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A Cationic Lipid for Rapid and Efficient Delivery of Plasmid DNA into Mammalian Cells

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Cationic lipids are widely used for gene transfer into cultured eukaryotic cells. However, lipids with potent transfection activity are often associated with high levels of cytotoxicity, and also require serum-free conditions for optimal performance. These characteristics in many cases result in unsatisfactory transfection efficiency. In this report, we describe a new cationic amphiphile, N-t-butyl-N′-tetradecyl-3-tetradecylaminopropionamidine (Amidine). Amidine requires only 1-2 hour incubation intervals to produce maximal transfection efficiency, and can transfect cells in the presence of serum. Such characteristics significantly minimize cytotoxicity, and also provide time flexibility for researchers. We routinely obtain over 80% transfection efficiency as evidenced by use of an enhanced green fluorescence protein (EGFP) as the reporter. These studies demonstrate the utility of Amidine for rapid and efficient transfection of mammalian cells.

The introduction of foreign genes into cells is of great interest for basic research and gene therapy. For example, transient transfection of plasmid DNA into mammalian cells is a commonly used method to investigate transcriptional regulation and gene expression. Transfection of cultured cells is also a common model system in gene therapy applications to develop DNA delivery systems and to optimize vector design. Over the past decades, extensive effort has been focused on the development of methods that produce high transfection efficiency both *in vitro* and *in vivo*. The earliest developed procedures, such as use of DEAE-dextran and calcium phosphate ($CaPO_4$), often produce low transfection efficiency and are also associated with variability between duplicate experiments (1). Although electroporation can be used with virtually any cell type, this method involves the use of an expensive instrument and may also reduce cell viability (1). In recent years, cationic lipid-mediated transfections have become popular. The cationic lipids are presumed to electronically interact with the negatively charged phosphates of nucleotides to spontaneously form lipid-DNA complexes with near 100% DNA entrapment (1). The lipid-mediated transfections can be used to deliver nucleic acids into a variety of cell types. However, the lipids with potent transfection activity are often associated with a high level of cytotoxicity, and thus in many cases limit the use of these lipids for transfections.

Here we describe a new cationic amphiphile, N-t-butyl-N′-tetradecyl-3-tetradecylamino-propionamidine (Amidine; 2), that can be used to efficiently transfect a variety of mammalian cells. Amidine is suitable for both transient and stable transfections, and requires only a 1-2 hr incubation interval for maximal transfection efficiencies. In addition, using Amidine transfections can be done in the presence of up to 2.5% serum with no significant loss in performance in comparison to transfections performed in the absence of serum. Taken together, these studies demonstrate the utility of Amidine as a general reagent for transfection of mammalian cells.

MATERIALS AND METHODS

Cell culture and supplements. CHO-K1 cells were cultured in Hams F12 medium supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney 293 cells were cultured in MEM supplemented with 10% heat-inactivated horse serum. Other cells were cultured in DMEM supplemented with 10% FBS. All types of cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). All media and culture supplements were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Media for all cultures routinely included 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cultures were maintained at 37°C with 5% CO_2/95% air.

Transfections. For transfection experiments, 1.5×10^5 cells per well were seeded in 12-well tissue culture plates (3.83 cm^2) one day prior to the transfections, and grown in the appropriate medium

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Abbreviations: Amidine, *N-t*-butyl-*N′*-tetradecyl-3-tetradecylaminopropionamidine; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SEAP, secreted alkaline phosphatase.

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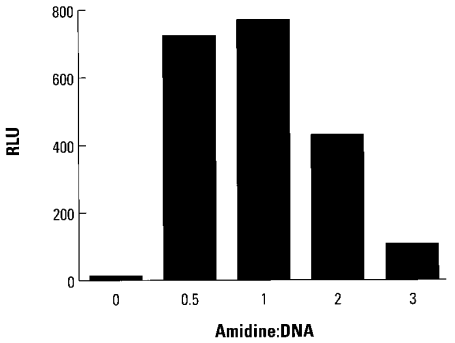

FIG. 1. Optimization of Amidine/DNA Ratio for Optimal Transfection. CHO-K1 cells grown in 12-well dishes were transfected with 1 μg modified pSEAP-Control expression vector using various Amidine/DNA ratios. SEAP activity was determined 48 hr posttransfection as described in Methods. The data show that an amidine/DNA ratio of 0.5–1:1 produced optimal transfection efficiency in CHO-K1 cells.

supplemented with 10% serum. The cultures were 60-90% confluent at the time of transfection. Cells were transfected with 1.0 μg plasmid DNA. Amidine was synthesized according to a method previously described (2,3) and also termed as CLONfectin for commercial distribution (CLONTECH Laboratories, Inc., Palo Alto, CA, USA). Transfections were routinely performed in serum-free medium for 1-4 hours unless otherwise indicated. Media was replaced with fresh complete medium and gene expression assayed 24-48 hours posttransfection.

Plasmid vectors and assays. In most experiments, we used a modified pSEAP-Control expression vector (CLONTECH) as a reporter to evaluate the transfection efficiency of Amidine. The modified pSEAP-Control vector contains a secreted alkaline phosphatase (SEAP, 4) gene under the control of the SV40 early promoter/enhancer unit. The secreted nature of the SEAP protein confers a major advantage since preparation of cell extracts is not required. For transfection experiments, the relative efficiency of transfection was assessed by measuring SEAP activity in the culture medium using a chemiluminescent assay contained in the Great EscAPe Detection Kit (CLONTECH, 4,5).

For the experiments shown in Figure 1, we transfected CHO-K1 cells for 2 hours with the pEGFP-C1 expression vector (CLONTECH, 6,7). After 24 hours, cells were analyzed for green fluorescence by flow cytometry using FACSCalibur (Becton Dickinson) and fluorescence microscopy using a Zeiss Akioskop fluorescence microscope (Carl Zeiss, Inc.). Photographs were taken under a 40× objective.

RESULTS AND DISCUSSION

To define the optimal ratio of Amidine/DNA for maximal delivery of plasmid DNA into eukaryotic cells, we first transfected CHO-K1 cells with 1 μg modified pSEAP-Control expression vector using various amounts of Amidine. Transfection efficiency was quantified by assaying for the secreted alkaline phosphatase (SEAP) reporter protein in the conditioned medium of transiently transfected cultures. The results shown in Fig. 1 illustrate the dose-dependent transfection activity of Amidine, with an optimal Amidine:DNA ratio of 1:1.

To determine whether Amidine can be used as a general reagent for delivery of plasmid DNA into cultured cells, we performed transfections of pSEAP-Control vector with a variety of mammalian cell lines derived from different tissues and species. Table I summarizes the cell lines that have been tested, all of which can be successfully transfected using Amidine. In each transfection, the SEAP activity was at least 100 fold higher in the transfected cultures than in the mock-transfected control culture. In addition, we also successfully transfected an insect cell line, SF-21 (Invitrogen, San Diego, CA), using the Amidine reagent (Table I). Taken together, these results demonstrate that Amidine can be used as a general reagent for efficient transfection of both mammalian and insect cells.

The SEAP reporter assay, as described above, provides a somewhat indirect measurement of the efficiency of gene transfer as it is based on activity of the secreted alkaline phosphatase in the conditioned media. In order to better estimate the transfection efficiency obtained using Amidine, we performed transfections in CHO-K1 cells using an EGFP expression vector, pEGFP-C1 (CLONTECH, 6,7). Fig. 2 shows that 85% of cells in transfected cultures express EGFP as analyzed by flow cytometry (panel B compared to panel A). Figure 1C illustrates a representative microscopic field for the EGFP expression. Similar transfection efficiency was also observed in other mammalian cells tested for expression of EGFP, such as BHK-21 and 293 cells (data not shown). These results demonstrate that Amidine can efficiently transfect various mammalian cells.

Since Amidine can efficiently transfect various mammalian cells, we asked whether shorter transfection intervals could be used to obtain optimal efficiency.

TABLE I

Cell Lines Successfully Transfected Using Amidine

Origin	Cell lines	Cell types
Hamster	CHO-K1	Fibroblast
Hamster	BHK-21	Fibroblast
Human	293	Kidney transformed
Human	A431	Epidermoid carcinoma
Human	HeLa	Epitheloid carcinoma
Human	Neuroblastoma	Neuron
Mink	Mv1Lu	Fibroblast
Monkey	COS-1	Epithelial
Monkey	CV-1	Epithelial
Mouse	NIH3T3	Fibroblast
Rat	L6	Skeletal muscle myoblast
Insect	SF-21	*Spodoptera frugiperda* ovarian

Cells were transfected for 2 hrs with the modified pSEAP-Control expression vector (see Methods for details) using Amidine. SEAP assays were performed 48 hr posttransfection using the Great EscAPe SEAP Detection kit as described in Methods. SEAP activities in the transfected cultures were at least 100 fold higher than that in the control culture.

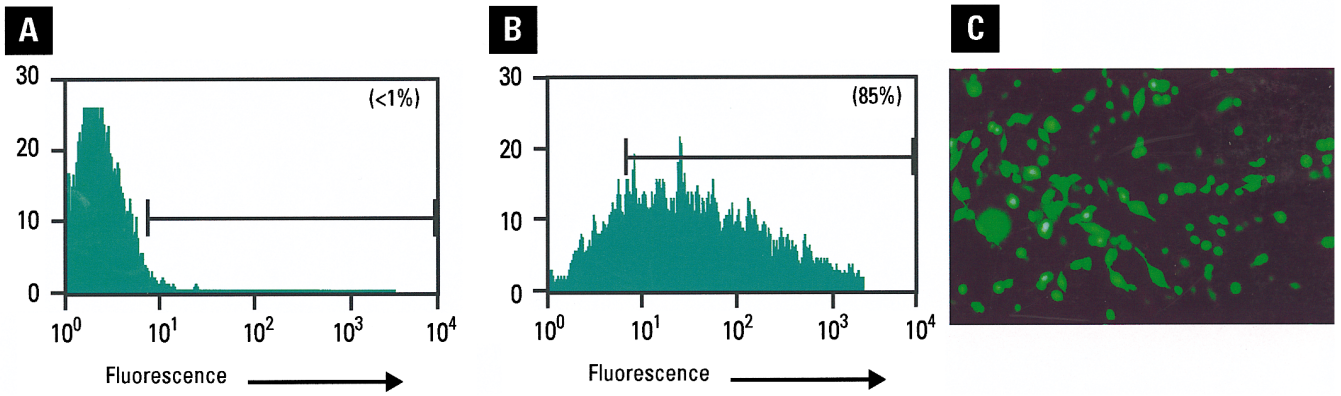

FIG. 2. Transfection of pEGFP-C1 in CHO-K1 Cells. Transfections were performed for 2 hours using Amidine and the pEGFP-C1 vector (CLONTECH). After 24 hours, cells were analyzed for green fluorescence by flow cytometry (panel A and B) using FACSCalibur (Becton Dickinson) and fluorescence microscopy (panel C) using a Zeiss Akioskop fluorescence microscope (Carl Zeiss, Inc.). Photographs were taken under a 40× objective.

Time-dependent transfections were performed in CHO-K1 and COS-1 cells using pSEAP-Control vector for various transfection intervals as indicated. Significantly, we found that as little as 1-2 hr of transfection time is sufficient for maximal transfection efficiency in each cell line (Fig. 3). These results demonstrate that, using Amidine, transfections can be done in less than two hours with no loss in efficiency. The potential for using shorter transfection times to obtain optimal transfections provides researchers with significant time flexibility and convenience, in addition to minimizing toxic effects due to prolonged exposure of the cells to the lipid/DNA complexes.

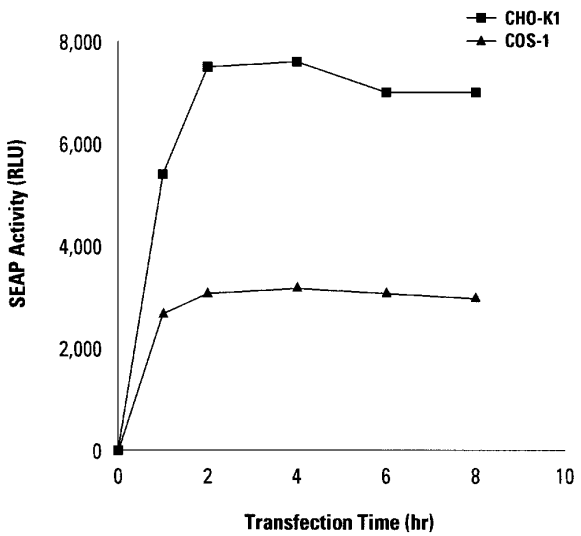

FIG. 3. Time Requirement for Transfections Using Amidine. Transfections were performed in CHO-K1 and COS-1 cells using a modified pSEAP-Control vector (CLONTECH). At the indicated incubation time, the transfection media were replaced with regular culture media and SEAP activities assayed 48 hours posttransfection using the Great EscAPe Detection Kit (CLONTECH) as described in Methods. RLU, relative light unit.

In summary, we have demonstrated that Amidine can efficiently deliver plasmid DNA into a variety of mammalian cells. Amidine acts fast, requiring only 1-2 hr for optimal transfection efficiency. In addition, Amidine can transfect cells in the presence of up to 2.5% serum with no loss in efficiency (data not shown). The ability to transfect cells in the presence of serum is of significant benefit to those working with cell lines incapable of tolerating the toxic effects due to the absence of serum. Moreover, Amidine is also effective for stable transfection of mammalian cells as demonstrated by transfection of CHO-K1 cells with a internal ribosome entry site (IRES) containing expression vector, pIRES1hyg (8). These properties illustrate the utility of Amidine as a rapid, efficient, and general reagent for delivery of plasmid DNA into a variety of mammalian cells. Further studies are required to evaluate whether Amidine will be effective for *in vivo* delivery of plasmid DNA for gene therapeutic applications.

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