High Temperature Stabilization of DNA in Complexes with Cationic Lipids

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ABSTRACT The influence on the melting of calf thymus and plasmid DNA of cationic lipids of the type used in gene therapy was studied by ultraviolet spectrophotometry and differential scanning calorimetry. It was found that various membrane-forming cationic lipids are able to protect calf thymus DNA against denaturation at 100°C. After interaction with cationic lipids, the differential scanning calorimetry melting profile of both calf thymus and plasmid DNA revealed two major components, one corresponding to a thermolabile complex with transition temperature, $T_{m(labile)}$, close to that of free DNA and a second corresponding to a thermostable complex with a transition temperature, $T_{m(stable)}$, at 105 to 115°C. The parameter $T_{m(stable)}$ did not depend on the charge ratio, $R(\pm)$. Instead, the amount of thermostable DNA and the enthalpy ratio $\Delta H_{(stable)}/\Delta H_{(labile)}$ depended upon $R(\pm)$ and conditions of complex formation. In the case of *O*-ethyldioleoylphosphatidylcholine, the cationic lipid that was the main subject of the investigation, the maximal stabilization of DNA exceeded 90% between $R(\pm) = 1.5$ and 3.0. Several other lipids gave at least 75% protection in the range $R(\pm) = 1.5$ to 2.0. Centrifugal separation of the thermostable and thermolabile fractions revealed that almost all the transfection activity was present at the thermostable fraction. Electron microscopy of the thermostable complex demonstrated the presence of multilamellar membranes with a periodicity 6.0 to 6.5 nm. This periodic multilamellar structure was retained at temperatures as high as 130°C. It is concluded that constraint of the DNA molecules between oppositely charged membrane surfaces in the multilamellar complex is responsible for DNA stabilization.

INTRODUCTION

Complexes of DNA vectors with cationic lipids (lipoplexes) are widely used for transfection of cultural cells and have been successfully applied in modern gene therapy. Their physical properties and structure have been increasingly studied since the synthesis of the first effective cationic lipid transfection agent, DOTMA, in 1987 (Felgner et al., 1987). A number of investigators have found that the structure of lipoplexes and their transfection activity varies considerably, depending, for example, on aspects of the preparation protocol, such as the size of liposomes, positive to negative charge ratio, and even the sequence of addition of DNA and lipid (Chesnoy and Huang, 2000; Lin et al., 2000; Kennedy et al., 2000).

The first electron microscopy studies of DNA-cationic lipid complexes revealed the presence of various tube-like structures of lipid, the presence of which was initiated by interaction with DNA molecules (Gustafsson et al., 1995; Lasic et al., 1997; Bhattacharya and Mandal, 1998). Non-lamellar structures, coexisting with membrane vesicles, were observed later by many authors (Sternberg et al., 1994, 1998; Zabner et al., 1995; Wheeler et al., 1996; Xu et al., 1999). Increasingly, however, evidence has accumulated that indicates that multilamellar structures consisting of many parallel lipid bilayers alternating with monolayers of DNA helices predominate in a number of complexes of DNA with cationic lipids (Gustafsson et al., 1995; Lasic and Templeton, 1996; Lasic et al., 1997; Battersby et al., 1998; MacDonald et al., 1999). Hexagonally ordered lipid tubes containing DNA molecules were also observed in some cases (Ghirlando et al., 1992; Tarahovsky et al., 1996; Mel'nikova et al., 1999). The coexistence of multilamellar structures with hexagonally ordered lipid tubes was confirmed by small-angle x-ray scattering (Rädler et al., 1997; Lasic et al., 1997; Koltover et al., 1998). It is possible that a wide range of different structures normally coexists in a given sample, and different experimental approaches will be needed to reveal them. Furthermore, the structure of complexes appears somewhat variable and can depend on conditions of sample preparation and the proportion of positive and negative charge. For example, multilamellar complexes predominated in samples with excess of cationic lipid, whereas with excess negative charged DNA, unilamellar coated vesicles were present (Huebner et al., 1999).

It is known that cationic substances influence the physical properties of DNA. For example, the DNA melting process displays considerable sensitivity to the presence of mono- and especially polyvalent cations. A large number of metal cations, including the naturally abundant K^+, Na^+, Ca^{2+}, and Mg^{2+}, can significantly influence the thermodynamic parameters of the DNA melting process (Dove and Davidson, 1962; Eichhorn and Shin, 1968; Gruenwedel, 1974; Duguid et al., 1993, 1995). These effects may be assumed to be due to differential interactions of the metal ions, i.e., in general, any ligand that interacts more strongly with double-stranded than with single-stranded DNA will influence the thermodynamic parameters of helix-coil transition and

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hence also the DNA melting process by shifting the equilibrium toward stabilization of the helix form (Bloomfield et al., 2000). Thus, low and moderate concentrations of metal cations stabilize DNA and increase the temperature of melting. There are limits to such effects, however, because high concentrations of alkaline earths and the transition metal ions cause rupture of hydrogen bounds, base unstacking, and ultimately decrease of thermal stability of DNA.

The thermal stability of DNA, its base pairing, as well as the electrostatic interactions of DNA with some ligands are also sensitive to the aqueous environment. The extent of hydration of DNA influences interactions of cations with the DNA phosphate groups and the strength of base pairing. Some uncharged molecules, polyethylene glycol for example, can alter water activity and, as consequence, significantly affect the temperature and free energy of DNA melting (Spink and Chaires, 1999). The hydration shell of DNA consists of a specifically ordered array of ~15 to 20 water molecules per base pair (Rentzeperis et al., 1993; Chalikian et al., 1999). DNA melting is accompanied by release of four water molecules per base pair and 0.3 to 0.5 Na^+ ions per phosphate charge, depending on the salt concentration (Spink and Chaires, 1999).

Here we describe thermal stabilization of DNA molecules by cationic amphiphiles. We observed that thermal stabilization of DNA by membrane-forming cationic lipids depends on the charge ratio in a way that it is substantially different from that of the case of water-soluble cationic substances like metal cations. This indicates a fundamental difference in interaction with DNA of cationic water-soluble compounds and cationic membrane surfaces formed by lipid. It is assumed that the structural characteristics of DNA-lipid complexes are important for understanding their physical properties.

MATERIALS AND METHODS

Materials

Tetradecyltrimethylammonium bromide (TTMA), dimethylaminoethanecarbamoyl cholesterol (DC-Chol), and highly-polymerized calf thymus DNA were purchased from Sigma (St. Louis, MO). Dioleoyltrimethylammoniumpropane (DOTAP), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylethanolamine (DOPE) were from Avanti Polar Lipids (Alabaster, AL). Ethyldioleoylphosphatidylcholine (EDOPC) was either from Avanti as the chloride salt or synthesized as previously described as the trifluoromethysulfonate salt (Rosenzweig et al., 2000). Plasmid pCMVSport β-gal (7853 bp) was purchased from Bayou Biolabs (Harahan, LA) in quantities sufficient for physical characterization.

DNA preparation

Highly polymerized calf thymus DNA was hydrated overnight in milli-Q water and treated in a 250-W laboratory ultrasound bath for 5 h at 40°C in a sealed glass vial filled with argon. Electrophoresis in 1% agarose of DNA treated in this way revealed the presence of predominantly 6000- to 7000-bp fragments. The DNA fragments were diluted 2 times with 10 mM sodium cacodylate, pH 7.0, or double concentrated Dulbecco phosphate-buffered saline (DPBS), pH 7.2, which contained 1 mM Ca^{2+}, 4 mM K^+, 5 mM Mg^{2+}, and 150 mM Na^+.

Preparation of liposomes and DNA-lipid complexes

The appropriate volume of a chloroform solution of lipid was transferred to a glass vial and the bulk of the solvent removed under an argon stream. Subsequently the lipid was placed under high vacuum for ~2 h. The dried film was then hydrated in either 5 mM cacodylate, pH 7.0, or DPBS, pH 7.2 and vortexed for a few minutes to yield an apparently homogenous white suspension with a final lipid concentration of 10 mM. Sonicated liposomes were prepared from vortexed liposomes by immersing glass vials containing a few milliliters of sample in an 80-kHz ultrasound bath (Laboratory Supplies Co., Hicksville, NY) for a few minutes. The suspension was saturated with argon before sonication. Stock dispersions of liposomes were added to DNA fragments or plasmid DNA under conditions of continuous magnetic stirring. Sometimes the complexation was accompanied by the formation of a heavy precipitate, in which case, the precipitated complexes were homogenized by 10 to 100 passes through a syringe until a homogenous suspension was formed.

Differential scanning microcalorimetry

Solutions of DNA or dispersions of DNA-lipid complexes were prepared as described above. Complexes prepared from sonicated calf thymus DNA (1 mg/ml) or plasmid DNA (0.5 mg/ml) and a corresponding amount of lipid were analyzed with a VP-DSC Micro Calorimeter (MicroCal Inc, Northampton, MA) at a scan rate of 10°C/h. Additional details on preparation of individual samples are provided below in the Results section. Computer analysis of the differential scanning calorimetry (DSC) data was performed using Origin Scientific plotting software, version 5. Data were analyzed after subtraction of the baseline obtained by scanning with the corresponding buffers in both sample and reference cell. Repeated scans revealed good reproducibility in both the transition maxima and their thermodynamic parameters.

Ultraviolet absorption measurements

The concentration of DNA was determined from ultraviolet (UV) absorption at 260 nm using the relationship 1.0 absorbance units (A) = 50 μg/ml DNA. For determination of DNA denaturation in DNA-lipid complexes, the appropriate amount of lipid suspension was added to 2 ml of stock solution containing 50 μM DNA in 5 mM sodium cacodylate, pH 7.0. Glass tubes containing the complexes were immersed in boiling water for 10 min, cooled to room temperature, and then 1 M sodium dodecyl sulfate (SDS) was added to a final concentration of 25 mM. The percentage of native DNA (DNA%nat) was calculated according to: DNA%nat = 100%(DNAden − DNAs)/(DNAden + DNAnat), in which DNAden, DNAnat, and DNAs are absorption of denatured, native, and sample DNA at 260 nm, respectively, in the presence of 25 mM SDS.

Separation and assay of DNA-lipid complexes

Complexes were separated into two fractions by centrifugation in an Eppendorf centrifuge (15,000 rpm at 4°C for 30 min). After removal of the supernatant, the sediment was vigorously suspended in the initial volume of buffer. The amount of DNA in both sediment and supernatant was estimated spectrophotometrically at 260 nm in the presence of 25 mM SDS to diminish light scattering. To assay for lipid content, 1% (lissamine Rhodamine B sulfonyl)-DOPE was added to the liposomes and its concentration determined by measuring absorbance at 560 nm.

Electron microscopy and image analyses

For thin section electron microscopy, complexes of DNA were prepared with cationic lipids containing the unsaturated oleic acid. Complexes were prepared according to the procedure described above. Samples with a 1-mg/ml DNA concentration and a corresponding concentration of lipid in 5 mM cacodylate or DPBS buffers were fixed with 1% osmium tetroxide overnight. Fixation at temperatures above 100°C was performed in screw cap vials heated to the desired temperature in a dry block heater. After 10 to 15 min incubation at the necessary temperature, the osmium tetroxide solution was injected by syringe through a rubber septum. After fixation, the sample was sedimented by centrifugation at 5000 to 8000 rev/min in an Eppendorf centrifuge, postfixed overnight with 1% tannic acid in the buffers mentioned above, dehydrated in series of alcohol and propylene oxide, and embedded in Epon resin according to standard procedures. Thin sections of ~50-nm thickness were cut on a MT6000-XL microtome (RMC, Inc., Tucson, AZ) and observed with a JEM-100CX (JEOL, Peabody, MA) electron microscope. The micrographs (magnification = 250,000) were digitized, and fast Fourier transforms of the selected regions were performed with Scion Image (Scion Corporation Imaging Software, Frederick, MD) for Windows 98.

Transfection assay

The transfection procedure has been described elsewhere (MacDonald et al., 1999). In brief, lipid at 1 mg/ml was quickly added to pCMVSport-β-gal plasmid at 0.1 mg/ml to give a 1:1 weight ratio (corresponding to a 3% positive charge excess). Both compounds were in the DPBS buffer. In some experiments, complexes were separated by centrifugation as described above. Transfection using both whole complex and its fractions (supernatant and sediment) was performed on BHK cells. Cells were grown in 96-well plates, and complex containing 3 μg of DNA was added to each well. β-Galactosidase was assayed after 20 to 24 h using a microtiter plate assay described elsewhere (Rakhmanova and MacDonald, 1998).

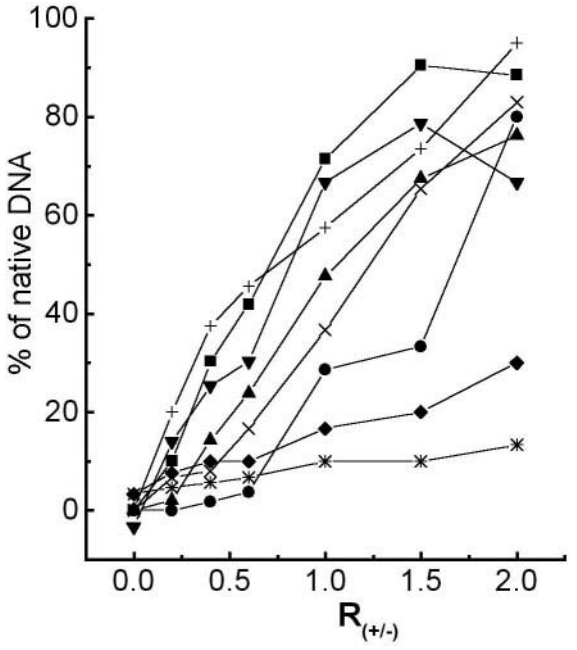

FIGURE 1 Percent denaturation of native calf thymus DNA after 10 min boiling DNA-lipid complexes that were prepared with EDOPC (■), DOTAP (●), DC-Chol + DOPC (1:1) (▲), DC-Chol + DOPE (1:1) (▼), TTMA (◆), TTMA + DOPE (1:1) (+), TTMA + DOPC (1:1) (×), and DOPC (*). The data were obtained from UV spectrophotometry at 260 nm of samples prepared in 5 mM cacodylate (pH 7.0). Lipid was added before boiling. SDS (25 mM) was added at room temperature after boiling and then cooling the sample to room temperature. All liposomes were ultrasound treated. The limits, 100% and 0%, of native DNA are based on the UV absorbance of the control solution of DNA obtained before and after boiling.

RESULTS

UV spectrophotometry of DNA-lipid complexes after boiling

When calf thymus DNA was held at 100°C for 10 min and then cooled to room temperature, an increase in absorption at 260 nm was observed. As is well known, this is the hyperchromic effect due to denaturation-induced unstacking of base pairs. In contrast to the significant hyperchromicity of free DNA, changes in UV absorption were much smaller when samples of DNA were treated with various cationic lipids before heating, i.e., the presence of the lipids greatly reduced the extent of DNA denaturation at 100°C. The effect was observed only in the samples treated with SDS after cooling the sample to room temperature; if SDS was added before or during heating, no protection of the DNA against thermal denaturation was observed and its denaturation was close to 100%.

The extent of protection depended strongly on the charge ratio of DNA-lipid mixtures, $R(\pm)$. As shown in Fig. 1, for most cationic lipids and lipid mixtures (containing the neutral lipids DOPC or DOPE) used in our experiments, the protection was above 75% in the range of $R(\pm) = 1.5$ to 2.0. In the absence of a cationic component, DOPC had no protective activity. The effect of the cationic detergent TTMA alone was considerable smaller than that of the cationic lipids, EDOPC or DOTAP, however, mixtures of TTMA with the bilayer-forming neutral lipids DOPC or DOPE expressed a protective activity similar to that of the other cationic lipids.

It should be noted that above $R(\pm) = 2.5$ to 3.0 we observed such a dramatic increase of UV absorption, evidently due to extensive aggregation, which led to high levels of light scattering, that reliable data could only be obtained in the range shown in Fig. 1.

DSC assay of boiled DNA-lipid complexes

Because of the light scattering limitation of absorbance methods for determining DNA denaturation, we used DSC to further characterize the protection of DNA by the cationic lipid, EDOPC, i.e., the compound that exhibited the largest effect according to Fig. 1. In the presence of EDOPC and SDS, control samples of calf thymus DNA melted in the temperature range of 60 to 85°C with a maximum at 71°C (Fig. 2 *A*). The shape of the curve shown is typical for calf thymus DNA, although the position of the maximum de-

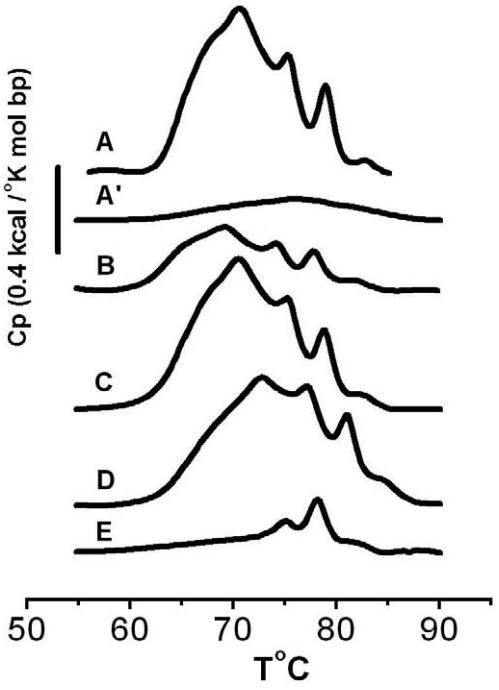

FIGURE 2 Thermal protection of calf thymus DNA/EDOPC complexes revealed by DSC. Samples were prepared in 5 mM cacodylate buffer, pH 7.0. (*A*) Control sample of calf thymus DNA in presence of 25 mM SDS. (*A′*) Sample "A" after 10 min boiling. (*B–D*) DNA/EDOPC complexes after 10 min boiling followed by cooling to room temperature and treatment with SDS to 25 mM. $R(\pm) = 0.7$ (*B*); $R(\pm) = 1.7$ (*C*); $R(\pm) = 3.3$ (*D*). (*E*) This sample was heated for 10 min at 110°C ($R(\pm) = 1.7$). Refer also to Fig. 3.

pends on the amount and type of electrolyte present. As shown in Fig. 2 *A′*, when the sample was first heated to 100°C and then scanned in the calorimeter, no heat absorption was revealed because denaturation of calf thymus DNA is effectively irreversible on the time scale of these experiments. In contrast to the results of Fig. 2, *A* and *A′*, when membrane-forming lipid EDOPC was added to DNA before first heating at 100°C, its protective effect was readily apparent in the significant heat absorption that was revealed in the subsequent heating scan (Fig. 2, *B–D*). Thus, the DNA from the complex that had been heated to 100°C was clearly native because it could be subsequently denatured after being released from the lipid by SDS treatment. It may be seen that the extent of DNA protection depended upon the charge ratio $R(\pm)$ (Fig. 3), and a 1.5-fold excess of positive lipid over negative DNA charge of was required for maximal protection. It may also be observed in Fig. 2 *E* that a distinct fraction of native DNA was present even after heating up to 110°C, although the melting profile of DNA was distorted and the principal transitions occurred at somewhat elevated temperatures. In general, the DSC experiments confirm the results of the UV measurements described above. The

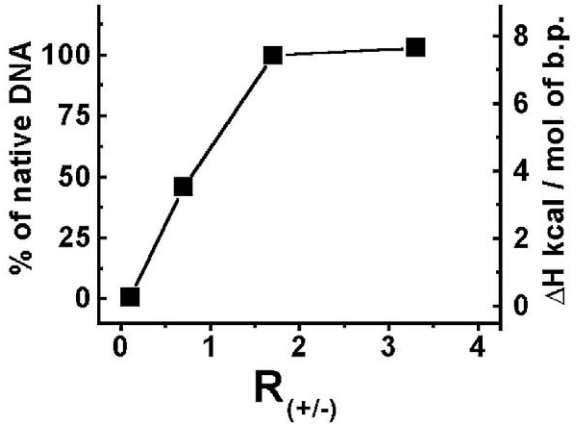

FIGURE 3 Protective effect of EDOPC on calf thymus DNA. The relationship between the percentage of native DNA and $R(\pm)$ was calculated from the data presented in Fig. 2

protective effect depended on $R(\pm)$ in a similar manner for both experiments (compare Figs. 1 and 3).

DSC assay of naive DNA-lipid complexes

Although the experiments described above indicated that DNA in complexes with cationic lipid denatures at a much higher temperature than does a control solution of free DNA, they did not reveal the actual value of that denaturation temperature. To characterize the DNA melting profile after complexation with cationic lipid, DSC scans were carried to a considerably higher temperature than 100°C. Such scans are presented in Fig. 4 for both calf thymus and plasmid DNA in complexes with EDOPC. These samples were examined at two different ionic strengths. In addition, two different sizes (vortexed and sonicated) of liposomes were used to form the complex with DNA. The quantitative data from these scans is given in Table 1. Both types of DNA revealed the presence of two fractions with very different melting temperatures. One fraction had a denaturation temperature, $T_{m(labile)}$, close to that of the control sample; it is termed the thermolabile component. The other fraction, with a denaturation temperature in the range above 100°C, $T_{m(stable)}$, is termed the thermostable component. The shape of melting curve and the position of maxima of both thermostable and thermolabile components, but especially the latter, depended up the nature of DNA and the electrolyte composition of solution, as may be seen in the three panels of Fig. 4. Calf thymus exhibited its typical denaturation profile of a broad major peak with three high-temperature shoulders. It also exhibited the expected shift to a higher temperature at a higher ionic strength; for example, the melting curve of calf thymus DNA in 5 mM cacodylate had a maximum at ~65°C, whereas in high ionic strength DPBS buffer, the maxima moved up to ~85°C (Fig. 4, *(1) A* and *(2) A*;

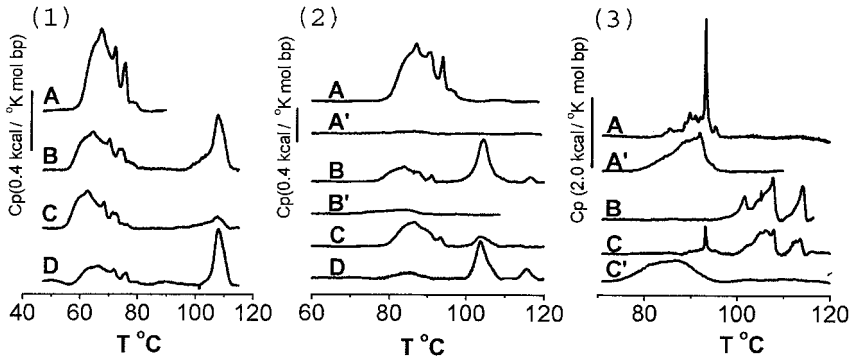

FIGURE 4 DSC melting profiles of EDOPC complexes prepared with DNA from different sources. (*1*) Calf thymus DNA in 5 mM cacodylate buffer, pH 7.0. (*2*) Calf thymus DNA in DPBS buffer. (*3*) Plasmid DNA in DPBS buffer. (*A*) DNA control samples. (*B*) Complexes made from sonicated lipid with $R(\pm) = 1$. (*C*) Complexes made from vortexed liposomes with $R(\pm) = 1$. (*D*) Complexes with ultrasound liposomes with $R(\pm) = 1.7$. (*A′–C′*) Second scans of corresponding samples, *A* through *C*, after heating to 120°C. See also Table 1.

Table 1). Most of the DNA in the lipid complex was thermostable and denatured with maxima in the range 103 to 107°C (Fig. 4, *(1) B* and *(2) B*). The thermostable melting profiles were narrower that those of thermolabile DNA and lacked the shoulders, although a small, separate peak, centered at 115°C, is visible at high ionic strength.

When the complex was formed from sonicated lipid, more thermostable DNA was present than when it was formed from vortexed lipid (compare Fig. 4, *(1) B* and *C*, see also enthalpies $\Delta H_{(\text{labile})}$, $\Delta H_{(\text{stable})}$ and enthalpy ratio $\Delta H_{(\text{stable})}/\Delta H_{(\text{labile})}$; Table 1), and when the ratio of sonicated lipid to DNA was increased from $R(\pm) = 1{:}1$ to $R(\pm) = 1.7{:}1$, the amount of thermostable DNA was increased by almost two times (compare Fig. 4, *(1) B* and *D* and *(2) B* and *D*; see Table 1). Upon rescanning, both the control DNA and the complexed DNA were seen to be completely denatured (see absence of endotherm in Fig. 4, *(2) A′* and *B′*). Calf thymus DNA by itself is too complex to significantly renature on the time scale of our experiments. Apparently, interactions with the lipid are unable to prevent the loss of alignment of the single strands, and the DNA remains denatured after heating to a high temperature even in the lipid complex.

The melting profile of plasmid DNA (Fig. 4 *(3) A*) was very different from that of the more complex chromosomal DNAs, and individual melting domains (Volker et al., 1999)

TABLE 1 Parameters of DSC melting profiles of samples presented in Fig. 4

Sample Fig. 4	$T_{m(\text{labile})}$*	$T_{m(\text{stable})}$*	$\Delta H_{(\text{labile})}$**	$\Delta H_{(\text{stable})}$**	$\Delta H_{(\text{stable})}/\Delta H_{(\text{labile})}$	$\Sigma\Delta H$* **
(*1*)						
A	67.5	–	5.9	–	–	5.9
B	64.5	107.8	3.4	2.1	0.6	5.5
C	62.9	107.8	3.0	0.6	0.2	3.6
D	66.5	107.8	1.8	2.0	1.1	3.8
(*2*)						
A	87.0	–	6.2	–	–	6.2
B	83.9	104.5 116.5	1.6	2.4	1.5	4.0
C	86.5	103.8 115.7	2.7	0.6	0.2	3.3
D	85.6	103.7 115.7	0.5	2.5	5.0	3.0
(*3*)						
A	93.2	–	5.7	–	–	5.7
A′	91.8	–	8.4	–	–	8.4
B	–	107.7 114.2	–	7.5	∞	7.5
C	93.2	107.9 113.6	1.1	4.8	4.4	6.9
C′	86.8	–	7.7	–	–	7.7

*, $T_{m(\text{labile})}$ and $T_{m(\text{stable})}$ are transition maxima in °C of thermolabile and thermostable DNA. Some complexes had two maxima of thermostable DNA.
**, $\Delta H_{(\text{labile})}$ and $\Delta H_{(\text{stable})}$ are the enthalpy in kcal/mol of base pairs of thermolabile and thermostable DNA. In the case where $T_{m(\text{stable})}$ was split into two maxima, we present $\Delta H_{(\text{stable})}$ as the sum of both.

were clearly resolved. Heating and then rescanning the DNA revealed considerable renaturation of this DNA, for the scan (Fig. 4 *(3) A′*) shows heat absorption in the same range as native plasmid, although the annealing was imperfect, because the profile lost its characteristic features. In any case, the general consequence of plasmid DNA complexation with EDOPC was similar to that for calf thymus DNA, namely a shift of the transition to above 100°C (Fig. 4 *(3) B*; Table 1). In this case, several maxima were seen, principally at 107 and 114°C. In all experiments with calf thymus and plasmid DNA, the high-temperature melting fraction was observed only when EDOPC was present. In all samples, the thermostable fraction melted in the temperature range 100 to 120°C, usually with more than one peak. With plasmid, as with thymus DNA, sonicated lipid afforded more thermal protection than did vortexed lipid. When the plasmid complex was first heated to 120°C, and then rescanned, a thermolabile fraction was again seen (Fig. 4 *(3) C′*). This behavior was consistent with the partial renaturability of this DNA on the time scale of these experiments, although the profile was even more distorted than that of the free plasmid that had previously been heated to 120°C (Fig. 4 *(3) A′*).

In the case of calf thymus DNA, the total enthalpy ($\Sigma\Delta H$) of all fractions of DNA-lipid complex was somewhat smaller than the enthalpy of the free DNA. This is illustrated in Table 1, where all $\Sigma\Delta H$s of the B and C scans of Fig. 4, *(1)* and *(2)*, were somewhat smaller than corresponding $\Sigma\Delta H$s of the corresponding A scans. In contrast, $\Sigma\Delta H$ of lipid complexes with plasmid DNA were larger than $\Sigma\Delta H$ of free plasmid DNA (compare scans Fig. 4 *(3) A–C*; Table 1). A difference is not unexpected, however, because the environment of neutralized DNA in the complex is much different than that of DNA free in solution, i.e., the charge is neutralized by a surface and it is relatively dehydrated (Choosakoonkriang et al., 2001). It should be noted, however, that others have described problems during sample transfer of other DNA-cationic lipid complexes; because such complexes can be adhesive, quantitative transfers are difficult, which can lead to uncertainties in calorimetric measurements (Zantl et al., 1999).

Separation and analysis of thermostable and thermolabile complexes

Complexation of DNA with cationic lipids was accompanied by formation of aggregates, which was especially pronounced in the samples with an excess of cationic lipid and $R(\pm)$ $\beta 1$. The aggregates were easily sedimented by centrifugation and hence could be easily collected for further investigation. Analysis, in particular by DSC, revealed that the pellet contained essentially the entire fraction of thermostable DNA, whereas the transparent supernatant contained the major portion of the thermolabile fraction (Fig. 5). It may be seen that the thermostable fraction contained

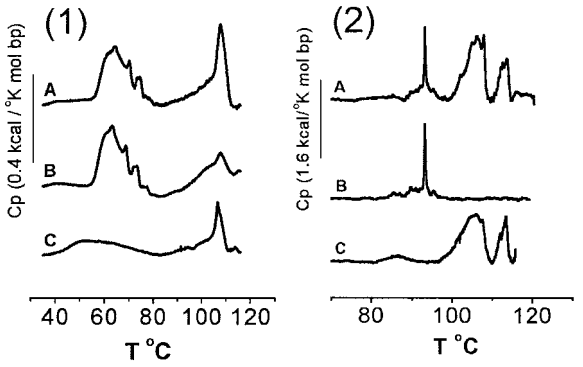

FIGURE 5 Melting profiles of DNA-lipid fractions separated by centrifugation. (*1*) Calf thymus DNA complexed with ultrasound-treated EDOPC liposomes in 5 mM cacodylate buffer. (*2*) Plasmid DNA complexed with vortexed EDOPC liposomes in DPBS buffer. (*A*) Whole sample before centrifugation. (*B*) Supernatant of sample *A*. (*C*) Sediment of sample *A*. For both (*1*) and (*2*), $R(\pm) = 1$.

more than one peak (Fig. 5; see also Fig. 4). It is possible that this material is not homogenous and contains a mixture of different complexes of DNA with lipid, but if so, they did not readily separate by centrifugation. More than 95% of lipid and ~80% of DNA was present in the sediment, whereas in the supernatant the concentration of DNA was much larger than that of lipid (Fig. 6). Transfection assays revealed that almost all of the DNA delivery activity was present in the sediment and that in the supernatant was very low. The transfection activity per unit weight of DNA was 31 times larger for the pellet than for the supernatant.

Thin section electron microscopy of DNA-lipid complexes fixed with osmium tetroxide revealed the presence of multilamellar membrane structures in the pellet, as shown in Fig. 7. The periodic lamellar structures were very stable and were retained even at 130°C, i.e., above the presumptive denaturation temperature of the thermostable DNA. Fourier analysis of the images revealed that the repeat distance

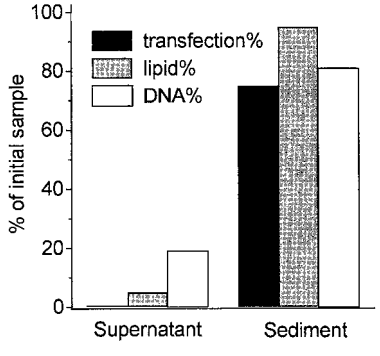

FIGURE 6 Transfection activity and composition of supernatant and sediment obtained from complex prepared from plasmid DNA and vortexed EDOPC liposomes in DPBS buffer with $R(\pm) = 1$. DSC analyses of these samples are given in Fig. 5. The data are presented as a percent of the initial sample value.

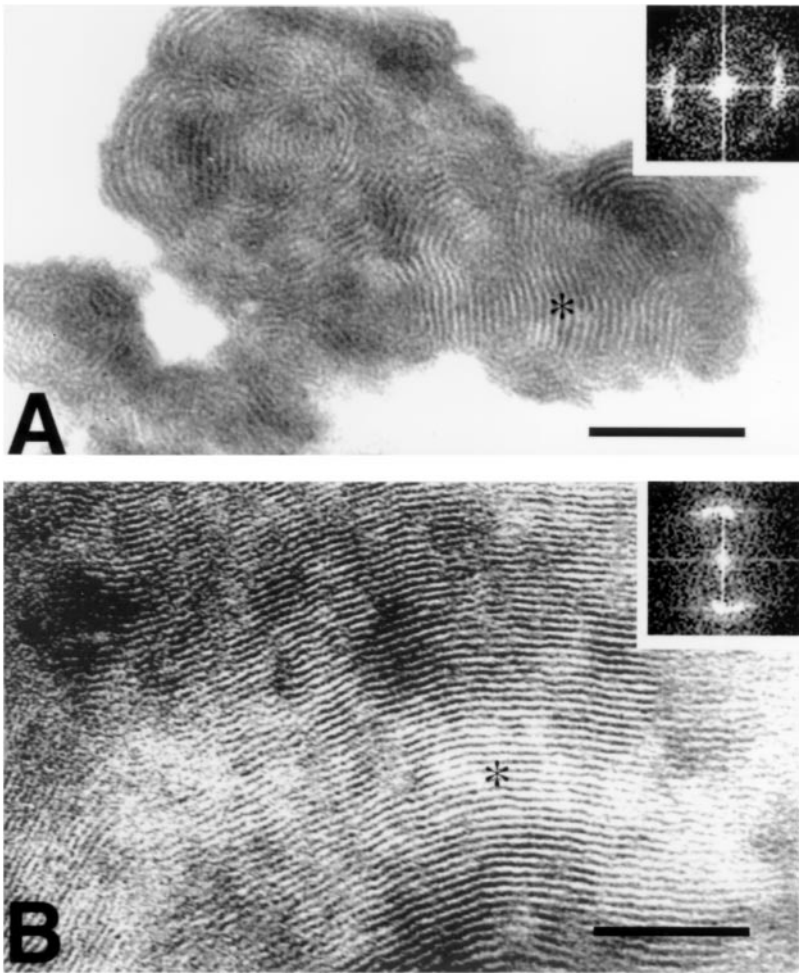

FIGURE 7 Thin-section electron microscopy of sediment obtained by centrifugation of complex prepared by addition of sonicated EDOPC liposomes to calf thymus DNA in 5 mM cacodylate at $R(\pm) = 1.7$ (refer also to Figs. 5 *(1) C* and 6). Samples were fixed with 1% osmium tetroxide at room temperature (*A*) and at 130°C (*B*). Fourier transforms of the selected regions (denoted by *arrows*) are presented in the upper right corners. Bars = 200 nm.

between lamellae, both at room temperature and at 130°C, was 6.0 to 6.5 nm. This indicates that, in the whole range of temperatures used in our experiments, the multilamellar structure was present and is thus a likely candidate for the basic structure of thermostable DNA.

DISCUSSION

Two kinds of experiments demonstrated the existence of DNA stable to heating above 100°C in complexes with cationic lipids. The first set of experiments revealed that several common cationic lipid transfection agents protected DNA against denaturation and also provided evidence for the presence of native DNA after exposure to 100°C. The DNA-lipid complexes were treated with the anionic detergent SDS to release DNA from its complex with cationic lipids (Bhattacharya and Mandal, 1998) and to eliminate light-scattering interference during UV spectrophotometry. It was found that if SDS was added before or during the thermal treatment, the cationic lipids did not protect DNA against denaturation. As shown in Fig. 1, native DNA was found only in the samples treated with SDS after cooling to room temperature; i.e., the protective effect required that the lipid be in its normal self-associated array. UV spectrophotometry revealed that the thermal protection of DNA in the interval $1.5 < R(\pm) < 2.0$ exceeded 75%, and for some lipids, like EDOPC and the TTMA-DOPE mixture, it was above 90%. High protection was exhibited both by the potent DNA delivery agents EDOPC, DOTAP, and DC-Chol/DOPE, as well as by the weak transfection agents DC-Chol/DOPC, TTMA/DOPE, and TTMA/DOPC. All these lipids or lipid mixtures exist in the bilayer structure. The cationic micellar detergent, TTMA, exhibited considerably lower protective activity than the lamellar phase lipids, although this class of cationic detergents can electrostatically interact with DNA and finally form a hydrophobic complex (Spink and Chaires, 1999; Wang et al., 2000). It thus seems clear that the bilayer structure of lipids is important for DNA protection. It is equally clear that interaction of DNA molecules with the cationic charge of the membrane surfaces is also necessary for stabilization of DNA against thermal denaturation, for the bilayer-forming, neutral DOPC did not exhibit detectable protective activity.

To confirm and extend the UV absorbance measurements, DSC was applied to DNA complexes formed with the cationic lipid EDOPC, which exhibited the largest protective effect. Using an experimental protocol similar to that of the spectrophotometric experiments, we observed a similar protective effect (Figs. 2 and 3) and moreover, were able to extend the determination to a higher $R(\pm)$. Indeed, with this technique we found that the protective effect of EDOPC was close to 100% at $R(\pm) = 1.5$ to 3.0 and some native DNA was even detectable even after heating to 110°C. Although EDOPC has very good transfection activity, in general, the protective effect of cationic lipids did not correlate with their transfection activity.

The presence of native DNA after boiling could be explained either by a reversibility of DNA denaturation or by an increase of the DNA melting temperature to above 100°C. A second set of experiments, using DSC, was performed to distinguish between the two possibilities. DSC analyses of DNA-EDOPC complexes over a wide range of temperatures (Fig. 4; Table 1) directly demonstrated the appearance of two fractions of DNA: a thermolabile DNA with a melting profile and melting temperature, $T_{m(labile)}$, quite similar to that of the control DNA solution, and a thermostable DNA with a greatly increased melting temperature, $T_{m(stable)}$. Cationic lipids stabilized both DNAs that were examined, namely, calf thymus (Fig. 4, *(1)* and *(2)*) and bacterial plasmid DNA (Fig. 4 *(3)*); however, the denaturation of plasmid DNA, unlike that of calf thymus DNA, was substantially reversible.

On the basis of the parameter $\Delta H_2/\Delta H_{(labile)}$ (Table 1), it is apparent that the procedure for complex formation and the size of liposomes are both important for DNA stabilization. Small ultrasound treated liposomes were consistently more effective in the stabilization of DNA than large vortexed liposomes (Fig. 4; Table 1). The difference could be related to different extent of aggregation of liposomes observed after addition of lipid to DNA. Large liposomes aggregated more intensively and produced larger aggregates than did small liposomes. Aggregation could well prevent uniform mixture of DNA and lipid because some free lipid could be trapped inside the lipid aggregates with the consequence that some free DNA is left in solution. Generally, the conditions favorable for better mixing of DNA and lipid, such as fast stirring during addition lipid to DNA, use of small ultrasound treated liposomes, and ultrasound fragments of DNA, enhanced formation of the thermostable complex. Fractionation of complexes by centrifugation revealed that the thermostable component contained the major proportion of lipid. The thermolabile fraction was relatively enriched in DNA and contained a small amount of lipid (Figs. 5 and 6). Although the simplest interpretation is that the thermolabile fraction is essentially free DNA, given the present data, we cannot rule out that it is a DNA-lipid complex with a different structure and much higher DNA content than the thermostable complex. Presumably the excess of DNA did not interact with bulk of the lipid and remained in the solution as free thermolabile DNA. Ultrastructural analyses revealed that the thermostable fraction contained multilamellar structure with periodicity of ~6 nm, which is close to the periodicity found early by several methods of electron microscopy and by small angle x-ray scattering (Rädler et al., 1997; Huebner et al., 1999; MacDonald et al., 1999). This periodic structure was very stable and was retained at all temperatures involved in our experiments. Because this multilamellar structure consists of parallel surfaces of bilayer membranes alternating with monolayers of ordered DNA cylinders, then, if the diameter of a DNA molecule, $D_{DNA} = 2$ nm, and the linear charge density of DNA is $LCD_{DNA} = 0.17$ nm/negative charge (i.e., 0.34 nm/bp), the projection of a DNA cylinder onto the membrane surfaces is: $S = 2D_{DNA}\,LCD_{DNA} = 2 \times 2$ nm $\times$ 0.17 nm/negative charge = 0.68 nm^2/negative charge. Because the surface area of one EDOPC molecule is a few squared Ångstroms larger than the surface area of a DOPC molecule and approaches 0.7 nm^2/mol (MacDonald, Momsen, Brockman, unpublished results), the projection of one negative charge of a DNA cylinder should be approximately equal to one positive charge of one surface of an EDOPC membrane, hence a 1:1 stoichiometry would imply that the DNA chains were essentially close packed. However, there is interchain repulsion that holds the chains apart, so the stoichiometry of the complex should be >1:1. In fact, based on the plateau values of the DNA denaturation temperature (Figs. 1 and 3), the stoichiometry of our complex with EDOPC was actually $R(\pm) = 1.5$ to 2.0, which corresponds to an intercylinder distance of: $d = D_{DNA}\,R(\pm) = 3.0$ to 4.0 nm. This distance is, in fact, close to that found by small angle x-ray scattering and electron microscopy for other cationic lipid complexes (Rädler et al., 1997; Lasic et al., 1997; Battersby et al., 1998; Koltover et al., 1998; Huebner et al., 1999). It thus appears that the thermostable complex with stoichiometry of approximately $R(\pm) = 1.5$ to 2.0 corresponds to the known multilamellar periodic structure containing ordered DNA molecules with fixed distances between axes of DNA cylinders.

In itself, the thermal stabilization of DNA by cationic lipids is not unexpected, for many other cations are known to have this effect. For example, mono- and bivalent metal cations can considerably increase the temperature of DNA melting (Duguid et al., 1993, 1995). In general, the stabilization of DNA by cations can be explained by electrostatic interactions between positive charges and DNA phosphate oxygens, the result of which is reduced coulombic repulsive forces between phosphates.

Although DNA stabilization by cationic lipids exhibits some resemblance to that of water-soluble cations, there are also some very significant differences. Indeed, these differences constitute some of the most significant aspects of this study. The most obvious difference has to do with concentration dependencies. Stabilization by cationic lipids is char-

acterized by a stoichiometric interaction in which the denaturation temperature rises to a maximum and remains constant thereafter, independent of the ratio of lipid to DNA. In contrast, metal cation effects are highly concentration dependent with stabilization increasing with concentration in the low and moderate ranges. Moreover, in some cases (particularly transition metals), high metal ion concentrations induce destabilization of the double helix by causing improper base pairing and backbone disorder, and A-conformations and even Z-form elements may appear (Anderson and Record, 1990; Duguid et al., 1993, 1995; Kornilova et al., 1997; Andrushchenko et al., 1997). The only reported effect on DNA of a cationic lipid has been some small changes in polar region that could be explained by partial dehydration (Choosakoonkriang et al., 2001). Our results revealed that the position of $T_{m(stable)}$ was not only largely independent of $R_{(\pm)}$, but was also uninfluenced by conditions of sample preparation and buffer composition. Furthermore, $T_{m(stable)}$ was approximately the same for both calf thymus and plasmid DNA (Fig. 4; Table 1).

These fundamental differences between the concentration-dependent DNA stabilization by metal cations and the highly cooperative step-like changes in the melting temperature induced by cationic lipids are explicable by the basic structural differences between the associated charges of lipid bilayers and the dispersed charges of the water soluble agents. One important characteristic of the self-assembled nature of the lipid bilayer is that its multiple charges are "preassociated" and need not undergo a large concentration increase (and an associated entropy decrease) to associate with DNA. This must significantly reduce the free energy barrier for DNA condensation and account for a considerable part of the high association constant of bilayer-forming cationic lipids with DNA (Pozharski and MacDonald, manuscript in preparation). The second important characteristic of the lipid bilayer has to do with the behavior of the two interacting surfaces. Once lipid is in excess, the DNA faces two cationic surfaces along its entire length, and dissociation is impossible without breaking large numbers of interactions simultaneously. Although there may be other influences, the two differences described appear to adequately explain the significant differences between DNA stabilization by lipids and those of dispersed cations.

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