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Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytofectin complexes

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Abstract

Cytofectins are positively charged lipophilic molecules that readily form complexes with DNA and other anionic polynucleotides. Normally, cytofectins are combined with an activity-augmenting phospholipid such as dioleoylphosphatidylethanolamine (DOPE), and a film of dried, mixed lipid is prepared and hydrated to form cationic liposomes. The liposome solution is then mixed with a plasmid DNA solution to afford cytofectin-DNA complexes which, when presented to living cells, are internalized and the transgene is expressed. One of the most potent cytofectins, dimyristoyl Rosenthal inhibitor ether (DMRIE), is presently being used to deliver transcriptionally active DNA into human tumor tissues. Here we report the remarkable consequences of replacing the alcohol moiety of DMRIE with a primary amine. The resulting cytofectin, called β-aminoethyl-DMRIE (βAE-DMRIE), promoted high level transfection over a broad range of DNA and cationic lipid concentrations. A comparison of in vitro transfection activity between DMRIE and βAE-DMRIE in 10 cell types revealed that βAE-DMRIE was more active than DMRIE, and that βAE-DMRIE, unlike DMRIE, was maximally effective in the absence of colipid. The consequences of the alcohol-to-amine conversion on the structure of the cytofectin/DNA complex was also examined by Atomic Force Microscopy. Strikingly dissimilar images were found for plasmid DNA alone and for the plasmid complexes of βAE-DMRIE and DMRIE/DOPE.

Keywords: Liposome; Reporter gene; Gene expression; LacZ; β-Galactosidase; Ultrastructure; Cytofectin; Atomic force microscopy

1. Introduction

Cytofectins are used increasingly to deliver functionally active polynucleotides into cells. Several cytofectins are available commercially for the transfection of cultured cells (e.g., Lipofectin®, LipofectACE®, LipofectAMINE®, Transfeactam®, and DOTAP®). Two cytofectins, DMRIE (dimyristoyl Rosenthal inhibitor ether) and DC-cholesterol (dimethylaminoethylcarbamoyl cholesterol) are currently being used in human clinical gene therapy trials to deliver DNA into cells [1–9]. It is presently unclear which chemical and physical properties of cytofectins are responsible for their ability to deliver polynucleotides into cells [10]. Most cytofectins reported to date possess long chain hydrocarbon groups attached to a polar head group. The more active cytofectins possess a net positive charge, and associate into layered structures containing multiple positive charges on the surface. The cytofectins described to date require mixing with 'neutral', zwitterionic lipids such as DOPE (dioleoylphoshatidylethanolamine) in order to elicit optimal polynucleotide delivery activity [11–14]. By combining cytofectins with neutral lipids, a liposome is formed that possesses properties of both amphipathic substituents. Such composite liposomes are usually much more effective at transfection than either substituent alone. The net polycationic surface of cationic liposomes is likely to

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interact strongly with the polyanionic phosphate backbone of polynucleotides. If cationic charges are present on the surface of the polynucleotide/cytofectin/neutral lipid complex, then the complex should interact strongly with the predominantly negative charge of the cell surface.

DMRIE, one of the most active cytofectins described to date, has a quaternary nitrogen adjacent to a primary alcohol, thus imparting a pH independent positive charge. It was predicted that substituting a primary amine group for the alcohol of DMRIE would: (1) maintain and perhaps enhance the cytofectin's ability to hydrogen bond with DNA, a property which has been shown to be important [10,11]; (2) increase the cytofectin's net positive charge; (3) allow bidentate interactions between the quaternary nitrogen/primary amine and polyanionic structures. This amino compound, termed βAE-DMRIE (β-aminoethyl-DMRIE), was synthesized and compared with DMRIE. βAE-DMRIE was found to form structurally distinct complexes with DNA compared to DMRIE, and βAE-DMRIE, unlike DMRIE, was generally able to transfect cells effectively in the absence of helper co-lipids.

2. Materials and methods

2.1. DNA preparation and quality control

The two plasmid DNAs used in these studies, pVCL-1005 (4951 bps) and pRSV-LacZ (5319 bps), code for, respectively, human HLA-B7 + β2 microglobulin proteins [1,2] and bacterial β-galactosidase [6]. Eucaryotic expression of both plasmids is regulated by the RSV promoter. Bulk amounts of plasmid were prepared as described [15,16]. Briefly, the plasmid DNAs were transformed into *Escherichia coli* DH5a competent cells and grown in Terrific Broth supplemented with 100 μg/ml ampicillin in a 5L Fermenter (Applikon). Temperature was controlled at 30°C ± 0.5°C, pH was controlled at 7.0 ± 0.5, stirring at 600 rpm ± 50 rpm, and air flow was set at 5 l/l/min. Cells were harvested by centrifugation at the end of exponential growth phase. Covalently closed circular plasmid DNA was isolated by a modified lysis procedure (no use of enzymes, but the use of salt precipitation to reduce RNA contamination) followed by standard double CsCl-ethidium bromide gradient ultracentrifugation. Endotoxin was determined by the *Limulus* Amebocyte Lysate (Associates of Cape Cod) assay and protein was determined by the Bicinchoninic acid (BCA; Pierce) All plasmid preparations were free of detectable chromosomal DNA and RNA and from protein impurities based on gel analysis and BCA assay, respectively. Endotoxin levels were less than 60 EU/μg of plasmid DNA. The A_{260}/A_{280} ratios were between 1.75 and 2.00. Plasmids were ethanol precipitated and the DNA was resuspended in USP saline at 4°C until dissolved. Aliquots of DNA were stored at −76°C until use.

2.2. Cytofectin components

2.2.1. (±)-N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE)

The intermediate from which the cationic lipids were made, (±)-*N*,*N*-dimethyl-(2,3-bis(tetradecyloxy))propylamine, or (DMP-DMA), was prepared as follows. Racemic 1-dimethylamino-2,3-propanediol (1.96 g; Janssen Chimica) was converted to the disodium salt in situ by treatment with sodium hydride (60% in oil, 1.65 g) in tetrahydrofuran (110 ml). Condensation with tetradecyl methane sulfonate (10.1 g; NuChekPrep) afforded crude DMP-DMA. This material was purified to homogeneity by extraction and then silica gel chromatography employing a step gradient of ether in hexane. The structure and purity were confirmed by ^{1}H-NMR and IR. ^{1}H-NMR ($CDCl_3$, TMS): δ 3.6–3.4 (overlapping m, 7H), 2.38 (m, 2H), 2.26 (s, 6H), 1.54 (m, 4H), 1.25 (s, 44H), 0.88 (t, $J = 6.7$ Hz, 6H). IR (film): cm^{-1} 2900, 1450, 1110, 725.

For DMRIE preparation, DMP-DMA (5.12 g) was treated with bromoethanol (2.19 g) in dimethylformamide (15 ml) at elevated temperature (110°C, overnight) to effect quaternization of the amine. Evaporative removal of the dimethylformamide followed by silica gel chromatography employing chloroform/methanol/aqueous ammonia as eluant afforded homogenous material. The expected structure and purity were confirmed by ^{1}H-NMR, IR, and elemental analysis. ^{1}H-NMR ($CDCl_3$, TMS): δ 5.01 (t, $J = 5.8$ Hz, 1H), 4.16 (br. s, 2H), 4.08 (m, 1H), 3.95–3.60 (overlapping m, 4 H), 3.55 (d, $J = 4.1$ Hz, 2H), 3.51–3.3 (overlapping m and s, 9 H), 1.55 (m, 4H), 1.26 (s, 44H), 0.88 (t, $J = 6.7$ Hz, 6H). IR (KBr): cm^{-1} 3300 (br), 1460, 1060, 955, 725. Elemental analysis [$C_35H_{74}O_3NBr$]: calc. C: 66.00, H: 11.71, N: 2.20, Br: 12.55; found C: 66.13, H: 11.58, N: 2.10, Br: 12.75.

2.2.2. (±)-N-(2-Aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (βAE-DMRIE)

DMP-DMA (4 g) was treated with 2-bromoethylphthalimide (4 g; Aldrich) in dimethylformamide (15 ml) at elevated temperature (110°C, overnight) to effect quaternization of the amine. Evaporative removal of the dimethylformamide followed by silica gel chromatography employing chloroform/methanol/aqueous ammonia as eluant afforded TLC homogenous material. The structure of the β-phthalimido intermediate was confirmed by ^{1}H-NMR and IR. ^{1}H-NMR ($CDCl_3$, TMS): δ 7.86 (m, 2H), 7.76 (m, 2H), 4.3–4.0 (overlapping m, 6H), 3.7–3.6 (overlapping m and s, 11 H), 3.35 (t, $J = 6.7$ Hz, 2H), 1.54 (m, 2H), 1.47 (m, 2H), 1.25 (s, 44H), 0.88 (t, $J = 6.7$ Hz, 6H). IR (KBr): cm^{-1} 2800, 2730, 1760, 1700, 1010, 725. Deprotection of the primary amine was accomplished by treatment of the phthalimide (1.8 g) with anhydrous hydrazine (2.7 g) in anhydrous ethanol (40 ml). Filtration, evaporative removal of ethanol and extraction afforded the crude product which was purified to homo-

3. Results

The molecular structures of the lipid reagents used in this study are shown in Fig. 1. In βAE-DMRIE, a primary amino group replaces the primary alcohol in DMRIE. This substitution should confer a positive charge of roughly 1.5 on βAE-DMRIE at physiological pH (7.4) based on the pK_a values reported for ethylenediamine [20]. Thus, on average, βAE-DMRIE has a greater net positive charge than DMRIE (about +1.5 vs. +1.0). The primary amine moiety may possibly possess an enhanced hydrogen bonding capability compared to DMRIE as a consequence of its various protonation states.

We compared the structure of plasmid DNA/βAE-DMRIE, DNA/DMRIE and DNA/DMRIE-DOPE cytofectin complexes by Atomic Force Microscopy. The results are shown in Fig. 2. Fig. 2A and B shows the appearance of naked, uncomplexed plasmid DNA. All of the naked plasmid DNA images that attached to the mica were observed to be uniformly sized, overlapping loop images that are characteristic of a highly purified preparation of intact covalently closed circular plasmid DNA. Regions of the images that show increased height (whitish areas) are apparently areas of plasmid crossover and/or supercoiling [17,18]. Condensation of plasmid DNA with βAE-DMRIE or DMRIE gave rise to distinct structural changes in the apparent plasmid backbone. All of the attached DNA/βAE-DMRIE images appeared as compacted, irregularly shaped branches and loops (Fig. 2C). All of the images observed when DNA/DMRIE complexes were examined with AFM appeared similar to images of plasmid alone (not shown). By contrast, all of the attached DNA/DMRIE-DOPE images appeared as irregularly shaped globules (Fig. 2E). Under higher magnification, both DNA/βAE-DMRIE and DNA/DMRIE-DOPE complexes appeared to contain closely juxtaposed plasmid strands (Fig. 2D,F), although the interstrand juxtapositions appeared more frequent in DNA/DMRIE-DOPE preparation.

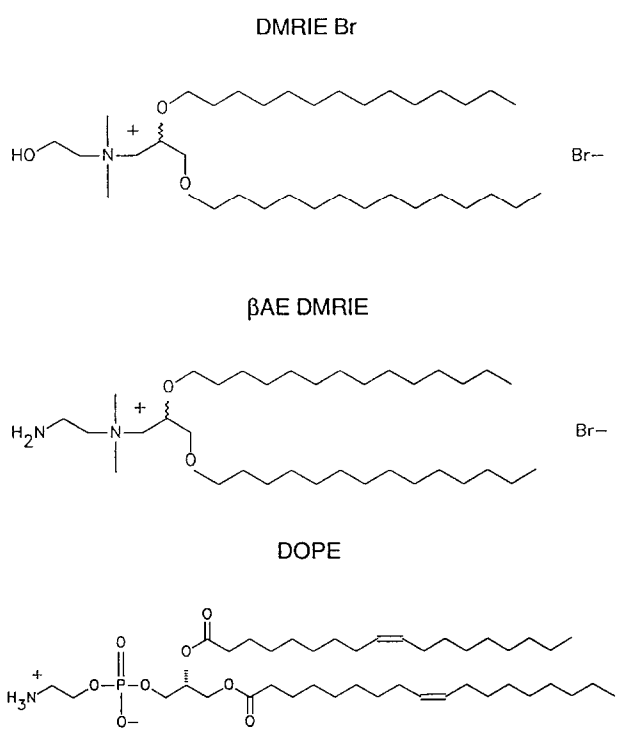


Fig. 1. Cytofectin structural components. The chemical structures of the reagents used in this paper are shown. βAE-DMRIE and DMRIE were synthesized from a common intermediate. The βAE-DMRIE contains a primary amine group while DMRIE contains a primary alcohol group. The neutral co-lipid used in this work was DOPE.

Virtually all cationic lipids used to deliver DNA into cells to date have required some proportion of a neutral lipid to be present in order to achieve optimal transfection activity [11–14,21]. Rather than assume a uniform co-lipid requirement, the transfection activity of βAE-DMRIE and DMRIE was compared with 0, 25, 50, or 75 mol% of the neutral lipid, DOPE. These four formulations for each of the two cationic lipids (i.e., βAE-DMRIE and DMRIE alone and in combination with DOPE) were mixed with plasmid DNA in a 96 well plate before being presented in 100 μl of serum-free culture medium to cells cultured in microwells arranged in an 8 × 8 matrix. Fig. 3 presents the format for transfection used in all the experiments reported in this study. Shown are cationic lipid and DNA amounts per well in μg units (Fig. 3, numbers in italics) and μM units (Fig. 3, italicized numbers in parenthesis) as well as the resulting well-by-well DNA/cationic lipid mass ratios (Fig. 3, bold numbers) and molar ratios (Fig. 3, bold numbers in parentheses). Thus, replicate cell cultures received DNA and cationic lipid amounts ranging from 2.000 to 0.016 μg and 10.7 to 0.08 μg, respectively. The resulting DNA/cationic lipid mass ratios covered a 12000-fold range from 0.002 (well H1) to 23.82 (well A8). Past experience with other cytofectins has shown that optimal DNA/cationic lipid ratios for cells generally falls within this range.

The malignant mouse melanoma cell line, B16F10, was transfected with 64 different nCMVintlacZ plasmid DNA/cationic lipid mixtures according the scheme outlined above and the procedures stated in Section 2. The resulting typical well-by-well expression levels of β-galactosidase in pg per well are shown in Fig. 4. For presentation purposes, values in each well were categorized into three transfection groups, high, medium and low. For example, using DNA mixtures containing 100% βAE-DMRIE, well A1 shown in Fig. 4A had a maximum transfection value of 11410 pg β-galactosidase. Values highlighted in black are those pg values that are from 66–100% of this maximum plate value (i.e., range 7606–11410 pg). Those values highlighted as grey wells fall between 33–66% of maximum (range 3802–7605 pg) and the unhighlighted values represent the bottom 33% (range 0–3801 pg). Fig. 4A–D and F–I shows individual pg values per well. Fig. 4E and J shows the μg sums con-

geneity by recrystallization from hexane. The structure and purity were confirmed by ^{1}H-NMR, IR, high resolution mass spectroscopy, and elemental analysis. ^{1}H-NMR ($CDCl_3$, TMS): δ 4.1 (m, 1H), 4.0–3.8 (overlapping multiplets, 3H), 3.7 (m, 1H), 3.6–4.0 (overlapping m and s, 12 H), 3.29 (t, $J = 6.1$ Hz)2H), 1.55 (m, 4H), 1.26 (s, 44H), 0.88 (t, $J = 6.7$ Hz, 6H). IR (KBr): cm^{-1} 3400 (b), 2900, 2840, 1460, 1370, 1120, 725. High Resolution mass spectrum M^+ [M − Br]: calc. 555.5828; found 555.5807. Elemental analysis [$C_{35}H_{75}O_2N_2Br$]: calc. C: 66.11, H: 11.89, N: 4.41, Br: 12.57; found C: 66.61, H: 12.38, N: 4.42, Br: 12.33.

2.2.3. *1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)*

Material of > 99% purity was purchased from Avanti Polar Lipids as a chloroform solution and used without further purification.

2.3. *Cytofectin formulation*

Solutions of DMRIE and βAE-DMRIE in chloroform were prepared on a weight to volume basis. Aliquots of cationic lipid and DOPE (when required) were transferred aseptically to sterile vials in amounts calculated to provide the relative and absolute lipid concentrations desired upon reconstitution with 1 ml of aqueous vehicle. Bulk chloroform was removed with a stream of dry nitrogen, and the vials were treated with high vacuum overnight to remove any residual solvent.

2.4. *DNA-lipid complexes*

The dried, formulated cytofectin-neutral lipid mixture was suspended by vortexing in OPTIMEM (Gibco BRL). The Plasmid DNA was also diluted in OPTIMEM. The cytofectin-neutral lipid and DNA were diluted in OPTIMEM to make a 2 × solution and 50 μl of each were mixed together with a multichannel pipette in 96 well plates at the desired mass/molar ratios. No precipitation occurred at the dilute solutions used as monitored by light microscopy. The DNA-lipid complexes were added to the cells within 2 h after mixing.

2.5. *Atomic force microscopy*

A 1.0 mg βAE-DMRIE/ml, 1.0 mg DMRIE or 1.0 mg DMRIE + 1.0 mg DOPE/ml were reconstituted into liposomes by vortexing the appropriate amount of thin dried lipid films in distilled water. An aliquot of either cationic liposome preparation was diluted to 0.1 mg cationic lipid/ml and 75 μl of it was mixed with 50 μl 0.2 mg pVCL-1005 DNA/ml in water. This 125 μl solution contained 10 μg DNA and 7.5 μg cationic lipid, representing a DNA/cationic lipid mass ratio of 1/0.75. A 2 μl aliquot of DNA or DNA/cytofectin complex was diluted with 200 μl of adhesion buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol) and 20 μl was deposited onto freshly cleaved mica, allowed to remain in place for 90 sec, and the unadsorbed material was washed off with 6 drops of distilled water. The mica surface was then dried with a stream of nitrogen. The samples were imaged in air using a NanoScope II atomic force microscope Digital Instruments Inc., Santa Barbara, CA) [17,18]. Commercially available silicon nitride cantelivers, 100 μm in length with a spring constant of 0.38 N/m (Digital Instruments) with electron-beam deposited micro tips were used [19]. At least 10 separate fields were examined for each sample and representative images are presented in Section 3.

2.6. *Cell lines*

All but three of the cell lines used in this study were obtained from the American Type Tissue Culture Collection (ATCC) as follows: BHK-21 (Syrian hamster kidney, #CCL 10), COS7 (African Green monkey SV40 transformed kidney, #CRL 1651), C2C12 (mouse muscle myoblast, #CRL 1772), CV-1 (African Green monkey kidney, #CCL 70), B16-F0 (mouse melanoma, #CRL 6322), HeLa (human epitheliod cervical carcinoma, #CCL 2) and 3T3 (mouse fibroblast, #CRL 1658). The Renca mouse adenocarcinoma cells were a kind gift from Dr. Drew Pardoll at the Johns Hopkins University. The UM-449 human melanoma and CT26 murine colon adenocarcinoma cells were kindly provided by Mark Cameron and Dr. Gary Nabel, respectively, at the University of Michigan. All cells were passaged 1:5 to 1:10 in 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM). All cells were expanded through 10 doubling passages upon receipt and aliquots were stored frozen. Upon re-expansion, all cells were used for transfection studies before another 10 passages.

2.7. *Transfection assays*

On day 0, 20 000 cells in 100 μl 10%FBS/90%DMEM were seeded into each well of 96-well culture plates (Nunc) and cultured overnight in a 5% CO_2 incubator at 37°C. On day 1, the medium was aspirated carefully without dislodging cells, and 100 μl of the DNA-cationic lipid formulation in serum-free OPTIMEM (Gibco BRL) was added. After 4 h of culture, 50 μl 30% FBS/70% OPTIMEM was added to each well. On day 2, each well received 100 μl 10% FBS/90% OPTIMEM. On day 3 the medium was removed and 50 μl lysis buffer (0.1% Triton X-100 in 250 mM Tris, pH 8.0) was added and the plates were stored at −70°C for least 2 h. After thawing, the well media were assayed for their content of β-galactosidase enzyme activity [11].

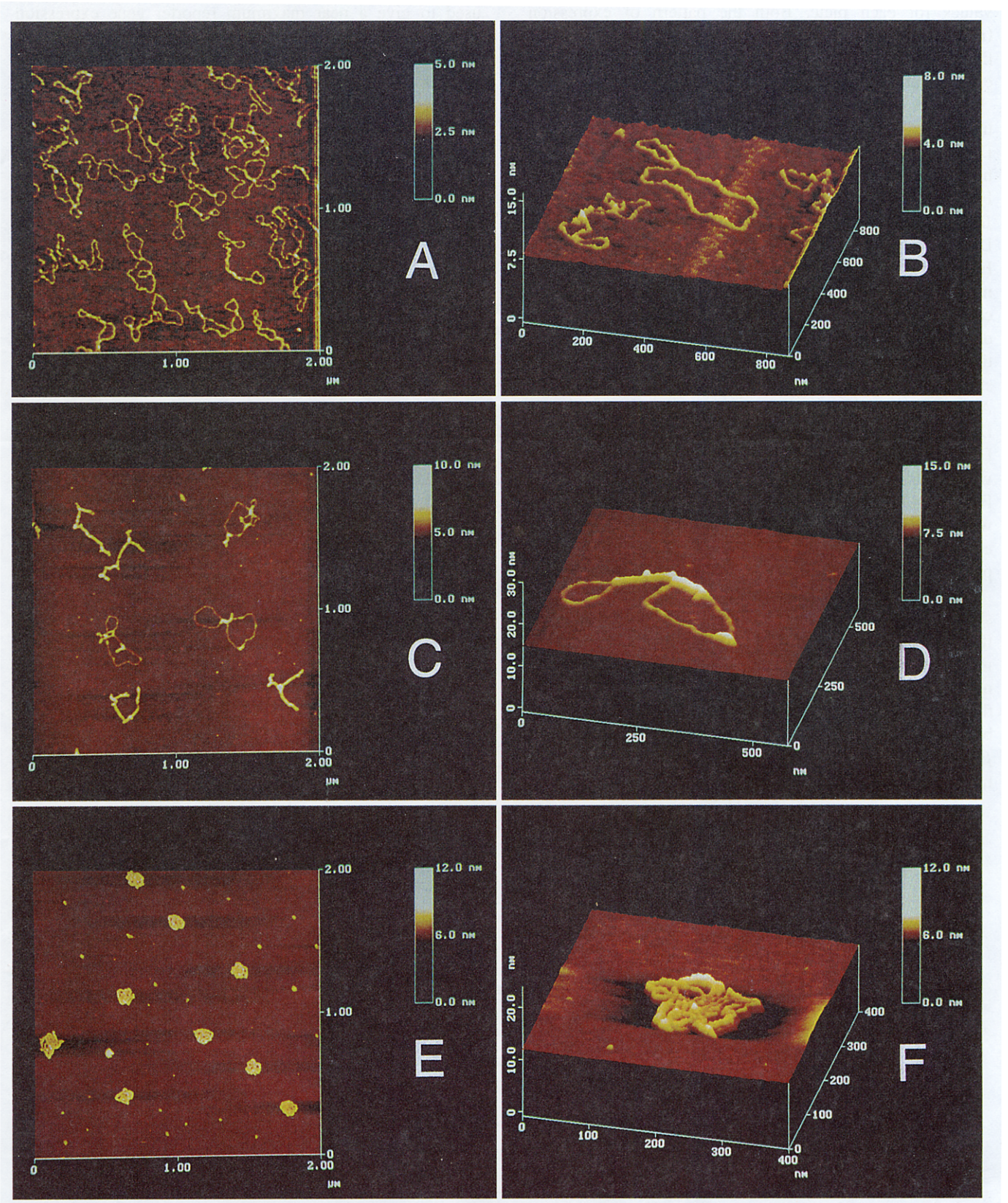


Fig. 2. Atomic force microscopy of plasmid DNA-cationic lipid complexes. Plasmid DNA alone (A,B) and plasmid DNA complexed to either βAE-DMRIE (C,D) or DMRIE-DOPE (E,F). Higher magnifications are shown in B,D, and F. Scales at the bottom of individual images denote dimension in the mica plane and vertical (color) scale is representative of relative heights. Image width to height ratios do not represent actual proportions due to differential resolution in the planar versus vertical dimensions (see Refs. [17,18,23] for a more detailed explanation).

tributed from the corresponding solid, shaded and open values for each plate. Both the pattern of expression (reflected as solid or shaded wells) and the total transfection levels (Fig. 4E,J) varied less than 20% in replicate experiments (data not shown).

In the absence of DOPE as co-lipid, βAE-DMRIE transfected B16F10 cells over a much broader range of DNA/cytofectin ratios than did DMRIE (compare the number of solid or shaded values in Fig. 4A with those in F). The total levels of transfection achieved with 100% βAE-DMRIE was about 6-fold higher than that of 100% DMRIE (compare the 0% DOPE values in Fig. 4E and J). Even when formulated with DOPE, the βAE-DMRIE mediated high to medium level transfections of B16F10 cells over a wider range of DNA/cationic lipid ratios than did the corresponding DMRIE-DOPE mediated transfections (compare the number and position of the solid and shaded wells in Fig. 4B with G, C with H, and D with I). As was true when comparing with cationic lipids without DOPE, total transfection levels using βAE-DMRIE with DOPE were consistently higher than those using DMRIE with DOPE (compare the 75%, 50% and 25% values in Fig. 4E and J). And finally, the transfection levels obtained with βAE-DMRIE at 100% were higher than those achieved with the optimal DMRIE/DOPE mixture (compare Fig. 4E, 0% DOPE with Fig. 4J, 50% DOPE).

The presentation of transfection data in the manner shown in Fig. 4 easily allows one to choose optimal transfection conditions for this particular B16F10 melanoma cell type. For example, using βAE-DMRIE, a near maximum transfection can be obtained by choosing the conditions in well E5 in Fig. 4A. Well E5, referring back to Fig. 3, contained 0.125 μg of DNA and 0.67 μg of βAE-DMRIE in the 100 μl transfection volume. The transfection conditions in well E5 might be preferred because the least amount of DNA and cationic lipid were used to give a near maximum reporter gene expression (shown as 10 330 pg in well E5 in Fig. 4A). Similarly for DMRIE, one might choose well D6 in Fig. 4H where DMRIE was formulated with 50% DOPE and maximum transfection (7385 pg/well) was obtained using 0.25 μg of DNA and 0.33 μg of cationic lipid (about 0.66 μg of total lipid).

To compare βAE-DMRIE and DMRIE more rigorously, nine additional sets of tumor and non-tumor producing cells lines were transfected. The transfection data has been arranged in the same manner as with the B16 melanoma cells shown in Fig. 4, except that, in order to save space, the graphs have been miniaturized and the actual well-by-well pg values have been excluded. The data are shown in Fig. 5 for eight of the nine cell types comprising UM-449 (A), BHK (B), COS7 (C), 3T3 (D), C2C12 (E), Renca (F), HeLa (G), and CV-1 (H) cells. As was the case for B16 melanoma cells, βAE-DMRIE generally transfected these tumoral and non-tumoral cell lines over a wider range of lipid and DNA concentrations and afforded as much or more total gene product than did DMRIE (in Fig. 5A–H compare the amount of solid and shaded areas in the left columns, #1–#5, with the right columns, #6–#10). In all cases, βAE-DMRIE without co-lipid transfected cells much better than did DMRIE without co-lipid (in Fig. 5A–H, compare #5 and #10, 0% DOPE bar values).

The data in Fig. 5 also reveal that the range of DNA and cationic lipid concentrations that elicit the highest transfection (i.e., the black areas in each set of plates in Fig. 5) varies considerably between different cell types. For example, very low amounts of DNA and lipid are required to optimally transfect UM-449 cells (eg., Fig. 5A-1, well H-7 and Fig. 5A-6, well F-6) while rather high

		Column:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Row:		*μg Cat. Lipid (μM Cat. Lipid)*	*10.7 (168)*	*5.34 (84)*	*2.67 (42)*	*1.34 (21)*	*0.67 (10.5)*	*0.33 (5.25)*	*0.17 (2.63)*	*0.08 (1.31)*
A	*μg DNA:*	*2.000*	**0.187**	**0.374**	**0.749**	**1.497**	**2.995**	**5.989**	**11.95**	**23.82**
	(μM DNA):	*(60.600)*	**(0.361)**	**(0.721)**	**(1.443)**	**(2.886)**	**(5.771)**	**(11.54)**	**(23.04)**	**(45.91)**
B	*μg DNA:*	*1.000*	**0.094**	**0.187**	**0.374**	**0.749**	**1.497**	**2.995**	**5.978**	**11.91**
	(μM DNA):	*(30.300)*	**(0.180)**	**(0.361)**	**(0.721)**	**(1.443)**	**(2.886)**	**(5.771)**	**(11.52)**	**(22.96)**
C	*μg DNA:*	*0.500*	**0.047**	**0.094**	**0.187**	**0.374**	**0.749**	**1.497**	**2.989**	**5.955**
	(μM DNA):	*(15.150)*	**(0.090)**	**(0.180)**	**(0.361)**	**(0.721)**	**(1.443)**	**(2.886)**	**(5.761)**	**(11.48)**
D	*μg DNA:*	*0.250*	**0.023**	**0.047**	**0.094**	**0.187**	**0.374**	**0.748**	**1.494**	**2.976**
	(μM DNA):	*(7.575)*	**(0.045)**	**(0.090)**	**(0.180)**	**(0.361)**	**(0.721)**	**(1.442)**	**(2.878)**	**(5.735)**
E	*μg DNA:*	*0.125*	**0.012**	**0.023**	**0.047**	**0.094**	**0.187**	**0.375**	**0.748**	**1.490**
	(μM DNA):	*(3.788)*	**(0.023)**	**(0.045)**	**(0.090)**	**(0.181)**	**(0.361)**	**(0.722)**	**(1.441)**	**(2.871)**
F	*μg DNA:*	*0.063*	**0.006**	**0.012**	**0.024**	**0.047**	**0.094**	**0.188**	**0.375**	**0.747**
	(μM DNA):	*(1.894)*	**(0.011)**	**(0.023)**	**(0.045)**	**(0.091)**	**(0.181)**	**(0.362)**	**(0.722)**	**(1.439)**
G	*μg DNA:*	*0.031*	**0.003**	**0.006**	**0.012**	**0.024**	**0.047**	**0.094**	**0.187**	**0.373**
	(μM DNA):	*(0.947)*	**(0.006)**	**(0.011)**	**(0.023)**	**(0.045)**	**(0.091)**	**(0.181)**	**(0.361)**	**(0.720)**
H	*μg DNA:*	*0.016*	**0.002**	**0.003**	**0.006**	**0.012**	**0.024**	**0.047**	**0.095**	**0.189**
	(μM DNA:)	*(0.473)*	**(0.003)**	**(0.006)**	**(0.011)**	**(0.023)**	**(0.046)**	**(0.091)**	**(0.183)**	**(0.364)**

Fig. 3. DNA/cationic lipid ratios used for in vitro transfection. An 8 × 8 titration matrix was set up in 96 well microplates by adding the indicated μg (italics) and μM (italics in parenthesis) amounts of premixed RSVlacZ DNA/cationic lipid complexes to each well. The resulting mass ratios (bold) and micromolar concentrations (bold in parentheses) are indicated for each well in the matrix. DNA 'molarity' is based on M_r of 330 per nucleotide monomer. These solutions in 100 μl were presented to cells in corresponding microplates for all the transfection assays used in this report.

A. ßAE DMRIE only

	1	2	3	4	5	6	7	8
A	11410	8805	7561	6080	7046	7262	5394	3734
B	11133	8812	7565	7470	7729	6098	4007	3245
C	8911	10078	10793	7597	8108	8057	6058	4602
D	4722	9630	10100	9148	10246	7149	6627	3920
E	1435	3252	8721	9969	10330	7258	5131	1406
F	589	1045	2439	4040	3686	4037	1435	2172
G	100	169	1235	1318	2209	786	629	487
H	177	104	162	260	665	859	479	0

F. DMRIE only

	1	2	3	4	5	6	7	8
A	1090	1651	740	867	935	420	337	211
B	247	2156	553	993	1104	654	395	222
C	31	478	2865	1785	1990	1619	1101	589
D	6	384	2779	5112	2127	1410	921	427
E	13	56	586	2980	4982	1803	1115	557
F	0	17	42	380	1533	1605	496	550
G	0	103	96	64	67	438	283	229
H	0	0	0	0	161	197	197	42

B. 25% DOPE

	1	2	3	4	5	6	7	8
A	6919	6120	6523	7352	4474	5020	3191	1502
B	5852	6814	6245	6292	8009	6797	3967	1885
C	8991	7765	5656	7731	8127	8100	6110	2887
D	8168	11459	9424	10426	9580	7850	4999	3479
E	3587	9126	11208	11540	10193	8392	5636	2805
F	706	5013	8615	10785	9326	6056	3438	1336
G	564	1986	5747	6083	6546	3598	2030	764
H	314	1089	2298	3337	2463	2128	909	510

G. 25% DOPE

	1	2	3	4	5	6	7	8
A	1598	1565	1096	1244	2450	1908	1092	648
B	977	1392	995	2172	2804	1992	1139	778
C	489	1923	3173	2284	2804	2606	1638	1034
D	146	800	3982	3761	5495	3010	1692	1269
E	0	359	3942	6481	6091	3346	1381	670
F	0	0	2548	3122	4805	2988	1110	381
G	0	0	623	355	937	821	319	204
H	0	0	518	316	222	265	124	135

C. 50% DOPE

	1	2	3	4	5	6	7	8
A	1829	3337	2197	3586	3691	2116	1885	3054
B	3131	4079	3915	3624	3152	3100	2480	1861
C	4800	4272	3327	3397	4254	2809	1731	2382
D	4331	5587	6413	4699	3960	3565	3170	4223
E	3435	4842	6522	5874	4055	3820	2718	2263
F	1343	4926	5391	3726	3939	3775	1962	997
G	983	2876	2925	1987	2106	3239	888	727
H	559	1441	1399	1007	934	1693	829	773

H. 50% DOPE

	1	2	3	4	5	6	7	8
A	717	1826	2142	2903	5943	6202	4264	2411
B	409	2609	2726	2889	6212	6000	3027	1142
C	58	1249	2861	3304	6025	6616	2861	1189
D	0	402	2358	3282	4951	7385	2971	1543
E	0	51	1426	2705	3654	3325	3034	671
F	0	0	409	1103	1529	1323	2053	795
G	0	0	182	1008	515	321	416	154
H	0	0	104	335	434	30	65	58

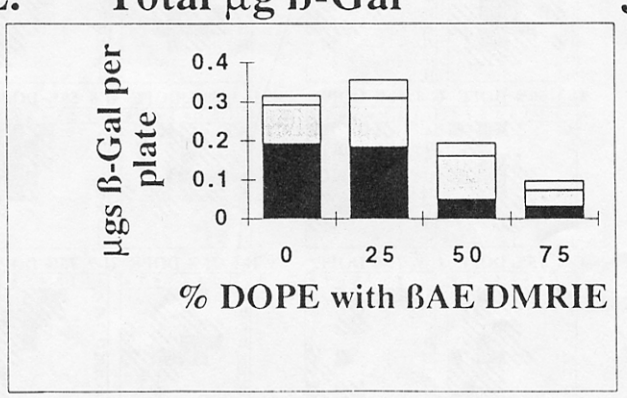
D. 75% DOPE

	1	2	3	4	5	6	7	8
A	1244	1303	520	350	487	505	598	705
B	1480	1188	897	697	860	790	845	1137
C	2196	3297	2458	804	989	1181	1295	1262
D	2410	3511	3470	2204	1690	1462	2030	2067
E	450	2366	3463	3861	3603	2927	2684	3153
F	321	398	1576	2573	2170	2658	2396	2429
G	59	343	372	572	1041	1591	2322	3178
H	40	107	14	339	498	731	963	1059

I. 75% DOPE

	1	2	3	4	5	6	7	8
A	1205	2295	1857	1733	1836	1340	1365	833
B	3119	3539	2076	2353	1821	2266	2011	1482
C	599	1219	4622	3032	1726	1723	1336	1496
D	0	286	1121	2525	3134	1766	1748	1686
E	0	38	173	1394	3236	2667	2211	1471
F	0	38	89	169	789	1912	2259	2011
G	0	0	0	34	238	596	1124	1584
H	45	23	0	0	0	0	92	246

E. Total µg ß-Gal



J. Total µg ß-Gal

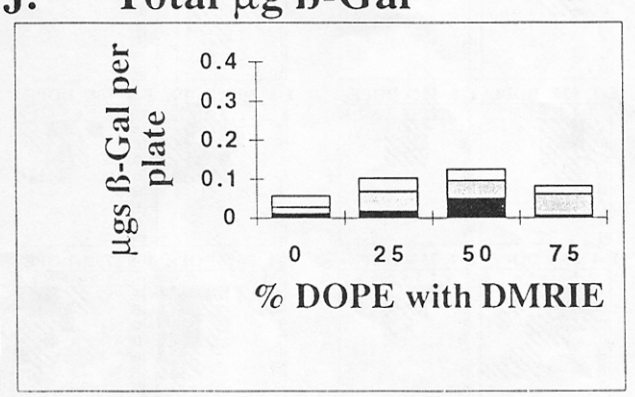
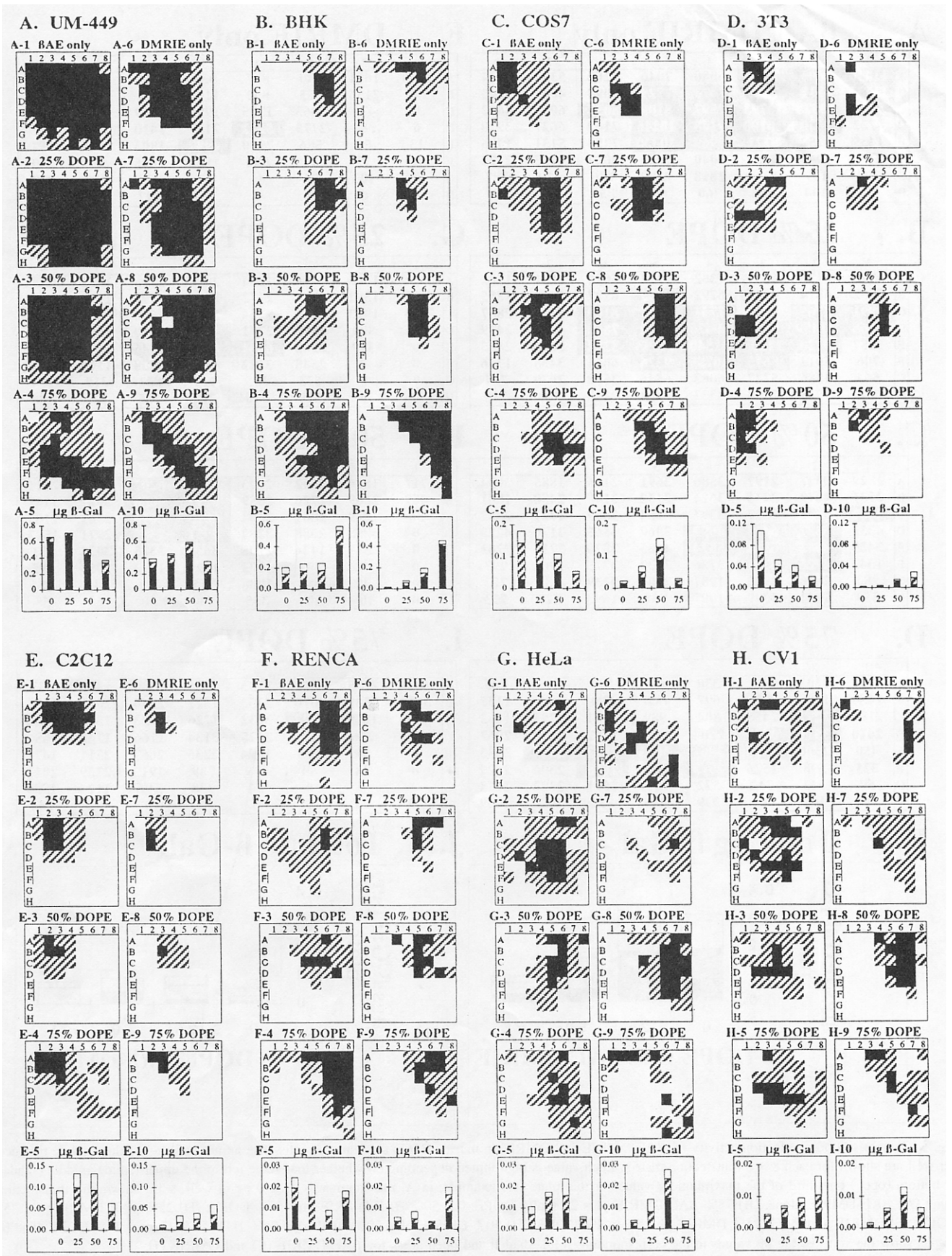


Fig. 4. Comparison of transfection activity of βAE-DMRIE and DMRIE in murine B16F10 melanoma cells. The number of pg β-galactosidase produced per well are shown for each 8 × 8 transfection matrix. Each value is highlighted depending on whether that value fell in the upper (solid), middle (shaded) or bottom (open) one-third of the maximum pg value for that plate (for example, in A, maximum was 11410 pg in well A-1). Shown are transfections using (A) βAE-DMRIE alone; (B) 75% βAE-DMRIE plus 25 mol% DOPE; (C) 50% βAE-DMRIE plus 50% DOPE; (D) 25% βAE-DMRIE plus 75% DOPE; (F) DMRIE alone; (G) 75% DMRIE plus 25 mol% DOPE; (H) 50% DMRIE plus 50% DOPE; (I) 25% βAE-DMRIE plus 75% DOPE; The bar graphs show the sums of the pg values in the corresponding solid, shaded and open areas for βAE-DMRIE (E) and DMRIE (J).


A. UM-449
A-1 ßAE only
A-6 DMRIE only
A-2 25% DOPE
A-7 25% DOPE
A-3 50% DOPE
A-8 50% DOPE
A-4 75% DOPE
A-9 75% DOPE
A-5 µg ß-Gal
A-10 µg ß-Gal
B. BHK
B-1 ßAE only
B-6 DMRIE only
B-3 25% DOPE
B-7 25% DOPE
B-3 50% DOPE
B-8 50% DOPE
B-4 75% DOPE
B-9 75% DOPE
B-5 µg ß-Gal
B-10 µg ß-Gal
C. COS7
C-1 ßAE only
C-6 DMRIE only
C-2 25% DOPE
C-7 25% DOPE
C-3 50% DOPE
C-8 50% DOPE
C-4 75% DOPE
C-9 75% DOPE
C-5 µg ß-Gal
C-10 µg ß-Gal
D. 3T3
D-1 ßAE only
D-6 DMRIE only
D-2 25% DOPE
D-7 25% DOPE
D-3 50% DOPE
D-8 50% DOPE
D-4 75% DOPE
D-9 75% DOPE
D-5 µg ß-Gal
D-10 µg ß-Gal
E. C2C12
E-1 ßAE only
E-6 DMRIE only
E-2 25% DOPE
E-7 25% DOPE
E-3 50% DOPE
E-8 50% DOPE
E-4 75% DOPE
E-9 75% DOPE
E-5 µg ß-Gal
E-10 µg ß-Gal
F. RENCA
F-1 ßAE only
F-6 DMRIE only
F-2 25% DOPE
F-7 25% DOPE
F-3 50% DOPE
F-8 50% DOPE
F-4 75% DOPE
F-9 75% DOPE
F-5 µg ß-Gal
F-10 µg ß-Gal
G. HeLa
G-1 ßAE only
G-6 DMRIE only
G-2 25% DOPE
G-7 25% DOPE
G-3 50% DOPE
G-8 50% DOPE
G-4 75% DOPE
G-9 75% DOPE
G-5 µg ß-Gal
G-10 µg ß-Gal
H. CV1
H-1 ßAE only
H-6 DMRIE only
H-2 25% DOPE
H-7 25% DOPE
H-3 50% DOPE
H-8 50% DOPE
H-5 75% DOPE
H-9 75% DOPE
I-5 µg ß-Gal
I-10 µg ß-Gal

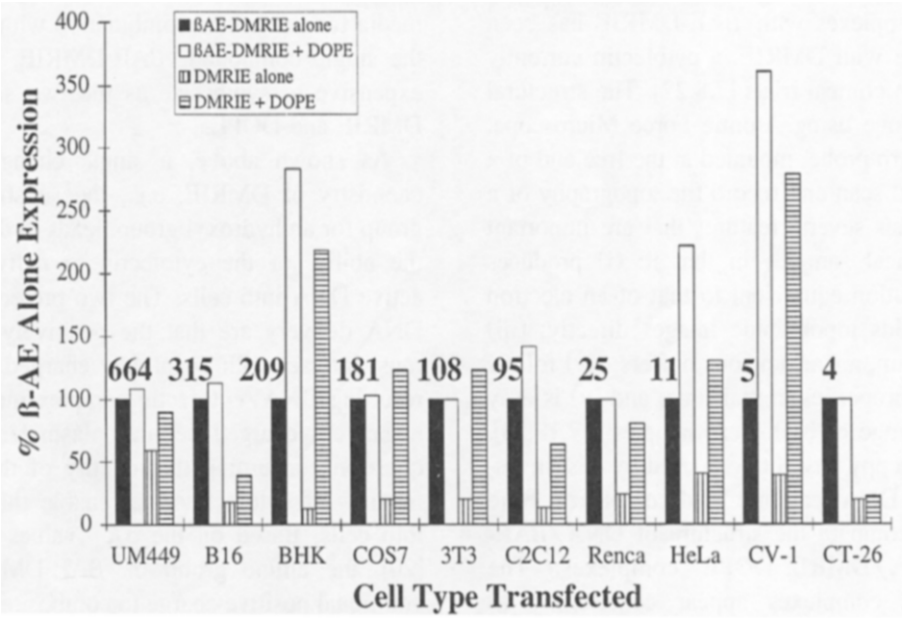


Fig. 6. Comparison of total transfection activities of βAE-DMRIE and DMRIE and their optimal formulations with DOPE among different cell types. The sum of pg β-galactosidase values from each 8 × 8 matrix from Figs. 4 and 5 were determined and expressed as the percentage of the pg sum value for βAE-DMRIE alone (value shown in bold above each set of solid bars). Bars are shown for βAE-DMRIE alone (solid, = 100%); the optimal βAE-DMRIE-DOPE formulation (shaded); DMRIE alone (vertical hatching); and the optimal DMRIE-DOPE formulation (horizontal hatching).

DNA and lipid amounts are required to maximally transfect 3T3 cells (Fig. 5E-1, well 5C and Fig. 5E-6, well C3). As was the case for B16 cells and the data in Fig. 4, the data shown in Fig. 5 can be used to choose DNA and cationic lipid levels and DNA/lipid ratios needed for maximal transfection of each of the eight cell types shown. For the best transfections, one would generally choose DNA and cationic lipid concentrations and ratios present in the solid wells in Fig. 5.

The total amounts of reporter gene product from each cell line, expressed as the percentage of product from transfections with βAE-DMRIE alone as shown in Figs. 4 and 5 were plotted together to allow direct comparison. The results are shown in Fig. 6. The various cell types exhibited a wide variation in the absolute amount of reporter gene expression as indicated by the pg/64 well total transfection value typed above each set of solid bars in Fig. 6. CT26 cells (the cells that expressed the least reporter gene product) exhibited a total transfection value of 4 ng/64 wells, while UM-449 melanoma cells (the cells that expressed the most gene product) exhibited a 141-fold higher transfection value of 664 ng/64 wells. The remaining cells showed transfection levels ranging between 4 and 664 ng/64 wells.

Despite this wide range of transfection efficiencies, βAE-DMRIE and DMRIE differed in four major ways from one another in their transfection efficiencies. First, βAE-DMRIE without co-lipid transfected better than DMRIE without co-lipid in 10 out of the 10 cell types examined (Fig. 6, compare solid bars with vertically hatched bars). Second, βAE-DMRIE without co-lipid transfected 8 out of 10 cell types as well or better than DMRIE optimally formulated with co-lipid (Fig. 6, compare solid bars with horizontally hatched bars). Third, βAE-DMRIE with co-lipid transfected cells as well or better than DMRIE with co-lipid (Fig. 6 compare shaded bars with horizontally hatched bars). Fourth, DMRIE alone never transfected cells better than DMRIE optimally formulated with DOPE (Fig. 6, compare vertically hatched bars with horizontally hatched bars). Overall, then, βAE-DMRIE represents a stand-alone cationic lipid molecule that can generally elicit high transfection activity in the absence or presence of a co-lipid and over a wide range of cationic lipid and DNA concentrations.

4. Discussion

The synthesis of a novel cytofectin reagent, βAE-DMRIE, has been achieved. The structure and transfection

Fig. 5. Comparison of transfection activity of βAE-DMRIE and DMRIE in selected tumoral and non tumoral cell lines. Eight sets of transfections were carried out in the same manner as that shown for B16 cells in Fig. 4, except that the actual well-by-well pg values are not shown. Cells transfected include UM-449 (A), BHK (B), COS7 (C), 3T3 (D), C2C12 (E), Renca (F), HeLa (G) and CV-1 (H) cells. Each well is highlighted depending on whether the pg β-galactosidase value from that well falls in the upper (solid), middle (shaded) or bottom (open) one-third of the maximum pg value for that plate. Shown at the bottom of each column of plates are bar graphs representing the sums of the corresponding solid, shaded and open pg/well values shown in the columns of data above each bar graph.

activity of DNA complexes with βAE-DMRIE has been compared with those with DMRIE, a cytofectin currently being used in human clinical trials [7,8,22]. The structural comparisons were done using Atomic Force Microscope. The AFM uses a sharp probe, mounted at the free end of a flexible cantilever, to scan and record the topography of a sample. The AFM has several features that are important for studying biological samples in that it: (i) produces images with a resolution equivalent to that of an electron microscope; (ii) yields topographic images directly; (iii) can operate in vacuum, air or aqueous buffers; (iv) follow dynamic molecular properties in real time; and (v) is easy to use for a wide range of biological samples [17,18,23]. Atomic force microscopy has not been used by past investigators to visualize DNA/cationic lipid complexes. Here AFM was used to compare the structure of DNA/βAE-DMRIE and DNA/DMRIE-DOPE complexes. The DNA/βAE-DMRIE complexes appear consistently as compacted, irregularly shaped branches and rods, while DNA/DMRIE complexes could not be distinguished from images of free plasmid. The appearance of DNA/DMRIE-DOPE complexes by AFM was highly variable from one preparation to another, although a predominant image was irregularly sized and shaped globules. Therefore, a single functional group change (OH of DMRIE changed to NH_2 of βAE-DMRIE) resulted in a dramatic change in the ultrastructural appearance of DNA-liposome complexes, at least in those that bound to the mica surface during AFM processing. Further work will be needed to determine if the AFM images observed represent the majority of structures present in these DNA/cationic lipid complexes.

We conclude that βAE-DMRIE is superior to DMRIE in mediating in vitro cellular transfection for the following reasons. βAE-DMRIE does not require the use of a neutral co-lipid in order to express high transfection activity, a feature that has the following advantages: (i) formulation with DNA is less complex, thereby decreasing work and expense; (ii) upon storage, unsaturated co-lipids can undergo hydrolytic and oxidative decomposition; (iii) co-lipids or their hydrolysis or oxidation products (such as lyso-phosphatidyl ethanolamine from DOPE) may have unwanted biological activities [24–27]; and (iv) future studies designed to reveal DNA-lipid structure and elucidate the cellular mechanism of transfection should be considerably simplified by the use of a single cationic lipid species.

In addition to not requiring formulation with co-lipids, βAE-DMRIE has other advantages. βAE-DMRIE has a broader range of DNA/cationic lipid ratios that elicit optimal transfection, thus allowing more flexibility in choosing the concentrations to present to cells. βAE-DMRIE appears to mediate the transfection of a wide range of tumoral and non-tumoral cell types. Also, solid βAE-DMRIE, unlike the individual solid components, DMRIE and DOPE, can be dissolved directly in aqueous media for immediate combination with DNA. And finally, the single compound, βAE-DMRIE, is simpler and less expensive to synthesize as the two separate compounds, DMRIE and DOPE.

As shown above, a single change in the structural chemistry of DMRIE, e.g., the substitution of an amino group for an hydroxyl group, leads to dramatic increases in the ability of the cytofectin to deliver transcriptionally active DNA into cells. The two prerequisites for effective DNA delivery are that the positively charged cytofectin must interact with negatively charged DNA, and that the resulting DNA/cytofectin complex must interact with the negatively charged cellular plasmalemma. This raises a question concerning the identity of the critical structural features of cytofectins that enable them to deliver DNA into cells. Based on the pK_a values of ethylenediamine [20], the amine group of βAE-DMRIE should confer additional positive charge to composite liposome structures compared to DMRIE. At the same time, the amine group in βAE-DMRIE should hydrogen bond at least as well as the hydroxyl of DMRIE. Thus, the additional cationic charge of βAE-DMRIE may enhance the ability of liposomes containing βAE-DMRIE to interact with DNA. If the βAE-DMRIE positive charge is maintained in excess over the negatively charged phosphates of DNA, the DNA/βAE-DMRIE liposomal complex should maintain a high electrostatic binding affinity to the negative cell surface. These properties may explain why βAE-DMRIE is able to deliver DNA to cells effectively over a greater range of concentrations than does DMRIE. Alternatively, βAE-DMRIE may be more effective than DMRIE for other, yet to be discovered reasons.

βAE-DMRIE consistently transfects cells effectively in the absence of a co-lipid, while DMRIE and other cationic lipids examined to date, show significantly better transfection activity when combined with neutral phospholipids ([21]; unpublished observations). Previous reports [28–30] have rationalized this need for co-lipids as the requirement for an easy transition between bilayer and hexagonal II phase states which the co-lipid is thought to confer to the liposome complex. The presence of a primary amine function at the liposome surface has also been invoked as important in optimizing DMRIE activity, although this may not be generalizable since DOPC (which possesses a $(CH_3)_3N^+$ in lieu of the NH_3^+ in DOPE) is an effective co-lipid for some cationic lipids [11]. By analogy, βAE-DMRIE is an analog of DMRIE (in that NH_2 replaces OH), and an analog of DOPE (in that $-N(CH_3)_2^+-$ replaces $-O-PO_2^--O-$).

Thus, βAE-DMRIE may provide similar important coulombic, hydrophobic and hydrophilic properties to the macroscopic liposome as those provided by the combination of DMRIE and DOPE. This would not, however, explain why βAE-DMRIE actually elicits better transfection than the optimal DMRIE-DOPE formulation. The complex relationship between lipid physico-chemistry and

efficiency of cellular transfection is unclear. Investigations are currently underway in this and other laboratories to shed more light on this important issue.

The fact that a single substitution in the cytofectin skeleton can result in drastic changes in transfection properties suggests that other modifications may also allow leaps in the improvement of gene delivery. This issue is currently being addressed by synthesizing and testing other analogues including the related propylene and butylene homologues of βAE-DMRIE. Structure–activity studies with such series of compounds may ultimately lead to the development of cytofectins with even more desirable properties than βAE-DMRIE.

Finally, the studies presented in this paper report only in vitro transfections. The mechanism of action and structural properties of cytofectins that enable them to deliver DNA into cells in vivo is similarly not well understood. Thus, it is important to compare the transfection activity of DMRIE with that of βAE-DMRIE and other DMRIE analogues using in vivo transfection assays, and we are actively engaged in such pursuits.

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References

[1] Lew, D., Parker, S.E., Latimer, T., Abai, A., Kuwahara-Rundell, A., Doh, S.G., LaFace, D., Gromkowski, S.H., Nabel, G.J., Manthorpe, M. and Norman, J. (1995) Hum. Gene Ther. 6, 553.

[2] Horn, N.A., Meek, J.A., Budahazi, G. and Marquet, M. (1995) Hum. Gene Ther. 6, 565.

[3] Parker, S.E., Vahlsing, L.H., Serfilippi, L.M., Franklin, C.J., Doh, S.G., Gromkowski, S.H., Lew, D., Manthorpe, M. and Norman, J. (1995) Hum. Gene Ther. 6, 575.

[4] Caplan, N.J., Alton, 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. and Geddes, D.M. (1995) Nature Med. 1, 39.

[5] Nabel, G.J., Chang, A.E., Nabel, E.G., Plautz, G.E., Ensminger, W., Fox, B.A., Felgner, P., Shu, S. and Cho, K. (1994) Hum. Gene Ther. 5, 57.

[6] Sorcher, E.J., Logan, J.J., Frizzell, R.A., Lyrene, R.K., Bebok, Z., Dong, J.Y., Duval, M.D., Felgner, P.L., Matalon, S., Walker, L. and Wiatrak, B.R. (1994) Hum. Gene Ther. 5, 1259.

[7] Vogelzang, N.J., Lestingi, T.M., Sudakoff, G. and Kradjian, S.A. (1994) Hum. Gene Ther. 5, 1357.

[8] Hersch, E.M., Akporiaye, E., Harris, D., Stopeck, A.T., Unger, E.C., Warneke, J.A. and Kradjian, S.A. (1994) Hum. Gene Ther. 5, 1371.

[9] Rubin, J., Charboneau, J.W., Reading, C. and Kovach, J.S. (1994) Hum. Gene Ther. 5, 1385.

[10] Felgner, P.L. (1990) Adv. Drug Deliv. Rev. 5, 163.

[11] Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M. and Felgner, P.L. (1994) J. Biol. Chem. 269, 2550.

[12] Rose, J.K., Buonocore, L. and Whitt, M.A. (1991) Biotechniques 10, 520–525.

[13] Leventis, R. and Silvius, J.R. (1990) Biochim. Biophys. Acta 1023, 124–132.

[14] Pinnadwage, P., Schmitt, L. and Huang, L. (1989) Biochim. Biophys. Acta 985, 33.

[15] Manthorpe, M., Cornefert-Jensen, F., Hartikka, J., Felgner, J., Rundell, A., Margalith, M. and Norman, J. (1993) Hum. Gene Ther. 4, 419.

[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1992) Molecular Cloning: A Laboratory Manual, 2nd. Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor.

[17] Bustamente, C., Keller, D. and Yang, G. (1993) Curr. Opin. Struct. Biol. 3, 363.

[18] Vesenka, J., Guthold, M., Tang, C.L., Keller, D., Delaine, E. and Bustamente, C. (1992) Ultramicroscopy 42–44, 1243.

[19] Akama, Y., Nishimura, Sakai and Murakami, H. (1990) J. Vac. Sci. Technol. A. 8, 429.

[20] Gordon, A.J. and Ford, R.A. (1972) The Chemist's Companion, John Wiley and Sons, New York.

[21] Jiao, S., Ascadi, G., Jani, A., Felgner, P.L. and Wolff, J.A. (1992) Exp. Neurol. 11, 400.

[22] Nabel, G.J., Chang, A.E., Nabel, E.G., Plautz, G.E., Ensminger, W., Fox, B.A., Felgner, P., Shu, S. and Cho, K. (1994) Hum. Gene Ther. 5, 57.

[23] Bustamente, C., Erie, D. and Keller, D. (1994) Curr. Opin. Struct. Biol. 4, 750.

[24] Farhood, H., Bottega, R., Epand, R.M. and Huang, L. (1992) Biochim. Biophys. Acta 1111, 239.

[25] Sinisterra, G. and Epand, R.M. (1993) Arch. Biochem. Biophys. 300, 378–383.

[26] Slater, S.J., Kelly, M.B., Taddeo, F.J., Ho, C., Rubin, E. and Stubbs, C.D. (1994) J. Biol. Chem. 269, 4866.

[27] Gawrisch, K., Parseegian, V.A., Hajduk, D.A., Tate, M.W., Gruner, S.M., Fuller, N.L. and Rand, R.P. (1992) Biochemistry 31, 2856.

[28] Duzgunes, N. (1985) Subcell. Biochemistry 11, 195.

[29] Gruner, S.M. (1987) in Liposomes (Ostro, M.J., ed.), Marcel Dekker.

[30] Litzinger,, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1113, 210.