Gene Transfer *In Vitro* and *In Vivo* by Cationic Lipids Is Not Significantly Affected by Levels of Supercoiling of a Reporter Plasmid

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Purpose. It is a common preconception that supercoiled plasmid DNA is more desirable for the transfection of cells that the relaxed form of the plasmid. This notion has led to the recommendation that a specification for the minimum amount of plasmid in the supercoiled form should exist in a gene therapy product. We have tested this notion by examining the effects of the degree of supercoiling on cationic lipid-mediated gene transfer *in vitro* and *in vivo.*

Methods. An ion-exchange high performance liquid chromatography (HPLC) method was developed to accurately quantitate the relative amounts of supercoiled DNA in purified plasmid. A sample of the purified plasmid was fully relaxed using topoisomerase. Next, the ability of various levels of supercoiled plasmid to transfect mammalian cells was measured.

Results. This study suggests that there is no relation between the degree of supercoiling and lipofection efficiency. Subsequent transfection using several different lipofection agents, different cell types, and an *in vivo* model support these results.

Conclusions. In considering a specification for the amount of supercoiled plasmid in a gene therapy product, it must be noted that the relaxed forms of the plasmid are no less efficient at gene delivery than the supercoiled forms.

KEY WORDS: cationic lipids; transfection; DNA supercoiling; HPLC; lipofection; gene therapy.

INTRODUCTION

The ability to efficiently transfer genes into cells has made the prospect of gene therapy possible. Gene transfer methods currently being developed can be broadly divided into viral and non-viral approaches (1). Viral systems can be highly efficient at gene delivery; however, limitations including viral tropism, viral titer, immunogenicity, difficulty of large-scale production, and regulatory constraints can complicate their application (2). Non-viral techniques have generated an intense amount of work due to the possibility of avoiding the constraints of the viral approach. One of the more successful non-viral approaches is the cationic lipid-based delivery system (3). Since the development of the first cationic lipid carrier, many cationic lipids have been synthesized and cationic lipid-based formulations have been optimized for both in vitro and in vivo gene transfer. Many different cationic lipid-based formulations are currently commercially available for in vitro gene transfer. In the last ten years, the efficiency of gene transfer by cationic lipids has increased 3 orders of magnitude (4). Consequently, lipofection has become a standard technique in the molecular biology laboratory. Furthermore, cationic lipid based gene delivery approaches are currently being tested in the clinic as gene therapy agents (3,5–9).

In spite of these advances, there is a limited understanding of the basic mechanisms of gene transfer by cationic lipids. Recent work has focused on the cellular mechanisms involved in transfection (10). The primary route of entry of the lipid-DNA complex appears to be endocytosis, followed by an inefficient disaggregation of the lipid-DNA complexes and transport of the DNA into the nucleus. It is reasonable to expect the smallest, most compact particles to be more efficient at entry, since endocytosis is involved.

It would follow logically that supercoiled DNA is more desirable for the transfection of cells than the relaxed form of the plasmid. Furthermore, in the production of gene therapy plasmid-based drugs, a specification for the minimum amount of plasmid product in the supercoiled form is recommended by the regulatory agencies (11,12). While several studies appear to be consistent with the notion that supercoiled plasmid transfects cells better, it is difficult to separate the effects of particle size from other factors, such as salt concentration, pH and electrostatic effects (13–15). Arguments have also been made against a specification for high levels of supercoiled plasmid, since the plasmid is likely to be "nicked" and relaxed during the transport of the plasmid into the nucleus, regardless of the level of input supercoils (16). No direct comparison of the lipid-mediated transfection activities of relaxed and supercoiled plasmid has been done previously. The goal of this work was to directly compare the transfection activities of supercoiled and relaxed DNA complexed with cationic lipids in order to characterize the requirement for supercoiling in cellular transfection. We have found no difference in the ability of supercoiled and relaxed DNA to transfect a variety of cells using an array of cationic lipid transfection reagents. Supercoiled and relaxed DNA are equally active in transfection both in vitro and in vivo.

MATERIALS AND METHODS

Plasmid Construction

The plasmid pCMV-InLuc was constructed from pGL3-Basic (Promega, Madison, WI), which contains a modified firefly luciferase gene and pCMVβ (Clontech, Palo Alto, CA), which contains the immediate early promoter/enhancer of CMV, an intron and polyadenylation site from SV40, a bacterial origin of replication from pUC and the β-lactamase gene encoding ampicillin resistance. Standard molecular techniques (17) were used in the construction. Both pCMVβ and pGL3-Basic were digested with SmaI and SalI. The plasmid pCMVβ was further digested with EcoRI to facilitate agarose gel purification of the 3682 base pair SmaI/SalI fragment. The 3682 base pair fragment from pCMVβ, and the 1982 base pair fragment from pGL3-Basic were isolated by agarose gel electrophoresis and ligated

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ABBREVIATIONS: HPLC, high performance liquid chromatography; DC-chol, 3β[N,N′,N′-dimethylaminoethane)-carbamoyl]cholesterol); DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine).

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together. The ligation reactions were used to transform competent DH5α bacteria (Gibco/BRL, Gaithersberg, MD) Positive transformants were identified by restriction digestion of plasmid mini-preps. A positive clone was isolated, plasmid purified by a Qiagen Endo-free kit (Qiagen, Chatsworth, CA) and the junctions of the construct were confirmed by sequencing.

Plasmid HPLC Analysis

The column used was a TSK-GEL DNA-NPR, 4.6 mm × 7.5 cm, 2.5 μm (Toso-Haas, Montgomeryville, PA). A guard column was also used (TSK-GEL, DEAE-NPR, 4.6 mm × 5.0 mm, 5.0 μm, Toso-Haas). TRIZMA base and NaCl were from Sigma. Water was purified on a Milli-Q system (Millipore, Bedford, MA). The HPLC system was a Waters 626LC, with a Waters 717plus autosampler (Waters, Milford, MA). Column flow was monitored continuously with a Waters 996 photodiode array detector outfitted with an 8 μl flow-cell. The pumps were controlled and the data were collected and analyzed by Waters Millennium 2010 software. Both the column and the autosampler were operated at ambient temperature. The column was equilibrated in 0.56 M NaCl, 50 mM Tris, pH 9.0. After loading the plasmid on the column, the column was developed with a linear gradient of 0.56 M to 1.2 M NaCl in 50 mM Tris, pH 9.0 over approximately ten column volumes at a flow rate of 0.5 ml/min.

Topoisomerase Reaction

Plasmid DNA was relaxed with topoisomerase I (Gibco/BRL) to generate a sample of completely relaxed plasmid. Initially, a time course of the topoisomerase reaction was performed to determine conditions which result in the complete relaxation of the plasmid. The relaxation was done in a 1 ml reaction, containing 350 μg pCMV-InLuc, 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithioerythritol, and 700 units of topoisomerase I (one unit catalyzes the conversion of 0.5 μg superhelical ϕX174 RF DNA to a relaxed state in 30 minutes at 37°C). Aliquots of 50 μl were removed at 0, 5, 10, 20, 30, and 60 minutes. The reaction at each time point was quenched by adding 0.1% SDS and placing on ice. Samples were then analyzed by agarose gel electrophoresis. Gels consisted of 1% agarose in TBE buffer (Tris-borate, EDTA). Each lane was loaded with 0.5 μg plasmid (by A_{260}). The gel was then electrophoresed at 100 volts for 2 hours. After electrophoresis, the plasmid was visualized by staining the gel with ethidium bromide.

For generating relaxed plasmid standard, 200 μg of plasmid was treated with 500 units of topoisomerase at 37°C for one hour, in the buffer described above. After one hour, the reaction was extracted once with phenol, once with a 1:1 mixture of phenol:chloroform, and once with chloroform. Finally, the plasmid DNA was precipitated with isopropanol, dried, and resuspended in 400 μl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The removal of topoisomerase activity was confirmed by incubating supercoiled pCMV-InLuc with the relaxed standard and finding no loss of supercoil from the input supercoiled plasmid (not shown).

In Vitro Transfection

Cells were seeded at 5–8 × 10^4 cells/well in a flat bottom, 48 well plate (Costar, Cambridge, MA) and grown overnight in the media appropriate for each cell type (see below) at 37°C. Mixtures of supercoiled and relaxed pCMV-InLuc were prepared to achieve desired percentages of supercoiling. Lipid was resuspended and added to the DNA in the formulation ratio of lipid to DNA specific for each lipid (see below). The complete media was replaced with serum-free media and the desired dose of formulation added. Doses of pCMV-InLuc between 0.05 and 0.25 μg in a total volume of 0.25 ml were used to transfect the cells, each performed in triplicate. These doses were chosen to be submaximal, i.e., they resulted in levels of expression that were less that the maximum level that can be achieved. The cells were transfected for 4–5 hours, then the transfection media was replaced with complete media.

Cells were harvested 48 hours after transfection. Each well was aspirated and rinsed once with 500 μl of PBS. 100 μl of 1X lysis buffer (Promega) was added and used to harvest the cells into microcentrifuge tubes. The lysates were flash-frozen and thawed three times, then spun in a microcentrifuge at 16,000 × g for 10 minutes. The supernatant was then assayed for luciferase expression using an EG&G Berthold Autolumat LB 953 luminometer (EG&G Instruments, Oak Ridge, TN). Protein concentrations in the lysate were determined by a Bradford assay (Pierce, Rockford, IL). The luciferase activity was normalized to extracted protein amounts. Data were plotted using DeltaGraph 4.0 (DeltaPoint, Monterey, CA) on a Macintosh computer.

Lipofection Ratios

Mixtures of lipofection agents and pCMV-InLuc (0% and 100% supercoiled) were prepared in different formulations, as follows:

Lipofectamine (Gibco BRL) 7:1 lipid:DNA (nmol:μg)

pFX-6 (Invitrogen, San Diego, CA) 6:1 lipid:DNA

Cellfectin (GibcoBRL) 6:1 lipid:DNA

Lipofectin (GibcoBRL) 6:1 lipid:DNA

DC-chol/DOPE (Targeted Genetics) 10:1 lipid:DNA

The composition of each transfection reagent, where known is: Lipofectamine, DOSPA/DOPE (3:1 w/w, DOSPA is 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N,dimethyl-1-propanaminiumtrifluoroacetate. DOPE is 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine), Cellfectin, TM-TPS/DOPE (1:1.5, w/w, TM-TPS is N,N′, N″, N‴-tetramethyl-N,N′,N″,N‴-tetrapalmitylspermine), Lipofectin, DOTMA/DOPE (1:1, w/w, DOTMA is N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DC-chol/DOPE (1:1, w/w, DC-chol is 3β[N,N′,N′-dimethylaminoethane-carbamoyl]cholesterol).

Cell Culture Conditions

All media were from Bio-Whittaker (Walkersville, MD). FBS is fetal bovine serum (Hyclone, Logan, UT). PSG is 5000 U/ml penicillin, 5 mg/ml streptomycin, 0.2 M glutamine. Several cell types were grown under the following conditions:

HeLa: DMEM, 10% FBS, 1% PSG, at 10% CO_2

CHO-3: DMEM/F12, 10% FBS, 1% PSG, at 10% CO_2

SKOV-3: DMEM/F12, 10% FBS, 1% PSG, at 10% CO_2

MCF-7: RPMI, 10% FBS, 1% PSG, at 5% CO_2

293-1: DMEM/F12, 10% FBS, 1% PSG, at 5% CO_2

In Vivo Transfection

MCA-205 cells were grown in culture in DMEM, 10% FBS, 1% PSG, 10% CO_2 at 37°C. Cells were harvested from subconfluent plates and resuspended at a concentration of 1×10^7 cells/ml. 100 μl (1×10^6 cells) were injected sub-cutaneously into each flank of C57/B16 mice. Tumors were allowed to grow for 12 days. Plasmid pCMV-InLuc (25 μg), either relaxed or supercoiled, was formulated with 25 nmole DC-chol/DOPE and directly injected into the tumors. After 16 hours, tumors were biopsied, snap-frozen and analyzed for luciferase expression in the same way as the in vitro transfectants (see above).

RESULTS

HPLC Analysis of Relaxed and Supercoiled Plasmid

The standard assay for percent supercoiled plasmid is an agarose gel electrophoresis-based assay. However, relaxed and supercoiled plasmid could take up different amounts of dye, skewing the quantitation of the relative intensities of the relaxed and supercoiled forms of the plasmid. Therefore, we developed an HPLC-based method for the quantitation of supercoiled and relaxed plasmid. By agarose gel, plasmid purified by the Qiagen Endo-free kit appears as two bands, a rapidly migrating, intense band and a slower migrating, faint band (Fig. 1A). When this plasmid is treated with topoisomerase, the more rapidly migrating band is progressively reduced in intensity and slower migrating bands, consistent with more relaxed topoisomers of the plasmid, appear. For subsequent experiments, a reaction time of one hour was used, to completely relax the plasmid.

When the purified plasmid, predominantly composed of the fully supercoiled form, was analysed by HPLC, as described in Materials and Methods, a large peak eluted from the column approximately one minute after a smaller peak (Fig. 1B, top panel). The two peaks are sufficiently resolved to allow integration of each. The larger peak comprised 99% of the material absorbing UV at 260 nm. A third peak elutes after the largest peak. This peak may represent multimer forms of the plasmid, however this has not been confirmed. Other preparations of plasmid, with varying amounts of this peak also contain varying amounts of multimer plasmid, as judged by agarose gels (not shown). We confirmed that the larger peak is supercoiled plasmid and the smaller peak is the relaxed form by completely relaxing the plasmid with topoisomerase. This fully relaxed plasmid eluted with a retention time identical to the smaller peak in the previous chromatogram (Figure 1B, bottom panel). Subsequently, mixtures of the fully relaxed and the predominantly supercoiled plasmid resulted in peaks corresponding to the amounts predicted by the mixing ratios (Fig. 1B, middle panels). We concluded that our pCMV-InLuc, purified by the Qiagen Endo-free kit consisted of 99% fully supercoiled plasmid.

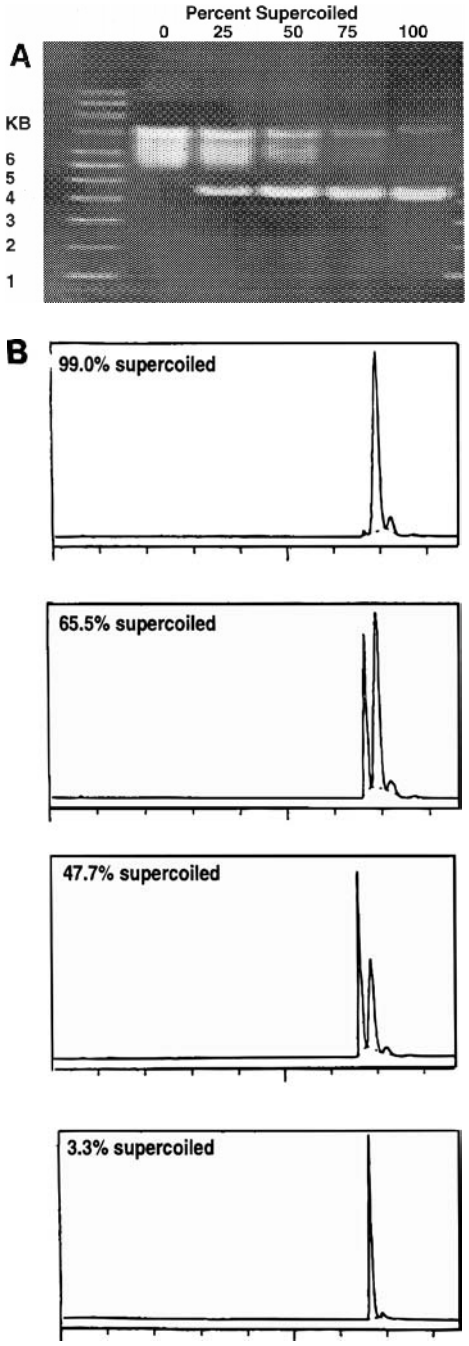

Fig. 1. Analysis of supercoiled and relaxed plasmid. Plasmid pCMV-InLuc was treated with topoisomerase for one hour, as described in Materials and Methods. The topoisomerase was removed from the reaction by phenol extraction and the completely relaxed plasmid mixed with fully supercoiled plasmid. Each sample was analysed on A) a 1% agarose gel and B) HPLC as described in Materials and Methods. On the agarose gel photograph, the percentage of "fully" supercoiled added to the "fully" relaxed plasmid, by volume, is indicated above each lane of the gel. The labels on the HPLC chromatograms indicate the actual percentages of each of these mixtures as calculated from the integration of the peak areas. The chromatogram for the "25% supercoiled" mixture is not shown.

Transfection of SKOV-3 Cells—Amount of Supercoiled Plasmid

We measured transfection efficiency of SKOV-3 cells with pCMV-InLuc to find a range of concentrations of plasmid which give a linear dose-activity response, when using DC-cholesterol/DOPE as the transfection reagent. We found that 0.5 μg per well on a 48-well plate was a saturating amount of plasmid (not shown). Next, we used sub-saturating concentrations (0.05, 0.1 and 0.25 μg per well) to analyze the effect of different ratios of supercoiled and relaxed plasmid. We mixed supercoiled and fully relaxed plasmid to give ratios of supercoiled to relaxed plasmid of 0:1, 1:3, 1:1, 3:1, and 1:0. Each mixture was tested in triplicate transfections. The ratios of supercoiled and relaxed plasmid were confirmed by HPLC (not shown). We found very similar levels of activity at each plasmid concentration, regardless of the amounts of supercoiled or relaxed plasmid in the transfection (Fig. 2).

Transfection of SKOV-3 Cells—Different Lipids and Amount of Supercoiled Plasmid

We next asked if the amount of supercoiled plasmid affected the SKOV -3 cell transfection activity of various lipid-based transfection reagents. We tested a total of five transfection reagents, Lipofectamine (DOSPA/DOPE, GibcoBRL), pFX-6 (PerFect Lipid/DOPE, Invitrogen), Cellfectin (TM-TPS/DOPE), Lipofectin (DOTMA/DOPE, GibcoBRL), and DC-chol/DOPE (Targeted Genetics). The ratios of each lipid to plasmid were set according to the manufacturers' protocols. For each lipid, we used the amount of plasmid previously determined to be sub-optimal for DC-chol/DOPE. Using DC-chol/DOPE (above), we saw the transfection activity to be independent of the proportion of supercoiled plasmid in the transfection mixture. Therefore, we chose to compare only completely relaxed and predominantly supercoiled plasmid to compare the effect of supercoiling on different lipids. With varying levels of absolute activity, we found no difference in the transfection activities of completely relaxed and supercoiled plasmids when using any of the lipids tested (Fig. 3).

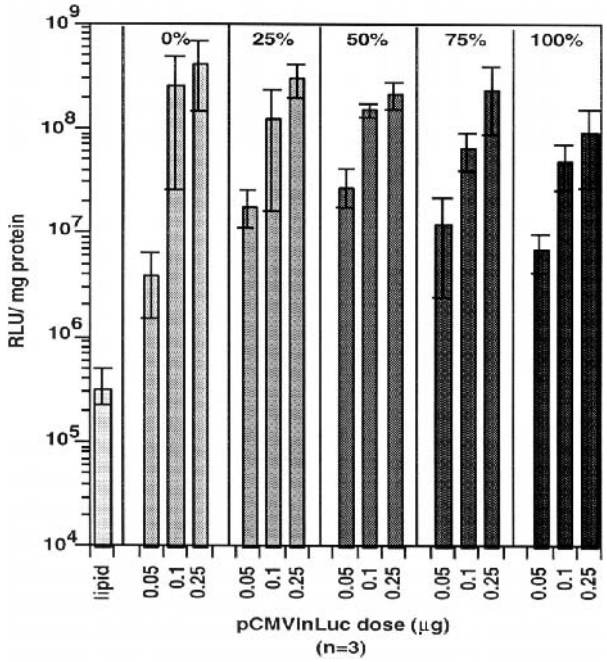

Fig. 2. Transfection of SKOV-3 cells by various ratios of relaxed and supercoiled plasmid. Plasmid pCMV-InLuc was relaxed with topoisomerase, mixed with fully supercoiled plasmid, and used to transfect SKOV-3 cells, as described in Materials and Methods. At each ratio of relaxed and supercoiled plasmid, three amounts of plasmid (0.05, 0.1, and 0.25 μg/well) were used, demonstrating that the amount used was sub-maximal for transfection. All transfections were done in triplicate. Error bars represent ± S.D.

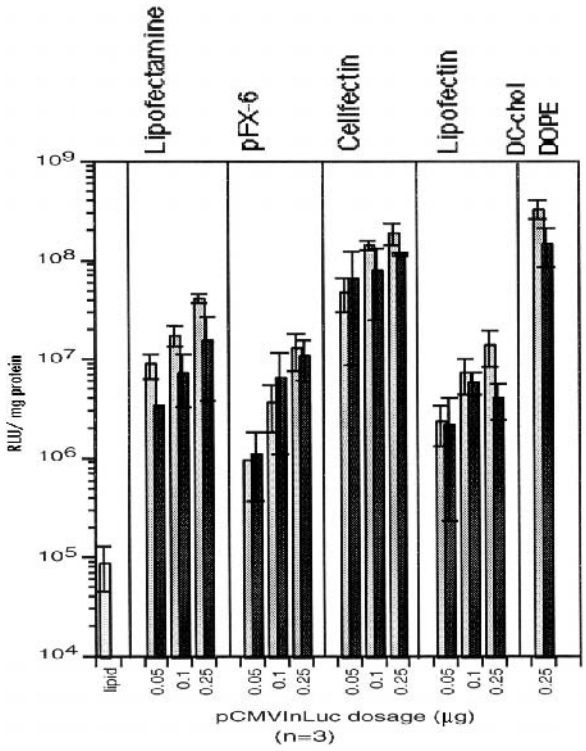

Fig. 3. Transfection by various cationic lipids. SKOV-3 cells were transfected with 0.05, 0.1, and 0.25 μg/well of either fully relaxed (grey bars) or fully supercoiled (black bars) plasmid pCMV-InLuc using Lipofectamine, pFX-6, Cellfectin, Lipofectin, or DC-chol/DOPE as described in Materials and Methods. In this experiment, only the 0.25 μg/well amount was used for DC-chol/DOPE, as the complete range was used in previous experiments (see Figure 2). Each transfection was done in triplicate. Error bars represent ± S.D.

Transfection of Different Cell Types

We tested a panel of five different mammalian cell types. Using DC-chol/DOPE as the transfection agent, we compared the transfection activity of completely relaxed and predominantly supercoiled plasmid at sub-saturating levels (Fig. 4). The cell types we tested were, HeLa (human cervical carcinoma), SKOV-3 (human ovarian carcinoma), MCF-7 (human breast carcinoma), CHO-3 (chinese hamster ovary cells) and 293-1 (transformed human embryonal kidney cells). While there was a range in total transfection activity seen, there was no difference in the transfection activity of completely relaxed and predominantly supercoiled plasmid in these various cell types.

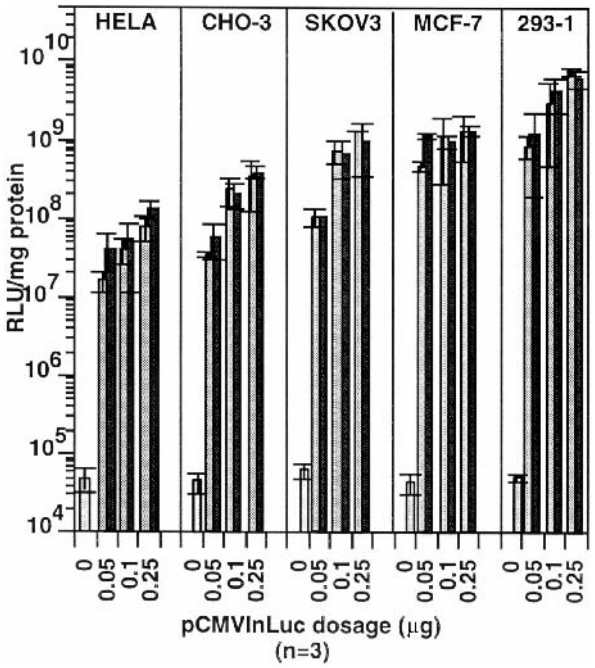

Fig. 4. Transfection of various cell types: DC-chol/DOPE and 0.05, 0.1, or 0.25 μg/well of plasmid pCMV-InLuc was used to transfect the indicated cell lines, as described in Materials and Methods. Plasmid was either fully relaxed (grey bars) or fully supercoiled (black bars). All transfections were done in triplicate. Error bars represent ± S.D.

Transfection of Tumors *In Vivo*

We next used a mouse tumor model to ask if the amount of supercoiled plasmid had an effect on the transfection activity of tumors in vivo. Mice (C57/B16) were injected subcutaneously with MCA-205 (mouse melanoma) cells. Tumors were allowed to grow for 12 days, at which point they were palpable. Completely relaxed, predominantly supercoiled, and a mixture of 33:67 (supercoiled:relaxed) plasmid were formulated with DC-chol/DOPE in a 1:1 (nmole lipid:μg DNA) ratio. These formulations were then directly injected into the tumors. After allowing adequate time for expression (16 hours), the tumors were excised and analysed for luciferase activity. By a paired t-test analysis, we saw no significant difference in the amount of luciferase activity when comparing the completely relaxed to the predominantly supercoiled plasmid ($p < 0.015$). Furthermore, by a separate paired t-test analysis, no significant difference was seen when comparing the completely relaxed to the mixture of relaxed and supercoiled plasmid ($p < 0.05$) (Fig. 5).

DISCUSSION

We have measured the transfection activity of relaxed and supercoiled plasmid using several lipofection agents and cell types, in vitro and in vivo. Under the experimental conditions tested, we found no difference between the transfection activity of fully supercoiled and fully relaxed plasmid DNA. Therefore, whether the plasmid is fully relaxed or supercoiled has little effect on the formation of active lipid-DNA complexes. Differences were seen in the transfection activities of the different lipids (Figure 3) and in the transfection abilities of different cells (Fig. 4); however, no difference could be attributed to the supercoiled nature of the plasmid.

There is an important difference between the relaxed plasmid that we have analyzed here and that which is likely to appear in purified plasmid samples. The plasmid which has been enzymatically relaxed by topoisomerase would be covalently closed open circular DNA. Topoisomerase relaxes plasmid by repeatedly hydrolyzing and re-ligating one strand of the DNA. In the absence of an exogenous energy source, the torsional strength of the supercoiled plasmid is the only energy present in the system. Therefore, the reaction proceeds until the plasmid is completely relaxed, resulting in covalently closed open circles (18). In a purified plasmid sample, a portion of the relaxed plasmid would be nicked (i.e., not covalently closed), by either mechanical shearing or chemical cleavage of the plasmid during the purification process. This nicked DNA may be less active in transfection than the relaxed DNA we have used in this study; however, we have demonstrated that the supercoiling of the plasmid itself has no effect on the transfection activity.

In other studies, the superhelical density of DNA appears to have an effect on its transcriptional activity (19,20). This suggests the possibility that in our study the plasmid entry is more efficient for relaxed DNA, yet the transcription is more active for supercoiled DNA. Therefore, in the examples in which completely relaxed DNA is used, a greater number of molecules may be entering the cells, resulting in the observed luciferase activity. In the predominantly supercoiled transfections, a smaller number of molecules may enter the cell, yet these are transcriptionally more active. In order for this to be the case, there would have to be a linear inverse correlation

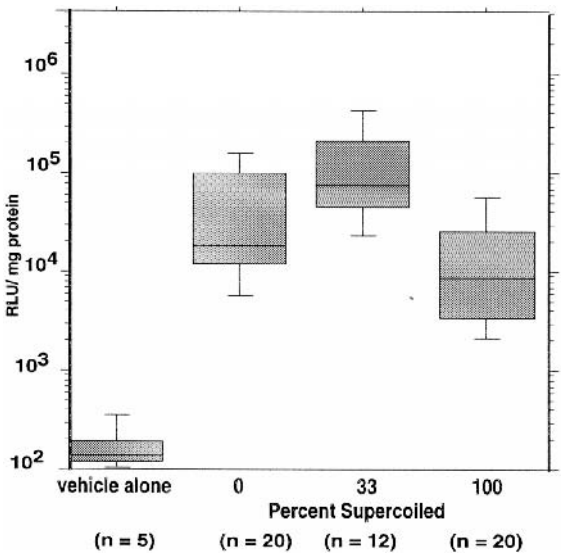

Fig. 5. Box plot representation of *in vivo* gene delivery. DC-chol/DOPE and the indicated ratios of relaxed and supercoiled pCMV-InLuc was used to directly inject subcutaneously implanted MCA-205 tumors in C57/B16 mice, as described in Materials and Methods. The indicated numbers of tumors (n) were used at each relaxed/supercoiled ratio. Data were analyzed on a Macintosh computer, using DeltaGraph 4.0. In the plot, the top line of the box represents the top quartile of the data, the bottom line represents the bottom quartile, and the middle line represents the median of the data. Whiskers on the top and bottom of the boxes extend from the 10th to the 90th percentile.

between the more efficient entry of relaxed plasmid and the greater transfection activity of supercoiled plasmid. This remains a formal possibility, however, it seems unlikely since no effect was seen in the various cell types and with the various transfection reagents that we tested. Further work, with labeled DNA, needs to be done to address this possibility.

Previous work with bacteria (21,22) and in mammalian cells by the formation of an HMG1-DNA complex (23), have also shown no effect of supercoils on gene transfer. We have extended these findings here to cationic lipid-based transfections and to an in vivo gene delivery system. The binding of the cationic lipid to the plasmid results in the formation of a lipoplex. This lipoplex may contain single, or multiple copies of the plasmid. A variety of structures have been observed for the lipoplex (13,24–29), and no structure has been identified to be more active in transfection than others. From the results of the study presented here, there appears to be little structural constraint on the topological state of the plasmid in forming an active lipoplex.

No clear correlation has been demonstrated between transfection activity in vitro and in vivo. In vitro studies have not always been predictive of in vivo gene delivery results. Therefore, we extended our in vitro results to an in vivo tumor model. We found that by direct injections, with a DC-chol/DOPE-plasmid complex, no significant differences were found between supercoiled and relaxed DNA. For gene therapeutic applications, it has been a common goal to specify a high amount of supercoiled plasmid in the drug formulation (11,12,30). While the supercoiled amount is still an important stability indicator, we see no reason to regard a plasmid sample with a greater percentage of supercoiled plasmid as being more active. Changes in the supercoil amount over time are a valuable indicator of progressive DNA damage. Therefore, careful quantitation and monitoring of the supercoiled amounts must be done for a plasmid drug. However, optimizing conditions for limiting these changes is more important than maximizing the amount of supercoiled plasmid in the drug product.

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