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Double long-chain amidine liposome-mediated self replicating RNA transfection

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Abstract We present experimental evidence that a complex made of a double long chain cationic amphiphile and recombinant mRNA facilitates the entry and expression of genetic material into cells. Combining the properties of the self replicating recombinant mRNA driven by the Semliki Forest Virus (SFV) replicon and the transfection potentialities of a new cationic amphiphile (*N*-*t*-butyl-*N*-tetradecyl-3-tetradecylaminopropionamidine) yields a highly efficient mRNA transfection system conferring up to 100% infectivity. The preparation and characterization of the long chain amidine cationic amphiphile–mRNA complex as well as the influence of the diC14-amidine/RNA ratio on the infective activity are described.

Key words: Cationic liposome; diC14-amidine; mRNA transfection; Self replicating recombinant mRNA

1. Introduction

Positively charged liposomes have been described to associate spontaneously with DNA [1–9] and RNA [10,11] and facilitate its entry and expression into eukaryotic cells. In vivo applications are being developed and some of them are promising for gene therapy [12–20].

RNA transfection can be an alternative to the use of DNA. Indeed, mRNA can be translated to corresponding proteins in virtually all eukaryotic cells without integrating into the host genome, thus offering a safe way for in vivo gene transfer. Nevertheless, mRNA is most sensitive to degradation and will not be replicated in the cell. This limitation has been shown to be alleviated by using self replicating recombinant mRNA driven by the Semliki-Forest Virus (SFV) replicon [21]. In this system, a DNA sequence coding for the protein to be expressed is cloned into SFV plasmid that serves as template for recombinant mRNA in vitro synthesis. The resulting recombinant mRNA carries the viral replicase gene and the foreign coding sequence under the viral subgenomic promoter. When introduced into cells, the recombinant mRNA allows expression of the viral replicase, which in turn drives efficient replication of the recombinant mRNA. In this respect, a method allowing an efficient and functional mRNA transfer into cells would achieve the goal of mRNA transfection. Electroporation was shown to be efficient in transfecting the SFV recombinant mRNA into BHK21 cells [21], but is restricted to in vitro use. We provide here the first experimental evidence that *N*-*t*-butyl-*N'*-tetradecyl-3-tetradecyla minopropionamidine (diC14-amidine) forms a complex with self-replicating recombinant mRNA capable of transfecting BHK21 cells with a high efficiency (virtually 100% of the cells were transfected). The diC14-amidine–mRNA complex has been characterized by sucrose gradient ultracentrifugation. Its protection against nuclease and transfection efficiency depends on the diC14-amidine/mRNA ratio.

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2. Materials and methods

2.1. Materials

*Spe*I restriction enzyme, CTP, ATP, GTP, UTP, m7G(5′)ppp(5′)G, RNasin, RNase-free DNase I, SP6 RNA polymerase, RNaseA, proteinase K, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 5 bromo-4-chloro-β-D-galactopyranoside (X-gal) and β-galactosidase were purchased from Boehringer Mannheim. Spermidine and DTT were purchased from Sigma. [^{3}H]DPPC, [α-^{32}P]UTP and ^{35}S-labelled methionine were obtained from Amersham. BMV RNA and wheat germ extract were purchased from Promega. BHK21 medium, tryptose phosphate broth, glutamine, Hepes, penicillin-streptomycin and foetal calf serum (FCS) were obtained from Gibco-BRL.

2.2. Plasmid DNA preparation and RNA synthesis

The pSFV-LacZ plasmid was generously provided by Peter Liljeström (Sweden). It has been obtained by fusing the 8th codon of the *E. coli* β-galactosidase in frame with the initiating AUG of the capsid gene of SFV. The vector has been described in [21]. The plasmid DNA was prepared by alkaline lysis and cesium chloride-ethidium bromide equilibrium gradient centrifugation. For transcription, DNA was linearized by complete restriction digestion with *Spe*I at 2 U/μg DNA for 2 h at 37°C (enzyme was added at the beginning of incubation and 1 h after). DNA was then phenol-extracted, ethanol precipitated and finally resuspended in sterile water. Transcription was carried out as described [22] with the following modifications: a 100 μl reaction mixture was prepared to contain 80 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 1.5 mM ATP, 1.5 mM CTP, 1.5 mM UTP, 1.5 mM m7G(5′)ppp(5′)G, 0.75 mM GTP, 1 U/μl RNasin, 100 μg/ml DNA, 800 U/ml SP6 RNA polymerase. The mixture was incubated for 2 h at 37°C, then immediately mixed with RNase-free DNase I from bovine pancreas and incubated for a further 15 min at 37°C. RNA was purified by chromatography using a G50 column (Pharmacia) according to manufacturer's instructions and sterile water for the elution. Uncapped mRNA was prepared in a similar fashion, except that m7G(5′)ppp(5′)G was omitted and GTP concentration raised to 1.5 mM. Uncapped radioactive RNA was prepared as described above by adding 2 μl of [α-^{32}P]UTP (800 Ci/mmol) to the reaction medium.

2.3. Preparation of diC14-amidine suspension

DiC14-amidine was synthesized and vesicles were prepared as described [9] with the following modifications: 20 μl of 50 mg/ml ethanolic solution was injected into 1 ml of 10 mM Hepes, 150 mM NaCl buffer (pH 7.2) under vortexing. The resulting suspension was used within the day of preparation. The mean size of diC14-amidine vesicles, prepared by ethanol injection method in 10 mM Hepes–150 mM NaCl buffer at pH 7.2 has been measured by laser light scattering: a mean size of 300 nanometers diameter was calculated. At high magnification and phase contrast microscopy, the vesicles look spherical. This vesicle suspension is stable at 4°C for several weeks and is not subject to oxidation. ^{3}H-labeled diC14-amidine liposomes were prepared as described above by adding [^{3}H]DPPC (62 Ci/mmol) to the diC14-amidine ethanolic

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solution (1 : 1000 [^{3}H]DPPC/diC14-amidine molar ratio) before vesicles formation.

2.4. DiC14-amidine–mRNA complex characterization

2.4.1. Sucrose gradient ultracentrifugation. 5 μg of uncapped mRNA (containing a fraction of ^{32}P-radiolabeled mRNA to a total activity of 30,000 cpm) was mixed in 100 μl of 10 mM Hepes, 150 mM NaCl (pH 7.2) with various amounts of diC14-amidine liposomes (containing a fraction of ^{3}H-labeled diC14-amidine liposomes to 100.000 cpm) to give the desired diC14-amidine/RNA weight ratios (1 : 1, 2 : 1, 4 : 1 and 8 : 1). The suspension was mixed with 700 μl of 80% sucrose solution in 10 mM Hepes, 150 mM NaCl (pH 7.2) and layered under a 60%–2% continuous sucrose gradient. Tubes were centrifuged at 100,000 × *g* for 16 h (4°C) in a Sorval AH-650 rotor. After centrifugation, 200 μl fractions were sampled, mixed with 4 ml of Aquasol2 scintillation cocktail (Dupont) and the [^{3}H] (liposomes) and [^{32}P] (mRNA) radioactivity counted.

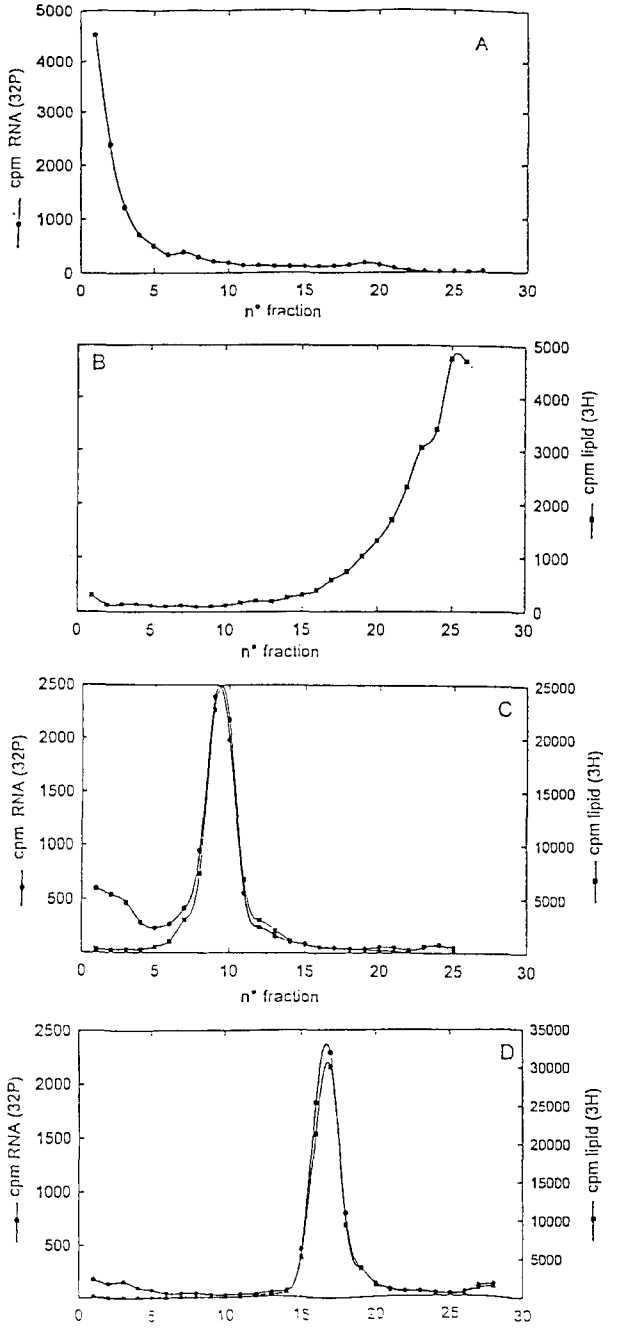

Fig. 1. Characterization of the diC14-amidine/mRNA complexes by sucrose gradient ultracentrifugation. Complexes were formed as described in section 2 and centrifuged on a 60–2% continuous sucrose gradient. [^{32}P]RNA radioactivity (●) and [^{3}H]lipid radioactivity (■) were measured along the gradient. Free mRNA (A). DiC14-amidine liposomes (B). 1 : 1 diC14-amidine/mRNA weight ratio (C). 2 : 1 diC14-amidine/mRNA weight ratio (D).

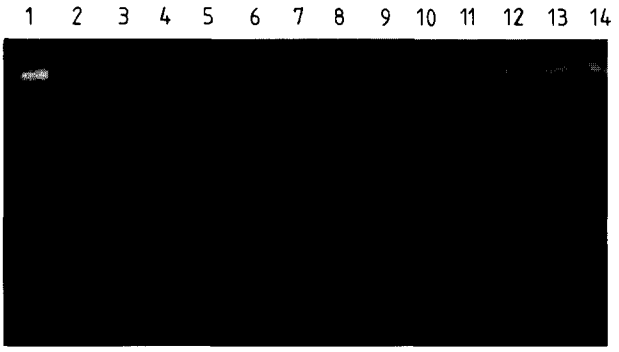

Fig. 2. Nuclease accessibility to the mRNA complexed with diC14-amidine. Free or liposomes complexed mRNA were subjected to RNaseA digestion as described in section 2. Products were run on agarose gel and stained with ethidium bromide. Lanes are numbered from 1 to 14 from the left. Lane 1: control untreated mRNA. Lanes 2–5: digestion kinetics of free mRNA with 5, 15, 30 and 60 min incubation times, respectively. DiC14-amidine/mRNA complexes were digested in the same conditions. Lanes 6–8: diC14-amidine/mRNA complexes with a weight ratio of 1 : 1 and 15, 30 and 60 min incubation times, respectively. Lanes 9–11: diC14-amidine/mRNA complexes with a weight ratio of 2 : 1 and 15, 30 and 60 min incubation times, respectively. Lanes 12–14: diC14-amidine/mRNA complexes with a weight ratio of 4 : 1 and 15, 30 and 60 min incubation times, respectively.

2.4.2. Nuclease protection assay. To investigate the accessibility of diC14-amidine complexed mRNA to the endonuclease RNase A, 3 μg of mRNA were diluted in 30 μl of 10 mM Hepes (pH 7.2) and mixed with 30 μl of 10 mM Hepes (pH 7.2) containing 3, 6 or 12 μg of diC14-amidine liposomes. The mixture was incubated for 15 min at room temperature, mixed with 6 μl of RNase A at 10^{-5} U/μl and incubated at 37 °C. After 15, 30 and 60 min, 22 μl of mixture was removed and the digestion stopped by adding proteinase K at 2 mg/ml final concentration. All samples were mixed with SDS (1% final), loading buffer (glycerol 3% final, Bromophenol blue 0.25% final) and run on a 0.6% agarose gel in TBE (45 mM Tris-borate, 1 mM EDTA, pH 8) at 5 V/cm. Free mRNA was hydrolyzed as a control.

2.5. mRNA transfection of BHK21 cells

BHK21 cells were obtained from the American Type Culture Collection and grown in BHK21 medium supplemented with: 10% tryptose phosphate broth, 2 mM glutamine, 25 mM Hepes pH 7.2, penicillin-streptomycin (50 IU/ml and 50 UG/ml, respectively) and 5% FCS (complete medium). DiC14-amidine–mRNA complexes were prepared and transfected into BHK 21 cells essentially as described [9] with the following modifications: cells were plated in a 24 well culture dish at 5×10^4 cells per well and grown overnight to reach approximately 70% confluency. mRNA–diC14-amidine complexes were prepared by separately diluting mRNA and diC14-amidine vesicles in 50 μl of 10 mM Hepes, 150 mM NaCl (pH 7.2). They were gently mixed in Polybutadiene Styrene tubes (Nunc Inc, Denmark) and incubated for 15 min at room temperature. The diC14-amidine/mRNA mixture was then added to 300 μl of medium in the absence or in the presence of serum. The cells were washed twice with medium without serum and incubated with complexes for 2 h tilting the plate every 15 min. The transfection medium was then removed and complete medium added.

2.6. β-Galactosidase assays

2.6.1. Percentage of transfected cells. 24 h after transfection, the cells were washed twice with PBS^{++} (PBS with 1 mM Ca^{2+} and 1 mM Mg^{2+}), fixed with methanol at −20°C for 5 min and washed 3 times with PBS^{++}. Staining was performed in PBS containing 5 mM $K_4Fe(CN)_6$,

5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-β-D-galactopyranoside (X-gal) for 4 h to overnight at 37°C. To determine the percentage of transfected cells, the blue stained cells were counted in 5 randomly chosen visual fields under the microscope and the mean was calculated. This number of cells was then divided by the number of cells contained in the observed field.

2.6.2. Total β-galactosidase expression in transfected cells. 24 h after transfection, the cells were washed twice with PBS, overlayered with 200 μl of PBS, harvested and transferred to Eppendorf tubes and centrifuged for 2 min. The pellets were resuspended in 100 μl of 250 mM Tris (pH 7.8) and cells were lysed with three freeze-thawing cycles. Lysates were diluted in 100 mM sodium phosphate buffer, pH 7.5 and 10 μl were mixed with 90 μl sodium phosphate buffer (100 mM, pH 7.5) containing 1 mM $MgCl_2$, 45 mM β-mercaptoethanol and 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in a 96 well microtiter plate. A standard curve was obtained in parallel using purified β-galactosidase ranging between 2.5 and 0.02 U per well. The reactions were carried out at 37°C for 1 to 2 h, stopped by adding 170 μl of 1 M Na_2CO_3 and absorbance was measured at 405 nm in a microtiter plate reader.

2.7. In vitro RNA translation

BMV (Brome Mosaic Virus) mRNA was chosen because of its high translation efficiency in a cell-free translation system. BMV mRNA was in vitro translated in a 25 μl reaction mixture containing 12.5 μl wheat germ extract, 130 mM potassium acetate, 1 U/μl RNasin, 2 μl amino acid mixture, 1.5 μl ^{35}S-labelled methionine and 1 μg mRNA or 1 μg mRNA complexed with diC14-amidine. The reaction mixture was then incubated for 20 min at 25 °C. Recombinant SFV-LacZ mRNA was translated the same way. To quantify the radioactivity associated with newly synthesized polypeptides, reaction mixtures were diluted in 800 μl of water, precipitated with 30% TCA (final), filtrated on nitrocellu-

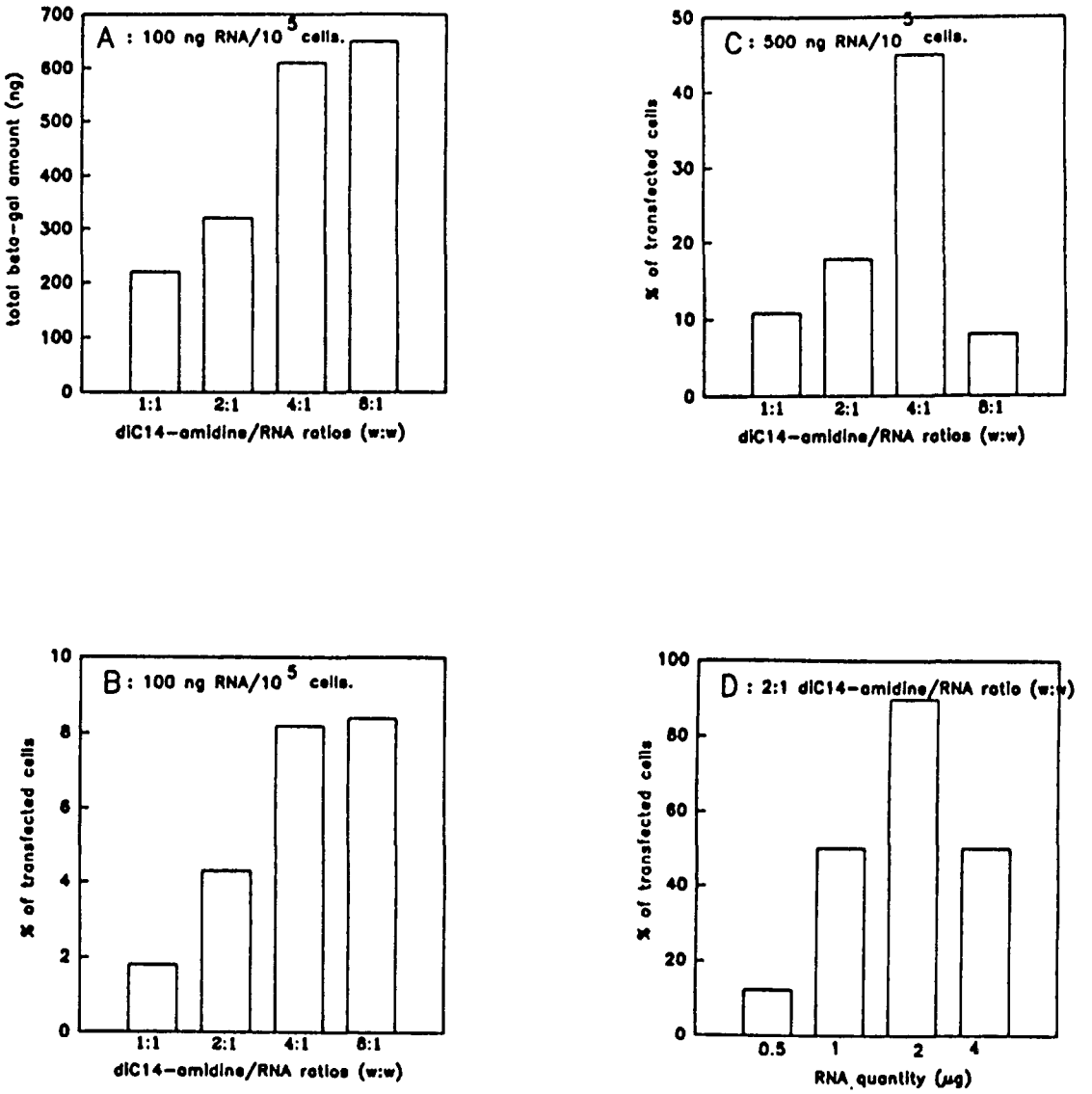

Fig. 3. Panels A and B: 10^5 BHK21 cells were transfected with 100 ng of β-galactosidase (β-gal) recombinant mRNA and increasing amounts of lipids (see section 2) in 24-well plates in duplicates. A: cells were homogenized and total β-gal activity quantified using the ONPG assay. The β-gal activity was converted to an absolute level of β-gal protein per well using the β-gal standard curve prepared in parallel. B: cells were in situ stained using the X-gal reagent and the percentage of positives cells counted. Data shown represent a typical result of the mean of 3 independent experiments. Panel C: effect of diC14-amidine/mRNA ratio on the percentage of transfected cells at higher mRNA dose. BHK21 cells (10^5) were transfected with 500 ng mRNA and increasing amounts of diC14-amidine. The percentages of transfected cells were plotted as a function of diC14-amidine/mRNA weight ratio. The experiment was repeated at different times. Panel D: effect of mRNA dose on the percentage of transfected cells. BHK21 cells were transfected with complexes formed at constant diC14-amidine/mRNA weight ratio (2 : 1) and increasing amount of mRNA (0.5–4 μg). The percentages of transfected cells were plotted as a function of the mRNA quantity used for transfection.

lose filters and washed to remove unincorporated labeled methionine. The filters were dried and counted for radioactivity in Ecoscint O scintillation liquid (National Diagnostics).

3. Results and discussion

3.1. mRNA/diC14-amidine complex characterization

Fig. 1 shows that in a continuous 60 to 2% sucrose gradient, free mRNA (Fig. 1A) was found in the bottom of the gradient whereas free diC14-amidine (Fig. 1B) migrated at the top. Mixed at 2:1 weight ratio prior to centrifugation, diC14-amidine and mRNA migrated at an intermediate density revealing complete association (Fig. 1D). At a 1:1 diC14-amidine/mRNA weight ratio (Fig. 1C), two populations of free and diC14-amidine-associated mRNA could be separated. The single homogenous peak corresponding to mRNA-associated diC14-amidine provides evidence of a homogenous population of complexes. At 4:1 and 8:1 diC14-amidine/mRNA weight ratios, the amount of material detectable in the gradient was too low to be measured (data not shown) mainly because of strong adhesion to the centrifuge tube walls.

3.2. Nuclease accessibility to the diC14-amidine-complexed mRNA

mRNA–diC14-amidine complexes and free mRNA were exposed to RNaseA. Free mRNA was completely degraded within 5 min incubation (Fig. 2, lanes 2–5). mRNA complexed with diC14-amidine at a lipid/mRNA weight ratio of 1:1 was partially accessible to the enzyme (Fig. 2, lanes 6–8). Increasing the diC14-amidine to mRNA ratio above 2:1 fully protected the mRNA from hydrolysis (Fig. 2, lanes 9–14). Pre-incubation of the RNase with increasing amounts of diC14-amidine followed by addition of mRNA leaded to complete degradation of the mRNA indicating that diC14-amidine is not an inhibitor of the RNase (data not shown).

3.3. Optimization of the transfection protocol

The percentage of transfected cells and the total β-galactosidase expression were measured at several diC14-amidine/mRNA ratios using 100 ng mRNA per 10^5 cells, both parameters were shown to increase with the diC14-amidine/mRNA ratio to reach a plateau at a 4:1 ratio (Fig. 3A,B).

Increasing the amount of mRNA from 100 ng to 500 ng to transfect the same amount of cells (10^5) enhanced the percentage of transfected cells by a factor of 4 to 5 at all diC14-amidine/mRNA weight ratios, except at the highest ratio (8:1) corresponding to a total amount of 4 μg diC14-amidine (Fig. 3C). The lower efficiency at the 8:1 ratio could be associated to the cytotoxicity of diC14-amidine at high doses. It can however be controlled. Indeed, Fig. 3D shows that the transfection efficiency depends both on the mRNA dose and the diC14-amidine/mRNA ratio. With a 2:1 ratio and 2 μg of mRNA, about 90 % of cells were transfected although 4 μg of diC14-amidine were used. It should be mentioned that the presence of serum in the transfection medium did not affect the transfection efficiency in these conditions.

3.4. In vitro translation experiments

Little is known about the mRNA accessibility to the translation machinery after its complexion with a cationic amphiphile. This point is very important to help discriminate between two possible pathways for transfection mechanism: entry of the complex in the cytoplasm or dissociation of the complex after initial contact with the membrane. Here, we have tried to evaluate the availability of mRNA for in vitro translation after complexion with diC14-amidine. The mRNA of BMV (Brome Mosaic Virus) was used instead of recombinant SFV-LacZ mRNA since the later gives lower in vitro translation efficiency. This is probably due to the fact that SFV-LacZ mRNA is a long (about 9 kb), in vitro capped mRNA whereas BMV RNA is a mixture of short mRNA, efficiently capped in BMV infected cells. As a consequence, the stability and translation efficiency of SFV-LacZ mRNA is lower. BMV mRNA was translated here in a cell-free translation system containing wheat germ extract. Pre-incubation of the BMV mRNA with diC14-amidine before addition to the reaction mixture reduced the translation efficiency by a factor of 2 at a diC14-amidine/mRNA ratio of 2:1 and completely at a 4:1 ratio (Fig. 4). On the contrary, sequential addition of the same amount of diC14-amidine and mRNA (to a final diC14-amidine/mRNA ratio of 4:1) to the translation reaction mixture yielded no more than 50% inhibition of translation efficiency. It is likely that mRNA binds competitively to liposomes and ribosomes, the uncomplexed mRNA being responsible for the residual translation activity. If diC14-amidine would act as an inhibitor of the translation machinery, a total inhibition of the translation efficiency would be observed regardless of sequential or preformed complex addition since diC14-amidine concentration was kept constant.

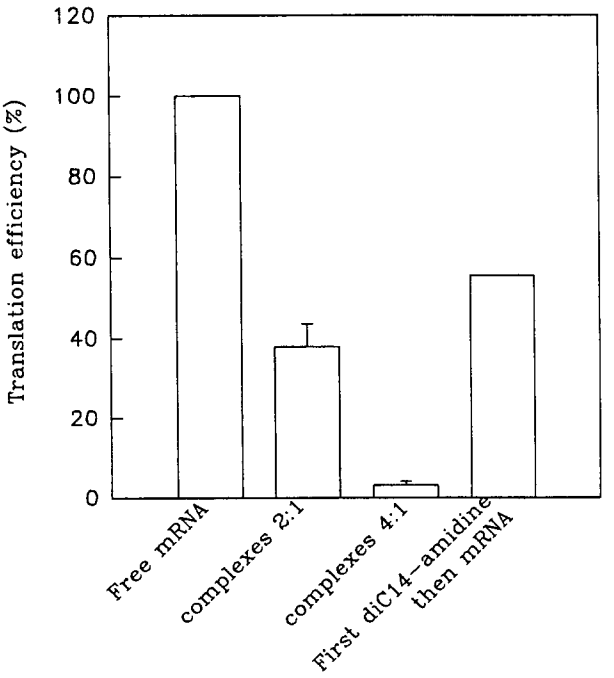

Fig. 4. Effect of diC14-amidine complexion on the translation efficiency of the BMV mRNA. 1 μg mRNA was used in each case. mRNA was used alone (free mRNA), preincubated with 2 μg of diC14-amidine (complexes 2:1) or 4 μg of diC14-amidine (complexes 4:1) before being translated in vitro in a 25 μl reaction mixture as described in section 2. In a control experiment, 4 μg of diC14-amidine were added to the reaction mixture before addition of the mRNA (first diC14-amidine then mRNA). The radioactivity associated with newly synthesized polypeptides was counted and the values obtained expressed as a percentage of the free mRNA value (the free mRNA translation efficiency was considered as 100%) after subtraction of the background (obtained for a reaction carried out without mRNA).

These cell-free translation experiments demonstrate that complexion of mRNA by diC14-amidine strongly reduces its accessibility to the translation machinery. It is therefore highly probable that in the cell context, the complex dissociates, at least partly, after its initial interaction with the cell membrane in order to make the mRNA available for efficient translation. Where and how this process takes place is unknown but surely merits further investigation.

In summary, we have developed a highly efficient mRNA transfection system using diC14-amidine containing liposomes which was previously used for successful DNA transfection [9]. DiC14-amidine has been previously shown to form vesicles that have been characterized [23]. In transfection reagents described so far, the polar head group contained an amine function. We demonstrate here that addition of another basic moiety (amidine) may also result in high transfection activity.

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