Evidence of Interlipidic Ion-Pairing in Anion-Induced DNA Release from Cationic Amphiphile−DNA Complexes. Mechanistic Implications in Transfection[†]

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ABSTRACT: Complex formation of DNA with a number of cationic amphiphiles has been examined using fluorescence, gel electrophoresis, and chemical nuclease digestion. Here we have addressed the status of both DNA and lipid upon complexation with each other. DNA upon binding with cationic amphiphiles changes its structure in such a way that it loses the ability to intercalate and becomes resistant to nuclease digestion. Fluorescence anisotropy measurements due to 1,6-diphenylhexatriene (DPH) doped in cationic liposomes demonstrated that upon complexation with DNA, the resulting complexes still retain lamellar organizations with modest enhancement in thermal stabilities. The lipid−DNA complexation is most effective only when the complexation was carried out at or around the phase transition temperatures of the cationic lipid employed in the complexation with DNA. The release of DNA from cationic lipid−DNA complexes could be induced by several anionic additives. Determination of fluorescence anisotropies (due to DPH) as a function of temperature clearly demonstrates that the addition of equivalent amounts of anionic amphiphile into cationic lipid−DNA complexes leads to the ion-pairing of the amphiphiles, the melting profiles of which are virtually the same as those obtained in the absence of DNA. In this process DNA gets released from its complexes with cationic lipids and regains its natural intercalation ability, movement, and staining ability on agarose gel and also the sensitivities toward nuclease digestion. This clearly suggests that combination of ion-pairing and hydrophobic interactions between cationic and anionic amphiphiles is stronger than the electrostatic forces involved in the cationic lipid−DNA complexation. It is further revealed that the DNA release by anions is most efficient from the cationic lipid−DNA complexes at or around the T_m of the cationic lipid used in DNA complexation. This explains why more effective DNA delivery is achieved with cationic lipids that bear unsaturated hydrocarbon chains than with their saturated hydrocarbon counterparts.

Cationic liposome mediated DNA delivery into various types of cells has been finding increasing attention in recent years (*1*−*6*). A variety of other synthetic nonviral vectors have also been suggested for similar applications due to their ability to complex with DNA. These include cyclic amphipathic peptides (*7*), dendrimers (*8*), various cationic and guanidinium derivatives of cholesterol (*9*, *10*), polylysine (*11*), lipopolysine (*12*), and lipospermine (*13*), among others. Aqueous formulations based on these systems are attractive because they are simple, chemically well-defined, and nonviral in character. However, the practical applications of most of the above-mentioned vectors for therapeutic purposes are still limited for several reasons. Some of them are the finite shelf life of such recipes, frequent inactivation of the transfection complexes by serum proteins, and requirements of helper lipids and toxicity associated with most of the above formulations.

The complexation of DNA with preformed cationic liposomes has been shown to induce structural changes at the level of both DNA and liposomes. Depending on the composition of lipid mixtures and their concentrations in a cationic lipid formulation, DNA-promoted vesicle fusion leading to the formation of heterogeneous particles has also been reported (*14*). But little was explored about the fate of cationic liposomes upon complexation with DNA. Consequently the following issues remain to be addressed in detail. What happens to the lipid order upon DNA complexation? How do the lipid melting properties influence the binding with cationic liposomes and subsequent release of DNA from these complexes? Is there any role of the hydrocarbon part of the lipid during DNA complexation or DNA release from the cationic lipid−DNA complexes? How exactly do the anionic amphiphiles induce the DNA release from the cationic lipid−DNA complexes? The last parameter is particularly important as the DNA has to retain its native form in order to effect its transcription in the cytoplasm (upon delivery).

Previous efforts in this laboratory were directed toward the comparison of the effects of surfactants of different charge types against salts and small organic ions on the DNA melting and intercalation properties (*15*). In the present paper, we systematically examine the DNA complexation with various cationic micelle- and vesicle-forming amphiphiles and also the release of DNA from such complexes.

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The complex formation and the DNA release were examined by EB intercalation assay (fluorescence spectroscopy), gel electrophoresis, chemical nuclease digestion, extraction into organic solvent, and circular dichroism spectroscopy. On the basis of these studies, we provide new evidences on the characterization of DNA−cationic amphiphile complex formation and the release of DNA from these complexes. The effects on the cationic lipid order−disorder transition upon complexation with DNA were determined by carefully following the changes in the fluorescence anisotropy due to doped 1,6-diphenylhexatriene (DPH). The dissociation of DNA from such complexes was induced by a range of negatively charged additives, and the processes were examined by the above cited methods.

EXPERIMENTAL PROCEDURES

Materials

Calf thymus DNA (CT DNA) was obtained from Sigma Chemical Co. (St. Louis, MO) and was purified by a published procedure (*16*) involving phenol/chloroform extraction followed by ethanol precipitation as sodium salts. This was then dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. The plasmid DNA PTZ19R used in this study was prepared according to procedures described previously (*17*). DNA concentrations were determined by measuring the absorptions at 260 nm using the relation 1.0 OD = 40 μg/mL.

All other compounds and solvents used in this study were of highest purity available from well-known commercial sources. Cholesterol, 1,6-diphenylhexatriene (DPH), cetyl trimethylammonium chloride (CTAC), sodium dodecyl sulfate (SDS), ethidium bromide (EB), dextran sulfate, dicetyl phosphate (DCP), palmitic acid, myristic acid, oleic acid, and cholic acid were purchased from Sigma Chemical Co. Dihexadecyl dimethylammonium bromide (DHDAB) and dioctadecyl dimethylammonium bromide (DODAB) were synthesized following a published procedure (*18*).

For this work, a new cationic amphiphile (HOHAB) was synthesized. *N*-(2-Hydroxyethyl)-*N*,*N*-dimethyl-*N*-hexadecylammonium bromide was allowed to react for 48 h at ambient temperature with an excess amount of oleoyl chloride (1:1.5 mol/mol) in dry chloroform in the presence of 1.5 equiv of dry pyridine. Evaporation of the solvents from the reaction mixture gave a gummy material which was chromatographically purified over silica gel to afford a highly viscous substance which has all the characteristic spectroscopic signals corresponding to HODAB. ^{1}H NMR ($CDCl_3$, 90 MHz): δ 5.4 (t, 2H, *H*C═C*H*), 4.5 (br t, 2H, OCH_2), 3.95 (br t, 2H, OCH_2CH_2), 3.4−3.5 (br s + m, 8H, $N(CH_3)_2$, $NCH_2(CH_2)_n$), 2.3 (t, 2H, OCOCH_2), 2.0 (br m, 4H, CHCH_2), 1.1−1.5 (br m, 50H), 0.9 (t, 6H, CH_2CH_3).

1,2-Dioleoyloxy-3-(trimethylammonio)propane (DOTAP) and 1,2-dipalmitoyloxy(trimethylammonio)propane (DPTAP) were synthesized following appropriate literature procedures (*19*).

Methods

Aggregate Preparation. The micellar solutions were prepared from the individual amphiphiles in water above their critical micellar concentrations (cmc). The cationic liposomes were prepared by the following method. A chloroform solution of the individual lipid was taken in a 2 mL wheaton glass septum vial, and the solvent was removed by rotary evaporation. Vials were then placed under vacuum for several hours to remove the last traces of solvent. This gave a lipid film in the vial to which the requisite amount of double-distilled water was added to initiate lipid hydration by vortexing. Then this suspension was sonicated above its phase transition temperature in a bath-type sonicator (Sidilu Ultrasonic bath) or in an immersion probe sonicator (at 25 W, Heat Systems, model XL-2020 Ultrasonic Processor) until a translucent lipid suspension was obtained.

Ethidium Bromide Intercalation Assay. Fluorescence emission due to ethidium bromide (EB) at 590 nm was monitored in a Hitachi model F-4500 spectrofluorimeter (excitation wavelength = 266 nm with slits of excitation and emission of 5 nm) immediately after addition of various micellar or liposomal aggregates into preformed EB−DNA complexes in 5 mM Tris-HCl (pH 7.4) buffer.

Agarose Gel Electrophoresis. Amphiphile−DNA association complexes were first prepared by mixing either the plasmid DNA PTZ19R (0.5 μg) or CT DNA (1.5 μg) with the appropriate concentration of cationic amphiphilic aggregates at pH 7.4 Tris-HCl (20 mM). Samples containing these mixtures were incubated at ambient temperature for 5 min. Subsequently, some of the cationic amphiphile−DNA complexes were treated with various concentrations of anionic additives such as SDS or DCP aggregates. The resulting mixtures were incubated again at 25 °C for 5 min. Finally all the reaction mixtures were directly loaded onto 1% agarose (Pharmacia) gel with 5 μL loading dye [20% glycerol, 25 mM EDTA, and 0.05% bromophenol blue and xylene cyanol FF (1:1)]. Specific control reactions, as mentioned under the figure legends, were subjected to the phenol/chloroform extractions followed by ethanol precipitations prior to loading onto the agarose gel. Electrophoresis was carried out in 0.05 M Tris, 0.05 M boric acid, and 1 mM EDTA buffer according to the standard procedure (*20*) under a constant electric field of 80 V for 90 min at room temperature. DNA was visualized after staining the gel with ethidium bromide (0.5 μg/mL) for 2 h. Bands of DNA were detected and photographed (Canon SLR camera with an orange filter) under UV light (Photodyne Transilluminator, 312 nm) in a darkroom.

DNA Sensitivity to Chemical Nuclease in Amphiphile−DNA Complexes. For this experiment, several cationic amphiphile−DNA complexes were prepared as described above. Then the release of DNA from individual cationic amphiphile−DNA complexes was attempted by addition of various concentrations of different anionic amphiphile−lipid aggregates followed by their incubation at 37 °C for 5 min. Then both type of samples (untreated and treated with anions) were subjected to chemical nuclease treatment using a mixture of a biscationic Ni−salen complex and magnesium monoperoxyphthalate (MMPP) as described (*17*), and the reaction mixtures were incubated at 37 °C for 5 min. The DNA digestion reactions were then quenched by addition of 10 mM β-mercaptoethanol, and finally each of these samples was individually loaded onto a 1% agarose gel with the gel loading dye and electrophoresed as described above. After electrophoresis, the bands in the gel were stained with ethidium bromide and photographed.

Circular Dichroism (CD) Measurements. The CD measurements were carried out in a Jasco J-500 A spectropolarimeter using a DP-500N data processor with cells of 0.5 cm path length equipped with a thermostated water-circulating bath. The effects of addition of increasing amounts of single- or double-chain amphiphilic aggregates on the CD spectra of DNA (72.7 μM, base molar) were monitored in 5 mM Tris-HCl (pH 7.4) buffer, and the spectral changes were recorded after each addition of the additives. Then the effects of addition of anionic aggregates onto preformed DNA−cationic amphiphile complexes were examined by CD spectroscopy in the above cited conditions.

Fluorescence Anisotropy Measurements. The fluorescence anisotropies of different vesicle-doped DPH (1 μM) in the presence and the absence of DNA were measured by excitation of DPH at 360 nm and emission at 430 nm. The slit widths were 5 nm for both the excitation and the emission. The fluorescence intensities of the emitted light polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excited light were recorded at various temperatures, and the fluorescence intensities were corrected for the scattered light intensities. The fluorescence anisotropy (r) values were calculated from the equation $r = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + 2GI_{\perp})$, where G is the instrumental grating factor.

The systemic gel (solid) to liquid-crystalline (fluid) phase transition (melting) temperatures of individual lipid vesicles or their complexes with DNA were obtained from the midpoints of the breaks related to the temperature-dependent fluorescence anisotropy values. The temperature ranges for the phase transition were calculated from the two temperature points for each experiment which marked the beginning and the end of the apparent phase transition process.

RESULTS

Ethidium Bromide Intercalation. Ethidium bromide (EB), a fluorophore which interacts with double-stranded DNA or RNA by intercalating within the adjacent base pairs, has been used in the present study. An aqueous solution of EB (13.9 μM) gave a weak fluorescence emission with a broad λ_{max} at ca. 590 nm when excited at 266 nm (Figure 2A, trace 1). In the presence of CT DNA [63.3 μM (base molar)], the fluorescence emission at 590 nm due to EB increased by more than 1 order of magnitude (Figure 2A, trace 2). Addition of an aqueous CTAC (1.6×10^{-4} M) solution into DNA-bound EB at 25 °C resulted in an immediate decrease of the fluorescence intensity to ca. 20% of the maximum value (Figure 2A, trace 3), suggesting the "dissociation" of EB from the CT DNA upon binding with cationic CTAC aggregates to CT DNA. Notably, the specific CTAC to CT DNA ratio at which the fluorescence quenching saturates, corresponded to a ± charge ratio of ∼1.2. Upon addition of an aqueous solution of anionic aggregates of SDS (1.6×10^{-4} M) into the above CTAC−DNA mixture at 25 °C, substantial (∼85%) extent of the EB fluorescence was recovered (Figure 2B). This suggests that the addition of SDS induces destabilization of the CTAC−DNA complexes, which in turn facilitates the reintercalation of EB into the released DNA. Further addition of SDS (up to 3.8×10^{-4} M) did not, however, induce any additional extent of fluorescence recovery (trace 10).

Similarly, the addition of a cationic, vesicular DHDAB into a DNA-bound EB solution also resulted in fluorescence quenching by ca. 89% of the maximum value (Figure 2C, trace 3). Addition of an aqueous SDS (1.1×10^{-4} M) solution at 25 °C into the DHDAB−DNA complex led to ∼88% recovery of the EB fluorescence, again indicating the release of DNA from the above complex. Further addition of SDS (up to 6.4×10^{-4} M) did not induce any additional release of DNA (Figure 2D). To examine the generality of the above observations, we then carried out complexation of DNA with a few other cationic lipids. The relevant data on the DNA release induced by SDS from various DNA−cationic amphiphile complexes are given in Table 1.

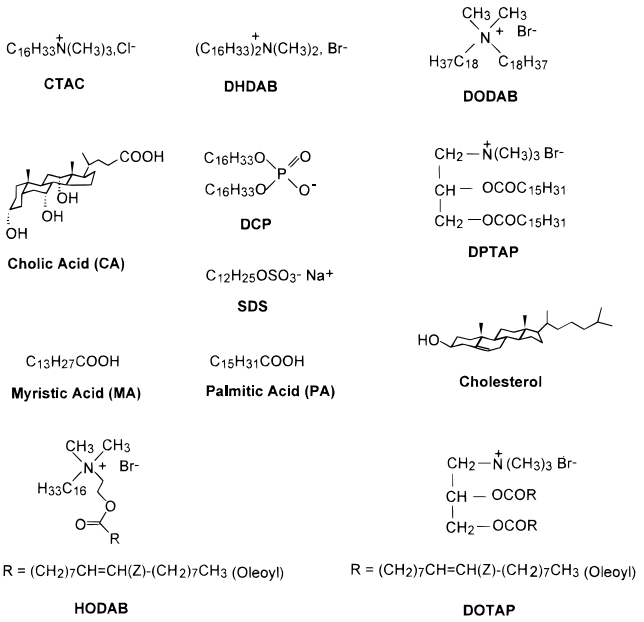

FIGURE 1: Chemical structures and abbreviations of various amphiphiles and lipids used in this study.

Table 1: Release of DNA by SDS from Various Cationic Amphiphile−DNA Complexes at 25 °C[a]

amphiphile[b]	10^4 M (concn)	[amphiphile]/[DNA][c]	[SDS] 10^4 M	[anion]/[cation][d]	% release[e,f]
CTAC	1.6	2.4	1.6	1.01 (2.42)	80
DHDAB	0.8	1.6	1.1	1.34 (7.61)	88
DPTAP	2.1	1.6	5.5	2.59 (7.64)	78.5
DOTAP	2.9	1.6	2.5	0.87 (1.41)	60
HODAB	3.3	1.8	3.2	0.98 (1.95)	77

[a] See text for experimental details. [b] See Figure 1 for abbreviations for individual amphiphile structures. [c] [DNA] expressed in base molarities. [d] The minimum (±) charge ratio of [SDS]/[cationic amphiphile] at which the DNA release was maximum and final ratio of the (±) charge used is shown inside the bracket. [e] Based on % fluorescence recovery (due to EB) upon addition of SDS. [f] These are averages of at least two independent experiments and are within ±2%.

Addition of other anionic additives, such as ATP and glutamic acid, and neutral amphiphilic aggregates, such as micellar Triton-x-100 or zwitterionic liposomes composed of DMPC, did not exert any noticeable effect on the fluorescence emission intensity even when added to EB−DNA−cationic amphiphile complexes at ∼10-fold molar excess over the cationic amphiphile.

Effects of Different Anions on DHDAB−DNA Complex. We then compared the effects of addition of different anions on the DNA release properties from a given cationic lipid (DHDAB)−DNA complex (Figure 3A). When the additives are fatty acids or contain a negatively charged headgroup and one hydrocarbon chain, the fluorescence (EB) recovery

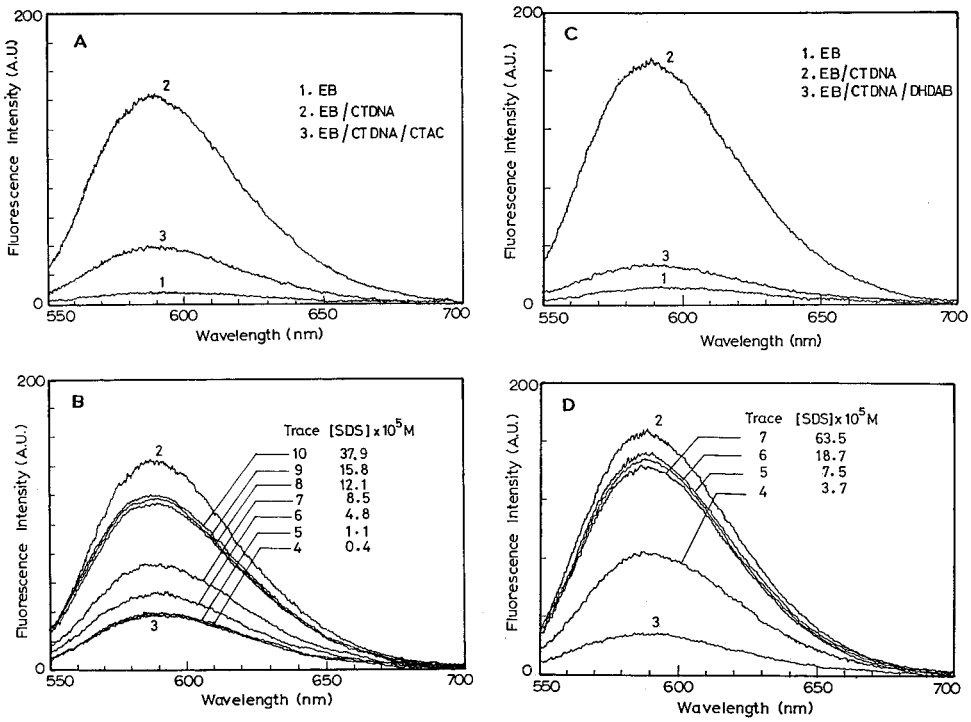

FIGURE 2: Ethidium bromide (EB) intercalation assay. (A) Effect of CTAC addition to the EB−DNA complex in 5 mM Tris-HCl (pH 7.4). Trace 1, EB alone (14 μM); trace 2, EB + CT DNA (65 μM); trace 3, 156 μM CTAC was added to the above EB−DNA complex solution. (B) Effect of addition of SDS to a solution containing CTAC−DNA−EB. Traces 2 and 3 are due to the same solution as described in panel A. Fluorescence changes due to progressive addition of SDS to the CTAC−DNA complex have been shown inside the figure. (C) Effect of DHDAB addition to the EB−DNA complex in 5 mM Tris-HCl (pH 7.4). Trace 1, EB alone (9 μM); trace 2, EB + CT DNA (53 μM); trace 3, 83.5 μM DHDAB was added to the above EB−DNA complex solution. (D) Effect of addition of SDS to DHDAB−DNA−EB complex solution. Traces 2 and 3 are due to the same solution as described in panel C. Fluorescence changes due to progressive addition of SDS to the DHDAB−DNA complex have been shown inside the figure.

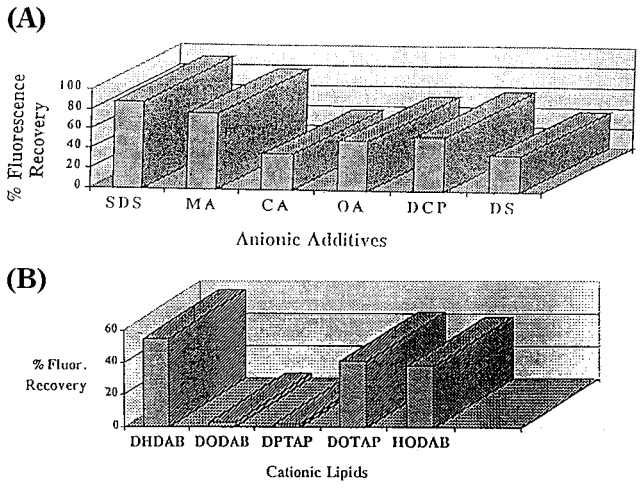

FIGURE 3: (A) Percent of CT DNA detached from the DHDAB−DNA complexes upon treatment with various anions at 25 °C. See Figure 1 for abbreviations. (B) Percent of CT DNA detached from various cationic lipid−DNA complexes upon treatment with vesicular DCP at 25 °C (MA = myristic acid, CA = cholic acid, OA = oleic acid, DS = dextran sulfate).

from the DHDAB−DNA complex was found to be significant (>50%) at 25 °C. The maximum release of DNA that is achievable at 25 °C from the DHDAB−DNA complex is virtually independent of the amount of anionic amphiphile added beyond an anion/cation (amphiphile) ratio of >1.0. However, the DNA release was less (~36%) when the additive was in the form of cholate aggregates or dextran sulfate oligomers. This suggests that the release of DNA from the DHDAB−DNA complex is dependent on the structural features of the anionic additive. Again, the amount of cholic acid or dextran sulfate needed to effect maximal DNA release from DHDAB−DNA complexes under comparable conditions was much higher than an anion/cation (amphiphile) ratio of ~1.0. Thus the DNA recovery from the cationic CTAC−DNA or DHDAB−DNA complexes as observed above occurs effectively when the last additive is a negatively charged amphiphilic aggregate.

Anion-Induced DNA Release. In this experiment, the DNA release induced by *anionic*, vesicular DCP at 25 °C from different cationic lipid−DNA complexes is examined (Figure 3B). At 25 °C, the DCP vesicles induced significant extent (37−55%) of DNA release from their complexes with either of the cationic lipids DHDAB, DOTAP, and HODAB, respectively. However, the DNA release in the presence of DCP vesicles from the DNA complexes with DODAB and DPTAP was negligible at 25 °C (≤3%).

Effects of Temperature. Thus the DNA release induced by a given anion was found to depend on the chemical structures of the cationic amphiphiles involved in the DNA complexation and also on the temperature at which such experiments were conducted. To understand such profound differences in the DNA release behavior from the two types of cationic lipid−DNA complexes, we then investigated the fluorescence recovery (EB) by vesicular DCP from both DODAB−DNA or DPTAP−DNA complexes as a function of temperature (Figure 4). Indeed, a significant extent of DNA release was observed at higher temperatures as evidenced from increases in fluorescence recovery upon rises in temperature for a given composition of each of the DCP−

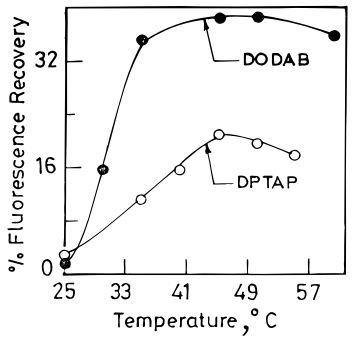

FIGURE 4: Effect of temperature on the anion-induced DNA release from cationic lipid−DNA complexes. The respective cationic lipid vesicles were mixed with DNA that intercalated EB (±ca. 1.1). The resulting complexes were then treated with vesicular DCP (at DCP/cationic lipid ± ratio 1.9), and the fluorescence emission (EB) from the resulting mixtures was monitored as a function of temperature. (●) Effect on DODAB−DNA−DCP: [DODAB] = 2.0×10^{-4} M, [DNA] = 1.8×10^{-4} M, [DCP] = 3.4×10^{-4} M; (○) effect on DPTAP−DNA−DCP, [DPTAP] = 2.0×10^{-4} M, [DNA] = 1.8×10^{-4} M, [DCP] = 3.4×10^{-4} M.

DODAB−DNA or DCP−DPTAP−DNA complexes, finally reaching a saturation at ∼40 and 45 °C, respectively. Further increase in the temperature did not lead to any additional fluorescence enhancement. Strikingly, the temperature values of 40−45 °C are close to the melting (phase transition) temperatures (T_m) of the respective cationic lipid membranes. A significant amount of precipitation was also observed in the case of DNA complexes with DPTAP at elevated temperature. The above observations indicate that the anion-induced DNA release is particularly effective when the cationic lipid employed for DNA complexation is in a fluid state.

DNA Release from Cationic Lipid−DNA Complex Containing Cholesterol. Cholesterol is ubiquitously present in all eukaryotic cells. The presence of cholesterol in cationic lipid bilayers has been shown to enhance the bilayer stiffness and abolish thermotropic phase transition process (*21*). How does the inclusion of cholesterol into the cationic liposomal aggregates used for DNA complexation influence the release efficiency of DNA induced by different anions? To answer this question, we included various amounts of cholesterol (0−30 mol %) into vesicular DHDAB and DODAB, respectively. The cationic lipid−cholesterol covesicles were first complexed with DNA and then subjected to treatment of different anionic additives using the fluorescence (EB) recovery assay as described earlier. When myristic acid was added to induce DNA release from DHDAB− or DODAB−cholesterol−DNA complexes, no significant alteration on the extent of DNA release from the corresponding lipid−DNA complexes was seen (figure not shown). However, when we examined the same with vesicular DCP aggregates as the anionic additive, small differences in the extent of DNA release could be seen. Thus, the addition of vesicular DCP to DNA complexes of cationic liposomal DHDAB which also contained 30% cholesterol effected only ∼37% of DNA release while ∼55% of DNA was released under comparable conditions from DHDAB−DNA complexes that did not contain any cholesterol (not shown). Small resistance to achieve DNA release induced by vesicular DCP was also seen with DNA−(DOTAP−cholesterol) complexes.

Fluorescence Anisotropy Measurements. To understand the effects of DNA binding on the lipid order−disorder

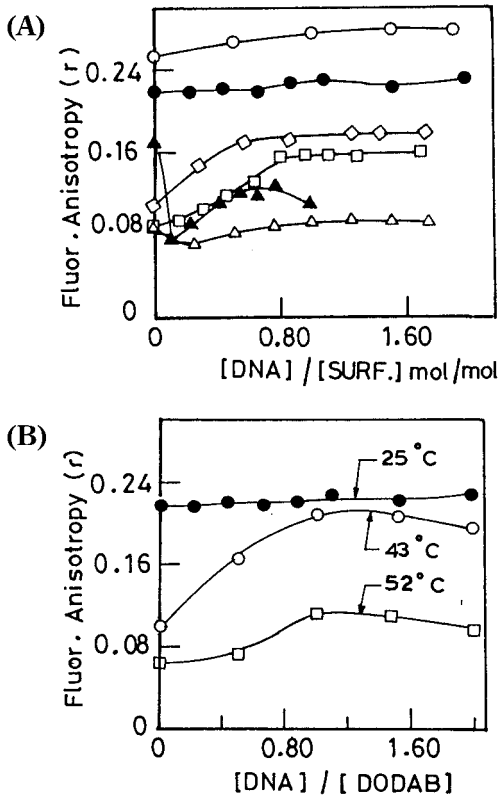

FIGURE 5: Fluorescence anisotropy measurements. (A) Fluorescence anisotropy (r) vs [DNA]/[surfactant] plot: Fluorescence anisotropies (r) were measured for specific amphiphilic aggregates (DPH-doped) as a function of [DNA] at 25 °C (except HODAB, at 15 °C). (○) DPTAP: [DPTAP] = 0.8×10^{-4} M; (●) DODAB: [DODAB] = 1.7×10^{-4} M; (◇) HODAB: [HODAB] = 1.9×10^{-4} M; (□) DHDAB: [DHDAB] = 1.9×10^{-4} M; (▲) CTAC: [CTAC] = 1.8×10^{-3} M; (△) DOTAP: [DOTAP] = 2.4×10^{-4} M. (B) Fluorescence anisotropy (r) vs [DNA]/[DODAB] plot at different temperatures (as mentioned in the curves). [DODAB] = 1.75×10^{-4} M.

(melting) transition, we then examined the effects of the addition of increasing amounts of DNA into various cationic amphiphilic aggregates using fluorescence anisotropy technique. The changes in the steady-state fluorescence anisotropy (r) due to DPH doped in various cationic amphiphilic aggregates were recorded as a function of [DNA] at 25 °C (Figure 5A). Interestingly, the addition of DNA into CTAC micelles led to an initial decrease in the r value, which was followed by its gradual increase upon DNA addition, finally reaching a saturation at ∼1:1 ratio of the CTAC to DNA. Further addition of DNA led to onset of precipitation. When progressively increasing amounts of DNA were added to vesicular DHDAB (T_m ∼ 26 °C), gradual increases in the fluorescence anisotropies were observed, finally reaching a saturation at the (±) charge ratio of DHDAB to nucleotide phosphate of ca. 1.0. Notably, the pure DHDAB vesicles, which are practically in the fluid phase at 25 °C, produce more ordered, tightly packed organization upon complexation with DNA (DNA/lipid ratio >1.0) as indicated by the enhancement in the r value. This suggests that the assembly of DHDAB gets more ordered upon binding with DNA. Interestingly, in the cases of vesicular DODAB (T_m ∼ 41 °C) and DPTAP (T_m ∼ 46 °C), the addition of DNA at 25 °C even in excess of 1:1 molar ratio over the lipid aggregates did not, however, lead to any significant enhancement in the

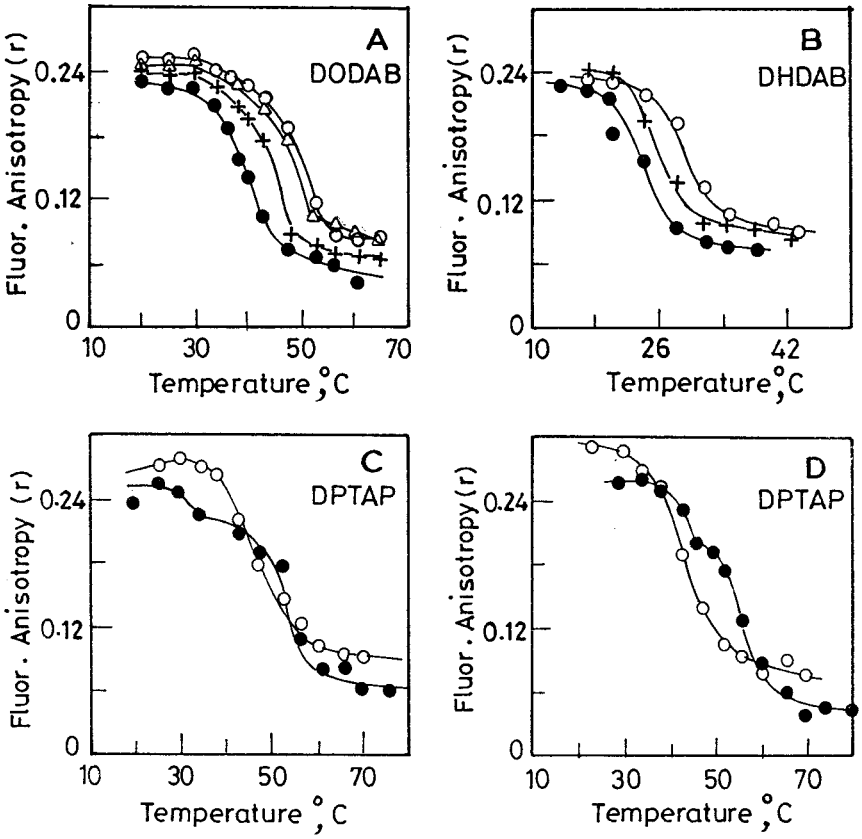

FIGURE 6: Effects of different cationic lipid:DNA ratio on the steady-state fluorescence anisotropy (*r*) profiles of DPH embedded in lipid or lipid−DNA aggregates as temperatures are varied. (Panel A) *r* values for DODAB vesicles only (●) and *r*−*T* profiles of the DODAB−DNA complexes at lipid:DNA ratios of 2:1 (+), 1:1 (△) and 1:2 (○). (Panel B) *r* values for DHDAB vesicles only (●) and *r* − *T* profiles of the DHDAB−DNA complexes at lipid:DNA ratios of 2:1 (+) and 1:1 (○). (Panel C) *r* values for DPTAP vesicles only (●) and *r*−*T* profiles of the DPTAP−DNA complexes at lipid:DNA ratio 1:1 (○), large multilamellar DPTAP liposomes were prepared by bath sonication method. (Panel D) Small unilamellar vesicles of DPTAP were prepared with probe sonication: *r* values for DPTAP vesicles only (●) and *r*−*T* profiles of the DPTAP−DNA complexes at lipid:DNA ratio 1:1 (○).

r value (Figure 6A). When examined with the cationic lipid, DOTAP (T_m < 5 °C), the aggregates of which are in the fluid state at 25 °C, we observed only a small enhancement in the fluorescence anisotropy upon DNA complexation. This enhancement was continued till the ratio of the cationic lipid to DNA (± charge ratio) reached ∼1.0. The measurement of fluorescence anisotropy of DPH doped in vesicular HODAB (T_m ∼ 12 °C) as a function of DNA concentration at 15 °C showed a significant increase in the *r* value till the lipid/DNA (±) charge ratio was close to 1.0, finally reaching a plateau.

The above phenomenon was then verified with a given lipid, DODAB, by systematically examining the effects on the fluorescence anisotropy as a function of DNA concentration at three temperatures below (25 °C), at (43 °C), and above (52 °C), the T_m of DODAB (Figure 5B). While the change in the *r*-value at 25 °C was virtually undetectable and at 52 °C was only modest, a substantial increase in *r*-value at 43 °C was observed which is around the melting transition temperature of the vesicular DODAB. Thus it is clear that the changes in the lipid packing upon DNA complexation becomes most pronounced at ±1 °C of the T_m of the cationic lipid vesicles.

To quantitate the effects of DNA complexation on the melting properties of cationic lipid vesicles, we then attempted to determine the apparent gel-to-liquid crystalline phase transition temperatures (T_m) of various cationic lipid aggregates before and after complexation with DNA. For a DPH-doped vesicular DODAB solution, a T_m of 41 °C (width ∼13K, see Table 2) was obtained from the midpoint of the breaks related to the *r* vs *T* plot (Figure 6A). This value for T_m of DODAB is in good agreement with the T_m values reported earlier (*18*). Similar examination with a DPH-doped 2:1 DODAB−DNA complex (± charge ratio ∼2.0) revealed an enhancement of the T_m value by ∼4 °C, while a 1:1 mixture of DODAB−DNA gave a T_m of ∼50 °C. However, further increases in the DNA contents of the DODAB−DNA

Table 2: Apparent Thermotropic Parameters for Cationic Lipid Aggregates and Their DNA Complexes As Determined by the Fluorescence Anisotropy Measurements[a]

lipid[b] (molar ratio)	T_m (°C)[c]	ΔT_m
DODAB	41	14
DODAB/DNA (2:1)	43	16
DODAB/DNA (1:1)	47	16
DODAB/DNA (1:2)	47.5	16
DHDAB	24	8
DHDAB/DNA (2:1)	26	11
DHDAB/DNA (1:1)	29	10
DPTAP[d]	29, 52	9, 18
DPTAP/DNA (1:1)	47	18.5
DPTAP[e]	35, 59	9, 26
DPTAP/DNA (1:1)	45	30

[a] See text for experimental details. [b] See Figure 1 for abbreviations for individual amphiphile structures; note that Lipid/DNA complexes also contained DPH (1 μM). [c] These values are within ±1 °C. [d] Prepared by injection method. [e] Prepared by probe sonication method.

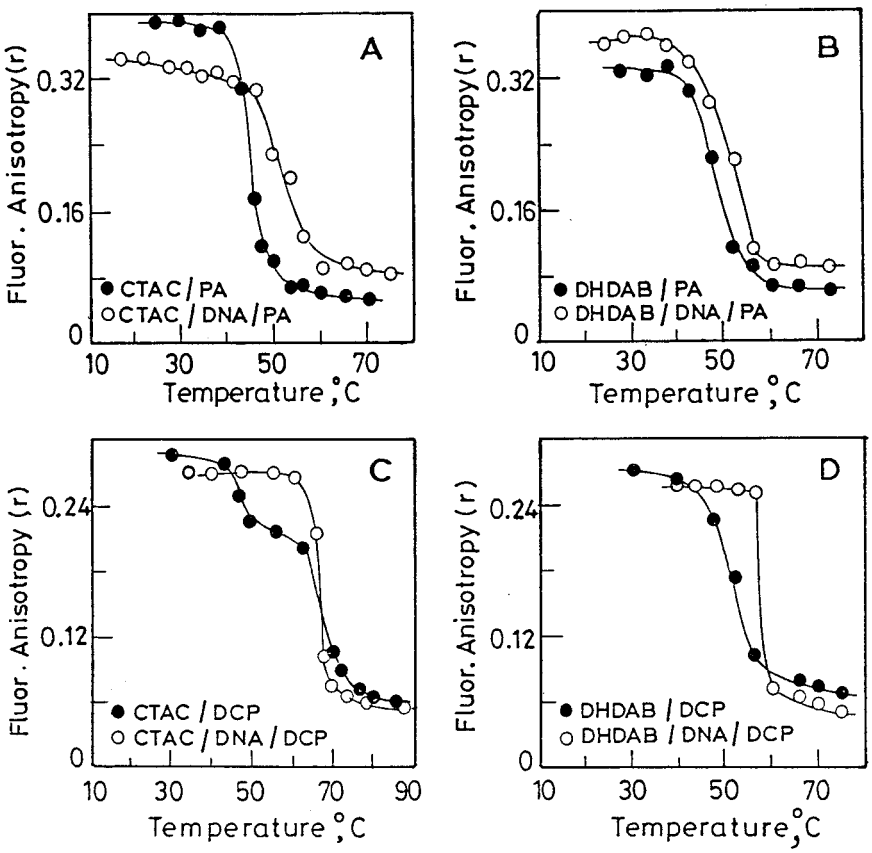

FIGURE 7: Effects on fluorescence anisotropies during anion-induced DNA release. The steady fluorescence anisotropies (*r*) were measured as function of temperature for either 1:1 (molar ratio) mixture of specific pure cationic and anionic amphiphile or for the mixture of 1:1:1 (molar ratio) cationic amphiphile, CT DNA, and anionic amphiphile, respectively. (Panel A) $r-T$ profile for 1:1 mixture of CTAC and PA (●) and 1:1:1 mixture of CTAC, CT DNA, and PA (○); (Panel B) $r-T$ profile for 1:1 mixture of DHDAB and PA (●) and 1:1:1 mixture of DHDAB, CT DNA, and PA (○); (Panel C) $r-T$ profile for 1:1 mixture of CTAC and DCP (●) and 1:1:1 mixture of CTAC, CT DNA, and DCP (○); (Panel D) $r-T$ profiles for 1:1 mixture of DHDAB and DCP (●) and 1:1:1 mixture of DHDAB, CT DNA, and DCP (○).

complexes did not lead to any significant rise in the T_m values for the resulting mixtures.

Similar examinations of the thermal phase transitions of the DHDAB−DNA complexes revealed that the inclusion of 2:1 and 1:1 equivalents of DNA resulted in enhancements in the phase transition temperatures of the individual complexes by ca. 4 and 8 °C, respectively (Figure 6B), relative to that of vesicular DHDAB alone, while additional DNA inclusion did not lead to any further enhancement of T_m except that it led to precipitation. In contrast to the observed DNA-induced changes in the phase transition properties of DHDAB and DODAB, the thermal phase transition behavior of the DPTAP in the presence of DNA was found to be affected only modestly. The T_m value for 1:1 DPTAP−DNA complex did not virtually change except that the thermogram was found to be a little different from that of the DPTAP vesicles alone. It was found that the initial sizes and differences in the lamellarities of the DPTAP vesicles as well as variation in their preparation protocol (vortexing vs sonication) did not result in any significant alteration in the melting properties either before or after complexation with DNA (Figure 6C and 6D). The phase transition parameters for various cationic lipid aggregates and their DNA complexes are given in Table 2.

Fluorescence Anisotropies during Anion-Induced DNA Release. To figure out what happens to the cationic lipid order after the release of DNA upon addition of anionic amphiphiles, we then examined specific cationic and anionic amphiphile mixtures in the presence and in the absence of DNA by measuring fluorescence anisotropies using DPH. In a typical experiment, a DPH-doped cationic vesicular or a micellar aggregate was first prepared to which an equimolar amount of an aqueous anionic amphiphilic aggregate was added. The fluorescence anisotropies experienced by DPH in such mixtures were then measured as a function of temperature. In parallel, first, a given amount of DPH doped, cationic amphiphilic aggregate was complexed with CT DNA. Then an aqueous dispersion of an anionic amphiphile was added into the cationic amphiphile−DNA complex. The resulting mixtures were allowed to equilibrate for 10 min, and then the fluorescence anisotropies (*r*) due to doped DPH were measured as a function of temperature. The *r* vs *T* plots for a particular cationic and anionic amphiphile combination were compared in the presence and absence of DNA (Figure 7).

Melting profiles of a physical mixture of CTAC and palmitic acid (PA) at pH 7.4 (1:1 mol/mol) gave a sharp break (Figure 7A) with an apparent T_m value at ∼42 °C which is in agreement with the value reported for cetyl trimethylammonium palmitate (CTAP) (*22*). The phase transition profile of a 1:1:1 ternary mixture of the CTAC−CT DNA−PA and the corresponding T_m values were found to be similar to that of the CTAC−PA ion-pair (CTAP). However, a 1:1 CTAC−DNA complex did not show any breaks in the *r vs T* plot, suggesting a lack of melting transition with CTAC−DNA complexes (not shown). This

indicates that while DNA from the CTAC−DNA complex is released, the cationic amphiphile CTAC gets tied up with added fatty acid anion palmitate for ion-pairing.

Similar experiments were performed with several other binary combinations of cationic and anionic amphiphiles with or without DNA. The thermogram and the cooperativity (sharpness) of the melting profile for an equimolar mixture of vesicular DHDAB and PA ($T_m \sim 52$ °C) were comparable to that of a 1:1:1 mixture of DHDAB−DNA−PA ($T_m \sim 48$ °C, Figure 7B). When a 1:1 mixture of CTAC and DCP was examined, the resulting transition profile was found to be complex (with minor and major transitions at ∼40 and ∼65 °C, Figure 7C). Chung and Regen (*23*) studied the melting profile of an equimolar mixture of CTAC and DCP by calorimetry and observed a pretransition at ∼39 °C and a main transition at ∼62 °C which is consistent with our findings. However, when the CTAC was first complexed with equimolar amount of DNA and then mixed with anionic DCP (1:1:1 mol/mol/mol), the melting profile became sharper and only the main melting transition (∼66 °C) was seen. On the other hand, a 1:1 mixture of cationic lipid (DHDAB) and anionic lipid (DCP) gave comparable melting transition patterns and breaks related to the T_m values irrespective of the presence or absence of DNA, except that the transition was little sharper in the presence of DNA.

Circular Dichroism Studies. The effects of addition of cationic amphiphilic aggregates into DNA were also studied by recording the CD spectra of CT DNA upon addition of progressively increasing amounts of the cationic amphiphile (Figure 8). Figure 8A, curve 1, stands for DNA alone (72.7 μM). Upon addition of a cationic vesicular DPTAP solution into DNA, both the positive and the negative CD bands due to native DNA got gradually reduced, finally reaching a saturation at [lipid]/ [DNA] ∼1.8 (Figure 8A, curves 2−9). Further addition of DPTAP led to increases in turbidity impeding further recording of the CD spectrum.

Addition of SDS into the above DPTAP−DNA complex, led to a gradual recovery of the CD spectral signatures of DNA. At a ratio of [SDS]/[DPTAP] ∼2.1, the original spectrum of DNA was nearly reproduced (Figure 8B). However, further SDS addition did not lead to any significant CD spectral change. A similar phenomenon was also observed with other cationic micellar or vesicular aggregates. In other words, addition of cationic amphiphilic aggregates mitigated the circular dichroism of native DNA. Inclusion of anionic aggregate to cationic amphiphile−DNA complexes led to a recovery of the CD spectrum of native DNA.

Gel Electrophoresis of the Cationic Amphiphile−DNA Complexes. To examine the complex formation of DNA with various cationic micelle- and vesicle-forming amphiphilic aggregates, various amounts of different aqueous cationic amphiphile or lipid dispersions were added separately into plasmid DNA pTZ19R or CT DNA, and the resulting mixtures were electrophoresed in agarose gel. The gel was then stained with EB to visualize the DNA. When either plasmid or CT DNA was complexed with CTAC, a retardation in the mobilities of the DNA bands in the gel (Figure 9A, lanes 2, 7) was seen. Addition of a micellar SDS solution (± CTAC/SDS charge ratio ∼1) into the CTAC−DNA complexes led to the recovery of the electrophoretic pattern observed from DNA alone. Thus upon SDS treatment, movement and stainability of DNA on agarose gel could be restored (Figure 9A, lanes 3, 4, and 8, 9, respectively, with plasmid DNA and CT DNA).

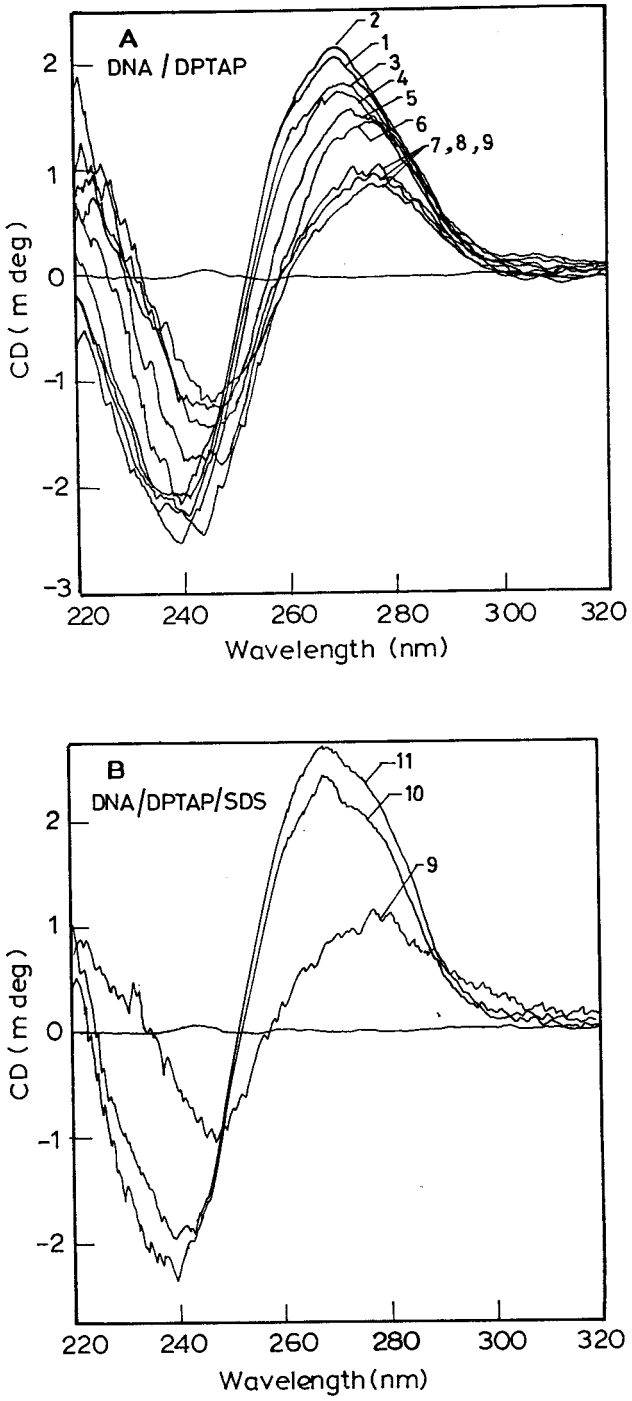

FIGURE 8: Circular dichroism studies. (A) Effect of DPTAP on CD spectra of CT DNA. Curve 1, DNA alone, [DNA] = 72 μM; curves 2−9 were obtained upon addition of progressively increasing amounts of liposomal solution of DPTAP. Final [DPTAP] = 2.39×10^{-4} M. (B) Effect of addition of liposomal aggregate SDS into the DPTAP−DNA complex. Curve 9 of Panel A is shown again in Panel B. Curves 10−11 were obtained upon addition of increasing amount of SDS into the DPTAP−DNA complex.

Complexation of DNA with various other cationic lipids was also examined. Addition of more than 1 equiv of vesicular DPTAP, DODAB, or DHDAB resulted in decreases in DNA migration and loss of staining on the agarose gel, as observed in the case of CTAC−DNA complexes (not shown). A small amount of DNA was found to be migrating near the well of the gel. This could have resulted from the

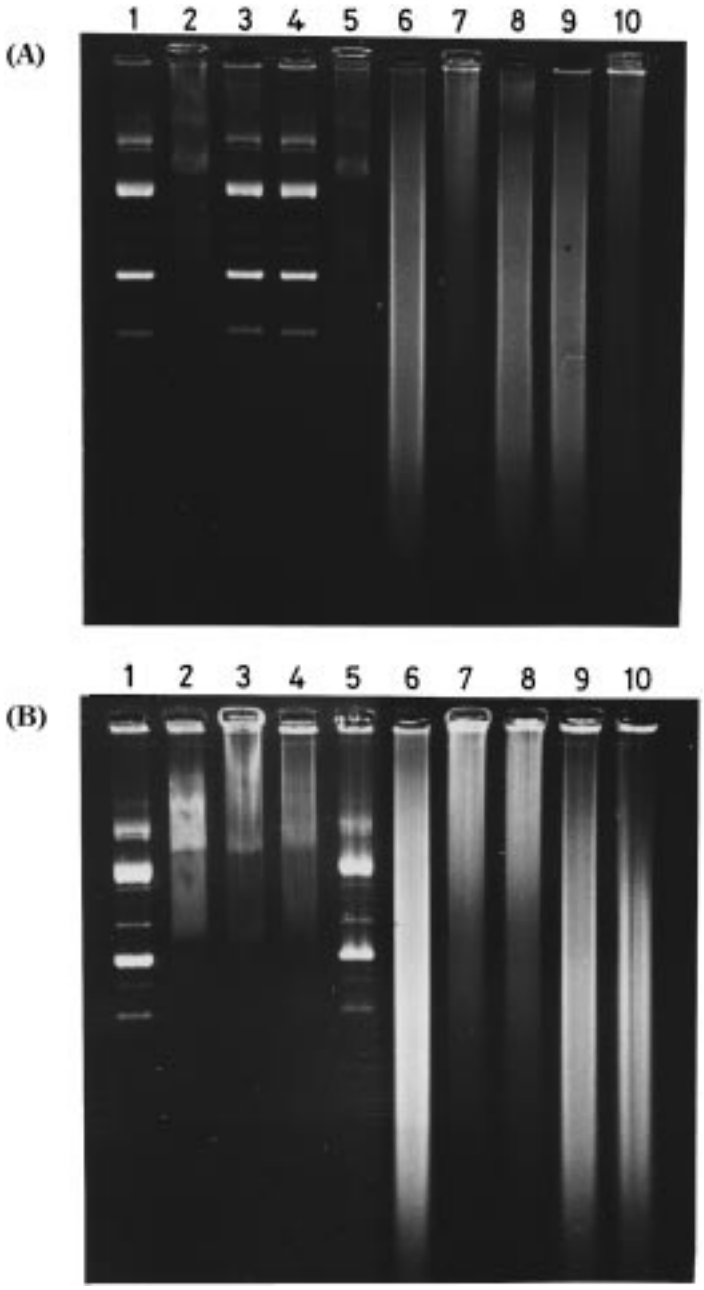

FIGURE 9: Gel electrophoresis of the preformed CTAC−DNA complexes upon incubation at 25 °C for 5 min (pH 7.4) with Tris-HCl and various anionic additives. In both the panels (A and B), (lanes 1−5) plasmid DNA pTZ19R, and (lanes 5−10) CT DNA were used, respectively. (A) Addition of SDS into CTAC−DNA complex: lane 1, plasmid DNA alone (0.5 μg); lane 6, CT DNA (1.5 μg) alone; lanes 2 and 7, respective DNA samples after complexation with CTAC at ± charge ratio 6.6 and 2.5, respectively; lanes 3−5, plasmid DNA−CTAC complexes that were treated with 11-, 1.1-, and 0.1-fold charge excess (over CTAC) of micellar SDS, respectively; lanes 8−10, CT DNA−CTAC complexes that were treated with 11-, 1.1-, and 0.11-fold charge excess (over CTAC) of SDS, respectively. (B) Addition of DCP into CTAC−DNA complex: lane 1, plasmid DNA alone (0.5 μg); lane 6, CT DNA (1.5 μg) alone; lanes 2 and 7, respective DNA samples after complexation with CTAC at ± charge ratio 6.6 and 2.5, respectively; lanes 3−5, plasmid DNA−CTAC complexes that were treated with 0.5-, 0.8-, and 1.2-fold charge excess of micellar DCP, respectively, at 25 °C for 5 min; lanes 8−10, CT DNA−CTAC complexes that were treated with 0.5-, 1-, and 1.5-fold charge excess (over CTAC) of SDS, respectively.

formation of a stable charge neutralized DNA−amphiphile complex in the presence of excess cationic amphiphile. At higher cationic lipid/DNA ratios, a positively charged complex is probably produced which may have prevented the EB staining of these complexes. Absence of DNA bands on the agarose gel under these conditions also may be due to the movement of the resulting complex toward the cathode.

The recovery of the staining ability and DNA mobility from the various cationic amphiphile−DNA complexes were also examined with anionic liposomes of DCP. DNA from the CTAC−DNA complex could be released upon incubation with >1 equiv of DCP at 25 °C (Figure 9B). However, from DODAB−DNA or DPTAP−DNA complexes only a negligible extent of DNA release was seen upon addition of DCP liposomes, even at DCP/cationic lipid ratio >1 at 25 °C, although at ∼40 °C, enhancement in the DNA release was evidenced. This might be due to more labile micellar aggregation of SDS over more rigid nature liposomal aggregates of DCP (T_m ∼ 66 °C).

DNA Sensitivity of Cationic Amphiphile−DNA Complexes to Chemical Nuclease. Evidence for the protection of DNA from DNase I digestion in the presence of cationic liposomes

has been recently demonstrated (*11, 24*). However, the presence of positive charges on the DNA−cationic lipid complexes may lead to denaturation of DNase I which could be responsible for its insensitivity to DNA in a cationic lipid complex. To unambiguously establish the resistance of DNA to cleavage upon its complexation with cationic lipid, the DNA−cationic amphiphile complexes were subjected to chemical nuclease digestion. Significant protection of the DNA molecules toward chemical nuclease degradation was observed with the complex in which the cationic amphiphile-to-DNA ratio exceeded 1 (Figure 10, lanes 3, 6). At over 10-fold higher concentration of the cationic amphiphile over DNA, complete protection of DNA toward degradation was observed (not shown). However, significant extent of DNA scission was observed when DNA−cationic amphiphile complexes were treated with anionic aggregates of either SDS or DCP when anionic/cationic amphiphile ratios were close to 1 (Figure 10A, lane 7, and Figure 10B, lanes 6, 7). In the case of Figure 10A (which included SDS) the nuclease activities on DNA were again lost when the SDS/CTAC exceeded 1. This could be due to preferential binding of the cationic nuclease reagents with anionic SDS aggregates over DNA (Figure 10A, lanes 8 and 9, respectively). Similar studies were also performed with other cationic lipids such as DHDAB or DPTAP. The results were similar as long as SDS was used as the anionic amphiphile. However, when the anionic additive was a vesicle forming amphiphile then the DNA release by the anion from the cationic lipid−DNA complexes was much less efficient. Hence the chemical nuclease-induced DNA scission could not be substantially enhanced upon the addition of anion into the cationic lipid−DNA complexes (not shown). A significant amount of precipitation was also noticed upon addition of the anionic vesicular DCP into either of the preformed complexes of cationic lipid, e.g., DODAB or DPTAP with DNA (not shown). These observations again point toward the importance of lipid order during the release of DNA from its complexes with cationic lipid.

To further confirm the above results, we also employed a known DNA cleaving reagent, $[Fe(EDTA)]^{2-}/H_2O_2$ (*25*) in parallel to the Ni−salen derivative/MMPP (*17, 26*) combination. It was found that the plasmid DNA, which was precomplexed with CTAC or HODAB, did not show any detectable DNA scission upon treatment with $[Fe(EDTA)]^{2-}/H_2O_2$ as was found with the Ni−salen/MMPP. When preformed cationic amphiphile−DNA complexes were incubated with either micellar SDS or vesicular DCP, DNA degradations were seen comparable to that in which the DNA alone was treated with any one of the above reagents (not shown). This showed that irrespective of the nature of the DNA cleaving agent, the findings on the DNA sensitivities are in agreement.

In other words, both types of cationic micellar and vesicular aggregates provide protection to DNA from the chemical nuclease digestion. Such protection, however, disappears when the cationic amphiphile−DNA complexes are treated with various anionic micellar or vesicular aggregates which promote the release of DNA to make them accessible to chemical nuclease reagents. Among the anionic additives examined, only anionic micelles (SDS), vesicles (DCP), and polyanionic dextran sulfate assisted in the DNA release from its complexes with cationic amphiphiles.

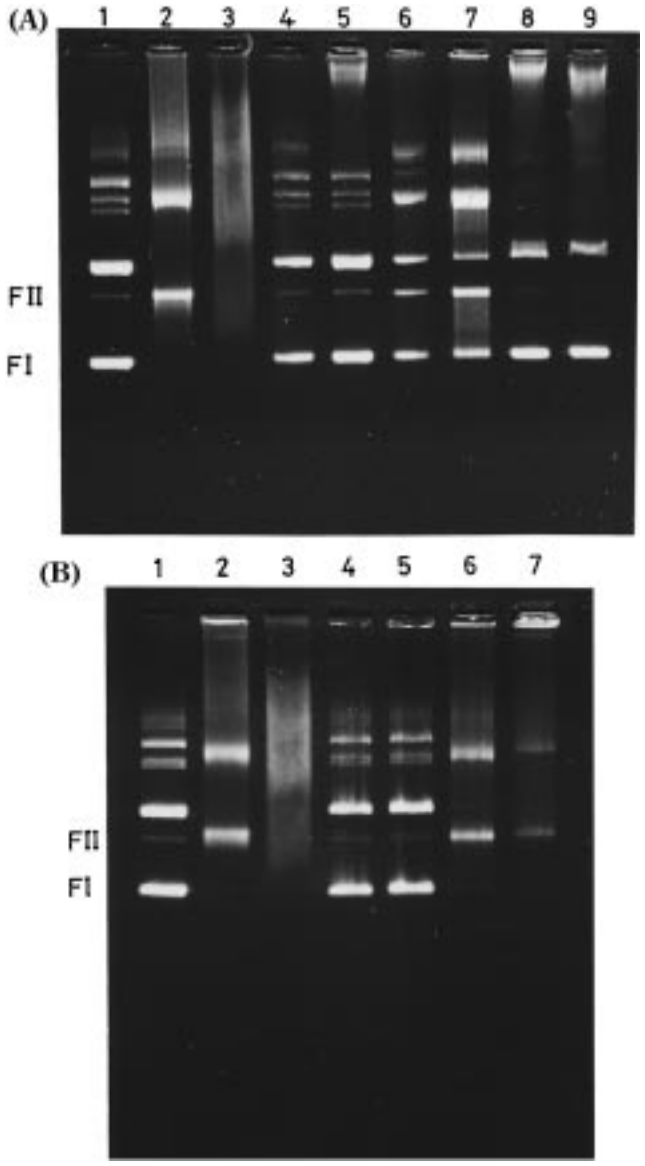

FIGURE 10: DNA sensitivity of cationic amphiphile−DNA complexes to chemical nuclease. (A) Preformed CTAC−DNA complexes (CTAC−DNA charge ratio 6.6) were mixed with various concentration of SDS. Selected reactions were subjected to chemical nuclease treatment [Ni−salen (20 μM), MMPP (500 μM)] digestion for 5 min at 37 °C.: lane 1, plasmid DNA alone; lane 2, DNA alone digested with [Ni−salen (20 μM), MMPP (500 μM) for 5 min]; lane 3, CTAC−DNA complexes; lane 4, DNA recovered from preformed CTAC−DNA complex by phenol/$CHCl_3$ extraction followed by ethanol precipitation; lane 5, preformed DNA−CTAC complex treated with SDS (SDS−CTAC ~1.1); lane 6, preformed DNA−CTAC complexes were first treated with chemical nuclease [Ni−salen (20 μM), MMPP (500 μM) for 5 min] and then subjected to phenol/$CHCl_3$ extraction followed by ethanol precipitation; lanes 7−9, DNA−CTAC complexes were treated with 0.6-, 1.1-, and 1.7-fold charge excess of SDS over CTAC, respectively. Then the individual reactions mixtures were digested with $Ni(salen)^{2+}$/MMPP for 5 min. (B) Effect of addition of addition of vesicular DCP into preformed CTAC−DNA complex: lane 1, DNA alone; lane 2, DNA alone digested with [Ni−salen (20 μM), MMPP (500 μM)] for 5 min]; lane 3, CTAC−DNA complex (± ratio 3.3); lanes 4 and 5, DNA−CTAC complexes treated with 1- and 1.5-fold charge excess of vesicular DCP over CTAC for 5 min, respectively; lanes 6 and 7, preformed CTAC−DNA, then with 1- and 1.5-fold charge excess of vesicular DCP over CTAC for 5 min at 37 °C, followed by treatment with chemical nuclease [Ni−salen (20 μM), MMPP (500 μM) for 5 min].

DISCUSSION

Vesicular or micellar aggregates composed of cationic amphiphiles form complexes with DNA. These can readily fuse with eukaryotic cell membranes. It is believed that such interaction leads to the delivery of DNA inside the cell. Several reports have appeared in the literature that address the DNA complexation by cationic lipids in detail; however, exactly how DNA is released from such complexes and what happens to the cationic lipids after DNA release remains inadequately understood. Understanding the mechanism of this event is required as this involves the participation of the biomolecules that are present in the cell membranes during DNA release and its subsequent entry into the nucleus for transcription. This also addresses the status of cationic lipids which are believed to be responsible for the toxic effects associated with transfection recipes (*4*, *7*, *27*).

While synthetic amphiphiles on their own can produce micellar, vesicular, or related aggregates, they can also be mixed with naturally occurring lipids such as dioleoylphosphatidylethanolamine (DOPE) prior to their aqueous dispersion. As a matter of fact, most of the presently studied commercially available formulations employ DOPE as a helper lipid for optimal transfection activity. The role of DOPE has been examined extensively (*7*, *9*, *28*, *29*). However, to clearly understand the nature of cationic amphiphile−DNA complexation and the mechanism of DNA release from such complexes, we confined the present investigation involving DNA complexation and release with pure, single-component micellar or vesicular aggregates.

The DNA complexations with cationic CTAC micelles were accompanied by increase in their order (more rigid). This suggests that the association of the cationic amphiphilic aggregates with DNA is highly cooperative in nature such that the nucleic acid polyelectrolyte chain binds with amphiphiles through electrostatic interactions. This result is consistent with the reported observation of an elongated coil to compact, globular state transition in DNA upon binding with CTAC (*30*).

Both naturally occurring and synthetic lipids have been shown to have characteristic thermotropic gel (solid) to fluid transition properties (*18*). Since small variations in the lipid order can be sensed by the thermotropic studies, we first sought to analyze cationic lipid order−disorder (melting) transitions in complexes with DNA. To elucidate the role of lipid chains on DNA complexation, cationic lipids containing full saturation or single olefinic unsaturation in the hydrocarbon chain were employed and the fluorescence anisotropies (r) of the DPH embedded in various cationic lipid bilayers or in their complexes with DNA at various cationic lipid to DNA phosphate charge ratios were measured as a function of temperature. These investigations reveal that DNA insertion into cationic lipid vesicles of DHDAB or DODAB gradually causes an increase in the apparent order of the lipid organization. The enhanced order of the DNA−cationic lipid complex system at T below and above the T_m is clearly reflected in the higher r values. Such cooperativity implies that the basic lamellar organization is maintained in DNA complexes of cationic lipids. Interestingly, DNA appears to interact more effectively at the melting temperatures of these vesicles. However, increase in the lipid order upon DNA binding is also dependent on lipid structure. Thus when the vesicles of a different (glycerol-based) molecular architecture (i.e., DPTAP) were employed in an analogous study, only modest effect in the order upon DNA binding was seen.

The observed differences in the melting behavior upon DNA complexation with the two types of cationic lipid systems employed herein appear to be intriguing. The lipid architectures with glycerol backbones as in DPTAP have been shown to be more tightly packed in membranes than in the membranes of their dialkyl dimethylammonium ion counterparts (DODAB or DHDAB). The presence of a diester chain/headgroup connector region in the pseudoglyceryl lipid DPTAP promotes additional intermolecular interaction presumably via water-promoted hydrogen bonding networks. This was inferred on the basis of the earlier studies involving cationic dialkyl dimethylammonium lipids and their corresponding diester analogues (*18*). To begin with, the amphiphile packing in the pure vesicles of DHDAB or DODAB is looser than that of the DPTAP. Since DNA binding of cationic lipids leads to compact particle formation (*11*), the hydrocarbon chain packing in such compact particles is considerably increased leading to a rise in the apparent order in the DODAB− or DHDAB−DNA complexes. However, in the case of DNA−DPTAP complexes, the hydrocarbon chain packing does not increase significantly as the DPTAP lipid monomers even in the absence of DNA are already tightly packed.

Our studies further indicate that the cationic lipids with high melting temperatures were less responsive to DNA binding. We believe that in the solid gel-like phase, the cationic lipid aggregates are not flexible enough to allow for efficient DNA interaction. From preformed DNA complexes of cationic lipids of $T_m \gg 25$ °C, the DNA release was also found to be significantly less efficient at ambient temperature (see below). Prior studies (*4*) have indeed shown that the transfection activities of the cationic lipid analogue are considerably affected by the changes in the composition of the hydrocarbon chain region. Thus, as the chain lengths in the cationic lipids in the saturated series were increased from 14 to 16 or 18 carbon atoms, the transfection activities of the resulting systems progressively declined. At the same time, introduction of unsaturations in one or two hydrocarbon chains of the lipids enhances the transfection capacities relative to their fully saturated chain analogues.

The addition of cholesterol to cationic bilayer vesicles abolishes their phase transition temperatures (*21*). Cholesterol is believed to influence the hydrocarbon chain conformations of the cationic lipid favoring a fully extended *s-trans* conformation rather than the coiled *s-gauche* conformation that predominate above T_m. Since the chains with *s-trans* conformation pack better, a more tightly organized array is formed in the presence of cholesterol, resulting in an increase in the stiffness of the bilayer. Consequently, we examined the DNA release from ternary complexes comprised of DNA−cationic lipid−cholesterol. While small effects in the DNA release were seen with anionic vesicles, the overall impact on the cholesterol inclusion in preformed complexes on the DNA release was not profound. These findings are in line with observations of Xu and Szoka (*24*). Thus the presence of cholesterol, even at concentrations found in endosomal surfaces, does not significantly influence the DNA release from its complexes with cationic lipids.

Endocytosis of a DNA−cationic liposome complex is believed to be the primary route of DNA uptake by cells during transfection (*4, 7, 12, 31*−*33*). Results from the previously described experiments clearly indicate that there exist strong interactions between the cationic amphiphiles and DNA to produce complexes of significant stability. Therefore, the obvious question arises as to how DNA dissociates from such complexes before entering into the nucleus. One would expect that the cationic amphiphile−DNA complexes owing to their hydrophobic character (*34)* would permeate through the outer membrane barrier to reach endosomal surfaces of the cell. The cytoplasmic layers of the plasma and endosomal membranes are unusually rich in negatively charged phospholipids. Such intrinsic lipid asymmetry associated with outer and inner surfaces of the cell membranes should be responsible for mediating the DNA release. Therefore, for effecting DNA release from the cationic amphiphile−DNA complexes, a range of anions of biological relevance was employed. These include saturated and unsaturated fatty acids, cholic acid (bile salt), anionic single- and double-chain amphiphiles capable of forming micellar or vesicular types of supramolecular organizations and a few polyanionic substances such as dextran sulfate or poly(Glu). It was found that the efficiencies of DNA release by the nonamphiphilic anions are much less than those of the negatively charged amphiphiles or lipids. Therefore it is clear that hydrophobic forces must play an important role in the DNA release process.

Anionic micellar and vesicular aggregates indeed demonstrated profound abilities to release DNA from its complexes with cationic amphiphilic aggregates. Thus, when a little over 1 equiv of anionic aggregate is added into a DNA−*cationic* lipid complex, the DNA becomes accessible to small intercalators such as EB (∼88% recovery of the intercalation capacity) and also becomes amenable to chemical nuclease digestion (>60% degradability). The last observation is consistent with earlier reports concerning DNA sensitivity to DNase I upon addition of anions to DNA complexes with cationic lipids (*11, 24)*. We also found that DNA−cationic lipid complexes either did not move on an agarose gel under electrophoretic conditions or lacked the ability to stain by the loss of capacities of intercalation of EB molecules. Upon treatment with >1 equiv of *anionic* liposomal or micellar solution, the DNA bands could be clearly seen on agarose gel under an applied electric field upon staining with EB. Polyanionic systems such as dextran sulfate or poly(Glu) (at high ± charge ratio) also showed limited abilities to displace DNA from its complexes with cationic liposomes. Notably however, small anions such as ATP, acetate, or even single-stranded DNA could not initiate any detectable dissociation of DNA from its complexes with cationic lipids. DNA release from its complexes with cationic lipid (DOTAP) vesicles induced by other anionic liposomes prepared from either PE, PS, PA, or PI lipids has been recently demonstrated (*24)*. Since zwitterionic lipids such as DOPC or DMPC or cholesterol and neutral single-chain micelle forming detergents such as Triton-x-100 did not effect any DNA release from the preformed cationic lipid−DNA complexes under comparable conditions, the charge on the additive amphiphile should have the key for the initiation of DNA release from its complexes with cationic liposomes. Thus only when the cationic amphiphile−DNA complexes sense the presence of anionic phospholipids in the vicinity of endosomal membranes do complex dissociation and the lipid mixing process begin (*19, 35*−*38)*.

Notably, the temperatures at which the anion-induced DNA release from its complexes with a given cationic lipid is conducted appear to be also important. Thus when the anionic vesicular DCP-induced release of DNA from its complexes with DODAB or DPTAP was examined as a function of temperature, optimal DNA release was observed only at the respective T_m values of the cationic lipids. This suggests that the nature of the hydrocarbon segment of the cationic lipid and the fluidities of the corresponding membranes play a key role in the anion-induced DNA release.

How does the interaction between DNA−cationic amphiphile complexes and the anionic additives induce DNA release? Electrostatic interaction is obviously important as only the anionic substances could promote DNA release. However, the electrostatic forces alone cannot drive DNA dissociation from DNA−cationic amphiphile complexes. Note that the complex formation of DNA and cationic amphiphiles is driven by strong electrostatic interactions. Although all the anionic substances do not induce DNA release, it is only when the additive is a negatively charged amphiphilic aggregate, the DNA release is very effective. Thus, the DNA release from its cationic amphiphile complexes by anionic amphiphiles must occur only when the electrostatic and hydrophobic interactions between amphiphilic cations and anions are reinforced. Addition of anionic amphiphiles in micellar or vesicular form triggered DNA release from its complexes with cationic amphiphiles and produced a thermogram specific for a given interlipid ion-pair (Figure 7). In crucial control experiments it was demonstrated that the ion-paired amphiphiles in the absence of DNA also confer similar thermotropic behavior. Mixtures of micellar solution of cationic amphiphile with micellar solution of anionic amphiphiles have been shown to form lamellar- and bilayer-type organizations. This is due to overall reduction in the headgroup area as a consequence of electrostatic attractions (ion-pairing) between the + and − charged components at the headgroup level and enhanced chain packing at the hydrophobic ends (*22, 23)*.

The retardation in DNA mobility in agarose gel upon complexation with CTAC suggests that the DNA−CTAC complex is less negatively charged relative to pure DNA which caused retardation in their movements toward the anode. This result is consistent with the earlier reports demonstrating that cationic liposome induced condensation of DNA leads to formation of a macromolecular complex that does not move within an applied electric field (*39)*. Alternatively, this could also be a consequence of the charge neutralization, compaction formation, and/or increases in molecular size during DNA−amphiphile complexation. One may expect that the restoration of DNA movement on the gel is possible if the amphiphile−DNA complex can be physically dissociated to release the free DNA capable of intercalating EB. Indeed the recovery of the DNA movement and attainability of its staining capacity upon addition of SDS into the CTAC−DNA complex suggest the release of DNA in its native form. Similarly, addition of adequate amounts of SDS to DHDAB−DNA or DPTAP−DNA complexes led to the release of DNA from the respective cationic double-chain surfactant−DNA complexes. But when similar DNA

release experiments were carried out by anionic DCP vesicles from the cationic lipid−DNA complexes at 25 °C, the release was not efficient. This again shows that the DNA release is dependent on the T_m of the cationic lipid employed for the DNA complexations.

To explore the DNA sensitivity in the presence of two different chemical nucleases in its complexes with cationic amphiphile aggregates, we employed $[Fe(EDTA)]^{2-}/H_2O_2$ (*25)* and Ni−salen derivative/MMPP (*17)*. These reagents avidly induce DNA cleavages by oxidative chemistry, and such reactivities are not affected due to the presence of cationic surfactant aggregates. It was found that the plasmid DNA, which was precomplexed with CTAC or HODAB, did not show any detectable DNA scission upon treatment with either of the above chemical nucleases. However, when the preformed cationic amphiphile−DNA complexes were incubated with either micellar SDS or vesicular DCP, DNA degradations comparable to that in which native DNA was treated with any one of the above reagents were seen (not shown). This showed that irrespective of the nature of the DNA cleaving reagent, the findings on DNA cleavages are consistent. Although, it has been demonstrated that DNA upon complexation with cationic liposome is rendered insensitive to DNase I digestion or chemical nuclease treatment, we found a small (<10%) DNA cleavage when the plasmid pTZ19R was complexed with CTAC at a CTAC/DNA ratio ∼6 (Figure 10A, lane 6). This observation suggests that at this CTAC/DNA ratio, perhaps the entire face of the helical DNA polymer is still not fully protected by electrostatic wrapping by CTAC, leaving a few DNA sites probably still accessible to chemical nuclease digestion. Similar kinds of interaction were seen when histone−DNA complexes were subjected to DNase I digestion (*40)*. Our assertion is buttressed by the fact that when a large excess of CTAC was used during DNA−CTAC complex formation, the resulting complex was found to be absolutely resistant toward nuclease degradation. Evidence for the DNA protection from natural enzyme, DNase I digestion in the presence of cationic liposomes has been already shown (*11*, *24*). Since the presence of positive charges on the DNA−cationic lipid complexes may lead to prior denaturation of DNase I, our findings unambiguously demonstrate that indeed DNA is rendered insensitive to nuclease degradation upon complexation with cationic lipids or amphiphiles and vindicate the findings reported earlier.

In summary, we provide for the first time an experimental evidence of interlipid or interamphiphilic ion-pairing during the DNA release from its complexes with cationic amphiphiles induced by anionic micelles or vesicles. Thus, the present findings provide experimental support to the hypothesis put forward on the putative DNA release mechanism during transfection by Xu and Szoka (*24*). We have further shown that the cooperative melting transitions of the cationic lipids are also retained after DNA complexation. Such cooperativity implies that the basic lamellar structure is maintained in the DNA complexes of the cationic lipids. These findings also explain why cationic lipids with unsaturated hydrocarbon chains (lower T_m values than the ambient temperature) improve cytofectin activity, and their saturated chain analogues either have no enhancing effects or are inactive. From a standpoint of design of new DOPE-independent cationic lipids for transfection, our findings also highlight the fact that there is considerable scope for fine-tuning such systems so that they are more effective in DNA delivery. Judicious control of the critical molecular design parameters such as headgroup size, lipophilic segments, and thermal state of the membranous surface should help in designing more effective transfection recipes. The fact that DNA interacts more effectively at the melting temperatures of the cationic lipid vesicles emphasizes the importance of the choice of the cationic lipid based on its T_m for optimal transfection.

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