Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6077–6081, August 1989
Biochemistry

Cationic liposome-mediated RNA transfection

[cationic lipid vesicles/*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/translation]

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Communicated by Giuseppe Attardi, May 12, 1989

ABSTRACT **We have developed an efficient and reproducible method for RNA transfection, using a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), incorporated into a liposome (lipofectin). Transfection of 10 ng to 5 μg of *Photinus pyralis* luciferase mRNA synthesized *in vitro* into NIH 3T3 mouse cells yields a linear response of luciferase activity. The procedure can be used to efficiently transfect RNA into human, rat, mouse, *Xenopus*, and *Drosophila* cells. Using the RNA/lipofectin transfection procedure, we have analyzed the role of capping and β-globin 5′ and 3′ untranslated sequences on the translation efficiency of luciferase RNA synthesized *in vitro*. Following transfection of NIH 3T3 cells, capped mRNAs with β-globin untranslated sequences produced at least 1000-fold more luciferase protein than mRNAs lacking these elements.**

The wide variety of methods to introduce genetic material into cells includes relatively simple manipulations like mixing high molecular weight DNA with calcium phosphate, DEAE-dextran, polylysine, or polyornithine. Other methods involve electroporation, protoplast fusion, liposomes, reconstituted viral envelopes, viral vectors, or microinjection. In nearly all cases DNA has been introduced into cells because of its inherent stability and eventual integration in the host genome. By comparison, progress in introducing RNA molecules into cells has been very slow and restricted to a few cases (1–4). Inability to obtain sufficient amounts of intact RNA and its rapid degradation have been a major hindrance in the past. The limitation of obtaining sufficient quantities of RNA can now be alleviated by synthesizing large amounts of RNA *in vitro*, using bacteriophage RNA polymerases (5).

Since we were interested in studying the cis- and trans-acting factors influencing both the translational efficiency and the stability of eukaryotic mRNAs, we undertook the development of a reliable method to efficiently introduce RNAs into cells. We report the use of RNA transfection mediated by lipofectin (a liposome containing a cationic lipid) for efficient and reproducible RNA introduction and expression in tissue culture cells. The RNA/lipofectin complex can be used to introduce RNA into a wide variety of cells, including fibroblasts, hematopoietic cell lines, F9 teratocarcinoma cells, JEG choriocarcinoma cells, PC12 pheochromocytoma cells, amphibian cells, insect cells, and a variety of cells grown in suspension.

MATERIALS AND METHODS

Tissue Culture and Plasmids. All the cell lines used were obtained from the American Type Culture Collection and grown in either Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum or RPMI 1640 medium + 10% fetal calf serum. Drosophila KC cells were obtained from Michael McKeown (Salk Institute) and maintained in D22 medium (Whittaker M. A. Bioproducts).

Cloning procedures were carried out essentially as described (6). T7 RNA polymerase transcription templates, as well as various mRNAs produced from them, are outlined in Fig. 1. *Xenopus laevis* β-globin sequences were derived from the plasmid pSP64 T (7), with the 5′ β-globin sequences obtained as the *Hin*dIII/*Bgl* II fragment and the 3′ β-globin sequences released as the *Bgl* II/*Eco*RI fragment. These 3′ sequences include a terminal polynucleotide tract of $A_{23}C_{30}$. The *Photinus pyralis* luciferase sequences were obtained as the *Hin*dIII/*Bam*HI fragment of pJD206 (8), and they include 22 bases of luciferase cDNA sequence preceding the open reading frame, as well as 45 bases of cDNA sequence downstream of the termination codon, but they are devoid of the luciferase polyadenylylation signal. The 30-nucleotide poly(A) tail of the plasmid Luc An was obtained from pSP64 An. All transcripts were generated from the T7 RNA polymerase promoter (9).

RNA Synthesis and Purification. The capped RNAs were transcribed from a linearized plasmid DNA in a reaction mixture containing 40 mM Tris·HCl at pH 8.0, 8 mM $MgCl_2$, 5 mM dithiothreitol, 4 mM spermidine, 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, 0.5 mM $m^7G(5')ppp(5')G$ (New England Biolabs), T7 RNA polymerase (New England Biolabs) at 4000 units/ml, RNasin (Pharmacia) at 2000 units/ml, and linearized DNA template at 0.5 mg/ml for 60 min at 37°C. Transcription reaction mixtures were treated with RQ1 DNase (2 units/μg of template; Pharmacia) for 15 min at 37°C, and, after extraction with phenol/chloroform, the samples were precipitated with ethanol/NaOAc. Uncapped RNAs were prepared in a similar fashion, except that $m^7G(5')ppp(5')G$ was omitted and the GTP concentration was raised to 1 mM. Radioactive RNA was prepared without capping as described above by adding 4 μCi (1 Ci = 37 GBq) of [^{32}P]UTP per μg of template DNA. All RNA species used for the data presented herein were prepared in bulk, using reactions yielding 0.1–1 mg of purified RNA.

RNA Transfection of Cells Mediated by *N*-[1-(2,3-Dioleyloxy)propyl]-*N,N,N*-trimethylammonium Chloride (DOTMA). DOTMA was prepared and incorporated into liposomes with dioleoyl phosphatidylethanolamine (DOPE) as described (10). Unless otherwise indicated, synthetic mRNA was mixed with uncapped carrier RNA to yield a total of 20 μg of RNA per transfection. The RNA was then added to 4 ml of Opti-MEM medium (GIBCO) containing 50 μg of lipofectin (DOTMA/DOPE 1:1, mol/mol) and the cells were incubated with the RNA/lipofectin/medium mixture for the indicated period (8 hr in most cases). Transfections of adherent cells were performed with 10-cm tissue culture plate monolayers which were about to reach confluency. Nonadherent cells were counted before transfection, and 10^7 cells

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Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride; DOPE, dioleoyl phosphatidylethanolamine; UT, untranslated.

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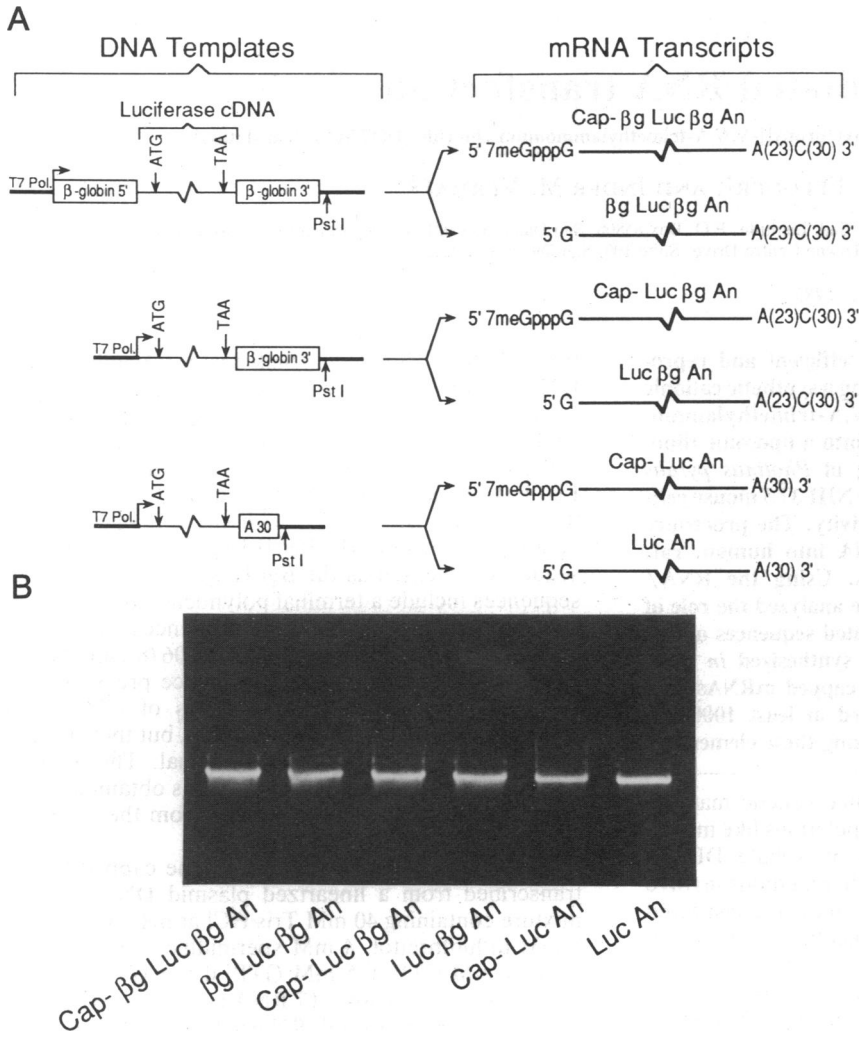

FIG. 1. mRNA transcripts synthesized *in vitro*. (*A*) Three DNA templates coding for the *P. pyralis* luciferase protein but varying in 5′ or 3′ untranslated regions were transcribed *in vitro* in the presence or absence of cap analog to generate the six mRNA species shown. (*B*) Ethidium bromide-stained 1% agarose Tris/borate/EDTA gel showing the integrity of the six RNA transcripts used for the comparative analysis summarized in Fig. 5 and Table 2. *In vitro* transcription conditions were as described, with 2 μg of purified mRNA loaded per lane. Transcripts of 1.7 to 1.9 kb are not resolved under these conditions.

were used with the above conditions for each transfection. Transfected cells were harvested by scraping at 8 hr after transfection unless otherwise indicated. Freeze–thaw cytoplasmic protein extraction, Bradford determination of protein yield, and *P. pyralis* luciferase activity assays were as described (8, 11). A total of 30 μg of total protein extract in a total volume of 150 μl of reaction mixture was used to perform each luciferase assay, using a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, CA) with automatic injection of substrate and integration of counts over a 30-s interval. Mock transfections were performed with carrier RNA, lipofectin, and Opti-MEM and were analyzed in parallel with corresponding experimental samples. Results are expressed as the difference of experimental and mock-transfected cell light emission specific activity (60-s light emission counts/30 μg of total extract protein). DEAE-dextran transfections were performed as described (3).

To determine the efficiency of association of the RNA/lipofectin mixture with its cellular target, uncapped ^{32}P-labeled RNA was mixed with Opti-MEM in the presence or absence of lipofectin as above and placed on NIH 3T3 mouse cells. At various times, transfected cell supernatant was aspirated and the cells were harvested by scraping in GIBCO phosphate-buffered saline (PBS). Cells were washed in PBS and lysed in Tris-buffered saline containing 1% Nonidet P-40, and the lysate was extracted with phenol/chloroform. The organic phase was reextracted with H_2O and the aqueous cell lysate phases were pooled. The radioactivities of samples were measured in Eco-Lite scintillation cocktail, and the fraction of cell-associated RNA was expressed as a ratio of the cell lysate radioactivity to the total recovered radioactivity. RNase-resistant radioactive material was determined as above, except RNase A was added to the harvested cells at 100 μg/ml and the mixture was incubated for 30 min at 37°C.

***In Vitro* Reticulocyte Translations.** Nuclease-treated rabbit reticulocyte lysate (Promega) was used for *in vitro* translation in a 50-μl reaction mixture with [^{35}S]methionine at 1 mCi/ml and 2 μg of mRNA. Following incubation for 60 min, the total translation mixture was diluted 1:100 prior to luciferase analysis, and 2 μl of the dilution was analyzed for light emission as above.

RESULTS

Optimization of Transfection: Lipofectin to RNA Ratio. Since loading cells with DOTMA, a positively charged lipid, is associated with cytotoxicity (10), optimization of the lipofectin-to-RNA ratio was performed for NIH 3T3 cells by varying the amount of lipofectin used to transfect 20 μg of RNA (15 μg of carrier plus 5 μg of capped βg Luc βg An runoff mRNA; see Fig. 1) and assayed for luciferase specific activity after 8 hr of transfection. The results shown in Fig. 2 indicate that the specific activity of luciferase protein is optimized at 50 μg of lipofectin under these conditions. This ratio (2.5 μg of lipofectin to 1 μg of RNA) was used for all further transfections reported here.

Kinetics of mRNA Transfection. To investigate the relationship between the quantity of mRNA transfected and the resulting protein translation, a total of 20 μg of RNA con-

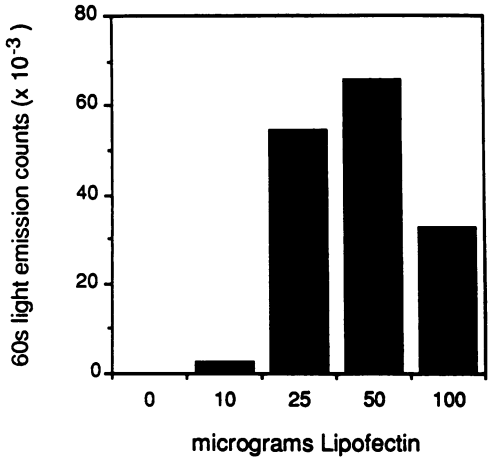

FIG. 2. Transfection efficiency varies with the ratio of RNA to lipofectin. Subconfluent 10-cm plates of NIH 3T3 cells were transfected with 20 μg of RNA (5 μg of Cap-βg Luc βg An + 15 μg of carrier) complexed with various quantities of lipofectin liposomes. Thirty micrograms of total cellular lysate protein from each transfection was then analyzed for luciferase light emission with integration over a 30-s counting interval. Data shown represent the average specific activity of two transfections after correction for background.

taining various amounts of Cap-βg Luc βg An mRNA was used to transfect NIH 3T3 cells. After transfection for 8 hr, a linear relationship was observed between specific activity of luciferase (within 2-fold experimental variation) and the quantity of transfected mRNA (Fig. 3*A*). It is worth noting that the assay is sensitive enough to detect protein synthesized after transfection of as little as 10 ng of RNA.

In contrast, cells transfected with RNA containing 20 μg of Cap-βg Luc βg An mRNA by using the DEAE-dextran procedure for 8 hr showed barely detectable levels of luciferase activity (0.01 pg of luciferase compared to 122 pg of luciferase with RNA/lipofectin). Our conservative estimate is that the RNA/lipofectin procedure is at least 100- to 1000-fold more efficient than the conventional DEAE-dextran method of RNA transfection.

Fig. 3*B* shows the time course of luciferase activity in cells transfected with the same mRNA in the absence of carrier. The data indicate an initial lag phase of about 30 min after transfection, and then the synthesis of the luciferase protein increases with time. Thus it appears that some fraction of the transfected RNA is functional *in vivo* for at least 5 hr.

Efficiency of RNA Transfection. To determine how efficiently the DOTMA/RNA complex associates with cells, labeled RNA was prepared without capping or polyadenylylation from *Eco*RV-linearized pIBI 31 (2×10^5 cpm/μg of RNA). About 10^6 cpm was transfected into NIH 3T3 cells and analyzed for association with the cells. Results summarized in Table 1 indicate that within 1 hr at least 60% of the labeled RNA was tightly associated with cells, and at least 20–30% of the radioactive material was RNase resistant. Moreover,

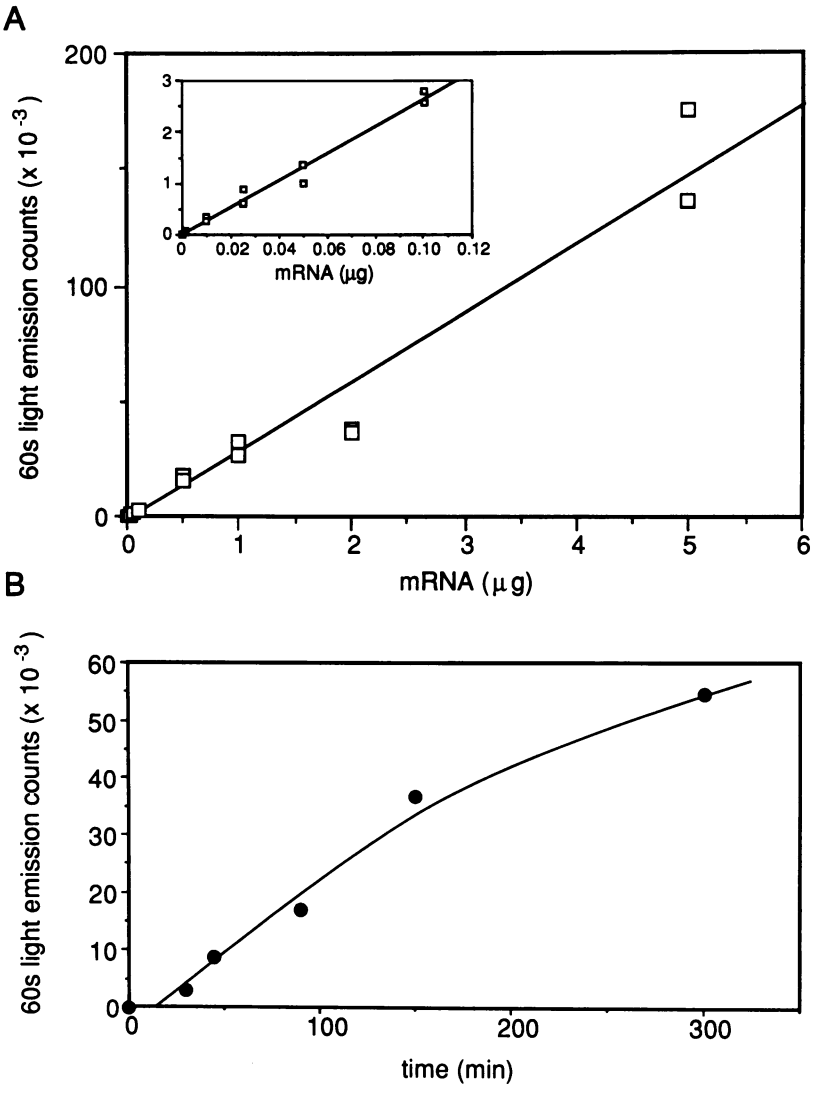

FIG. 3. Kinetics of mRNA transfection. (*A*) Subconfluent 10-cm dishes of NIH 3T3 cells were transfected with various quantities of mRNA (Cap-βg Luc βg An) with the addition of carrier RNA to a total of 20 μg per transfection under the standard conditions. Lysates were prepared 8 hr after addition of RNA/lipofectin complex to cells and analyzed for luciferase specific activity as described. (*Inset*) Data near the origin shown with expanded scales. All transfections were performed in duplicate as shown. (*B*) Transfections were performed as above with 5 μg of mRNA and no carrier. Addition of nonspecific RNA increases the overall luciferase protein production (about 2-fold), presumably by altering stability of mRNA (6). Lysates were prepared at the time indicated and then analyzed for luciferase specific activity.

Table 1. Efficiency of RNA transfection

Time after transfection, hr	RNA adherent to cells, %	RNase-resistant RNA, %
0	—	3.5
1.0	71	32
2.0	75	18
5.0	60	ND
8.2	ND	23
9.0	57	ND
15	ND	37

NIH 3T3 cells were transfected with 5 μg of ^{32}P-radiolabeled RNA for the indicated period of time, and then the fraction of ^{32}P which was tightly cell associated (adherent) or refractory to RNase release (RNase resistant) was determined. The 0 time point indicates the treatment of lipofectin/RNA complex with RNase before addition to the cells. ND, not determined.

this association appears to be very rapid, consistent with an ionic interaction between cell membrane and the DOTMA/RNA complex.

RNA Transfection in a Variety of Cell Types. To extend the utility of RNA transfection by lipofectin, a wide variety of cell types were transfected with 20 μg of Cap-βg Luc βg An RNA and 50 μg of lipofectin in 4 ml of Opti-MEM (optimal for NIH 3T3 cells; see Fig. 2) and incubated for 8 hr. Fig. 4 shows that nearly all cell types exhibited luciferase activity. The various efficiencies of RNA transfection in different cell types could be due to differential transfectability, suboptimal lipofectin-to-RNA ratios, translational efficiency, or stability of mRNA.

Role of Cap and 5′ and 3′ Untranslated (UT) Sequences. The RNA transfection technique developed here could be used to investigate various parameters influencing translational efficiency of mRNAs by directly transfecting mRNA transcripts generated *in vitro* from DNA constructs containing specific sequences. Fig. 1*A* shows (*i*) capped or uncapped 5′ UT β-globin–luciferase coding region and 3′ UT β-globin region containing an $A_{23}C_{30}$ tract (βg Luc βg An), (*ii*) capped or uncapped luciferase coding region and 3′ UT β-globin region with $A_{23}C_{30}$ (Luc βg An), and (*iii*) capped or uncapped luciferase coding region with a poly(A) tract of ≈30 A residues (Luc An). About 0.2, 1, 2.5, or 5 μg of each mRNA was transfected in a total of 20 μg of RNA (with appropriate amounts of the carrier RNA) into NIH 3T3 cells for 8 hr under the optimal conditions and analyzed for luciferase specific activity. Fig. 5 shows that maximal protein synthesis was obtained with the mRNA transcript which contains the cap and both the 5′ and 3′ UT regions of β-globin. Results obtained with all six transcripts were analyzed by linear regression and are tabulated (Table 2). The data indicated that the capped mRNA is nearly 40-fold more efficiently translated than the uncapped identical mRNA. The data in Table 2 also show that the 5′ UT region of β-globin mRNA imparts nearly 9-fold greater translational efficiency. Finally, the presence of β-globin 3′ UT region plus an $A_{23}C_{30}$ polynucleotide tract confers at least a 6-fold advantage over the transcripts containing only a poly(A) stretch of 30 residues.

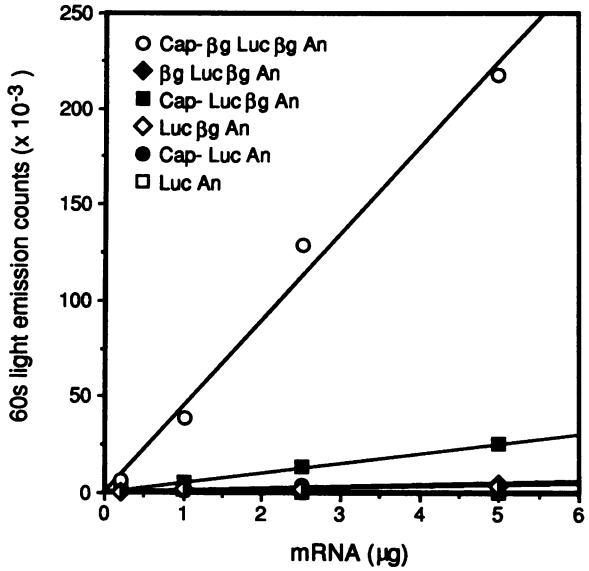

FIG. 5. Comparison of mRNA translations. The various RNA preparations seen in Fig. 1 were transfected into NIH 3T3 cells and analyzed for luciferase specific activity 8 hr after RNA/liposome complex addition. A total of 20 μg of RNA was used for each transfection, and the specific activity is indicated as a function of the mass of mRNA transfected. Regression lines were plotted by using the Cricket graph software package (Cricket Software, Malvern, PA).

As a control for the integrity of the T7 polymerase-transcribed mRNAs, we performed *in vitro* translation in rabbit reticulocyte extracts. Table 2 also shows that when the mRNA transcripts used for transfection were translated *in vitro* in a rabbit reticulocyte cell extract, they were all translated efficiently (within 2- to 3-fold) regardless of the presence of the cap or 5′ or 3′ UT sequences. However, the amount of luciferase synthesized in an *in vitro* translation system is at least 1000-fold greater (e.g., 7 ng in reticulocyte lysates per 2 μg of template as compared to 7.7 pg for 2 μg of transfected mRNA) than RNA/lipofectin.

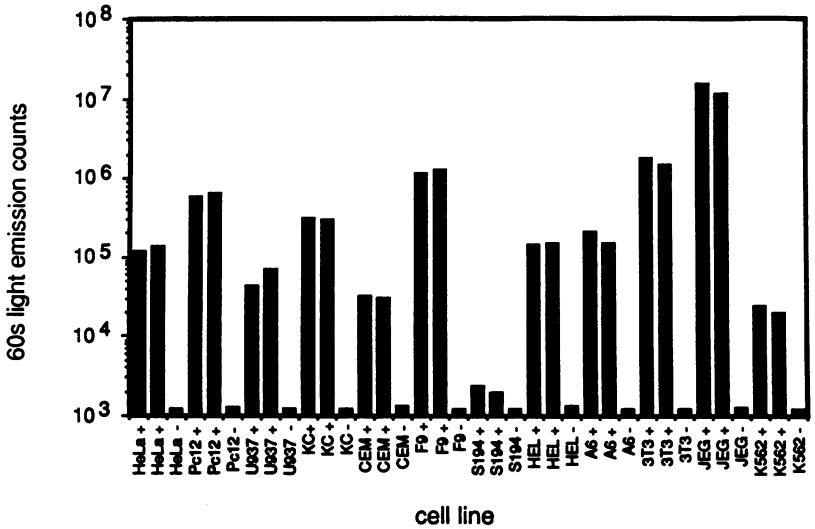

FIG. 4. RNA transfection in a variety of cell types. Fifty micrograms of lipofectin liposomes was used to transfect various cell lines either with (+) or without (−) 20 μg of mRNA (Cap-βg Luc βg An). Lysates were prepared 8 hr after addition of lipofectin and were analyzed for luciferase specific activity as before.

Table 2. Comparison of *in vivo* and *in vitro* translation

mRNA	*In vivo* translation: Slope of line, 60-s light emission counts/μg	*In vivo* translation: Correlation coefficient	*In vitro* translation, 60-s light emission counts
Cap-βg Luc βg An	44,798	0.99	85,280
βg Luc βg An	1,172	1.00	185,110
Cap-Luc βg An	5,124	1.00	134,294
Luc βg An	29	0.97	88,695
Cap-Luc An	807	1.00	263,815
Luc An	28	0.99	72,211

The various mRNAs diagrammed in Fig. 1 were translated after transfection into NIH 3T3 cells or by using rabbit reticulocyte lysate. Transfection data plotted in Fig. 5 are expressed as a linear slope and regression correlation coefficient. Reticulocyte lysate translation is expressed as luciferase activity of the total translation preparation.

DISCUSSION

We have developed a high-efficiency RNA transfection system using DOTMA-containing liposomes (lipofectin), which was previously used to transfect DNA into cells (10). The procedure is simple, reliable, and at least 100- to 1000-fold more efficient than the presently available DEAE-dextran method (refs. 1–4; S. Schlesinger and P.L.F., unpublished results). Furthermore, we show that the translation of *in vitro* synthesized mRNA in NIH 3T3 cells continues for at least 5 hr (Fig. 3*B*). It is thus feasible to study the parameters of translation machinery by direct RNA transfection, rather than by introducing DNA constructs. Using this method, we find that 70% of the transfected RNA is associated with the cells, and a large fraction of this RNA is taken up into the cells as judged by RNase resistance (Table 1).

A particularly attractive feature of the RNA/lipofectin procedure is the ability to transfect a wide variety of cell types. For example, conventional DNA transfection protocols have generally resulted in rather low levels of expression in hematopoietic cell lines such as U937, but the procedure described here using RNA is very efficient (Fig. 4). Furthermore, RNA can be transfected into human, mouse, rat, *Xenopus*, and *Drosophila* cells, thus enlarging the scope of this method. However, one limitation of the lipofectin procedure is the toxicity associated with the positively charged lipids. For this reason it is prudent to establish the optimal RNA-to-lipofectin ratio for the desired cell type.

The results establishing the role of 5′ and 3′ UT regions of β-globin sequences on protein production following transfection of *P. pyralis* luciferase mRNA directly demonstrate the utility of the RNA transfection procedure. These results are in agreement with a wide body of data that unmethylated mRNAs are poor templates for protein synthesis (12, 13). Presumably the m^7G-capped mRNA binds more efficiently to 40S ribosomal subunit (14). Surprisingly, the effect of methylation or appropriate polyadenylylation were undetectable in the *in vitro* rabbit reticulocyte translation system. It is, however, well established that effects on 5′ capping are more readily observed in the wheat germ extract (15).

The RNA/lipofectin procedure can also be used to study the effect of various sequence elements on mRNA stability (16). The technology developed here may eventually be extended to introduce antisense RNA into cells, including modified oligonucleotides containing methyl phosphonate or thiolated nucleotides, particularly to study the role of protooncogenes such as *fos* or *myc* whose transcripts have a short half-life of 30–60 min (17). The RNA/lipofectin method can be used to directly introduce RNA into whole tissues and embryos (R.W.M., C. Holt, and I.M.V., unpublished results), raising the possibility that liposome-mediated mRNA transfection might offer yet another option in the growing technology of eukaryotic gene delivery, one based on the concept of using RNA as a drug.

We thank Drs. Pablo Garcia and Don Helinski for materials, and Judy White and Tony Hunter for helpful discussions. We are grateful to BRL and The Institute of Bio-Organic Chemistry, Syntex Research, for their generous gift of lipofectin. This work was supported by grants from the National Institutes of Health and the American Cancer Society to I.M.V.

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