Lipid-based DNA transfer formulations are typically selected on the basis of in vitro transfection studies where the activity of specific formulations is defined by transgene expression. It is unclear, however, whether expression is directly related to the efficiency of DNA transfer. In an attempt to correlate DNA transfer with transgene expression, we used a simple assay consisting of measuring DNA (3H-plasmid encoding for β-galactosidase) binding to murine (B16/BL6) and human (KZ) melanoma cells in vitro at 4 and 37 °C. The difference in cell association at these temperatures was assumed to be a consequence of DNA uptake, an assumption that was confirmed by protease removal of cell surface-associated DNA. DNA associated with B16/BL6 melanoma cells (up to 30 ng or 12% of the added DNA) following incubation with dioleoyldimethylammonium chloride/dioleoylphosphatidylethanolamine (DOPE) liposome-DNA aggregates was comparable to that achieved with 1,2-dioleoylpropyl-3-trimethylammonium bromide/DOPE or dioleoyldimethylammonium bromide/DOPE liposomes; however, transgene expression was 2- and 5-fold less for the latter two formulations, respectively. Similarly, equivalent amounts of DNA delivery were achieved with B16/BL6 and KZ melanoma cells, yet the level of transgene expression in the KZ cells was undetectable. It was demonstrated that the lack of transgene expression was not a consequence of cell-specific differences in DNA degradation.

Although the understanding of the cellular and molecular mechanisms involved in cationic liposome-mediated gene transfer is limited, DNA delivered by this process must ultimately gain access to the nucleus if effective expression of the foreign gene is to take place (1). Experiments to date have focused on the development of various lipid formulations in an effort to identify an efficient carrier as measured by transgene expression. We designed a simple DNA delivery assay to complement these transfection studies. Delivery of intact DNA was determined as a function of the amount of DNA added and gene expression. Measurement of cell-associated DNA is not an accurate predictor of whether a specific cell line will be transfected or whether the cationic liposomes employed will be more or less effective than other cationic liposomes.

**EXPERIMENTAL PROCEDURES**

**Materials**

DDAB was purchased from Sigma, and DOPE was purchased from Avanti Polar Lípidos (Alabaster, AL). DODAC was synthesized and supplied by Steven Ansell of Inex Pharmaceuticals Corp. (Vancouver, BC). [14C]Cholesterylhexadecyl ether was purchased from Amersham Corp. (Oakville, ON). Lipofectin (DOTMA/DOPE, 50:50 mol %) was supplied by Life Technologies, Inc. Triton X-100 was purchased from Bio-Rad, and chlorophenol red β-galactopyranoside was obtained from Boehringer Mannheim (Laval, Quebec). All other chemicals used in this study were reagent grade and solvents used were high performance liquid chromatography grade.

The murine B16/BL6 melanoma cell line was obtained from NCI Tumor Repository 12-102-54 (Bethesda, MD) and was maintained in RPMI 1640 with 5% FBS. KZ, a human melanoma cell line, was a gift from Dr. Wright (Fred Hutchinson Cancer Center, Seattle, WA) and was maintained in RPMI 1640 with 10% FBS. Cell lines were main-
tained at 37 °C in a 5% CO2 atmosphere with no antibiotics.

The 7.2-kb plasmid pCMVβ (GenBank accession number U02451) encoding the Escherichia coli β-galactosidase was obtained from CLON-TECH Laboratories Inc. (Palo Alto, CA) and was used for analyzing standard molecular techniques (21) and purified using Qiagen Plasmid Purification Kit (Qiagen, Chatsworth, CA). Radiolabeled plasmids were generated by culturing E. coli pCMVβ or pInexCATv2.0 with [3H]-thymidine-5'-triphosphate (NEN Life Science Products) and purified using standard techniques. Specific activity of [3H]-pCMVβ and [3H]-pInex-CATv2.0 were approximately 50,000 and 200,000 dpm/saline at room temperature. The cells were lysed by the addition of 50 mM MgSO4, 10 mM KCl, and 50 mM b-galactosidase standard concentration curve and were expressed as milli- 

Methods

Preparation of Liposomes and Liposome-DNA Complexes—Prepared DOTMA/DOPE liposomes (50:50 mol %) were supplied under minor modifications. This procedure selectively precipitates genomic DNA in a 0.8% agarose gel. Plasmid DNAs were isolated using standard molecular techniques (21) and purified using Qiagen Plasmid Purification Kit (Qiagen, Chatsworth, CA). Radiolabeled plasmids were generated by culturing E. coli pCMVβ or pInexCATv2.0 with [3H]-thymidine-5'-triphosphate (NEN Life Science Products) and purified using standard techniques. Specific activity of [3H]-pCMVβ and [3H]-pInex-CATv2.0 were approximately 50,000 and 200,000 dpm/μg, respectively.

Prior to use, liposomes were diluted into sterile distilled H2O to a final lipid concentration ranging from 100 to 1000 μM. Plasmid DNA was diluted into sterile distilled H2O at concentrations ranging from 10 to 60 μg/ml. Ten μl of diluted liposomes were mixed with 10 μl of DNA in a microcentrifuge tube at the desired concentrations and incubated at room temperature for 30 min. The DNA-liposome complexes were used immediately for transfection.

Transfection Analysis—B16/BL6 and KZ cells were trypsinized, and 1×104 cells/well were seeded in 48-well flat bottom plates (Falcon Labware, Mississauga, ON) and incubated overnight. At the time of transfection, media were removed, and 80 μl of serum-free RPMI 1640 was added to each well. Twenty μl of prepared liposome-DNA complexes were added to each well and incubated at 37 °C in 5% CO2 for 4 h. The complex containing media was subsequently removed and replaced with 200 μl of RPMI 1640 containing 5% FBS. The cells were incubated for a further 48 h prior to assessment for β-galactosidase activity as described below.

Media were removed from each well of the microtiter plate, and the adherent cells were washed 2× with 100 μl of phosphate-buffered saline at room temperature. The cells were lysed by the addition of 50 μl of 0.1% Triton X-100, 250 mM sodium phosphate (pH 8.0) to each well. Subsequently, 150 μl of chloroform red β-galactopyranoside (1 mg/ml) in 60 mM sodium phosphate buffer (pH 8.0) containing 1 mM MgSO4, 10 mM KCl, and 50 mM β-mercaptoethanol was added to each well, and the samples were left to incubate at room temperature.

Absorbances at 570 nm were measured at regular time intervals using a Tittertek Multispec Type 310C microtiter plate reader (Flow Labora-

ysis—B16/BL6 and KZ cells were seeded at 2×104 cells/well in 48-well flat bottom plates (Falcon Labware, Missis-

sauga, ON) and incubated overnight. At the time of transfection, media were removed, and 80 μl of serum-free RPMI 1640 was added to each well. Twenty μl of prepared liposome-DNA complexes were added to each well and incubated at 37 or 4 °C for 1–4 h. At the appropriate time interval, the supernatant consisting of non-cell-associated DNA-lipo-

some complexes was removed by trypsinization at 4 °C for scintillation counting. Each well was rinsed carefully with 200 μl of serum-free RPMI 1640 and pooled with supernatant containing DNA-liposome complexes. Washed cells were lysed in 300 μl of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM MgCl2, 0.5% SDS for 5 min, and the lysate was retained for scintillation counting. Each well was rinsed with 100 μl of lysate and pooled with matched lysates. Pico-Fluor scintillant (Packard Instrument Co., Meriden, CT) was added to all samples, and the radioactivity ([3H]-pCMVβ) was measured in a Canberra Packard TR 1900 Scintillation Counter (Packard Instrument Co., Meriden, CT).

In an attempt to evaluate whether DNA associated with the cells at 37 °C was internalized, transfected B16/BL6 cells were protease-treated prior to sample collection. B16/BL6 cells were transfected for 4 h at 4 °C and 37 °C as described above using DODAC/DOPE liposomes complexed to [3H]-pInexCATv2.0. Adherent cells were removed from wells by trypsinization at 4 °C for 2 min using 0.25% trypsin (Life Technologies, Inc.) and diluted with 400 μl of Hanks’ balanced salt solution. Subsequently, cell-associated DNA was separated from unas-

associated DNA-liposome complexes by fractionation cells on a gradient centrifuged layered at densities of 1.022–1.077. Cells were collected at the 1.022–1.077 interface following centrifugation at 400 × g for 30 min at room temperature, and the amount of plasmid DNA associated with the cells was evaluated by scintillation counting. Control studies revealed that free plasmid and the liposome-DNA complexes were consistently found above the 1.022 fraction.

Assessment of Intact DNA—B16/BL6 cells were seeded at 2×104 cells/well, and KZ cells were seeded at 5×104 cells/well in 48-well flat bottom plates (Falcon Labware, Mississauga, ON) and incubated overnight in a 37 °C incubator. Liposome-DNA complexes were prepared using [3H]-pInexCATv2.0 as described previously. The 4.5-kb plasmid pInexCATv2.0 was used for these experiments because the migration of the three conformations of plasmid can readily be separated from genomic DNA in a 0.8% agarose gel. At the time of transfection, media were removed from all wells and replaced with 80 μl of serum-free medium. Twenty μl of prepared liposome-[3H]-pInexCATv2.0 complexes were added to each well and incubated in a 37 °C incubator for 4 h. Six hundred μl of media (with 5% FBS for B16/BL6 and 10% FBS for KZ) was added to each well and incubated for a further 24 h.

Medium containing [3H]-pInexCATv2.0-liposome complexes was re-

covered carefully with 200 μl of serum-free media, pooled with supernatant, and radioactivity ([3H]-pInexCATv2.0) evaluated by scintillation counting. Cells were lysed by the addition of 400 μl of 0.6% SDS, 10 mM EDTA. Cell lysates were collected in microcentrifuge tubes, the wells rinsed with 100 μl of lysis buffer, and pooled with respective cell lysates. Samples were further processed following the method of Hirt (24) with minor modifications. Total cellular DNA was isolated from genomic DNA leaving the smaller molecular weight plasmid DNA in the super-

nattant, thereby enhancing the detection of plasmid DNA following gel electrophoresis. NaCl was added to cell lysates at a final concentration of 1 M, and the samples were incubated at 4 °C overnight. Following centrifugation at 10,000 × g for 15 min at 4 °C, the supernatant was recovered and further purified. DNA was extracted from the supernatant once with equal volumes Tris-HCl-buffered phenol/chloroform (1:1) and precipitated with 0.1 volumes 3 M sodium acetate and 0.7 volumes isopropyl alcohol. DNA was recovered by centrifugation at 10,000 × g for 30 min at 4 °C and the pellet resuspended in 10 μl of 10 mM Tris-HCl (pH 8.0), 2 mM EDTA. Control samples were also evaluated by adding free DNA or DNA-liposome complexes to untransfected cell lysates and processed along with test samples. All experiments were performed in triplicate.

The integrity of the DNA was evaluated by two methods. First, the presence of intact plasmid DNA was assessed by agarose gel electrophoresis. Each DNA sample (containing DNA isolated from a single transfection from a 48-well plate) was loaded onto a 0.8% agarose gel and subjected to electrophoresis at 80 V in TBE buffer (90 mM Tris borate, 90 mM b-mercaptoethanol) for 2 h. The gel was stained with ethidium bromide (0.5 μg/ml) for 20 min and photographed with UV transillumi-

nation (Ultra Violet Products, San Gabriel, CA). Second, the amount of [3H]-pInexCATv2.0 associated with the three plasmid bands was quan-

ified by liquid scintillation counting. The area of the agarose gel encor-

passing the three [3H]-pInexCATv2.0 plasmid bands was excised and placed into a scintillation vial with 500 μl of H2O. Each sample was
boiled in a water bath for ~10 min until the gel slice was fully melted. Pico-Fluor scintillant was added to each sample and the radioactivity measured.

Statistical Analysis—Quantitative data generated for β-galactosidase activity, and the DNA uptake studies were statistically evaluated using regression analysis from the Microcal Origin version 3.5 computer program (Microcal Software Inc., Northampton, MA). Analysis of variance was used to determine statistical significance of β-galactosidase activity and DNA uptake in different liposome formulations and in different cell lines using Statistica (Statistica Software Inc., Tulsa, OK).

RESULTS

Although the mechanisms of liposome-mediated transfection are not understood, the ratio of cationic lipid to DNA is known to be important for optimal efficiency in vitro (25). The optimal ratio occurs when the number of positive charges incorporated into the cationic liposome is greater than the number of negative charges on the DNA (2), thereby promoting association of the liposome-DNA aggregates with the negatively charged surface of the cell. It is believed that cationic liposomes provide a multivalent surface to bind plasmid DNA, thus protecting the DNA from degradation (26). In addition, these liposomes are thought to effectively neutralize the negative charges (1). The quantity of DNA required and the optimal DNA phosphate to cationic lipid ratio for efficient transfection, however, varies with cell type (27, 28).

Initial experiments in this study evaluated the transfection efficiency of a mouse melanoma cell line (B16/BL6) using DODAC/DOPE liposomes and pCMVβ plasmid DNA. To determine the optimal cationic liposome:DNA ratio required for this cell line, pre-formed liposomes were added to plasmid DNA at various lipid and DNA concentrations as described under “Methods.” The results presented in Fig. 1 show that the transfection efficiency for this cell line was dependent on both factors. Regardless of the amount of DNA used, lipid concentrations of 50 µM showed β-galactosidase activity of <1 milliunit/well (Fig. 1A). The positive to negative charge ratio for these samples ranged from 1.62 to 16.23. The expression level increased more than 10-fold when 100–300 g/ml DNA ranged from 1.62 to 16.23. The expression level increased midway between the minimum (<1 milliunit/well) and maximum (>10 milliunits/well) activity. This ratio also falls within the range of optimal transgene expression achieved in our laboratory using a variety of other cell lines and is comparable to optimal charge ratios reported by other investigators (3). B16/BL6 cells, incubated at 37 °C, were exposed to this complex in serum-free media as described under “Methods” for times ranging from 1 to 4 h. At the appropriate time interval, the complexes were removed from the cells, and the amount of 3H-pCMVβ in the cell lysate was measured. As a control, the amount of cell-associated DNA obtained when cells were incubated at 4 °C was measured. Fig. 2A shows the amount of pCMVβ associated with B16/BL6 cells over the 4-h transfection period at 37 and 4 °C. For both temperatures, there was a time-dependent increase in the amount of DNA associated with these cells. However, this increase was less evident at 4 °C than at 37 °C. The difference in cell-associated DNA levels obtained at 37 °C minus 4 °C was defined as temperature-dependent cell-associated DNA. The data presented in Fig. 2B show that this value increased over a 3-h incubation period. Based on the addition of 200 ng of pCMVβ to the cells, a total of approximately 30 ng (12% of the added DNA) was bound to

![Fig. 1. A, β-galactosidase activity levels (milliunit/well) following transfection of B16/BL6 cells with DODAC/DOPE:pCMVβ complexes expressed as a function of lipid concentration. The amount of DNA added per well was 5.0 µg/ml (■), 10.0 µg/ml (○), 15.0 µg/ml (△), 20.0 µg/ml (□), and 30.0 µg/ml (▲). Lipid is expressed as total DODAC/DOPE:50:50 mol ratio. B, β-galactosidase activity levels (milliunits/well) following transfection of B16/BL6 cells with DODAC/DOPE:pCMVβ complexes expressed as a function of DNA concentration (µg/ml). The amount of lipid added per well was 50 µM (●), 100 µM (○), 300 µM (▲), and 500 µM (■). All data points are averaged from three replications and expressed as mean ± S.E.](image-url)
we completed a 37 °C control study where the cells were
lish whether the cell-associated DNA measured using the assay
ttributes of the aggregates at the two temperatures. To estab-
ferences observed were a result of subtle changes in the at-
37 °C are heterogeneous, and we cannot discount whether dif-
media resulted in additional lipid mixing and further aggrega-
assays3 demonstrated that significant lipid mixing occurred
ble in terms of lipid components. Specifically, lipid mixing
have demonstrated that once formed these aggregates are sta-
ter temperature. Studies in our laboratory, however,
be suggested from these data that 60–65% of the cell-associ-
the B16/BL6 cells in a temperature-dependent process. It can
be suggested from these data that 60–65% of the cell-associ-
da measured at 37 °C is a consequence of binding
events that occur in metabolically inactive cells maintained at
4 °C. Similar results were obtained using the plasmid pInex-
up 6.91 ± 0.45 ng of 3H-pInexCATv2.0 remained associated with the cells
at 4 °C, suggesting that 85% cell-associated DNA could be
removal of surface-associated liposome-DNA aggregates, we
evaluated cells that were measured at 4 °C. Following
trypsinization and separation on a NycoPrep gradient, 6.91 ±
trypsin was not significantly different than that shown in Fig.
level measured in the absence of trypsin was not significantly differ-
that shown in Fig. 2A. Furthermore, the level of cell-associated DNA recovered at
37 °C after trypsin (23.20 ± 3.51 ng) was not significantly
different from the cell-associated DNA measured using the
differences observed as a function of incubation temperature
(30.90 ± 8.21 ng). Although these data increase our confidence
in suggesting that temperature-dependent DNA cell associa-
tion is due to internalization, direct quantification of DNA
uptake is difficult, and the results obtained following
trypsinization of cells incubated with liposome-DNA complexes
at 4 °C suggest that it is difficult to achieve complete removal
of surface bound liposome-DNA complexes. For this reason, we
will define DNA associated with cells in these experiments as
temperature-dependent cell-associated DNA.

To evaluate whether cell-associated DNA is an important indicator of optimal transfection efficiency, the amount of
temperature-dependent cell-associated DNA was correlated with
β-galactosidase activity measured following addition of
different amounts of DNA to B16/BL6 cells (Fig. 4). Transfec-
tion was measured 48 h after DNA addition (Fig. 4A), and the
amount of cell-associated DNA was measured at 4 h (Fig. 4B).
The results demonstrate that under these conditions, the
amount of β-galactosidase expression increased linearly (r =

The assay described above is very simple and analogous to
standard endocytosis and phagocytosis assays used to measure
internalization of liposomes or latex beads. It is worth noting
that the cationic-liposome-DNA aggregates used here are het-
erogeneous and unstable. It can be argued, for example, that
the characteristics of aggregates may change as a consequence
of incubation temperature. Studies in our laboratory, however,
have demonstrated that once formed these aggregates are sta-
ble in terms of lipid components. Specifically, lipid mixing
assays3 demonstrated that significant lipid mixing occurred
when DNA was added to DODAC/DOPE liposomes. Dilution in
media resulted in additional lipid mixing and further aggrega-
Regardless, the samples incubated with cells at 4 and
37 °C are heterogeneous, and we cannot discount whether dif-
ferences observed were a result of subtle changes in the attributes
of the aggregates at the two temperatures. To estab-
lish whether the cell-associated DNA measured using the assay
described in Fig. 2 was not a consequence of differences at 4 °C,
we completed a 37 °C control study where the cells were

3 E. Wasan, unpublished observations.
0.99) with increased amounts of added DNA. As the amount of DNA added to cells increased, there was also a linear increase in temperature-dependent cell-associated DNA ($r = 0.88$) (Fig. 4B). These results were encouraging and suggest that a simple assay measuring cell association may be used to predict transfection efficiency at least for a given cell line and plasmid construct.

The influence of liposomal lipid composition and variations in cell line were assessed to establish the general utility of this approach. Relative $\beta$-galactosidase activity was measured in the B16/BL6 cells 48 h following transfection with pCMV$\beta$ complexed to three different pre-formed cationic liposome formulations as follows: DDAB/DOPE (50:50 mol %), DODAC/DOPE (50:50 mol %), and DOTMA/DOPE (50:50 mol %). Fig. 5A shows that DODAC/DOPE liposomes complexed with pCMV$\beta$ yielded the highest level of $\beta$-galactosidase activity. Expression levels achieved using DODAC/DOPE-DNA aggregates were significantly higher than achieved with either DOTMA/DOPE ($p < 0.05$) or DDAB/DOPE ($p < 0.001$) under the conditions used. Surprisingly, DNA accumulation by B16/BL6 cells, evaluated 4 h following addition of the cationic liposome-DNA aggregates, demonstrated that the level of cell-associated DNA was not significantly different ($p > 0.64$) among the formulations tested (Fig. 5B). These results suggest that the different liposome formulations tested are equally effective at delivering DNA to the cells. Subsequent processing of the cationic liposome-DNA aggregate must be critical to achieve transgene expression. We believe that this intracellular processing and dissociation of the DNA-liposome complex will be dependent on liposomal lipid composition.

Efficiency of gene transfection is also dependent on the cell line used (27, 28). To establish whether barriers to efficient transfection include DNA delivery, particularly in cell lines that are difficult to transfect, we evaluated $\beta$-galactosidase activity in cell lines derived from malignant melanomas in mouse (B16/BL6) and human (KZ). As shown in Fig. 6A, the $\beta$-galactosidase activity following transfection of the KZ cell line with DODAC/DOPE-pCMV$\beta$ aggregates was not detectable. In contrast, the $\beta$-galactosidase activity in the cell line derived from mouse melanoma peaked around 5 milliunits/well when the lipid concentration was between 100 and 200 $\mu$M and the DNA was 10 $\mu$g/ml. To determine whether the poor transfection of KZ cells was a consequence of inefficient DNA delivery, we measured temperature-dependent cell-associated DNA levels using these two cell lines. Fig. 6B shows that there was no significant difference in cell-associated DNA levels between the KZ cells and B16/BL6 cells ($p > 0.62$) when measured at the 4-h time point. These results suggest that 8% of the added pCMV$\beta$ was accumulated in the KZ cells. This is equivalent to

![Graph A](image1.png)  
**Fig. 4.** A, correlation between the amount of DNA added to B16/BL6 cells and the level of $\beta$-galactosidase activity per well when lipid:DNA ratio is 100 nmol of lipid/10 $\mu$g of DNA/ml. Data are averaged from three replications and expressed $\pm$ S.E. A linear regression was obtained with an $r = 0.99$. B, correlation between the amount of DNA added and the temperature-dependent cell-associated DNA. Data are averaged from three experiments and expressed $\pm$ S.E. A linear regression was obtained with an $r = 0.88$.

![Graph B](image2.png)  
**Fig. 5.** $\beta$-Galactosidase activity levels (A) and temperature-dependent cell-associated DNA (B) in B16/BL6 cells following a 4-h incubation with different liposome formulations used to complex pCMV$\beta$ (10 $\mu$g of DNA/100 nmol of lipid). All data are averaged from three replicates and expressed $\pm$ S.E. Statistical analysis of $\beta$-galactosidase activity was evaluated by analysis of variance and showed that DODAC/DOPE exhibit a significantly higher level than DDAB/DOPE ($p < 0.001$) and DOTMA/DOPE ($p < 0.05$). The charge ratio ($\pm$) for each of these liposome-DNA aggregates is 1.62. Statistical analysis of cell-associated DNA among the liposome formulations was evaluated by analysis of variance and showed no significant differences ($p > 0.64$).
CATv2.0 DNA was used instead of pCMV was evaluated by agarose gel electrophoresis. The pInex-the integrity of the isolated, cell-associated pInexCATv2.0 DNA observed for the KZ and B16/BL6 cells lines. To determine this, this could account for the differences in transfection efficiencies information, however, about whether the DNA is intact, and somes (used as a control) were subjected to electrophoresis on a DNA aggregates or DNA that had not been complexed to liposome-DNA ratio of 10:1 nmol/µg). B16/BL6 and KZ cells were transfected as described under “Experimental Procedures,” and plasmid DNA was extracted following Hirt (24) 24 h after transfection. Lane 1, DNA recovered from B16/BL6 cells transfected with pInexCATv2.0 DNA; lane 2, DNA recovered from B16/BL6 cells transfected with DODAC/DOPE-pInexCATv2.0 aggregates; lane 3, DNA recovered from KZ cells transfected with pInexCATV2.0 DNA; lane 4, DNA recovered from KZ cells transfected with DODAC/DOPE-pInexCATv2.0 aggregates; lane 5, DNA extracted following addition of DODAC/DOPE-pInexCATv2.0 aggregates to cell lysates from KZ; lane 6, DNA extracted following addition of pInexCATV2.0 to cell lysates from KZ; lane 7, molecular weight standard. Arrows indicate plasmid DNA.

approximately 10⁶ plasmids associated per cell compared with 15% (approximately 2 × 10⁶ plasmids) for B16/BL6 cells.

The DNA delivery assay used here measures the quantity of ³H-plasmid DNA associated with cells in vitro. It gives no information, however, about whether the DNA is intact, and this could account for the differences in transfection efficiencies observed for the KZ and B16/BL6 cells lines. To determine this, the integrity of the isolated, cell-associated pInexCATv2.0 DNA was evaluated by agarose gel electrophoresis. The pInexCATV2.0 DNA was used instead of pCMVβ because this plasmid could easily be resolved from genomic DNA on an agarose gel. DNA-liposome complexes were prepared at a lipid:DNA charge ratio of 1.62:1 and a DNA concentration of 10 µg/ml. The pInexCATV2.0 DNA has a size of 4.5 kb and is small enough such that the three DNA conformations (linear, relaxed, and supercoiled) can be readily separated from genomic DNA on an agarose gel. Furthermore, the amount of DNA in these bands could be quantified using radiolabeled DNA as a tracer. Transfections were executed as described under “Methods,” and 24 h after transfection as evidenced by the appearance of the three bands on the gel (lanes 1 and 3). DNA that was cell-associated following addition of free plasmid, however, did not reveal the presence of any bands, suggesting that associated plasmid was not intact.

The amount of intact DNA was semi-quantified by excising the plasmid bands from the gel and measuring the radioactivity by liquid scintillation counting. This analysis was completed following transfection of the B16/BL6 and KZ melanoma cell lines, and the results are shown in Table I. These data suggest for cells incubated with DODAC/DOPE-liposome-DNA aggregates that greater than 60% of the ³H counts (650–750 dpm) recovered from B16/BL6 and KZ were associated with the plasmid bands. The remainder of the counts were distributed throughout the lane and were found in association with the genomic DNA, small degraded fragments, and as DNA bound to protein retained in the wells. In contrast, as much as 90% of the radioactivity associated with B16/BL6 and KZ cells incubated with free DNA was degraded. It is important to point out that in comparison to the number of counts loaded onto the gel following transfection with liposome-DNA aggregates (1700 dpm), 400 dpm was loaded onto the gel when using cells transfected with free DNA. These data suggest that in the two cell lines that differ in ability to be transfected, DNA uptake is similar, and the proportion of intact DNA 24 h after transfection is comparable. The assay system used does not, however, allow discrimination between DNA bound at the cell surface and that internalized by the cell. It is possible, therefore, that the measure of DNA integrity reflects the stability of surface-associated DNA rather than internalized DNA.

**DISCUSSION**

If optimal non-viral DNA transfer formulations are going to be therapeutically useful, then the important parameters that control DNA delivery and transgene expression must be elucidated. This is, however, an extremely complicated proc-
addition of DNA to cationic liposomes varies with different liposomal lipid composition (6), and the lipid structures generated as a consequence of the aggregation events are also dependent on the cationic liposomes used. Differences in lipid composition did not, however, appear to influence the level of DNA delivery in vitro. These results suggest that the lipid composition may play a more important role in terms of effecting the ability of DNA to be processed following binding and entry into the cell. It has been suggested that the strength of the ionic/hydrophobic interactions of the lipid to the DNA will affect DNA stability as well as the manner in which DNA is directed to the nucleus and ultimately expressed (1, 4, 9, 29). With regard to cationic liposome-mediated protection of DNA, the sensitivity of internalized liposome-DNA aggregates to intracellular degradation may be dependent on lipid composition as well as cell line. In these studies, DNA degradation following addition of free plasmid to cells was significant in the cell lines tested. DNA delivered using DODAC/DOPA-DNA aggregates was shown to be significantly more stable (Fig. 7); however, we were unable to assess whether the cell-associated DNA was internalized or bound to the cell surface. Whether the stability of these aggregates is a function of lipid composition remains to be elucidated. Although the protection against enzymatic digestion is important, we believe a related parameter will involve dissociation of the lipid from DNA. It may, therefore, be necessary to derive lipid-based carriers that maintain strong ionic/hydrophobic interactions yielding complexes that are stable against circulating enzymes, yet allow dissociation of the lipid from the DNA after entry into the cell. We propose that evaluating DNA uptake as well as assessing complex stability will aid in developing formulations that exhibit such attributes.

Although lipid composition may play a role in efficient expression of the transgene, it is not unreasonable to suggest that different cell types will vary in the machinery used for expression of the transgene. For example, some cells may have higher levels of enzyme/proteins important in regulating DNA transport through the cell, transcription, stability of the message, and processing of the transgene product. Clearly, transfection in vitro and in vivo may not depend entirely on cellular delivery but on the inefficient expression of foreign DNA. Researchers have begun to investigate the role of specific promoters and enhancers in plasmid DNA on levels of transgene expression (30, 31). How these new constructs influence the expression of the transgene remains to be elucidated and may still prove to be cell type-dependent.

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![Table I](https://example.com/table.png)

|                  | Controls | Lysed KZ cells + pInExCATv2.0 | Lysed KZ cells + pInExCATv2.0 | pInExCATv2.0/cationic liposomes - B16 | Percent recovery % from three plasmid bands |
|------------------|----------|-----------------------------|-------------------------------|-------------------------------------|------------------------------------------|
| Cell-associated DNA |          |                             |                               |                                     |                                          |
| Free pInExCATv2.0 - B16 | 10.2     |                             |                               |                                     |                                          |
|  -Z               |          |                             |                               |                                     |                                          |
| pInExCATv2.0/cationic liposomes - B16 | 58.7     |                             |                               |                                     |                                          |
|  -KZ              |          |                             |                               |                                     |                                          |

![Graph](https://example.com/graph.png)

The role of liposomal lipid composition in influencing transfection efficiency may be related to promoting effective DNA cell membrane interaction. This may, in turn, be an important factor in designing effective lipid-based DNA delivery systems (3). However, the degree to which aggregation occurs following
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