosis (which results in limited internalization) because biological effects are produced even with very low numbers of bleomycin molecules inside the cell (500 molecules are sufficient to kill a cell [29]). Again, this is also the situation in the case of DNA, for which expression is largely amplified by the process of translation and transcription. Therefore, it is possible that a slow, saturable uptake mechanism, such as receptor-mediated endocytosis, could be operational for DNA as it is known for bleomycin.

In the light of this hypothesis, the following considerations could be made from the experiments reported here. First, inhibition of DNA expression by heparin may result from inhibition of polyanionic DNA uptake by polyanionic heparin, and this would implicate that the receptors that bind DNA also interact with heparin. It might be assumed that heparin does not inhibit DNA uptake by myofibers, and that the decreased luciferase expression by the cells is due to heparin activity within the cells (for example, by inhibiting plasmid entry into the nucleus, interacting with DNA binding proteins, and so on). However, our results demonstrate that the reduction in luciferase gene expression caused by heparin is not due to an effect of heparin presence inside the cells. Indeed, it is known that appropriate electric pulses permeabilize cell plasma membrane, *in vitro* as well as *in vivo*, and may significantly contribute to internalization of exogenous material into the cells [30,31]. Thus, if DNA can enter the cells, heparin (with a much lower molecular weight) can enter as well. Nevertheless, at a dose of 1 I.U. per muscle, heparin

doi:10.1006/mthe.2001.0465, available online at http://www.idealibrary.com on IDEAL

did not change gene expression in the presence of electric pulses, whereas the same amount of heparin in the absence of electric pulses reduced luciferase expression more than 100 times (Fig. 3). Thus, at low added amounts, heparin in the muscle exerts its activity not as an inhibitor of DNA expression but rather as an inhibitor of DNA uptake.

Second, among the polyanionic molecules, heparin is not the only one that possesses the ability to inhibit DNA uptake and expression. Other polyanions like dextran sulfate, nonexpressing DNA, sonicated salmon sperm DNA, polyglutamic acid, polycytidylic acid, and polyinosinic acid share such an activity both *in vitro* and *in vivo* [14,19]. Because all these compounds have negative charges, it could be assumed that electrostatic interaction might account for nonspecific binding to membrane proteins. However, not all polyanions inhibit plasmid DNA, suggesting that binding is not just due to nonspecific electrostatic interactions [14,19].

Third, the progressive internalization of DNA may reflect a limited number of DNA receptors or binding sites on the cell membranes, as well as the limited rate of endocytosis vesicle turnover in living cells, as in the case of bleomycin.

Slow DNA accumulation in muscle cells indicates that DNA should be present in the muscle for the entire time the uptake is taking place, that is, at least for 4 hours after its injection into the muscle. Moreover, DNA should stay in the muscle in an active form. To clarify this point, we applied electric pulses at various times after DNA injection and measured luciferase expression 48 hours later. Electric pulses are supposed to facilitate gene expression due to an increase in the amount of DNA transferred into the cells [6,11]. Therefore, this method allowed us to reveal the presence of DNA that has not yet been taken up by the cells (that is, the DNA located in the muscle, outside the fibers) but that could still be transferred and expressed. Our results showed constant luciferase expression if we applied the pulses up to 4 hours after the injection, at a level of DNA expression over 10-fold larger than the level reached after the injection of naked DNA alone. However, we lost the facilitation of DNA uptake by electric pulses when we delivered the electric pulses after 6 hours: luciferase activity was similar to that obtained after the simple injection of the DNA (Fig. 5). This demonstrates that, after plasmid intramuscular injection, extracellular DNA stays in the muscle in an active form for a maximum of 4 to 6 hours. This result is in agreement with previous reports indicating that DNA injected in the muscle was degraded only within 90 minutes after its injection [13,18]. Therefore, the kinetics of DNA uptake and the persistence of intact plasmid in the muscle suggest that the amount of DNA taken up by the muscle fibers is limited by the half-life of plasmid in that tissue. Any improvement of the protocol that would increase DNA half-life in muscle without altering its binding to the receptors should increase the amount of DNA internalized in the muscle fibers and therefore its overall level of expression. Nevertheless, previous attempts to block DNA degradation by adding DNase inhibitors did not increase muscle transfection efficiency [13,18].

Our results show that plasmid DNA is taken up slowly by the muscle fibers. A progressive accumulation of DNA in the fibers can be found for at least 4 hours after naked DNA injection, which is also the time for the persistence of intact DNA in the muscle. Heparin is a polyanionic molecule that inhibits DNA uptake. This inhibition is dose dependent and compatible with the competition for the binding to putative receptors or specific binding sites. Our results reinforce the assumption that the mechanism of plasmid DNA uptake is consistent with receptor-mediated endocytosis.

MATERIALS AND METHODS

Plasmid DNA. The plasmid pXL 3031 (pCMV-Luc+) contains the cytomegalovirus promoter (nt 229–890 of pcDNA3, Invitrogen) inserted upstream of the coding sequence of the modified cytosolic gene encoding firefly luciferase [32]. It was prepared as described [33]. As a general rule, at least 80% of the plasmid molecules were supercoiled.

Animals. For all experimental procedures we anesthetized female 7- to 9-week-old C57Bl/6 mice by intraperitoneal administration of the anesthetics ketamine (100 mg/kg; Ketalar, Panpharma, France) and xylazine (40 mg/kg; Rompun, Bayer, France). We shaved the legs of the mice using an electric shaver before DNA injection. Unless stated otherwise, 10 to 15 muscles were included in each experimental group.

Injection of DNA. We always used 3 μg of plasmid in all experimental conditions, prepared in 10 or 30 μl of 0.9 % NaCl. We injected the DNA into both tibial cranial muscles using a Hamilton syringe with a 26-gauge needle. For the consecutive injection of two different compounds (DNA and either DNase I (Sigma, Saint Quentin Fallavier, France) or heparin (Laboratoires Leo, Saint Quentin en Yvelines, France)), we prepared the DNA in 10 μl and the second compound in 20 μl. At defined times after DNA injection, we injected DNase I (6 mg/ml) or heparin (1, 3.6, 10, or 36 I.U. per muscle) into the treated muscles through the same injection path. In these experiments, 1 mg heparin (MW 10–12 kDa) corresponded to approximately 137 I.U.

DNA electrotransfer. We used a square wave electropulsator PS-15 (Jouan, St Herblain, France) to generate simple sequences of pulses (8 pulses, 200 V/cm, 20 ms, 1 Hz) as well as the HV pulses (1 pulse, 800 V/cm, 100 μs) in the combination of high voltage (HV) and low voltage (LV) pulses: HV + 4LV. To generate the LV pulses (4 pulses of 80 V/cm and 100 ms, delivered at 1-s intervals), a microprocessor-driven switch/function generator was built (at the Faculty of Electrical Engineering, University of Ljubljana, Slovenia) and connected to a power amplifier (Kepco, Inc., Flushing, NY). This switch also allowed keeping constant the lag of 1 s between the HV pulse and the first of the LV pulses in the HV + 4LV pulse sequences. We injected the DNA, 3 μg in 30 μl of 0.9% NaCl with or without heparin (1, 3.6, 10, or 36 I.U. per muscle), as described above. Soon after DNA injection (45 ± 15 s) or at precise times after DNA injection (10 s, 10 min, or 1, 2, 4, 6, and 8 h), we pulsed the muscles using simple or HV + 4LV pulse sequences. We applied conductive gel to assure good contact between the skin and the electrodes.

Luciferase activity measurement. We killed the mice 2 d after DNA treatment. We removed and homogenized the muscles in 1 ml Cell Culture Lysis reagent solution (10 ml Cell Culture Lysis reagent (Promega Charbonnières, France), diluted with 40 ml distilled water and supplemented with 1 tablet of the Protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). After centrifugation at 12,000 rpm for 10 min at 4°C, we assessed luciferase activity on 10 μl of the supernatant, using a Walac Victor^2 lumi-

nometer, by integration of the light produced during 1 s, starting after the addition of 50 μl Luciferase Assay Substrate (Promega) to the muscle lysate. We collected the results from the luminometer in relative light units (RLU). Calibration with purified firefly luciferase protein showed that 10^6 RLU correspond to approximately 70 ng of expressed luciferase. We expressed the final results as pg of luciferase per muscle.

Statistical analysis. Except otherwise stated (Figs. 1–4), we used the two tailed Mann-Whitney test for the statistical analysis of our data. In Fig. 5, we used the one-way ANOVA test to show that ther results for DNA alone after 6 h and 8 h are different from the data obtained at the other times, but that within these two groups there are not statistically significant differences. We repeated all experiments twice (4–6 muscles each time) and examined all experimental conditions using at least 10 muscles (except for "muscle alone" and "DNA - 1 h- DNAse I" in Fig. 1 we examined 4 and 9 muscles, respectively). We reported the data as means ± S.D. calculated using all the muscles treated under the same experimental conditions. We also reported all the individual experimental values in the figures.

ACKNOWLEDGMENTS

We thank Damijan Miklavčič and Marko Puc (Laboratory of Biocybernetics, Faculty of Electrical Engineers, University of Ljubljana, Slovenia) for providing electrical equipment to generate HV + 4LV pulse sequences, in the frame of the Cliniporator project (QLK3-1999-00484) funded by the EU; Rafael Davalos (Laboratory of Mechanical Engineering, University of California at Berkeley, USA) for revision of the paper; and the staff of the Service Commun d'Expérimentation Animale of the IGR for animal maintenance. S.S. was the recipient of a research grant from Aventis to L.M.M.

RECEIVED FOR PUBLICATION APRIL 23; ACCEPTED AUGUST 13, 2001.

REFERENCES

1. Wolff, J. A., *et al.* (1990). Direct gene transfer into mouse muscle *in vivo*. *Science* **247:** 1465–1468.
2. Wolff, J. A., *et al.* (1991). Conditions affecting direct gene transfer into rodent muscle *in vivo*. *Biotechniques* **11:** 474–485.
3. Suzuki, T., Shin, B. C., Fujikura, K., Matsuzaki, T., and Takata, K. (1998). Direct gene transfer into rat liver cells by *in vivo* electroporation. *FEBS Lett.* **425:** 436–440.
4. Rols, M. P., *et al.* (1998). *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nat. Biotechnol.* **16:** 168–171.
5. Mathiesen, I. (1999). Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*. *Gene Ther.* **6:** 508–514.
6. Mir, L. M., *et al.* (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* **96:** 4262–4267.
7. Somiari, S., *et al.* (2000). Theory and *in vivo* application of electroporative gene delivery. *Mol. Ther.* **2:** 178–187.
8. Jaroszeski, M. J., Heller, R., and Gilbert, R. (2000). *Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery*, Humana Press, Totowa, New Jersey.
9. Mir, L. M., Bureau, M. F., Rangara, R., Schwartz, B., and Scherman, D. (1998). Long-term, high level *in vivo* gene expression after electric pulse-mediated gene transfer into skeletal muscle. *C. R. Acad. Sci. III* **321:** 893–899.
10. Bettan, M., *et al.* (2000). High-level protein secretion into blood circulation after electric pulse-mediated gene transfer into skeletal muscle. *Mol. Ther.* **2:** 204–210.
11. Bureau, M. F., Gehl, J., Deleuze, V., Mir, L. M., and Scherman, D. (2000). Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. *Biochim. Biophys. Acta* **1474:** 353–359.
12. Wolff, J. A., *et al.* (1992). Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle. *J. Cell Sci.* **103:** 1249–1259.
13. Manthorpe, M., *et al.* (1993). Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* **4:** 419–431.
14. Budker, V., *et al.* (2000). Hypothesis: naked plasmid DNA is taken up by cells *in vivo* by a receptor-mediated process. *J. Gene Med.* **2:** 76–88.
15. Doh, S. G., Vahlsing, H. L., Hartikka, J., Liang, X., and Manthorpe, M. (1997). Spatial-temporal patterns of gene expression in mouse skeletal muscle after injection of lacZ plasmid DNA. *Gene Ther.* **4:** 648–663.
16. Yakubov, L. A., *et al.* (1989). Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc. Natl. Acad. Sci. USA* **86:** 6454–6458.
17. Loke, S. L., *et al.* (1989). Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA* **86:** 3474–3478.
18. Levy, M. Y., Barron, L. G., Meyer, K. B., and Szoka, F. C. (1996). Characterization of plasmid DNA transfer into mouse skeletal muscle: evaluation of uptake mechanism, expression and secretion of gene products into blood. *Gene Ther.* **3:** 201–211.
19. Takakura, Y., *et al.* (1999). Characterization of plasmid DNA binding and uptake by peritoneal macrophages from class A scavenger receptor knockout mice. *Pharm. Res.* **16:** 503–508.
20. Benimetskaya, L., *et al.* (1997). Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* **3:** 414–420.
21. Lew, D., *et al.* (1995). Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice. *Hum. Gene Ther.* **6:** 553–564.
22. Davis, H. L., Whalen, R. G., and Demeneix, B. A. (1993). Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. *Hum. Gene Ther.* **4:** 151–159.
23. Malone, R. W., *et al.* (1994). Dexamethasone enhancement of gene expression after direct hepatic DNA injection. *J. Biol. Chem.* **269:** 29903–29907.
24. Sikes, M. L., O'Malley, B. W., Finegold, M. J., and Ledley, F. D. (1994). *In vivo* gene transfer into rabbit thyroid follicular cells by direct DNA injection. *Hum. Gene Ther.* **5:** 837–844.
25. Meyer, K. B., Thompson, M. M., Levy, M. Y., Barron, L. G., and Szoka, F. C. (1995). Intratracheal gene delivery to the mouse airway: characterization of plasmid DNA expression and pharmacokinetics. *Gene Ther.* **2:** 450–460.
26. Pron, G., *et al.* (1999). Internalisation of the bleomycin molecules responsible for bleomycin toxicity: a receptor-mediated endocytosis mechanism. *Biochem. Pharmacol.* **57:** 45–56.
27. Pron, G., Belehradek, J., and Mir, L. M. (1993). Identification of a plasma membrane protein that specifically binds bleomycin. *Biochem. Biophys. Res. Commun.* **194:** 333–337.
28. Pron, G., Belehradek, J., Orlowski, S., and Mir, L. M. (1994). Involvement of membrane bleomycin-binding sites in bleomycin cytotoxicity. *Biochem. Pharmacol.* **48:** 301–310.
29. Poddevin, B., Orlowski, S., Belehradek, J., and Mir, L. M. (1991). Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. *Biochem. Pharmacol.* **42** (Suppl.)**:** S67–S75.
30. Orlowski, S., and Mir, L. M. (1993). Cell electropermeabilization: a new tool for biochemical and pharmacological studies. *Biochim. Biophys. Acta* **1154:** 51–63.
31. Neumann, E., Sowers, A. E., and Jordan, C. A. (1989). *Electroporation and Electrofusion in Cell Biology*, Plenum Press, New York and London.
32. Soubrier, F., *et al.* (1999). pCOR: a new design of plasmid vectors for nonviral gene therapy. *Gene Ther.* **6:** 1482–1488.
33. Ausubel, F. M., *et al.* (1994). *Current Protocols in Molecular Biology*, Wiley, New York.