Intracellular Compartmentalization of DNA Fragments in Cultured Airway Epithelial Cells Mediated by Cationic Lipids

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Purpose. The amount and intracellular distribution of DNA fragments (491-bp) was characterized after transfection *in vitro* with a commercially available cationic lipid. Localization of fragment to the nucleus, its subcellular distribution, and integrity within the cells was determined for various times after transfection.

Methods. Cystic fibrosis (CF) airway epithelial cells were transfected with ^{32}P and FITC labeled single-stranded (ss) or double-stranded (ds) DNA fragments complexed with Lipofectamine® at various charge ratios.

Results. A 5/1 (+/−) charge ratio was found to be the optimal ratio for transfection of both ss-and dsDNA. After a 5 h exposure, 7.51 ± 0.89% of the radioactivity was associated with the nuclear fraction whereas only 1.07 ± 0.23%, was found in the nuclear fraction when dsDNA was used. The nuclear radioactivity detected after a 24 h exposure was only 1/3 of that after 5 h. Analysis of fragment stability in the cytosolic and nuclear fractions showed the presence of intact fragment in each subcellular compartment. No intranuclear/intracellular fragment could be detected in control experiments with naked DNA.

Conclusions. The results from these experiments indicate that small fragments of DNA can be efficiently and rapidly transferred intact to the cell nucleus using cationic lipids and that ssDNA fragments are more effective than dsDNA fragments for nuclear delivery.

KEY WORDS: gene therapy; human somatic cells; CFTR.

INTRODUCTION

Oligonucleotides of different sizes have increasingly been used therapeutically as modulators of gene expression and mediators of gene targeting. Recently, an approach using oligonucleotides in the range of 500-bp, small fragment homologous replacement (SFHR), was used to correct a naturally occurring mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene of CF epithelial cells (1,2). The SFHR strategy uses relatively small fragments of DNA (400 to 800-bp) to target specific genomic loci. Unlike cDNA-based gene therapy approaches, this strategy is appealing as it has potential for maintaining gene integrity and retaining the relationship between endogenous regulatory elements and protein coding sequences for a given gene.

The effectiveness of SFHR, as well as other strategies interested in transfer of oligonucleotides into cells, is dependent on efficient delivery of therapeutic DNA fragments to the nucleus. In such cases, after the fragment has localized to the nucleus, it is also important to maintain fragment integrity for successful targeted replacement. A number of studies have investigated *in vitro* and *in vivo* delivery of antisense oligodeoxynucleotides (ODNs) in the size range of 10-30-nt (3–11). While incubation of naked, antisense ODNs with cultured cells has been successful for intracellular delivery of these ODNs, cellular uptake is generally poor due to rapid degradation of the ODNs by serum and cytoplasmic nucleases (8,9,12). To circumvent this problem, a variety of approaches, including microinjection, electroporation, and liposome-DNA complexes, have been used to enhance DNA delivery into cells. Liposomes, in particular, have been successfully employed to facilitate cellular uptake and stability as well as enhance the biological activity of antisense ODNs up to 30-nt (6,10–15). Numerous cationic lipid/liposome formulations have been described (16,17) and studies investigating cationic lipid-mediated ODN transfer have concentrated on the lipid formulations DOTMA, DDAB/DOPE, DOGS, and DOTAP (10,12–15). However, although cationic lipids have shown to be effective in delivering ODNs and plasmid DNA into cells (16–18), very little is known about the delivery and intracellular distribution of DNA fragments in the size range of 400- to 800-bp.

The majority of *in vitro* studies have evaluated the transfer of ODNs into well-established fibroblastic cell lines. While transfection into such cells can provide information about the general parameters important for ODN delivery, it does not provide insight into the specific conditions necessary for organ or cell type specific delivery. To improve conditions for efficient *in vivo* cell delivery (19–21) it is necessary that *in vitro* studies focus on clinically relevant cells. In the case of CF, the availability of numerous human airway epithelial cell lines (22) makes it possible to assess factors important for the transfer of DNA into the cells most closely associated with CF pathology (23–25).

Liposomal delivery and subcellular distribution of ~500-bp ^{32}P and FITC-labeled DNA fragments by a commercially available cationic lipid, Lipofectamine® (DOSPA: DOPE) was evaluated in CF airway epithelial cells. The ODN fragments were transfected into the ΣCFTE29o-cell line (24) previously used in SFHR studies (2). The intracellular integrity of both single-stranded (ss) and double-stranded (ds) fragments was evaluated following transfection. The results of the studies presented here indicate that small fragments of DNA can be efficiently transferred intact to the cell nucleus with cationic lipids and that ssDNA fragments are more effective than dsDNA fragments for nuclear delivery.

MATERIALS AND METHODS

Cell Culture

Studies were carried out in an immortalized CF airway epithelial cell line, ΣCFTE29o-(24). These cells are aneuploid,

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homozygous for the ΔF508 CFTR mutation, and display defective cAMP-dependent Cl ion transport. Cells were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics under humidified conditions at 37°C in 5% CO_2. Stock cultures were maintained in T75 flasks (Falcon) coated with an extracellular matrix of Vitrogen/fibronectin/bovine serum albumin and subcultured by trypsinization as described previously (24,26).

Synthesis and Labeling of DNA Fragments

A 491-bp DNA fragment, comprised of sequence for exon 10 and the 3′ and 5′ flanking intron regions of the wild-type (wt) CFTR gene, was generated by PCR as previously described (1,2). Briefly, primers CF1 and CF5 (1–2) were used in a PCR amplification (100 μl) to generate the fragment with the following conditions: Mg^{2+} 2.0 mM; primers, 0.5 μM; DNA template; 50–100 ng; 95°C/60 s, denaturation; 58°C/60 s, annealing; 72°C/90 s, extension; in a Perkin Elmer Thermal Cycler (9600) for 30 cycles with a 5 min extension on the last cycle. Fragment size was confirmed by agarose gel (1%) electrophoresis. The fragment was then isolated and used as a template for additional PCR amplifications for labeling with (α-^{32}P) dCTP (10 μCi/μl; ICN Pharmaceuticals, Inc., Costa Mesa, CA) or Fluorescein-12-dUTP (1nmol/μl; Boehringer Mannheim, Germany). In both cases, 0.5 μl of the labeling solution was added per 100 μl PCR amplification.

Unincorporated nucleotides and primers were removed from the PCR mixture with a 30,000 NMWL Millipore® Ultrafree-MC filter unit. The amount of fragment was determined on a 1% agarose gel using a Gibco-BRL DNA Mass Ladder. The specific activity (cpm/μg DNA) of the fragment was calculated after liquid scintillation counting (Packard). The FITC-labeled fragment was purified by ethanol precipitation and fragment concentration was determined spectrophotometrically.

Both double stranded (ds) and single stranded (ss) DNA fragments were used in the transfections. The ssDNA fragments were generated by heat denaturation followed by immediate cooling on ice as described previously (1,2).

Preparation of Liposome/Fragment Complexes

DNA-Lipofectamine complexes were made at specific charge ratios (+/−). A 3.5 μg aliquot of either single or double stranded ^{32}P-or FITC-labeled fragments was mixed with different amounts of Lipofectamine® (Gibco BRL, Gaithersburg, MD) according to manufacturer's specifications.

The mixture was then incubated at room temperature for 15 min and diluted to a final volume of 1 ml with serum-free MEM. The DNA-lipid complexes formed correspond to charge ratios (+/−) of 1.25/1, 5/1 and 15/1. DNA without liposome (naked) was used as a control in all experiments.

Transfection and Cell Fractionation

Approximately 10^5 cells were seeded in triplicate 6-well dishes 18–24 h before treatment. Cells were incubated with radioactively labeled fragments either as naked DNA or as DNA-lipid complexes for 5 h in serum-free medium at 37°C. After the initial incubation, cells were either harvested immediately (5 h exposure), or the cultures were supplemented with medium containing 10% FBS and harvested 19 hours later (24 h exposure). The number of cells transfected with radioactive fragment was determined using control dishes which were grown in parallel and transfected with unlabeled fragment. Cells were counted at the specific time points using a Model Z_F Coulter counter.

Cells were harvested by removing the culture medium and washing twice with ice cold phosphate buffered saline (PBS). Cells were then lysed in a solution containing: 1% NP40, 10 mM Tris (pH 7.8), 150 mM NaCl and 1.5 mM $MgCl_2$. The cytoplasmic lysate was collected and the remaining nuclei were washed twice with ice cold PBS. Dishes were viewed by light microscopy to insure that cytoplasmic material had been removed from the intact nuclei remaining on the dish. Nuclei were harvested by scrapping with a rubber policeman in 0.5 ml of the above lysis solution, transferred to a 1.5 ml Eppendorf tube, and pelleted by centrifugation. The amount of radioactivity in the nuclear and cytoplasmic fractions was determined by liquid scintillation counting.

Fragment integrity was assessed by polyacrylamide gel electrophoresis (PAGE) of cellular isolates. Cells were transfected as described above, except that after the initial five hour incubation, the transfection medium was completely removed and replaced with MEM containing 10% FBS. After a total of 24 h, DNA was isolated from both the nuclear and cytoplasmic fractions using DNAzol™ (Gibco BRL, Grand Island, New York) according to manufacturers instructions. The isolated DNA was analyzed on 10% polyacrylamide/8M urea denaturing gels. Following electrophoresis, gels were dried and exposed to X-ray film (Fuji).

Intracellular Distribution of FITC-Fragments

Approximately 10^5 cells were plated onto cover slips in 6-well dishes (Costar) 18–24 h before transfection with the DNA fragments. Cells were exposed to FITC-labeled fragments (3.5 μg), either alone or in a Lipofectamine® complex (5/1 (+/−) charge ratio) for various times (0.5, 5, and 24 h) in serum-free MEM. Media supplemented with 10% FBS was then added to the cells after the initial exposure. Following incubation, cells were washed twice with ice cold PBS and fixed for 10 min with 4% paraformaldehyde. The coverslips were immediately attached to slides and analyzed by fluorescence (450–490 nm) (Zeiss Axiophot) and confocal (Zeiss Confocal Laser Scanning) microscopy.

Statistics

The amount of fragment in each intracellular compartment (nuclear and cytoplasmic) is expressed as a percent of the total radioactivity and was calculated by dividing the radioactivity $(cpm)_n$ in the respective intracellular compartments by the total amount of radioactivity $(cpm)_T$ used in each experiment. Each experiment was performed 1-12 times and the data is presented as an average of the experiments ± S.E.M. Comparisons between groups were made using the Student t-test and differences were considered significant when $P < 0.05$.

RESULTS

Uptake of Radiolabeled DNA Fragments

Both ss- and dsDNA fragments were transfected into ΣCFTE29o- cells either as naked DNA or in a complex with

Lipofectamine. Their localization to the nucleus and cytoplasm was determined following exposure times of 5 and 24 hours. Unlike previous studies using smaller ODNs, the amounts of fragment found in the subcellular compartments of cells were negligible when incubated with DNA alone. Only trace amounts of radioactivity were detected in the cytoplasmic and nuclear compartments after 5 and 24 hour exposures to naked ss- and dsDNA and, were not substantially above background (Fig. 1). The only exception was in the nuclear fraction following a 5 h exposure to ssDNA in which case a small amount of radioactivity could be detected (0.17% of the total, Fig. 1C).

An increase in the charge ratio (+/−) of the lipid-DNA complexes (i.e., an increase in the relative amount of lipid used) generally coincided with an increase in radioactivity associated with the nucleus for both 5 and 24 h exposures (Fig. 1C and 1D). Cells transfected with ssDNA at a 1.25/1 charge ratio for 5 hours, showed 2.50 ± 0.67% of the total radioactivity to be localized in the nuclear fraction (Fig. 1C). However, at a charge ratio of 5/1, three times this amount (7.51 ± 0.89%, $P < 0.005$) was associated with the nucleus. There was no statistical difference observed between the 5/1 and 15/1 charge ratios for ssDNA at either 5 or 24 hours. On the other hand, nuclear localization was not as sensitive to changes in charge ratio after transfection with dsDNA. The only notable difference was that more fragment was localized to the nuclear fraction at a 15/1 charge ratio than at a 5/1 ratio after a 24 hour exposure ($P < 0.005$). Otherwise, there was no significant difference in the transfection ability of increasing charge ratio at either 5 or 24 hours (Fig. 1D). Besides transfection ability, the other noticeable effect of charge ratio was cytotoxicity. In experiments with both ss- and dsDNA-lipofectamine complexes, cytoxicity was observed after transfection using a charge ratio of 15/1. As such, a charge ratio of 5/1 was used in subsequent experiments.

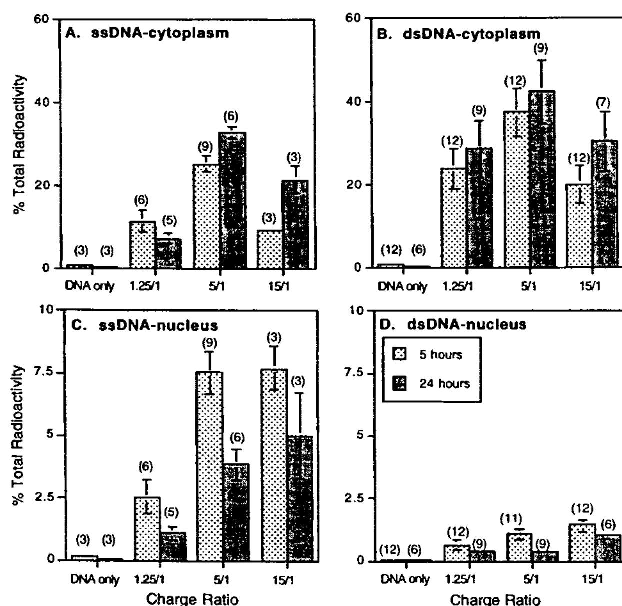

Fig. 1. The uptake and intracellular localization of ^{32}P labeled 491-bp ssDNA and dsDNA fragments. The percent of the total radioactivity associated with the cytoplasm after transfection with ssDNA (A) or dsDNA (B) and that associated with the nucleus following transfection with ssDNA (C) or dsDNA (D). Both 5 h and 24 h exposures to fragment are shown for 3 different charge ratios (+/−) of the lipid-DNA complexes. Numbers in brackets represent the number of experiments (n). Error bars are ± S.E.M.

In every experiment, the amount of intracellular radioactivity (i.e., nuclear and cytosolic) was not statistically different after transfection with dsDNA-lipid complexes than ssDNA-lipid complexes. Nevertheless, ssDNA complexes resulted in enhanced nuclear localization as compared with dsDNA complexes at the different charge ratios in both 5 and 24 h exposures (Fig. 1C and 1D, $P < 0.01$). The increase in nuclear-associated radioactivity for ssDNA ranged from a 3-fold increase (1.25/1 charge ratio, 24 h exposure) to a 10-fold increase (5/1 charge ratio, 24 h exposure). On the other hand, more radioactivity was generally recovered from the cytoplasmic fractions of cells transfected with dsDNA after 5 hours (Figs. 1A and 1B).

Following a 24 h exposure, the relative amount of radioactivity recovered from the nuclei of cells transfected with either ss- or dsDNA at a 5/1 charge ratio was consistently less than with the 5 h exposure ($P < 0.005$ and $P < 0.025$, respectively). The amount of radioactivity in the corresponding ssDNA cytoplasmic fractions showed a significant increase from the 5 h to the 24 h exposure ($p < 0.05$).

Fragment Stability

Intact fragment was observed in both nuclear and cytoplasmic fractions after transfection of ss- and dsDNA-Lipofectamine complexes (Fig. 2). The results of polyacrylimide gel electrophoresis of the cell extracts indicate: 1) no observable fragment in either nuclear of cytoplasmic compartments after transfection with naked ss- and dsDNA, 2) that there is more fragment in the cytoplasmic fraction than in the nuclear fraction after transfection with ssDNA and, 3) there is more fragment in the cytoplasmic fraction after transfection with dsDNA.

The nuclear extracts from cells transfected with both ss- and dsDNA indicate that a substantial portion of transfected fragment remains intact. Some degradation is observed in the nuclear fraction, however there appears to be more degradation in the cytoplasm fraction for both ss- and dsDNA. As such, these results, in addition to the fact that more fragment is found in the cytoplasmic fraction after 24 hours (Fig. 1), indicate that the break down of DNA fragments takes place in the cytosol.

Subcellular Localization of Transfected Fragments

To further characterize the intracellular distribution of transfected DNA fragment complexes, the distribution of FITC-labeled fragments was analyzed by fluorescence and confocal microscopy. Transfected cells were fixed after either a 30 min, 5 h, or 24 h exposure to the liposome-DNA complexes. Only background fluorescence was observed in cells transfected with naked DNA (data not shown). Cells transfected with the lipid-DNA complexes showed both nuclear and cytoplasmic staining with either ss- or dsDNA (Figs. 3B and 3D). Nuclear fluorescence appeared diffuse, while cytoplasmic fluorescence was primarily in bright perinuclear vesicular structures. These structures have been described previously (13,15,27), and are considered to be endosomal/lysosomal compartments. Thus, the appearance of these vesicular structures implicate the endocytic pathway in the delivery of small fragments of DNA into cells. Significant differences in nuclear fluorescence could not be

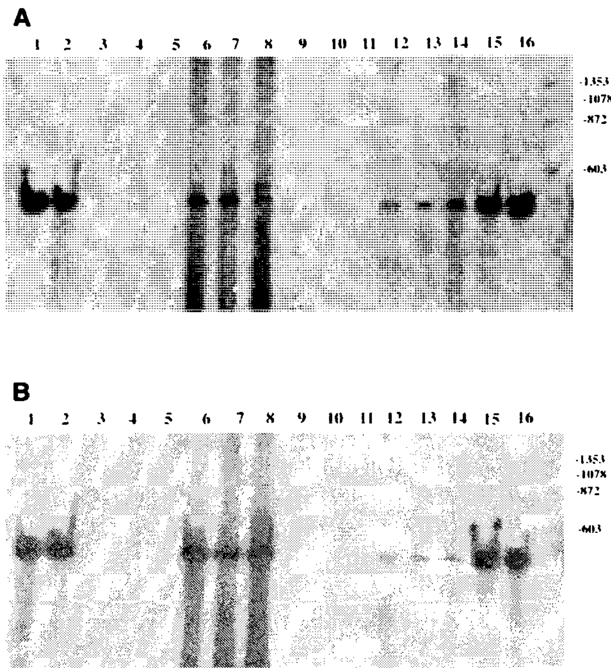

Fig. 2. Stability of transfected 491-bp fragment in a complex with Lipofectamine® at a 5/1 (±/−) charge ratio. (A) Extracts from cells transfected with ssDNA fragment. Lanes 1 and 15 are purified ssDNA fragment; Lanes 2 and 16 are purified dsDNA fragment; Lanes 3–5 are the cytoplasmic isolate from naked DNA transfection; Lanes 6–8 are the cytoplasmic isolate from lipid-DNA complex transfection; Lanes 9–11 are the nuclear isolate from naked DNA transfection; Lanes 12–14 are the nuclear isolate from lipid-DNA complex transfection. (B) Extracts from cells transfected with dsDNA fragment. Lanes 1 and 15 are purified dsDNA fragment; Lanes 2 and 16 are purified ssDNA fragment; Lanes 3–5 are the cytoplasmic isolate from naked DNA transfection; Lanes 6–8 are the cytoplasmic isolate from lipid-DNA complex transfection; Lanes 9–11 are the nuclear isolate from naked DNA transfection; Lanes 12–14 are the nuclear isolate from lipid-DNA complex transfection. In both panels a DNA ladder (123-bp) is shown in Lane 17. The smear above and below the fragment can be attributed to both fragment degradation and the subsequent incorporation of free nucleotides into genomic DNA.

detected when ss- and dsDNA microscopy images were compared. Confocal images of cells transfected as above gave similar results, although there appeared to be a stronger nuclear fluorescence after transfection with ssDNA (Fig. 4). No differences in fluorescence were detected for the other time points evaluated (data not shown).

DISCUSSION

The proportion of transfected fragment in the cytoplasmic and nuclear compartments of cystic fibrosis human airway epithelial cells was characterized using ^{32}P and FITC labeled 491-bp DNA fragments. Both ss- and dsDNA fragments were successfully delivered to the nucleus of cells with the cationic lipid, Lipofectamine. The amount of intracellular radioactivity (i.e., nuclear and cytosolic) was the same after transfection with both ss- and dsDNA-lipid complexes. However, based on quantitative assessments of radioactivity, delivery to the nucleus was most effective with a 5 hour exposure of ssDNA fragments at a 5/1 charge ratio. These studies provide evidence that cationic liposomes can be efficiently used for nuclear delivery of intact therapeutic DNA fragments in the 500-bp size range.

Subcellular fractionation has limitations due to the potential of contamination of the nuclear fraction with endosomes and lysosomes (28). By using internally labeled fragments, potential artifacts from digestion and catalysis by cellular phosphatases and exonucleases were circumvented (29). In addition, light microscopic analysis of subcellular fractionations insured the integrity of the samples.

These experiments showed no evidence for the significant uptake of free DNA. Less than 0.2% of the total radioactivity used was recovered from the nuclei of cells incubated with naked DNA and, essentially all the radioactivity recovered was found in the extracellular media (data not shown). The associated radioactivity found in the nuclear and cytoplasmic fractions in all likelihood represents minor contamination due to the cell fractionation procedure. This conclusion is supported by the fact that neither intact fragment nor degradation products were observable in either fraction (Fig. 1). Cellular uptake of free ODNs has been well documented. Several investigators have demonstrated that ODN size affects both the rate and extent of cellular uptake as well as nuclear delivery (4,5,12). In one study (5), intracellular uptake of ODNs was observed to be inversely proportional to the length of the oligonucleotide. Thus, it is possible that the relatively small amount of uptake of 491-bp fragment is a function of size. Another potential contributor to the low level of naked DNA fragment uptake could be due to cell-specific endocytic mechanisms.

The charge ratio of the cationic lipid to the negatively charged DNA (+/−) appears to be an important factor for effective *in vitro* transfection of DNA (16). Increased nuclear localization was observed following transfection with increasing positive charge and is consistent with previous reports which used cationic lipids and ODNs (14,15). However, this difference was less noticeable with both ss- and dsDNA for both 5 and 24 hour time points at the highest charge ratio (15/1). This may, in part, be due to cellular cytotoxicity or the inhibition of endocytic/lysosomal-pathways involved with trafficking the DNA-lipid complexes into and within the cell by the excess lipid. The results presented here reinforce the notion that the appropriate charge ratio for optimal transfection needs to be determined empirically for individual cell types and/or cell lines and will be a balance between DNA uptake and cytotoxicity of the lipid-DNA complex.

Another factor that will define the effectiveness of DNA fragments for SFHR is the fate and stability of the fragment over time. Previous studies have shown that fluorescently-labeled ODNs, microinjected into the cytoplasm of cells, rapidly accumulate in the nucleus (3,27,30). Within 10 hours, the intranuclear fluorescence diminishes and is accompanied by a concurrent increase in cytoplasmic fluorescence. A similar decrease in nuclear fluorescence has been observed when cationic lipids were used to deliver labeled ODNs. However, it took longer (48 h) for the fluorescence to completely dissipate (15). The working hypothesis is that the loss of intranuclear fluorescence represents nucleolytic degradation of the ODNs and the subsequent transport of fluorescent metabolites to cytoplasmic lysosomes (3). Because a substantial proportion of the

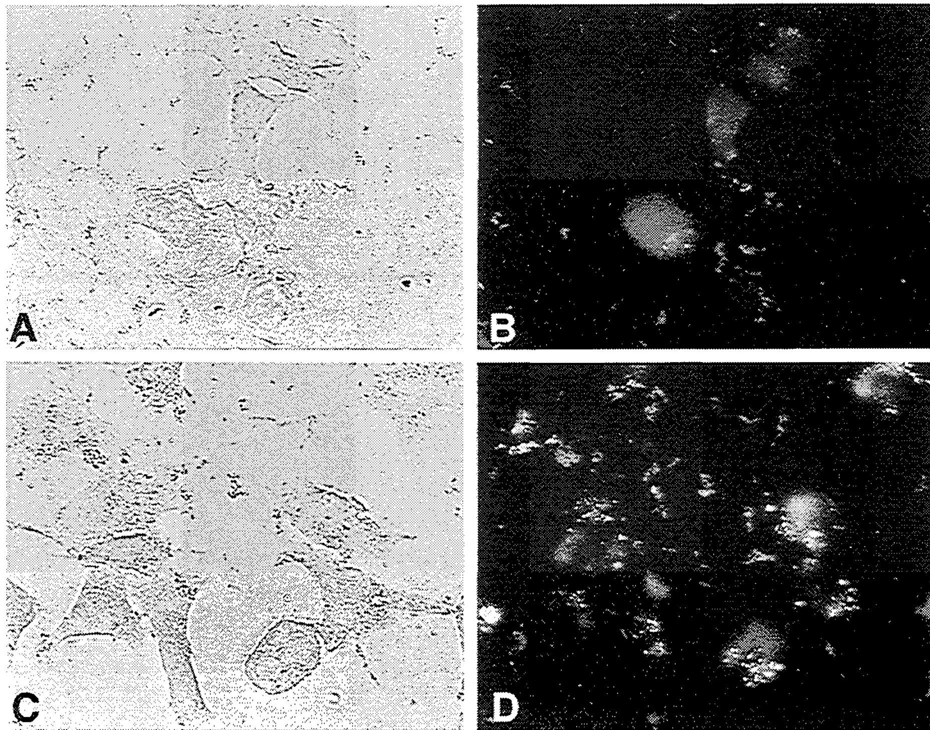

Fig. 3. Intracellular distribution of FITC-labeled 491-bp fragments. Cells were transfected with (A and B) dsDNA and (C and D) ssDNA. Both light and fluorescence microscopy images of the same field are shown.

fragments isolated from the nuclear fraction were intact (Fig. 2), the loss of nuclear associated radioactivity reported here may reflect transport of intact fragment into the cytoplasm where it is subsequently degraded. Further studies are required to elucidate the fate and kinetics of the loss of DNA fragments delivered to the nucleus by cationic lipids.

Unlike the studies investigating the compartmentalization of radiolabeled fragment, there was no significant difference in the fluorescence intensity in cells transfected for either 30 min, 5 h, or 24 h. This may be partially due to differences in the sensitivity of the two assays. The decay in nuclear fluorescence or radioactivity may be more dramatic after exposure to DNA is terminated. Ultimately, the use of the radiolabeled fragments may prove to be a more sensitive means to assay these kinetics and to give better understanding of intracellular fragment localization and degradation.

These experiments demonstrate that a commercially available cationic lipid can facilitate the effective delivery of DNA fragments to CF epithelial cells *in vitro* at levels much higher than can be achieved with DNA alone. A net positive charge of the lipid-DNA complexes, i.e., excess of lipid, appears to enhance the efficiency of DNA transfection. However because of the cytotoxic effects of the lipid, an excess of lipid may not be optimal overall. Cationic lipid-mediated enhancement of

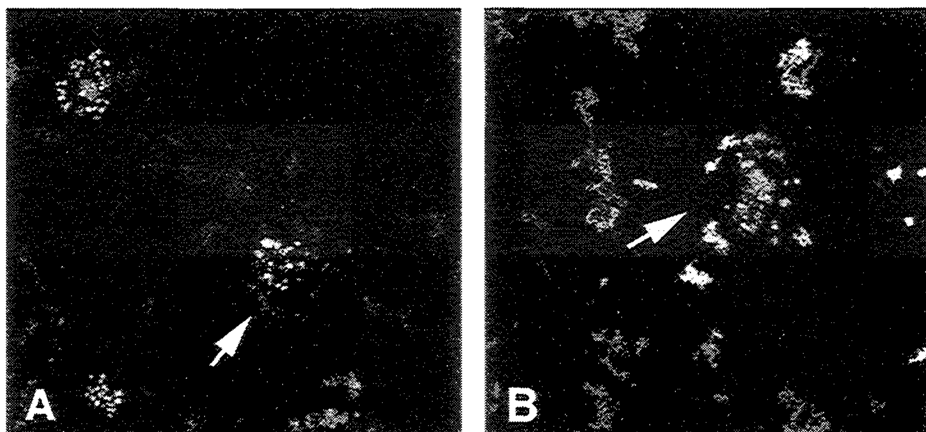

Fig. 4. Confocal images of the intracellular distribution of FITC-labeled 491-bp fragments. (A) Cells transfected with ssDNA. (B) Cells transfected with dsDNA. Arrows indicate the cell periphery.

ssDNA nuclear localization when compared to that of dsDNA fragments is important for defining the conditions for SFHR-based gene therapy. Further optimization of the transfer of DNA fragments in the 500-bp size range will occur by defining how lipids interact with the intracellular and degradative pathways involved with processing transfected DNA. Future studies evaluating these interactions will be critical for understanding nuclear uptake and stability of the transfected DNA fragments as well as the impact of these lipids on cell viability.

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