Cationic Lipid-Mediated Gene Delivery to Murine Lung: Correlation of Lipid Hydration with in Vivo Transfection Activity

Michael J. Bennett,[†] Alfred M. Aberle,[†] Rajiv P. Balasubramaniam,[‡] Jill G. Malone,[‡] Robert W. Malone,*[,‡,§] and Michael H. Nantz*[,†]

Departments of Chemistry and Medical Pathology, University of California, Davis, California 95616

Received March 10, 1997[⊗]

A panel of lipidic tetraalkylammonium chlorides has been prepared and screened in studies of both lipid hydration and in vivo mouse transfection. The effect of cationic lipid structure on liposome surface hydration was determined using differential scanning calorimetry. Increases in headgroup steric bulk and the inclusion of *cis*-unsaturation in the hydrophobic domain led to greater lipid hydration, indicative of a decrease in lipid polar domain associations. Cationic lipids containing hydrogen-bonding functionality in the polar domain exhibited a corresponding decrease in observed lipid hydration, indicative of an increase in lipid polar domain associations. To explore a potential correlation of the hydration data with transfection activity, we examined the in vivo transfection activity of the lipid panel by direct intratracheal instillation of cationic liposome−DNA complexes into BALB/c mice. The more active transfection agents were the lipids that featured headgroup structures promoting close polar domain association in combination with fatty acyl *cis*-unsaturation. The hydration data suggest that the more effective transfection lipids for mouse lung delivery are those possessing the greatest imbalance between the cross-sectional areas occupied by the polar and hydrophobic domains.

Introduction

The potential to treat diseases via intracellular delivery of exogenous polynucleotides continues to motivate research in the burgeoning field of gene therapy. Gene-based therapies have been proposed for a variety of diseases including cystic fibrosis,[1,2] malignant melanoma,[3,4] and Gaucher's disease.[5,6] These and other[7] applications of gene therapy, however, have been encumbered by inefficient in vivo delivery of polynucleotides or by accompanying immunological and/or toxicity complications.[8,9]

Synthetic cationic lipids have emerged as a promising alternative to viral-based technologies for gene therapy.[10,11] Cationic lipids have been investigated extensively for their ability to facilitate the transfer and expression of foreign genes in a variety of cell types, both in vitro and in vivo.[12,13] The relative simplicity associated with the preparation of an active cationic lipid−polynucleotide complex, referred to as a lipoplex, and the ease of lipoplex administration combine to afford a method that is amenable to therapeutic development. However, the problem of suboptimal gene delivery remains as the principal obstacle in many in vivo applications. In some studies, it has been revealed that cationic lipids do not significantly enhance the delivery of DNA in comparison to treatments using uncomplexed ("naked") DNA.[14] This problem, and the added difficulty in achieving reproducible levels of in vivo activity,[15] underscores a need for the continued development of cationic lipid formulations.

As part of our program aimed toward the development of lipoplex preparations for use in pulmonary gene delivery, we have focused on the relationship between cationic lipid structure and in vivo transfection activity.[12,16] The development of efficacious lipoplex preparations for the transfection of lung tissues potentially may be useful in the treatment of a variety of pulmonary diseases including cystic fibrosis.[17,18] Our previous studies have concentrated on optimizing pulmonary gene delivery in mice.[12,16] An analysis of hydrophobic domain structure indicates that cationic lipids possessing fatty acyl side chains that destabilize close lipid packing either through side chain dissymmetry or by the presence of *cis*-unsaturation have enhanced murine lung transfection activity.[12] Also, an analysis of the anionic counterion, a polar domain component, suggests that certain counterions improve plasmid DNA transfection by effecting a greater polar domain dehydration.[16] A possible interpretation of these separate observations is that either the destabilization of hydrophobic domain packing or a decrease in the extent of polar domain hydration may afford liposome preparations that are more prone to undergo a localized reorganization to form nonbilayer structures.[19] This propensity for lipid reorganization may facilitate the process of membrane fusion.[20,21] Hence, the noted increase in transfection activity may result from the improved ability of the lipid−DNA complex to undergo membrane fusion. Since the propensity of lipids to undergo bilayer reorganization is related to both the polar and hydrophobic domain structures, it is anticipated that a combination of polar domain features that promote surface dehydration and hydrophobic domain features that discourage close side chain packing may result in more effective in vivo transfection agents.

The present study seeks to confirm the influence of cationic lipid polar domain hydration on in vivo transfection activity. By measuring the extent of lipid hydration, the influence of structure on associations between neighboring lipids may be evaluated.[22] The extent of lipid hydration may be estimated from measurements of the percent unfrozen water associated with

[†] Department of Chemistry.
[‡] Department of Medical Pathology.
[§] Current address: Department of Pathology, University of Maryland at Baltimore, Baltimore, MD 21201-1192.
[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1997.

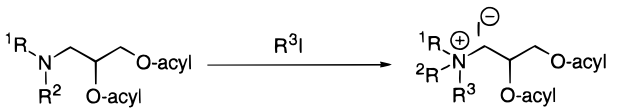
S0022-2623(97)00155-6 CCC: $14.00

Table 1. Cationic Lipid Preparation (eq 1) and Polar Domain Abbreviation

tertiary amine	R^3X	polar domain variant	abbr
a: $R^1, R^2 = CH_3$	CH_3I	**g**: $R^1, R^2, R^3 = CH_3$	−TAP
a: $R^1, R^2 = CH_3$	CH_3CH_2I	**h**: $R^1, R^2 = CH_3, R^3 = CH_2CH_3$	−EAP
a: $R^1, R^2 = CH_3$	$CH_3CH_2CH_2I$	**i**: $R^1, R^2 = CH_3, R^3 = CH_2CH_2CH_3$	−PAP
b: $R^1 = CH_3, R^2 = CH_2CH_2OH$	CH_3I	**j**: $R^1, R^3 = CH_3, R^2 = CH_2CH_2OH$	−RI
c: $R^1 = CH_3, R^2 = CH_2CH_2OCH_3$	CH_3I	**k**: $R^1, R^3 = CH_3, R^2 = CH_2CH_2OCH_3$	−MEP
d: $R^1, R^2 = CH_2CH_3$	CH_3I	**l**: $R^1, R^2 = CH_2CH_3, R^3 = CH_3$	−DEP
d: $R^1, R^2 = CH_2CH_3$	CH_3CH_2I	**m**: $R^1, R^2, R^3 = CH_2CH_3$	−TEP
e: $R^1, R^2 = CH_2CH_2OH$	CH_3I	**n**: $R^1, R^2 = CH_2CH_2OH, R^3 = CH_3$	−DHP
f: $R^1, R^2 = CH_2CH_2OCH_3$	CH_3I	**o**: $R^1, R^2 = CH_2CH_2OCH_3, R^3 = CH_3$	−DMP

cationic liposome preparations using subambient differential scanning calorimetry.[23] The use of cationic liposome preparations having similar composition to complex and deliver plasmid DNA (luciferase) into mouse lung would then establish the relative transfection efficacies. Consequently, the cationic liposome preparations were formulated using equimolar amounts of dioleoylphosphatidylethanolamine (DOPE):cationic lipid in both the hydration and transfection studies. In this way, the relationship between cationic lipid structure and hydration may be correlated with in vivo transfection activity. Herein we present the results of these hydration and murine lung transfection studies.

To influence the extent of cationic lipid hydrophobic and polar domain associations, a panel of dioleoyl and dimyristoyl tetraalkylammonium salts were prepared by quaternization of amines **1** and **2a**−**f** using various alkyl halides (eq 1, Table 1). The dioleoyl and dimyristoyl side chains were selected on the basis of conclusions from our previous study[12] of the hydrophobic domain influence on DNA transfection of murine lung. The spatial packing arrangement of the dimyristoyl lipids was expected to be more closely associated than the packing of corresponding dioleoyl analogues. The polar domain structures have been selected to independently assess the steric and hydrogen-bonding influences on lipid surface hydration. Cationic lipid polar domain steric influences on lipid hydration and murine lung transfection were determined by preparing unfunctionalized tetraalkylammonium salts varying in polar domain bulk and alkyl chain length (**g**−**i** and **l**−**m**). Increases in polar domain bulk and alkyl chain length were anticipated to decrease lipid association, thereby increasing the lipid surface hydration. The influences of hydrogen-bonding functionality in the cationic lipid polar domain were assessed by preparing hydroxy- and methoxy-functionalized tetraalkylammonium salts (**j**−**k** and **n**−**o**). The addition of hydrogen-bonding functionalities to the polar domain was anticipated to promote closer lipid−lipid associations, thereby decreasing the lipid surface hydration. By examining the analogous cationic lipids using an in vivo transfection model, it was anticipated that a correlation between lipid structure and transfection activity might be confirmed. Consequently, structural features of benefit in overcoming the limitations faced by nonviral gene transfer technologies might be identified.



1a-f **1** series: acyl = oleoyl **1g-p**
2a-f **2** series: acyl = myristoyl **2g-p** (1)

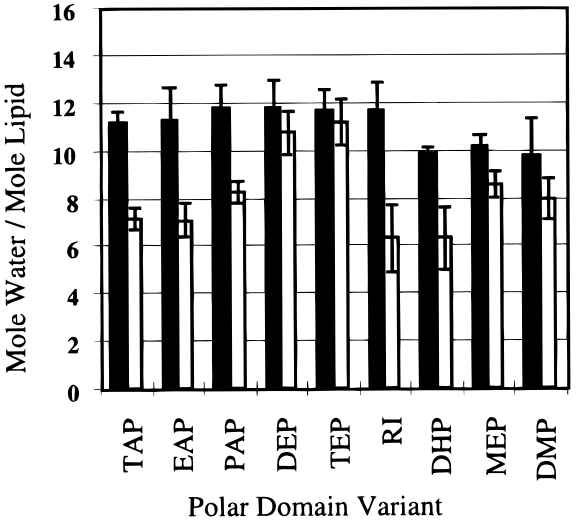


Figure 1. Lipid hydration as a function of modified polar domain structure for two hydrophobic domain series. The data are presented as the moles of water per mole of lipid (based on the average molecular weight of the cationic lipid and DOPE). Solid bars represent the dioleoyl series; open bars represent the dimyristoyl series.

Results

Influence of Cationic Lipid Structure on Lipid Surface Hydration. The lipid hydration study shows (Figure 1) that the dioleoyl derivatives (series **1**, Table 1) consistently had more lipid-associated water than the corresponding dimyristoyl derivatives (series **2**). The influence of the dioleoyl hydrophobic domain typically masked the polar domain influence on lipid−lipid association. This is evidenced by the insignificant changes in lipid hydration among the polar domain derivatives for the dioleoyl series. A lowering in lipid hydration is observed when hydroxyl or methyl ether functionality is introduced (e.g., DOMEP (**1k**), DODHP (**1n**), DODMP (**1o**) vs DOTAP (**1g**)). Statistical analysis of the hydration data for DOMEP and DODHP vs the prototypical unfunctionalized lipid DOTAP indicates the lowering is statistically significant at a 95% confidence level, and DODMP vs DOTAP is significant at an 80% confidence level.

For the dimyristoyl lipids, the influence of the polar domain structure on lipid−lipid associations was more pronounced. An increase in the chain length of one or more of the alkyl groups directly attached to nitrogen generally corresponded to an incremental increase in the observed mean hydration value. The increase in hydration is evident when the DMTAP (**2g**) hydration value is compared with those obtained for DMPAP (**2i**), DMDEP (**2l**), or DMTEP (**2m**). The dimyristoyl derivatives that contain hydroxyl functionality (DMRI (**2j**) and DMDHP (**2n**)) exhibited the lowest mean hydration

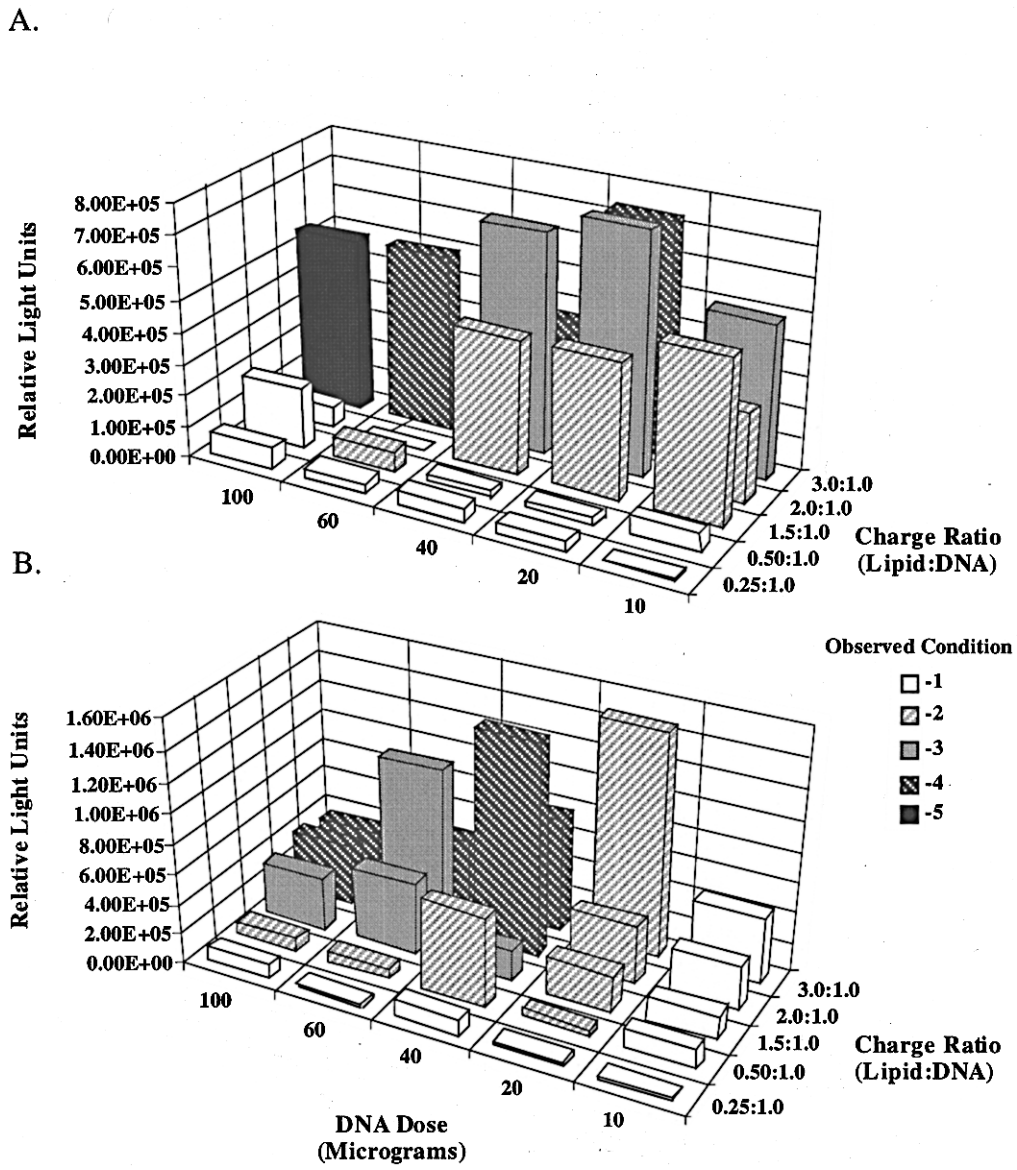


Figure 2. In vivo comparison of cationic lipid-mediated DNA transfection. Female BALB/c mice were transfected with the plasmid pNDCLux using (A) DODMP/DOPE and (B) DMDHP/DOPE cationic liposome preparations. Intratracheal installations of the lipoplexes were performed as described in the experimental procedure. Results are summarized in bar graph form as the mean (n = 4) and reported in relative luciferase light units obtained from samples of trachea/lung blocks lysed 24 h after treatment with the described amount of plasmid DNA. The condition of the mice after lipoplex delivery was scored as follows: 1, bright, alert, responsive; 2, eyes dull, significant piloerection, lethargic behavior; 3, significant piloerection, mice completely unresponsive with eyes closed, heavy labored breathing; 4, <50% fatality; 5, >50% fatality.

values. For these derivatives, conversion of the hydroxyl functionality to the corresponding methyl ether counteracted the hydroxyl effect and resulted in an increase in lipid hydration. For example, DMMEP (**2k**) and DMDMP (**2o**) were found to contain roughly 25% more lipid-associated water than their hydroxyl counterparts.

Optimization of Transgene Expression in Murine Lung. Two structurally dissimilar lipids (DODMP (**1o**) and DMDHP (**2n**)) were selected to represent the range of structural features present in the lipid panel. Lipoplexes containing DODMP and DMDHP were screened using a luciferase assay to determine optimal murine lung transfection conditions (Figures 2A and B). The DNA dose and the ratio of the cationic lipid to DNA (e.g. cationic lipid:DNAphosphate charge ratio) were varied to optimize the luciferase expression. We observed that these variables influenced both the level of measured transgene expression in murine lung tissue and the observed condition of the treated mice. Figures 2A and 2B show that lipoplex preparations containing DODMP or DMDHP with a cationic lipid:DNA charge ratio less than 0.5 exhibit low mean values of transfection activity. For the DNA doses investigated, the DODMP and DMDHP lipoplexes were generally most active at a lipid:DNA charge ratio of 2.0. The impact of treatment on the physiological condition of the mice was proportional to the cationic lipid:DNA charge ratio, an effect that was more pronounced at higher DNA doses.

By comparing the results presented in Figures 2A and 2B, some differences were observed in the levels of luciferase expression for a given formulation, and the concomitant effects on the physiological condition of the mice also varied. At charge ratios greater than 1.5, the observed luciferase expression in mouse lung after

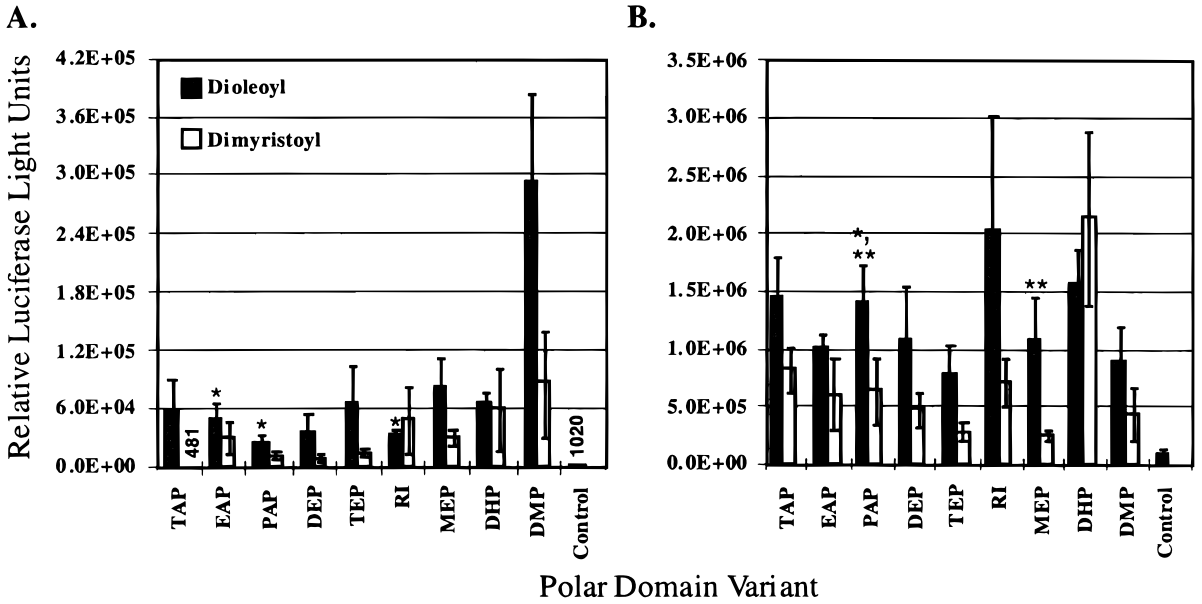


Figure 3. In vivo transfection comparison of cationic lipids. Cationic liposomes containing equimolar mixtures of the cationic lipid and DOPE were used to deliver (A) 20 μg or (B) 40 μg of pNDCLux plasmid DNA into BALB/c mouse lung. The activity is expressed as relative luciferase light units obtained from 30 μL of lysate obtained from a lung/trachea block 24 h after intratracheal administration of 200 μL of cationic liposome/DNA complexes. Groups of five mice were treated, and the data are depicted as the average observed transgene expression. The control for the experiment was instillation of uncomplexed "free" pNDCLux administered at the indicated dose. The error bars represent the standard deviation from the mean. (*) A single value was omitted because the animal expelled some of the fluid bolus after administration. (**) A single value was omitted due to fatality.

administration of lipoplexes containing DODMP was less responsive to the dose of DNA than analogous treatments using lipoplexes containing DMDHP. Likewise, lipoplexes with DODMP generally were more toxic when prepared at high DNA doses with high charge ratios than the corresponding DMDHP lipoplexes. For example, intratracheal administration of 100 μg of pNDCLux complexed with DODMP/DOPE at charge ratios of 2.0 or higher resulted in a greater than 50% fatalities. However, under identical formulation conditions, this mortality rate was not observed using lipoplex preparations containing DMDHP.

Plasmid DNA Transfection of Murine Lung. Noting that the DODMP and DMDHP lipoplex preparations were generally most active while least toxic at the 2:1 charge ratio, lipids **1**,**2g**−**o** were examined using lipoplexes containing 20 and 40 μg doses of plasmid DNA formulated at a 2:1 cationic lipid:DNA charge ratio. The comparative study was performed at two different DNA dose conditions to help minimize any bias toward a particular lipid or structural feature.

The results from the administration of 20 and 40 μg of luciferase reporter gene are given in Figures 3A and 3B, respectively. With the exception of the −EAP (at 20 μg), −RI (at 20 μg), and DHP (at 20 and 40 μg) variants, the dioleoyl derivatives were found to be significantly more active than the corresponding dimyristoyl derivatives. The relative differences in activity were dependent on DNA dose. The polar domain variations also led to changes in observed luciferase expression for a given hydrophobic domain. The most active lipids in both screens were those containing polar domain functionalization. The tetraalkylammonium lipids (unfunctionalized: −TAP, −EAP, −PAP, −DEP, and −TEP) exhibited an inverse correlation between the polar domain size and transfection activity. This trend was particularly evident among the dimyristoyl derivatives and also was observed with the dioleoyl derivatives at the 40 μg dose. With the exception of DMTAP at the 20 μg dose, the administration of free DNA was found to be significantly less active than any lipoplex preparation at either dose.

At the lower dose (20 μg, Figure 3A), the dioleoyl lipids possessing methyl ether functionality (DOMEP, DODMP) were more active than their hydroxyl counterparts. DODMP exhibited roughly a 4-fold improvement in activity vs DODHP, and likewise, DOMEP exhibited a similar 4-fold improvement vs DORI. Of the lipids studied, DODMP was the most active. DODMP lipoplexes were 4-fold more active than the second best lipoplex preparation comprised of DOMEP and 5-fold better than the lipoplex preparation comprised of the prototypic lipid DOTAP, an unfunctionalized cationic lipid. This result emphasizes the significance of adding additional polar functionality to the DNA-binding domain. Finally, close to 2 orders of magnitude increase in luciferase expression was observed when DODMP lipoplexes were used in lieu of 20 μg of uncomplexed DNA.

At the higher dose (40 μg, Figure 3B), the differences in transfection activity as a function of polar domain structure were less pronounced. Also, for a given headgroup structure, the hydrophobic domain composition did not influence transfection activity to the extent observed at the 20 μg dose. The dimyristoyl lipids generally had improved levels of transfection relative to their dioleoyl counterparts, yet in most instances the dioleoyl lipid remained the more active derivative. It is also interesting to note that the lipids containing hydroxyl functionality (DORI, DMRI, DODHP, and DMDHP) were on average 3-fold more active than their corresponding methyl ether derivatives (DOMEP, DMMEP, DODMP, and DMDMP, respectively). The inverse observation was noted at the 20 μg dose for the

dioleoyl derivatives. For example, the most active lipoplex preparation at the 40 μg dose contains the dimyristoyl lipid DMDHP. DMDHP promotes a 20-fold enhancement in luciferase expression relative to treatment with 40 μg of uncomplexed DNA. This result underscores the potential advantage in using lipoplex preparations to facilitate transfection of DNA into lung tissues.

Discussion

Investigations of lipid−lipid interactions and their influence on lipid aggregate properties have been focused principally on naturally occurring lipids. These studies have revealed that certain structural features promote close lipid associations. When these structural features are altered, the resultant change in lipid−lipid interactions has been shown to influence membrane properties such as the extent of surface hydration,[24] lipid polymorphism,[25] and phase transition temperatures.[26] It is likely that alterations in cationic lipid structure also will influence these properties and thereby influence the transfection process, particularly at stages involving membrane fusion. Thus, the incorporation of structural features to improve fusion events such as polynucleotide complexation[13,27] or cytoplasmic delivery[28] may lead to more active lipoplex formulations. Prior studies have shown that even minor structural changes to the lipid hydrophobic domain[12,29] or to the lipid polar domain[16,29,30,31] result in significant changes in transfection activity. Despite the attention devoted to understanding the physical bases for these observations, models to predict activity have not been developed. Studies correlating cationic lipid structure with the physical properties of derived liposome preparations and in vivo transfection activity have not been extensively published.[32] The present study has attempted to establish correlations between cationic lipid structure, lipid surface hydration, and cationic lipid-mediated in vivo transfection activity.

At present, the relationship between fusion ability and transfection activity remains unclear.[33−35] The fusion of cationic lipid−DNA complexes with cellular membranes is believed to be assisted by an initial electrostatic-mediated membrane association and surface dehydration and followed by bilayer reorganization to a nonbilayer lipid structure.[13] Membrane dehydration and imbalances between the effective cross-sectional area of the lipid polar and hydrophobic domains (Figure 4) are known to promote the localized bilayer reorganization.[36] In this regard, lipids exhibiting a cone-shaped molecular or hydration geometry should favor the formation of nonbilayer structures, as observed with other lipid systems.[37−39] Factors such as a small polar domain or close headgroup association decrease the effective cross-sectional area of the polar domain. These features would be expected to promote membrane fusion,[40] and they may be qualitatively identified by comparison of lipid hydration data. Likewise, factors such as *cis*-unsaturation or greater acyl chain hydration increase the effective cross-sectional area of the hydrophobic domain and thereby also would promote membrane fusion. Thus, the measurement of lipid hydration might causatively relate the lipoplex proclivity for membrane fusion.

Lipid hydration was measured to compare the relative strength of lipid associations. Studies using noncationic lipids show that lipid hydration is inversely related to the strength of the lipid−lipid interactions.[22] Hence, lipids containing functionality that promotes lipid association (e.g., hydrogen bonding) are expected to be less hydrated than lipids devoid of such functionality.[25] The decrease in lipid hydration occurs due to the greater exclusion of interstitial water, a consequence of increased lipid proximity. Conversely, lipids containing structural features that destabilize a close lipid association (e.g., steric bulk, *cis*-unsaturation) are expected to be more hydrated.[41]

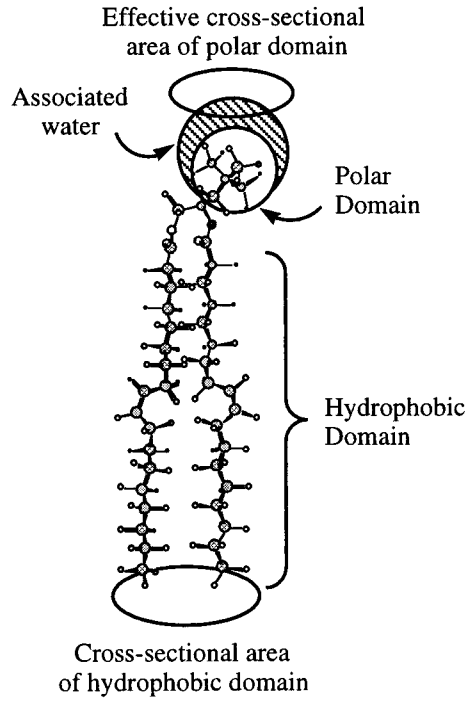


Figure 4. Lipid structural features. The representative cationic lipid DOTAP (**1g**) is depicted to illustrate the principal structural domains and related terminology.

The hydration studies presented in Figure 1 demonstrate that the changes in cationic lipid structure influence the lipid−lipid interactions in a predictable manner. The observed correlation between structure and lipid hydration is similar to that observed with natural phospholipids. As anticipated, cationic lipids containing oleoyl groups had more lipid-associated water than lipids containing myristoyl groups. The increase in observed lipid hydration is attributed to the presence of the *cis*-unsaturation which increases the cross-sectional diameter of the hydrophobic region and decreases lipid association relative to fully saturated fatty acyl domains.[36] In addition to accounting for a general increase in lipid hydration, the *cis*-unsaturation also appears to overshadow the influence of the polar domain on hydration.[42] This presumably accounts for the minor differences in lipid hydration observed between most members of the dioleoyl series. In regard to observable polar domain influences on lipid hydration, an increase in lipid hydration is noted as the bulk of the cationic lipid polar domain is increased. This trend is particularly evident among the dimyristoyl derivatives. The trend may be attributed either to an increase in steric repulsion between adjacent lipids or disruption of hydrogen bonding with DOPE, thereby decreasing close lipid packing. The observed correlation

of steric bulk with increased lipid hydration is consistent with previous reports.[43]

We noted a reduction in lipid hydration when hydroxyl functionality was incorporated into the cationic lipid polar domain. Again, the influence on hydration is more apparent among the dimyristoyl derivatives. The increase in lipid associations observed with these derivatives may be attributed to polar domain hydrogen bonding, particularly with the incorporated DOPE. It has been shown that the ammonium ion and the phosphate moiety of DOPE can participate in intermolecular hydrogen bonding.[44] The role of hydrogen bonding in increasing lipid associations is supported by the observed increase in lipid hydration that is noted when the hydroxyl group is converted to its corresponding methyl ether (DMRI → DMMEP and DMDHP → DMDMP). DMMEP and DMDMP both exhibit higher mean hydration values than their hydroxyl counterparts. This hydration result is consistent with the expected decrease in lipid association that occurs as a consequence of losing some hydrogen-bonding capability on alkyl ether formation. We also observed that the influence of hydrogen bonding overrides the influence of increasing steric bulk. For example, no change in lipid hydration was noted on increasing the polar domain bulk of DMRI by incorporating an additional hydroxymethyl group (DMRI → DMDHP).

The hydration data for the dioleoyl lipids DODHP, DOMEP, and DODMP show that these lipids possess the largest relative imbalance between the areas occupied by their polar and hydrophobic domains. The dioleoyl chains afford the larger hydrophobic domain cross-sectional area, and merger of this expanded hydrophobic domain with the least hydrated polar domains (DHP, MEP, and DMP) would assist the formation of a cone-shaped molecular geometry. Given this propensity for bilayer reorganization, these lipids should possess better fusion capabilities.[45]

We next examined the relationship between structure and transfection activity. To determine the influence of cationic lipid structure on optimal transfection conditions, the lipids DODMP and DMDHP were examined using a transfection matrix. Ideal transfection conditions would be those that confer the highest levels of DNA expression to the transduced cells with minimal treatment-associated toxicity. Optimization of transgene expression for a given transfection reagent is often obtained by varying the charge ratio of cationic lipid to DNA phosphate for a given DNA dose. While extensive in vitro research on these variables has been conducted,[29] in vivo studies that correlate both the cationic lipid:DNA charge ratio and the DNA dose with transfection activity have not been reported. To conduct the study, a matrix was designed to assess 25 different transfection conditions. Specifically, five cationic lipid:DNA charge ratios (0.25:1, 0.5:1, 1.5:1, 2:1, and 3:1) were examined at five different DNA doses (10, 20, 40, 60, and 100 μg).

The results of the optimization study (Figure 2) demonstrate that the lipoplex charge and DNA dose cooperatively influence in vivo transfection activity. The highest levels of luciferase expression using DODMP or DMDHP are observed when the lipoplex preparations have a net positive charge, an observation consistent with prior in vitro studies.[13] It is postulated that a positively charged lipoplex has a greater affinity for the negatively charged cellular membranes, thus facilitating lipoplex uptake.[11] The condition of the mice after treatment with either DODMP or DMDHP is impaired at high lipid:DNA charge ratios (>2) with high DNA doses (>40 μg). This is not unusual since cellular toxicity has been shown to generally correlate with increasing lipoplex dosage.[29,46] Increased incorporation of cationic lipids and DOPE within cellular membranes may affect cell membrane processes, such as signal transduction, and inhibits protein kinase C activity.[8,30,36] For the active (net positive) formulations, DODMP lipoplexes are more toxic than DMDHP lipoplexes at high DNA doses, a difference that may be attributed to any number of factors including physicochemical and colloidal properties of the lipoplexes. However, it should be noted that conditions exist for near optimal transfection activity without observable changes in the condition of the mice using either cationic lipid.

The transfection screen of the cationic lipid analogues (Figure 3) indicates a general correlation between cationic lipid structure and in vivo transfection activity. This study reveals that the effective geometric shape of the cationic lipid, as construed from the hydration data, strongly influences the efficiency of plasmid DNA transfection in murine lung. Generally, the more effective cationic lipids possess the greatest imbalance in the areas occupied by their polar and hydrophobic domains. Cationic lipids facilitating polar domain dehydration and hydrophobic domain destabilization exhibit the highest levels of luciferase expression. The correlation between hydration and transfection activity was observed with both functionalized and unfunctionalized cationic lipids. In cases where the hydration properties of unfunctionalized and functionalized polar domains were similar (e.g., DMTAP vs DMDHP), the functionalized lipid exhibited the greater transfection activity. This result suggests that the polar domain functionality may exert an additional beneficial effect and may be related to the greater ability of the functionalized cationic lipid to interact with cellular membranes via hydrogen bonding or by facilitating a greater electrostatic association. The strong hydrogen-bonding capabilities of these lipids apparently prevent the undesirable increase in headgroup hydration that would typically accompany an increase in effective headgroup charge. Thus, it may be most desirable to incorporate functionality that can inductively attenuate the polar domain yet still maintain minimal headgroup hydration. The observed correlation of transfection activity with lipid hydration suggests that membrane association and fusogenicity are primary determinants of lipid-mediated transfection activity. While this study suggests that membrane fusion may be an important step for in vivo transfection using lipoplex preparations, it should be noted that the fusion event, whether with plasma or endosomal membrane, is not the sole determinant of transfection efficiency.[35] Lipid mixing studies are needed to confirm the influence of our headgroup modifications and accompanying hydrated states on membrane fusion events.

Additional support for the correlations noted above may be found in the recent literature. The work of Solodin[32a] also has shown that the incorporation of unsaturation in the hydrophobic domain improves in

vivo lung transfection activity. Wheeler et al.[47] also have shown that the incorporation of headgroup functionality, specifically addition of a primary amine group to yield γAP-DLRIE, greatly improves in vivo lung transfection. It is interesting to speculate whether the lipid γAP-DLRIE enhances transfection relative to DOTAP in the same manner as the substitution of DOTAP with hydroxymethyl groups (e.g., DOTAP → DODHP).

The advantages imparted by certain cationic lipid structural features appear to be less pronounced at the higher DNA dose (40 μg). While this result may suggest that polar domain structural features impart minor advantages for transgene expression at high doses, it is important to note that the goal of lipoplex optimization for clinical applications necessarily dictates that cationic lipids be capable of effecting transgene expression with minimal toxic consequences. The use of an efficient cationic lipid at a lower concentration ultimately would be more desirable.

In summary, a panel of cationic lipids has been prepared to examine the relationships between lipid structure, lipid surface hydration, and plasmid DNA transfection activity. Correlation of lipid surface hydration with an in vivo transfection study reveals that cationic lipids possessing the greatest imbalance between the effective cross-sectional areas occupied by their polar and hydrophobic domains are the most active transfection agents. The present work in conjunction with our previous studies into the molecular effects of the hydrophobic and counterion domains suggest that the increase in transfection activity for these lipids may be due to enhanced membrane fusion properties. To examine the role of membrane fusogenicity as a next step toward optimizing in vivo transfection activity, we are pursuing studies on the relationship of cationic lipid hydration with the ability to undergo lipid mixing.

Experimental Section

Unless otherwise noted, all reagents were purchased from commercial suppliers and were used without further purification. The progress of reactions was monitored by thin-layer chromatography (TLC) using silica gel 60 F_{254} TLC plates. Visualization was accomplished by staining the plates with a 3% w/v phosphomolybdic acid/ethanol solution followed by charing on a hot plate. Silica gel 60 (230−400 mesh) was used for flash column chromatography. A Matteson Galaxy FTIR 3000 infrared spectrometer and a General Electric QE300 300 MHz nuclear magnetic resonance spectrometer were used to confirm structure and purity of the compounds. Melting point determinations were performed using a Thomas-Hoover capillary melting point apparatus and are uncorrected. Combustion analyses were performed by Midwest Microlabs (Indianapolis, IN).

The dioleoyl (series **1**) and the dimyristoyl (series **2**) lipids **1**,**2 a**−**f** (Table 1) were prepared in a manner analogous to the method previously reported.[48] Amine quaternizations were accomplished using one or the other of two synthetic methods, and an experimental procedure for each method is given below. Ion-exchange chromatography (iodide → chloride) was performed according to a literature procedure.[16] All ammonium chlorides were purified by column chromatography (silica gel, eluting with 5−10% methanol in dichloromethane).

Amine Quaternization. Method A (for derivatives g,j−**l).** The tertiary amine (0.5 g) was dissolved in iodomethane (5 mL), and the resulting solution was stirred for 8 h at room temperature. The residual iodomethane was removed by evaporation to afford a crude residue which was purified by column chromatography (silica gel), eluting with a gradient of 5−10% methanol in dichloromethane. **Method B (for derivatives h**−**i, and m**−**o).** A sealed tube was charged with the tertiary amine (0.5 g) and the appropriate alkyl iodide (5 mL). The tube was flushed with argon, sealed, and submersed in an oil bath maintained at 70 °C for 12 h. After the solution wsa cooled to room temperature, the residual alkyl iodide was removed by evaporation to afford the crude product. Purification was accomplished by column chromatography (silica gel), eluting with a gradient of 5−10% methanol in dichloromethane.

(±)-*N*,*N*,*N*-Trimethyl-*N*-[2,3-bis(9(Z)-octadecenoyloxy)-propyl]ammonium chloride (1g, DOTAP): mp 165−167 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.56 (m, 1H), 5.28 (m, 4H), 4.57 (d, *J* = 12 Hz, 1H), 4.47 (dd, *J* = 3, 12 Hz, 1H), 4.05 (dd, *J* = 6, 12 Hz, 1H), 3.73 (dd, *J* = 9, 14 Hz, 1H), 3.48 (s, 9H), 2.27 (m, 4H), 1.94 (m, 8H), 1.53 (m, 4H), 1.22 (m, 40H), 0.82 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 129.9, 129.5, 65.8, 65.6, 63.1, 54.1, 34.0, 33.7, 31.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.1, 27.0, 24.6, 24.5, 22.5, 14.0; IR (KBr) 2923, 2852, 1743 cm^{-1}. Anal. ($C_{42}H_{80}ClNO_4 \cdot H_2O$) C, H, N.

(±)-*N*,*N*,*N*-Trimethyl-N-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2g, DMTAP): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.59 (m, 1H), 4.59 (d, *J* = 14 Hz, 1H), 4.51 (dd, *J* = 4, 12 Hz, 1H), 4.09 (dd, *J* = 6, 12 Hz, 1H), 3.78 (dd, *J* = 9, 14 Hz, 1H), 3.52 (s, 9H), 2.32 (m, 4H), 1.57 (m, 4H), 1.23 (m, 40H), 0.86 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 172.7, 65.8, 63.1, 54.2, 34.2, 33.9, 31.8, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 24.7, 24.6, 22.6, 14.1; HRMS ($C_{34}H_{68}NO_4$) calcd 554.5148, found 554.5113.

(±)-*N*-Ethyl-*N*,*N*-dimethyl-*N*-[2,3-bis(9(Z)-octadecenoyloxy)propyl]ammonium chloride (1h, DOEAP): mp 156−158 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.58 (m, 1H), 5.31 (m, 4H), 4.50 (dd, *J* = 3, 12 Hz, 1H), 4.32 (d, *J* = 14 Hz, 1H), 4.09 (dd, *J* = 6, 12 Hz, 1H), 3.74 (m, 2H), 3.58 (m, 1H), 3.38 (s, 3H), 3.37 (s, 3H), 2.29 (q, *J* = 8 Hz, 4H), 1.97 (m, 8H), 1.55 (m, 4H), 1.41 (t, *J* = 7 Hz, 3H), 1.26−1.23 (m, 40H), 0.85 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 172.6, 129.9, 129.5, 65.5, 63.2, 62.4, 60.6, 51.1, 51.0, 34.0, 33.7, 31.8, 29.6, 29.5, 29.4, 29.2, 29.1, 29.0, 27.1, 27.0, 24.6, 24.5, 22.5, 14.0, 8.5; IR (KBr) 2923, 2854, 1741 cm^{-1}. Anal. ($C_{43}H_{82}ClNO_4 \cdot H_2O$) C, H, N.

(±)-*N*-Ethyl-*N*,*N*-dimethyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2h, DMEAP): mp 143−145 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.61 (m, 1H), 4.51 (dd, *J* = 3, 12 Hz, 1H), 4.32 (d, *J* = 14 Hz, 1H), 4.11 (dd, *J* = 6, 12 Hz, 1H), 3.82−3.63 (m, 3H), 3.45 (s, 3H), 3.43 (s, 3H), 2.30 (m, 4H), 1.56 (m, 4H), 1.42 (t, *J* = 7 Hz, 3H), 1.23 (s, 40H), 0.86 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 172.6, 65.6, 63.2, 62.3, 60.6, 51.2, 51.0, 34.0, 33.8, 31.7, 29.9, 29.7, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 24.6, 24.5, 22.5, 13.9, 8.5; IR (KBr) 2919, 2852, 1741 cm^{-1}. Anal. ($C_{35}H_{70}ClNO_4 \cdot H_2O$) C, H, N.

(±)-*N*,*N*-Dimethyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)-propyl]-*N*-propylammonium chloride (1i, DOPAP): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.59 (m, 1H), 5.32 (m, 4H), 4.50 (m, 2H), 4.11 (dd, *J* = 6, 14 Hz, 1H), 3.78 (dd, *J* = 9, 14 Hz, 1H), 3.50 (m, 8H), 2.31 (m, 4H), 1.90 (m, 8H), 1.81 (q, J = 7 Hz, 2H), 1.58 (m, 4H), 1.27 (m, 40H), 1.02 (t, *J* = 7 Hz, 3H), 1.86 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 129.9, 129.5, 66.4, 65.6, 63.2, 62.6, 51.9, 51.6, 34.1, 33.8, 31.8, 29.6, 29.4, 29.2, 29.1, 29.0, 27.1, 24.6, 24.5, 22.6, 16.3, 14.0, 10.5; IR (KBr) 2925, 2854, 1741 cm^{-1}. Anal. ($C_{44}H_{84}ClNO_4 \cdot H_2O$) C, H, N.

(±)-*N*,*N*-Dimethyl-*N*-propyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2i, DMPAP): mp 124−126 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.59 (m, 1H), 5.50 (dd, *J* = 3, 12 Hz, 1H), 4.39 (d, *J* = 14 Hz, 1H), 4.10 (dd, *J* = 6, 12 Hz, 1H), 3.77 (dd, *J* = 9, 14 Hz 1H) 3.52 (m, 2H), 3.41 (s, 6H), 2.29 (m, 4H), 1.80 (q, *J* = 9 Hz, 2H), 1.55 (m, 4H), 1.22 (s, 40H), 0.99 (t, *J* = 7 Hz, 3H), 0.84 (t, *J* = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 66.3, 65.6, 63.2, 62.9, 52.0, 51.6, 34.1, 33.8, 31.8, 29.5, 29.4, 29.2, 29.1, 29.0, 24.7, 24.5, 22.6, 16.2, 14.0, 10.5; IR (KBr) 2918, 2850, 1739 cm^{-1}; HRMS ($C_{36}H_{72}NO_4$) calcd 582.5461, found 582.5463.

(±)-*N*-(2-Hydroxyethyl)-*N*,*N*-dimethyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)propyl]ammonium chloride (1j, DORI[29]):

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.80 (m, 1H), 5.63 (s, 1H), 5.31 (m, 4H), 4.47 (dd, J = 3, 12 Hz, 1H), 4.38 (d, J = 14 Hz, 1H), 4.07 (m, 3H), 3.78 (m, 3H), 3.41, (s, 3H), 3.39 (s, 3H), 2.29 (q, J = 8 Hz, 4H), 1.97 (m, 8H), 1.56 (m, 4H), 1.25 (m, 40H), 0.85 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 130.0, 129.6, 129.5, 67.2, 65.7, 63.2, 55.7, 52.9, 52.7, 34.1, 33.8, 31.8, 29.7, 29.5, 29.5, 29.4, 29.3, 29.2, 29.1, 27.3, 27.2, 27.1, 24.7, 24.6, 22.6, 14.0; IR (KBr) 3222, 2923, 2854, 1741 cm^{-1}.

(±)-*N*−(2-Hydroxyethyl)-*N*,*N*-dimethyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2j, DMRI): mp 173−174 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.77 (m, 1H), 5.65 (m, 1H), 4.48 (dd, J = 3, 12 Hz, 1H), 4.36 (d, J = 14 Hz, 1H), 4.10 (m, 5H), 3.78 (m, 5H), 3.41 (s, 3H), 3.39 (s, 3H), 2.31 (q, J = 18 Hz, 4H), 1.57 (m, 4H), 1.23 (m, 40H), 0.86 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 172.7, 67.2, 65.7, 64.9, 63.3, 55.8, 52.9, 52.7, 34.2, 33.9, 31.9, 29.9, 29.8, 29.7, 29.6, 29.5, 29.5, 29.3, 29.3, 29.2, 29.1, 24.7, 24.6, 22.7, 14.1; IR (KBr) 3143, 2919, 2850, 1745 cm^{-1}. Anal. ($C_{35}H_{70}ClNO_5$) C, H, N.

(±)-*N*-(2-Methoxyethyl)-*N*,*N*-dimethyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)propyl]ammonium chloride (1k, DOMEP): mp 114−116 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.63 (m, 1H), 5.32 (m, 4H), 4.46 (dd, J = 3, 12 Hz, 1H), 4.20 (d, J = 14 Hz, 1H), 4.05 (dd, J = 6, 12 Hz, 1H), 3.96−3.77 (m, 5H), 3.42 (s, 3H), 3.78 (s, 6H), 2.30 (m, 4H), 1.98 (m, 8H), 1.57 (m, 4H), 1.27−1.25 (m, 40H), 0.86 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.7, 129.9, 129.5, 66.2, 65.7, 64.9, 63.9, 63.2, 59.0, 52.6, 52.5, 52.4, 34.0, 33.8, 31.8, 29.6, 29.5, 29.4, 29.2, 29.1, 29.0, 27.1, 27.0, 24.6, 24.5, 22.5, 14.0; IR (KBr) 2923, 2854, 1743 cm^{-1}. Anal. ($C_{44}H_{84}ClNO_5$) C, H, N.

(±)-*N*-(2-Methoxyethyl)-*N*,*N*-dimethyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2k, DMMEP): mp 109−110 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.62 (m, 1H), 4.5 (dd, J = 4, 12 Hz, 1H), 4.35 (d, J = 14 Hz, 1H), 4.12−3.77 (m, 4H), 3.50 (s, 3H), 3.45 (s, 3H), 3.37 (s, 3H), 2.30 (q, J = 7 Hz, 4H), 1.55 (m, 4H), 1.22 (s, 40H), 0.85 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 172.6, 66.1, 65.6, 64.8, 63.9, 63.1, 59.0, 52.5, 52.4, 52.3, 34.0, 33.7, 31.7, 29.0, 29.8, 29.8, 29.7, 29.5, 29.3, 29.2, 29.1, 28.9, 24.6, 24.5, 22.5, 13.9; IR (KBr) 2917, 2850, 1747 cm^{-1}. Anal. ($C_{36}H_{72}ClNO_5$) C, H, N.

(±)-*N*,*N*-Diethyl-*N*-methyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)propyl]ammonium chloride (1l, DODEP): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.54 (m, 1H), 5.26 (m, 4H), 4.48 (dd, J = 4, 12 Hz, 1H), 4.42 (d, J = 16 Hz, 1H), 4.09 (dd, J = 6, 12 Hz, 1H), 3.62 (m, 5H), 3.29 (s, 3H), 2.24 (m, 4H), 1.92 (m, 8H), 1.51 (m, 4H), 1.36 (m, 6H), 1.20 (m, 20H), 0.80 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 172.5, 129.9, 129.5, 129.4, 65.3, 63.3, 60.0, 57.5, 57.1, 48.0, 34.0, 33.7, 31.7, 29.6, 29.4, 29.2, 29.1, 29.0, 28.9, 27.1, 27.0, 24.6, 24.5, 22.5, 14.0, 8.2; IR (KBr) 2925, 2854, 1741 cm^{-1}. Anal. ($C_{44}H_{84}ClNO_4\cdot H_2O$) C, H, N.

(±)-*N*,*N*-Diethyl-*N*-methyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride (2l, DMDEP): mp 126−128 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.50 (m, 1H), 4.41 (m, 2H), 4.05 (dd, J = 6, 12 Hz, 1H), 3.54 (m, 5H), 3.22 (s, 3H), 2.19 (q, J = 7 Hz, 4H), 1.43 (q, J = 6 Hz, 4H), 1.32 (m, 6H), 1.12 (m, 40H), 0.74 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 65.4, 63.3, 60.1, 57.5, 57.1, 48.1, 34.1, 33.8, 31.8, 29.6, 29.4, 29.2, 29.2, 29.0, 24.7, 24.6, 22.6, 14.0, 8.3, 8.2; IR (KBr) 2917, 2850, 1739 cm^{-1}. Anal. ($C_{36}H_{72}ClNO_4\cdot H_2O$) C, H, N.

(±)-*N*,*N*,*N*-Triethyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)-propyl]ammonium chloride (1m, DOTEP): isolated as a wax; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.66 (m, 1H), 5.31 (m, 4H), 4.48 (dd, J = 4, 12 Hz, 1H), 4.34 (d, J = 15 Hz, 1H), 4.15 (dd, 6, J = 12 Hz, 1H), 3.83 (dd, J = 8, 15 Hz, 1H), 3.52 (m, 6H), 2.31 (m, 4H), 1.98 (m, 8H), 1.56 (m, 4H), 1.43 (t, J = 7 Hz, 9H), 1.27−1.24 (m, 40H), 0.86 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8, 172.4, 129.8, 129.4, 65.1, 63.1, 56.8, 54.3, 33.9, 33.7, 31.7, 29.5, 29.4, 29.3, 29.1, 29.0, 28.9, 27.0, 24.5, 24.4, 22.5, 13.9, 8.0; IR (KBr) 2924, 2852, 1739 cm^{-1}. Anal. ($C_{45}H_{86}ClNO_4\cdot H_2O$) C, H, N.

(±)-*N*,*N*,*N*-Triethyl-*N*-[2,3-bis(tetradecanoyloxy)propyl]-ammonium chloride (2m, DMTEP): mp 138−140 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.56 (m, 1H), 4.47 (dd, J = 4, 12 Hz, 1H), 4.20−4.10 (m, 2H), 3.78 (dd, J = 9, 15 Hz, 1H), 3.48 (m, 9H), 2.28 (m, 4H), 1.55 (m, 4H), 1.42 (t, J = 7 Hz, 9H), 1.21 (s, 40H), 0.84 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 172.6, 65.2, 63.2, 54.4, 34.0, 33.8, 31.8, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 24.6, 24.5, 22.6, 14.0, 8.0; IR (KBr) 2918, 2850, 1739 cm^{-1}. Anal. ($C_{37}H_{74}ClNO_4$) C, H, N.

(±)-*N*,*N*-[Bis(2-hydroxyethyl)]-*N*-methyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)propyl]ammonium chloride (1n, DODHP): isolated as a wax; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.68 (m, 1H), 5.33 (m, 4H), 4.46 (dd, J = 3, 12 Hz, 1H), 4.22−4.03 (m, 6H), 3.90−3.60 (m, 5H), 3.32 (s, 3H), 2.31, (q, J = 7 Hz, 4H), 2.00 (m, 8H), 1.58 (m, 4H), 1.29−1.26 (m, 40H), 0.87 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 172.7, 130.0, 129.6, 65.6, 65.1, 63.7, 63.6, 63.3, 55.7, 50.7, 34.1, 33.8, 31.9, 29.9, 29.7, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.2, 24.7, 24.6, 22.6, 14.1; IR (KBr) 32.53, 2924, 2854, 1741 cm^{-1}. Anal. ($C_{44}H_{84}ClNO_6$) C, H, N.

(±)-*N*,*N*-[Bis(2-hydroxyethyl)]-*N*-methyl-*N*-[2,3-bis(tetradecanoyloxy)propyl] ammonium chloride (2n, DMDHP): mp 170−172 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.66 (m, 1H), 5.43 (m, 2H), 4.44 (dd, J = 3, 12 Hz, 1H), 4.21 (d, J = 15 Hz, 1H), 4.06 (m, 5H), 3.77 (m, 5H), 3.31 (s, 3H), 2.28 (d, J = 8 Hz, 4H), 1.51 (m, 4H), 1.21 (m, 40H), 0.83 (t, J = 6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 172.7, 65.6, 65.5, 65.0, 63.5, 63.4, 63.3, 55.6, 50.7, 34.1, 33.8, 31.8, 30.31, 30.2, 30.2, 30.1, 30.1, 30.0, 29.9, 29.6, 29.5, 29.3, 29.1, 29.0, 24.7, 24.6, 22.5, 14.0; IR (KBr) 3261, 2919, 2852, 1741 cm^{-1}. Anal. ($C_{36}H_{72}ClNO_6$) C, H, N.

(±)-*N*,*N*-Bis(2-methoxyethyl)-*N*-methyl-*N*-[2,3-bis(9(*Z*)-octadecenoyloxy)propyl]ammonium chloride (1o, DODMP): isolated as a wax; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.64 (m, 1H), 5.31 (m, 4H), 4.30 (dd, J = 3, 12 Hz, 1H), 4.13−3.75 (m, 9H), 3.37 (s, 9H), 2.29 (m, 4H), 1.97 (m, 8H), 1.56 (m, 4H), 1.27−1.23 (m, 40H), 0.85 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8, 172.6, 129.8, 129.4, 66.0, 65.6, 63.5, 63.1, 62.9, 58.9, 49.7, 34.0, 33.7, 31.7, 29.5, 29.4, 29.3, 29.1, 29.0, 28.9, 27.0, 26.9, 24.5, 24.4, 22.4, 13.9; IR (KBr) 2925, 2854, 1741 cm^{-1}. Anal. ($C_{46}H_{88}ClNO_6\cdot H_2O$) C, H, N.

(±)-*N*,*N*-Bis(2-methoxyethyl)-*N*-methyl-*N*-[2,3-bis(tetradecanoyloxy)propyl] ammonium chloride (2o, DMDMP): mp 84−86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.65 (m, 1H), 4.42 (dd, J = 3, 12 Hz, 1H), 4.20−3.77 (m, 11H), 3.39 (s, 3H), 3.38 (s, 6H), 2.35−2.27 (m, 4H), 1.56 (m, 4H), 1.34−1.18 (m, 42), 0.86 (t, J = 7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 172.7, 66.1, 66.0, 65.7, 63.7, 63.6, 63.3, 63.2, 63.1, 63.0, 59.0, 50.0, 49.8, 34.1, 33.8, 31.7, 30.0, 29.9, 29.8, 29.7, 29.5, 29.3, 29.2, 29.1, 28.9, 24.6, 24.5, 22.5, 14.0; IR (KBr) 2919, 2850, 1741 cm^{-1}. Anal. ($C_{38}H_{76}ClNO_6\cdot H_2O$) C, H, N.

Hydration Studies. High-sensitivity, low-temperature differential scanning calorimetry (DSC) was performed using a Perkin-Elmer DSC 7 instrument with automatic data collection and analysis. Sample preparation was conducted as follows: a lipid thin film containing equimolar amounts of the cationic lipid and dioleoylphosphatidylethanolamine (DOPE) (total lipid mass of 4−6 mg) was dissolved in CH_2Cl_2 (100 μL), and the resultant solution was added to an aluminum DSC pan via microsyringe. The solvent was removed by warming the pan using a dry heating block heated to 30−50 °C. Any residual solvent was removed under vacuum overnight. The lipid sample was hydrated by addition of sterile deionized water to give a 40−60% w/w suspension. The DSC sample pan was then sealed and weighed on a Cahn electrobalance to the nearest microgram. The sealed lipid sample was annealed by repeated thermal cycling (3×) between −90 and 60 °C to ensure complete equilibration of the liposome suspension. Complete freezing of the bulk water was ensured by maintaining the sample at −90 °C for at least 5 min before warming. The sample was then warmed to −20 °C and allowed to equilibrate for ca. 3−5 min prior to scanning. The DSC scan was performed between −20 and 10 °C with a heating rate of 2 °C/minute. Each sample was recooled to −90 °C between scans. On completion of the data collection, the DSC pan was reweighed to determine any leakage, followed by dehydrating the lipid sample by puncturing the pan and heating in an oven at 80 °C for at least 48 h. Residual water was then removed by placing the sample in a flask under vacuum for an additional 48 h. Final lipid content was

determined by releasing the flask under vacuum in a drybox connected to an air dryer and immediately weighing the pan.

Hydration values were determined by the change in enthalpy under the ice melt peak in comparison to the enthalpy change observed for the melting of pure water. The ratio of unfrozen water (a measure of the lipid-associated water) in a given lipid sample was determined by dividing the average heat capacity of three scans by the total water removed (measured by weighing the punctured pan containing the dry lipid sample and subtracting from the weight of the sealed pan prior to scanning), and this value was then divided by the molar heat capacity of pure water. The quotient was subtracted from one to give the ratio of unfrozen water, and this value was multiplied by the total water removed to give the mass of unfrozen water. This value was then converted to moles of unfrozen water and divided by the moles of dry lipid sample, a technique which affords a measure of the moles of water per mole of lipid.[23] Three independent samples of each lipid were scanned three times according to the aforementioned procedure. Figure 1 was generated by plotting the average of the three means obtained for each lipid.

Liposome Formulations. The cationic lipid (1 mmol) and DOPE (1 mmol) were added as chloroform solutions to 1.9 mL sample vials. The chloroform was removed via rotary vacuum evaporation at 37 °C. The resulting thin lipid films were placed under vacuum overnight to ensure that all traces of solvent were removed. Sterile water (1 mL) was then added to each vial, and the lipid thin films were hydrated by being briefly warmed at 60 °C under argon with subsequent vortex mixing. The resultant 1 mM lipid suspensions were used within 12 h of hydration.

Plasmid DNA. The plasmid pNDCLux was prepared in our laboratories and consists of a modified *P. pyralis* luciferase open reading frame under the control of the human cytomegalovirus immediate early gene (hCMV IE) promoter/enhancer. Additional elements include the hCMV IE intron A and a polyadenylation sequence derived from the bovine growth hormone gene. The plasmids were transformed into competent *E. coli* DH5-α cells, amplified in Terrific Broth, and prepared by base lysis with the isolation of covalently closed circular plasmid DNA using two rounds of CsCl−EtBr gradient ultracentrifugation. The plasmid DNA was subsequently treated with DNAse-free RNAse, the phenol/chloroform was extracted, andthe solution was purified by precipitation from an ethanol/sodium acetate solution. DNA purity was determined by agarose gel electrophoresis and optical density (OD 260/280 greater than or equal to 1.8), and samples were routinely monitored for significant endotoxin contamination.

Lipoplex Preparations. Cationic lipid−DNA complexes were prepared as previously described.[49] pNDCLux DNA (10−100 μg) was slowly added to a diluted (sterile water) quantity of the cationic lipid suspension in a polystyrene tube (Falcon no. 2058) to give a final volume of 200 μL. Immediately after DNA addition, the resultant mixture was sequentially heated (30−60 s at 56 °C), sonicated (10−30 s) in a bath sonicator (Laboratory Supplies Inc., Hicksville, NY), and reheated (30−60 s at 56 °C). The lipoplex preparations were used in transfection experiments within 5−10 min of formation.

Intratracheal Instillation of DNA or Lipid/DNA Complexes. Female BALB/c mice (specific pathogen free, Charles River Laboratories) weighing approximately 20−21 g were used in the transfection experiments. Anesthesia was provided for invasive procedures. Neck dissections were performed on anesthetized mice using a 1 cm incision through the skin of the anterior neck. Instillation of 200 μL of the lipoplex preparation was performed using a $^1/_2$ in. 30 gauge needle inserted 1−3 tracheal ring interspaces inferior to the larynx. For comparison, free DNA solutions (200 μL containing 10−100 μg of DNA) were prepared in sterile water and instilled in a similar manner. After injection, the superficial neck wound was closed with staples. The mice were terminated 24 h after treatment by CO_2 in accordance with UCD guidelines. A tracheal/lung block was dissected and then homogenized in chilled lysis buffer (1 mL of lysis buffer from Enhanced Luciferase Assay Kit diluted (3×) with sterile water). An aliquot of the homogenate was assayed for luciferase protein as described below.

Luciferase Assays. Relative luciferase activity was determined using a Monolight 2010 luminometer (Analytical Luminescence Laboratories) by measuring luciferase light emissions from a 30 μL aliquot of the lysis homogenate. Light emissions were measured over a 10 s period. Activity is expressed as relative light units, which are a function of assay conditions, luciferase concentration, luminometer photomultiplier tube sensitivity, and background.

Acknowledgment. This research was supported by grants from the Cystic Fibrosis Foundation, the California Tobacco Related Disease Research Program (4KT-0205), U.S. Army (DAMD 17-94-J-4436), and the Promega Corporation (95K076). The authors thank Ms. Tracie Collier and Ms. Rola Bou-Salman for assistance in cationic lipid synthesis and purification, Mr. Phillip Montbriand for plasmid DNA preparation, and Prof. John H. Crowe and Dr. Lois M. Crowe (Department of Cellular and Molecular Biology, University of California, Davis) for their generosity in providing the differential scanning calorimeter and for useful discussions.

References

(1) (a) Caplen, N. J.; Kinrade, E.; Sorgi, F.; Gao, X.; Gruenert, D.; Geddes, D.; Coutelle, C.; Huang, L.; Alton, E. W. F. W.; Williamson, R. In Vitro Liposome-Mediated DNA Transfection of Epithelial Cell Lines Using the Cationic Liposome DC−Chol/DOPE. *Gene Ther.* **1995**, *2*, 603−613. (b) Caplen, N. J.; Alton, E. W. F. W.; Middleton, P. G.; Dorin, J. R.; Stevenson, B. J.; Gao, X.; Durham, S. R.; Jeffery, P. K.; Hodson, M. E.; Coutelle, C.; Huang, L.; Porteous, D. J.; Williamson, R.; Geddes, D. M. Liposome-Mediated CFTR Gene Transfer to the Nasal Epithelium of Patients With Cystic Fibrosis. *Nature Med.* **1995**, *1*, 39−46.

(2) (a) Hay, J. G.; McElvaney, N. G.; Herena, J.; Crystal, R. G. Modification of Nasal Epithelial Potential Differences of Individuals With Cystic Fibrosis Consequent to Local Administration of a Normal CFTR cDNA Adenovirus Gene Transfer Vector. *Hum. Gene Ther.* **1995**, *6*, 1487−96. (b) Crystal, R. G.; McElvaney, N. G.; Rosenfeld, M. A.; Chu, C. S.; Mastrangeli, A.; Hay, J. G.; Brody, S. L.; Jaffe, H. A.; Elissa, N. T.; Danel, C. Administration of an Adenovirus Containing the Human CFTR cDNA to the Respiratory Tract of Individuals With Cystic Fibrosis. *Nature Genet.* **1994**, *8*, 42−51.

(3) Nabel, G. J.; Nabel, E. G.; Yang, Z. Y.; Fox, B. A.; Plautz, G. E.; Gao, X.; Huang, L.; Shu, S.; Gordon, D.; Chang, A. E. Direct Gene Transfer With DNA-Liposome Complexes in Melanoma: Expression, Biologic Activity, and Lack of Toxicity in Humans. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11307−11311.

(4) Stingl, G.; Brocker, E. B.; Mertelsmann, R.; Wolff, K.; Schreiber, S.; Kampgen, E.; Schneeberger, A.; Dummer, W.; Brennscheid, U.; Veelken, H. Phase I Study to the Immunotherapy of Metastatic Malignant Melanoma By a Cancer Vaccine Consisting of Autologous Cancer Cells Transfected With the Human IL-2 Gene. *Hum. Gene Ther.* **1996**, *7*, 551−63.

(5) Dunbar, C.; Kohn, D. Retroviral Mediated Transfer of the cDNA for Human Glucocerebrosidase Into Hematopoietic Stem Cells of Patients With Gaucher Disease. A Phase I Study. *Hum. Gene Ther.* **1996**, *7*, 231−253.

(6) Wells, S.; Malik, P.; Pensiero, M.; Kohn, D. B.; Nolta, J. A. The Presence of an Autologus Marrow Stromal Cell Layer Increases Glucocerebrosidase Gene Transduction of Long-Term Culture Initiating Cells (LTCICs) From the Bone Marrow of a Patient With Gaucher Disease. *Gene Ther.* **1995**, *2*, 512−520.

(7) Herrmann, F. Clinical Application of Gene Transfer. *J. Mol. Med.* **1996**, *74*, 213−221.

(8) Felgner, P. L.; Gadek, T.; Holm, M.; Roman, R.; Chan, W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielson, M. Lipofection: A Highly Effiecient Lipid-Mediated DNA Transfection Procedure. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413−7417.

(9) Kozarsky, K.; Grossman, M.; Wilson, J. M. Adenovirus-Mediated Correction of the Genetic Defect In Hepatocytes From Patients With Familial Hypercholesterolemia. *Somat. Cell Mol. Genet.* **1993**, *19*, 449−458.

(10) Alton, E. W.; Geddes, D. M. Gene Therapy for Cystic Fibrosis: A Clinical Perspective. *Gene Ther.* **1995**, *2*, 88−95.

(11) Behr, J.-P. Gene Transfer with Synthetic Cationic Amphiphiles: Prospects for Gene Therapy. *Bioconjugate Chem.* **1994**, *5*, 382−389.

(12) Balasubramaniam, R. P.; Bennett, M. J.; Aberle, A. M.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Structural and Functional Analysis of Cationic Transfection Lipids: The Hydrophobic Domain. *Gene Ther.* **1996**, *3*, 163−172.
(13) Felgner, P. L.; Tsai, Y. J.; Sukhu, L.; Wheeler, C. J.; Manthorpe, M.; Marshall, J.; Cheng, S. H. Improved Cationic Lipid Formulations for In Vivo Gene Therapy. *Ann. New York Acad. Sci.* **1995**, *772*, 126−139.
(14) Meyer, K. B.; Thompson, M. M.; Levy, M. Y.; Barron, L. G.; Szoka, F. C. Intratracheal Gene Delivery to the Mouse Airway: Characterization of Plasmid DNA Expression and Pharmacokinetics *Gene Ther.* **1995**, *2*, 450−460.
(15) Alton, E. W.; Middleton, P. G.; Caplin, N. J.; Smith, S. N.; Steel, D. M.; Munkonge, F. M.; Jeffery, P. K.; Geddes, D. M.; Hart, S. L.; Williamson, R. Non-Invasive Liposome-Mediated Gene Delivery Can Correct the Ion Transport Defect in Cystic Fibrosis Mutant Mice. *Nat. Genet.* **1993**, *5*, 135−142.
(16) Aberle, A. M.; Bennett, M. J.; Malone, R. W.; Nantz, M. H. The Counterion Influence on Cationic Lipid-Mediated Transfection of Plasmid DNA. *Biochim. Biophys. Acta* **1996**, *1299*, 281−283.
(17) Curiel, D. T.; Pilewski, J. M.; Albelda, S. M. Gene Therapy Approaches for Inherited and Acquired Lung Diseases. *Am. J. Respir. Cell Mol. Biol.* **1996**, *14*, 1−18.
(18) (a) Sorscher, E. J.; Logan, R. A.; Frizzell, R. A.; Lyrene, R. K.; Bebok, S.; Dong, J. Y.; Duval, M. D.; Felgner, P. L.; Matalon, S.; Walker, L.; Wiatrak, B. R. Gene Therapy for Cystic Fibrosis Using Cationic Liposome Mediated Gene Transfer: A Phase I Trial of Safety and Efficacy in the Nasal Airway. *Hum. Gene Ther.* **1994**, *5*, 1259−1277. (b) Brigham, K. L.; Meyrick, B.; Christman, B.; Magnuson, M.; King, G.; Berry, L. C., Jr. In Vivo Transfection of Murine Lungs with a Functioning ProKaryotic Gene Using a Liposome Vehicle. *Am. J. Med. Sci.* **1989**, *298*, 278−281.
(19) Israelachvili, J. N.; Marcelja, S.; Horn, R. G. Physical Principles of Membrane Organization. *Q. Rev. Biophys.* **1980**, *13*, 121−200.
(20) Bentz, J.; Ellens, H.; Lai, M.-Z.; Szoka, F. C. On the Correlation Between Hexagonal II Phase and the Contact-Induced Destabilization of Phosphatidylethanolamine Containing Membranes. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5742−5745.
(21) Siegel, D. P. Inverted Micellar Intermediates and the Transitions Between Lamellar, Cubic and Inverted Hexagonal Lipid Phases II. *Biophys. J.* **1986**, *49*, 1171−1183.
(22) Crowe, J. H.; Crowe, L. M. in *Biological Membranes*; Chapman. D., Ed.; Academic Press: London, 1984; pp 57−102.
(23) Crowe, L. M.; Spargo, B. J.; Ioneda, T.; Beaman, B. L.; Crowe, J. H. Interaction of Cord Factor (Alpha, Alpha′-Trehalose-6-6′-Dimycolate) With Phospholipids. *Biochim. Biophys. Acta* **1994**, *1194*, 53−60.
(24) Epand, R. M.; Bryszewska, M. Modulation of the Bilayer to Hexagonal Phase Transitions and Solvation of Phosphatidylethanolamines In Aqueous Salt Solutions. *Biochemistry* **1988**, *27*, 8776−8779.
(25) Boggs, J. M. Lipid-Intermolecular Hydrogen-Bonding: Influence on Structural Organization and Membrane Function. *Biochim. Biophys. Acta* **1987**, *906*, 353−404.
(26) Blume, A. A Comparative; Study of the Phase Transitions of Phospholipid Bilayers and Monolayers. *Biochim. Biophys. Acta* **1979**, *557*, 32−44.
(27) (a) Lasic, D. D.; Strey, H.; Stuart, M. C. A.; Podgornik, R.; Frederik, P. M. The Structure of DNA-Liposome Complexes. *J. Am. Chem. Soc.* **1997**, *119*, 832−833. (b) Rädler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. Structure of DNA-Cationic Liposome Complexes: DNA Intercalation in Multilamellar Membranes in Distinct Interhelical Packing Regimes. *Science* **1997**, *275*, 810−814. (c) Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A. Mode of Formation and Structural Features of DNA-Cationic Liposome Complexes Used for Transfection. *Biochemistry* **1993**, *32*, 7143−7151.
(28) Xu, Y.; Szoka, F. C. Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection. *Biochemistry* **1996**, *35*, 5616−5623.
(29) Felgner, J. H.; Kumar, R.; Sridhar, C. N.; Wheeler, C. J.; Tsai, Y. J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P. L. Enhanced Gene Delivery and Mechanism Studies With a Novel Series of Cationic Lipid Formulations. *J. Biol. Chem.* **1994**, *269*, 2550−2561.
(30) Farhood, H.; Bottega, R.; Epand, R. M.; Huang, L. Effect of Cationic Cholesterol Derivatives on Gene Transfer and Protein Kinase C Activity. *Biochim. Biophys. Acta* **1992**, *1111*, 239−246.
(31) Remy, J.-S.; Sirlin, C.; Vierling, P.; Behr, J.-P. Gene Transfer With a Series of Lipophilic DNA-Binding Molecules. *Bioconjugate Chem.* **1994**, *5*, 647−654.
(32) (a) Solodin, I.; Brown, C. S.; Bruno, M. S.; Chow, C.-Y.; Jang, E.-H.; Debs, R. J.; Heath, T. D. A Novel Series of Amphiphilic Imidazolinium Compounds for In Vitro and In Vivo Gene Delivery. *Biochemistry* **1995**, *34*, 13537−13544. (b) Egilmez, N. K.; Iwanuma, Y.; Bankert, R. B. Evaluation and Optimization of Different Cationic Liposome Formulations for In Vivo Gene Transfer. *Biochem. Biophys. Res. Commun.* **1996**, *221*, 169−173.
(33) Farhood, H.; Serbina, N.; Huang, L. The Role of Dioleoylphosphatidylethanolamine In Cationic Liposome Mediated Gene Delivery. *Biochim. Biophys. Acta* **1995**, *1235*, 289−295.
(34) (a) Wrobel, I.; Collins, D. Fusion of Cationic Liposomes With Mammalian Cells Occurs After Endocytosis. *Biochim. Biophys. Acta* **1995**, *1235*, 296−304. (b) Friend, D. S.; Papahadjopoulos, D.; Debs, R. J. Endocytosis and Intracellular Processing Accompanying Transfection Mediated by Cationic Liposomes. *Biochim. Biophys. Acta* **1996**, *1278*, 41−50.
(35) Stegmann, T.; Legendre, J.-Y. Gene Transfer Mediated By Cationic Lipids: Lack of a Correlation Between Lipid Mixing and Transfection. *Biochim. Biophys. Acta* **1997**, *1325*, 71−79.
(36) Epand, R. M. Relationship of Phospholipid Hexagonal Phases to Biological Phenomena. *Biochem. Cell Biol.* **1990**, *68*, 17−23.
(37) Brown, P. M.; Steers, J.; Hui, S. W.; Yeagle, P. L.; Silvius, J. R. Role of Headgroup Structure in the Phase Behavior of Amino Phospholipids. 2. Lamellar and Nonlamellar Phases of Unsaturated Phosphatidylethanolamine Analogues. *Biochemistry* **1986**, *25*, 4259−4267.
(38) Cullis, P. R.; De Kruijff, B. Lipid Polymorphism and the Functional Roles of Lipids in Biological Membranes. *Biochim. Biophys. Acta* **1979**, *559*, 399−420.
(39) Cullis, P. R.; Hope, M. J.; Tilcock, C. P. S. Lipid Polymorphism and The Roles of Lipid in Membranes. *Chem. Phys. Lipids* **1986**, *40*, 127−144.
(40) Kinnunen, P. K. J. On the Mechanisms of the Lamellar → Hexagonal H_{II} Phase Transition and the Biological Significance of the HII Propensity. In *Nonmedical Applications of Liposomes*, 1st ed.; Lasic, D. D., Barenholz, Y., Eds.; CRC Press: Boca Raton, FL, 1996; Vol. 1, pp 153−171.
(41) Jendrasiak, G. L; Hasty, J. H. The Hydration of Phospholipids. *Biochim. Biophys. Acta* **1974**, *337*, 79−91.
(42) Slater, S. J.; Ho, C.; Taddeo, F. J.; Kelly, M. B.; Stubbs, C. D. Contribution of Hydrogen Bonding to Lipid-Lipid Interactions in Membranes and the Role of Lipid Order: Effects of Cholesterol, Increased Phospholipid Unsaturation, and Ethanol. *Biochemistry* **1993**, *32*, 3714−3721.
(43) Lafrance, D.; Marion, D.; Pezolet, M. Study of the Structure of N-Acyldipalmitoyl-phosphatidylethanolamine in Aqueous Dispersions By Infrared and Raman Spectroscopies. *Biochemistry* **1990**, *29*, 4592−4599.
(44) Papahadjopoulos, D.; Weiss, L. Amino Groups at the Surfaces of Phospholipid Vesicles. *Biochim. Biophys. Acta* **1969**, *183*, 417−426.
(45) Cone-shaped lipids have been shown previously to improve DNA transfer, see: Ibáñez, M.; Gariglio, P.; Chávez, P.; Santiago, R.; Wong, C.; Baeza, I. Spermidine-condensed DNA and Cone-Shaped Lipids Improve Delivery and Expression of Exogenous DNA Transfer By Liposomes. *Biochem. Cell. Biol.* **1996**, *74*, 633−643.
(46) Templeton, N. S.; Lasic, D. D.; Frederik, P. M.; Strey, H. H.; Roberts, D. D.; Pavlakis, G. N. Improved DNA: Liposome Complexes for Increased Systemic Delivery and Gene Expression. *Nat. Biotechnol.* **1997**, *15*, 647−652.
(47) Wheeler, C. J.; Felgner, P. L.; Tsai, Y. J.; Marshall, J.; Sukhu, L.; Doh, S. G.; Hartikka, J.; Nietupski, J.; Manthorpe, M.; Nichols, M.; Plewe, M.; Liang, X.; Norman, J.; Smith, A.; Cheng, S. H. A Novel Cationic Lipid Greatly Enhances Plasmid DNA and Expression in Mouse Lung. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11454−11459.
(48) Bennett, M. J.; Malone, R. W.; Nantz, M. H. A Flexible Approach to Synthetic Lipid Ammonium Salts for Polynucleotide Transfection. *Tetrahedron Lett.* **1995**, *36*, 2207−2210.
(49) Bennett, M. J.; Aberle, A. M.; Balasubramaniam, R. P.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Considerations for the Design of Improved Cationic Amphiphile-Based Transfection Reagents. *J. Liposome Res.* **1996**, *6*, 615−624.

JM970155Q