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November 19, 1991

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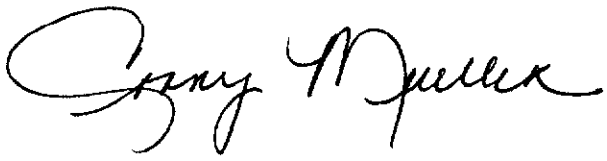
Re: U.S. Serial No. 326,620
Filed March 29, 1989
Verma and Malone
CATIONIC LIPID-MEDIATED
RNA AND DNA TRANSFECTION
Our File No. S48014

Dear Bob:

Pursuant to your telephone request, enclosed for your file is a copy of the referenced application.

Best regards.

Very truly yours,



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CATIONIC LIPID-MEDIATED RNA AND DNA TRANSFECTION

Field of the Invention

The present invention relates to methods of recombinant DNA technology. More particularly, the invention concerns the introduction of exogenous nucleic acids into target cells via cationic lipid-mediated transfection.

In one aspect, the present invention, it is believed for the first time, provides an efficient, reproducible method for the transfection of ribonucleic acids (RNAs) into target cells. This aspect of the invention arises from the surprising discovery that RNA, notably messenger RNA (mRNA) can be efficiently introduced into target cells utilizing a cationic lipid-mediated transfection procedure, similar to the "DOTMA"-mediated transfection (also referred to as "lipofection") technology recently developed for deoxyribonucleic acid (DNA) transfection. The transfected mRNA translates to the corresponding protein, thereby providing a convenient method, for example, for the short-term bulk production of foreign proteins in the transfected cells.

According to another aspect, the invention provides for the introduction of nucleic acids (RNA and DNA) into tissue cells such as, germ cells, including the cells of pre-implantation embryos.

After quick cultivation, the pre-implantation embryos transfected with a desired DNA can be re-transplanted into a host female animal to generate transgenic animals.

Background of the Invention

It is known that, under appropriate conditions, exogenous DNA can be introduced into the nucleus of target cells. This phenomenon is generally referred to as "DNA transfection". The delivery of the DNA into the nucleus is

a complex process. The large DNA molecules having a negatively charged phosphate backbone need to cross a multitude of enzymatic and membrane barriers before they can enter the nucleus. Due to these barriers resultant material losses are encountered; hence, spontaneous entry of a desired DNA into the nucleus of a target cell is very inefficient. Therefore, the spontaneous process is not suitable for the expression of foreign genes in transfected cells for any practical purpose. To improve the efficiency of the delivery of DNA molecules into the nuclei of cells and of the subsequent expression process, numerous methods, including the use of retroviruses, microinjection, protoplast fusion, etc. have been developed and brought partial success.

Techniques like microinjection require sophisticated equipments and skills, and in most cases the transfection efficiency of DNA is low and shows substantial differences for different cell lines.

Of the currently employed transfection protocols, the most widely used are perhaps the calcium phosphate-precipitation technique [Graham et al., Virology 52, 456 (1973)] and the DEAE dextran method [Danielsen et al., EMBO J. 5, 2513 (1986)].

A DNA-transfection protocol based on the use of a new synthetic cationic lipid has recently been developed and reported by Felgner et al., Proc. Natl. Acad. Sci. USA 84, 7413 (1987). This article is hereby expressly incorporated herein by reference. The lipid, (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA), is disclosed, along with other structurally closely related surfactants, in the European Patent Application published 16 July 1986, under No. 0,187,702, which is expressly incorporated herein by reference. DOTMA, either alone or in combination with neutral phospholipids, spontaneously forms multilamellar liposomes from which unilamellar vesicles can be obtained by sonication. The small unilamellar liposomes containing

DOTMA interact spontaneously with DNA, forming DOTMA-DNA complexes. The complex formation is probably due to ionic interactions between the negatively charged phosphate backbone of the DNA and the positively charged quaternary amine of DOTMA. On the other hand, there is an ionic interaction between the DOTMA-DNA complexes and the charged outer membrane of the target cells that facilitates fusion of the complex with the plasma membrane, resulting in both uptake and expression of the transfected DNA. The article reports the introduction of pSV2cat and other plasmids into various cell lines in in vitro experiments.

Further details of DOTMA-mediated DNA transfection into a variety of cell lines are disclosed by Chang et al. in Focus 10:4, 66 (1988). The authors found that the method was successful with a number of cell cultures that were normally resistant to transfection by other procedures.

Although Felgner et al., Supra suggest that their technique may be useful for the introduction of other molecules, e.g. RNAs into target cells, no experimental results or even theoretical considerations are disclosed in support of this speculation.

Chang et al., Supra report that, according to personal communication from other scientists including inventors of the present invention, RNA can be complexed with cationic liposomes for uptake by cells. No information is provided as to an RNA transfection protocol or the efficiency of RNA uptake by cells.

According to the earlier art, primarily due to the highly unstable nature of RNA molecules, exogenous genes have been introduced into target cells by transfecting the desired DNA into the cell nuclei, from which the corresponding mRNA was transcribed and the desired protein was expressed in the target cells. The introduction of DNA into the nuclei of target cells is very inefficient, usually only about 0.1-1 % of the cells is transfected with the desired DNA. In addition,

transcription of DNA into the corresponding mRNA requires rather stringent conditions, and in many cell types transcriptional controls are poorly understood and transcription from foreign genes is difficult to demonstrate.

Although RNA transfection of self-replicating viral RNAs generated in vitro has previously been demonstrated using the DEAE dextran technique [van der Werf et al., Proc. Natl. Acad. Sci. USA 83, 2330 (1986); Mizutani et al., Journal of Virology 56, 628 (1985)], efficient, reproducible mRNA transfection protocols have been unavailable. The main reason for this is rapid RNA degradation and the inefficiency of DEAE dextran.

It would, therefore, be desirable to find a way to increase the half life of RNA (mRNA) and to use directly RNA for transfection. Since RNA needs to be introduced into the cytoplasm of cells instead of nuclei, its transfection should be more efficient. Moreover, translational controls are by far less stringent than transcriptional controls, accordingly protein production should be more efficient from transfected mRNA.

It has been shown that capping of synthetic mRNA effects both its stability and translational efficiency, following microinjection into Xenopus oocytes [Furuichi et al., Nature 266, 235 (1977), Kreig et al., Nucleic Acids Research 12, 7057 (1984)]. Kreig et al., Supra also investigated the effect of the beta globin 3' flanking region on the stability of the injected mRNA but found only a slight effect.

The findings concerning the role of 5'-untranslated sequences on mRNA stability are contradictory. According to the scanning model which is currently the most widely accepted model for eukaryotic translation [Kozak , Cell 22, 7 (1980)] 5'-untranslated sequences devoid of "AUG" codons upstream of the translational start site used to generate a given protein should have little effect on the translational efficiency of an eukaryotic mRNA. Other

researchers have shown that specific sequence motifs can modulate translation [Parkin et al., Molecular and Cellular Biology 8, 2897 (1988); Aziz et al., Proceedings of the National Academy of Science USA 84, 8478 (1987); McGarry et al., Cell 42, 903 (1985)].

There is no method known in the art for the efficient increase of the stability of mRNA molecules. Similarly, there is no efficient, reproducible technique available for the transfection of mRNA.

For practical applications, for example for generating transgenic animals, nucleic acids need to be introduced not only into cultured cells but also into whole tissues, and specifically into germ cells, such as the cells of pre-implantation embryos. The current technology involves isolation of the animal pre-implantation embryo following fertilization, removing the zona pellucida and microinjecting the DNA into the male pronucleus.

The microinjection of plasmids into germinal vesicles of mouse oocytes or pronuclei of fertilized mouse ova are disclosed in Brinster et al., Science 211, 396 (1981). Illmensee et al., Cell 23, 9 (1981), and Gordon et al., P.N.A.S. 77, 7380 (1981) report injection of nuclei into nucleated mouse eggs and introduction of plasmids containing the herpes thymidine kinase gene and SV40 (Simian virus) sequences into mice.

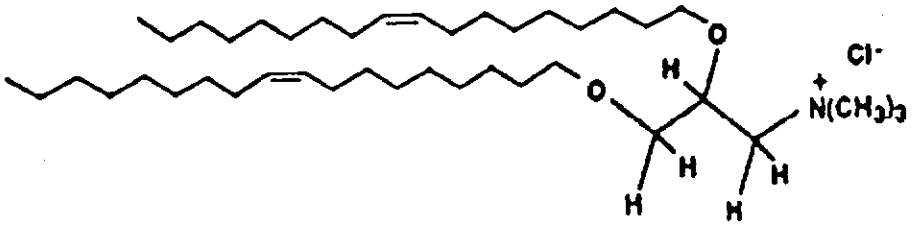
Basic microinjection techniques are disclosed in Graessmann, M. & Graessmann, A. Microinjection and Organelle Transplantation Techniques, eds. Celis, J. E., Graessmann, A. & Loyter, A. (Academic, London) pp. 3-13 (1986).

The method of micromanipulation of the embryo requires sophisticated technology and also can cause physical damage to the embryo. Thus, the number of animals surviving are relatively low and the procedure is complicated. In lack of a fast and efficient transfection protocol, the generation of transgenic animals is currently

more of a scientific curiosity than a reproducible technology.

Summary of the Invention

The present invention is based upon the discovery that ribonucleic acid (RNA) can be introduced into target cells (including cultured cells, tissues, germ cells, etc.) by making use of cationic lipids that give rise to physically stable liposomes and are capable of spontaneous complex formation with RNA. The lipid-RNA complexes enter the cytoplasm of target cells where translation takes place. Typical representatives of cationic lipids that can be used in the RNA transfection process of the present invention are synthetic, bilayer forming cationic lipids, including (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA) of the formula (I)



(I)

and other non-toxic salts of the same and related compounds.

Using a different nomenclature, DOTMA is also called N-[2,3-di-(9-(Z)-octadecenyloxy)]-prop-1-yl-N,N,N-trimethylammonium chloride.

In one aspect, the present invention relates to a process for the introduction of ribonucleic acid (RNA) into the cytoplasm of target cells, comprising:

(a) complexing said RNA with liposomes of a cationic lipid, and

(b) transfecting said target cells with the obtained complexes.

Preferably, RNA is complexed with liposomes of a bilayer-forming cationic lipid, such as a non-toxic salt of racemic or optically active (N-[1-(2,3-dioleyloxy)propyl]-N,N-dimethylamine) incorporated into

liposomes, and the target cells are transfected with the obtained complex.

It has been found that in addition to capping, the presence of an appropriate, translationally efficient 5'-untranslated region and a poly A-tailed 3'-untranslated region devoid of any destabilizing element consistently increase RNA stability and therefore improve protein yields. The translational enhancing or modulating role of the 5'-untranslated context is contradictory to the scanning model (Kozak, Supra) and indicates that surprisingly, mammalian fibroblasts are much more sensitive to the 5'-untranslated region than previously believed. Similarly, the strong effect of the 3'-flanking region on RNA stability and translation efficiency is entirely unexpected in view of the prior art (Krieg et al., Supra). This is an important feature of the invention.

In a preferred embodiment, the invention relates to a process for the introduction of mRNA into the cytoplasm of target cells, comprising:

(a) complexing mRNA comprising sequences encoding a polypeptide, cap, a translationally efficient 5'-untranslated region and a poly A-tailed 3'-untranslated region devoid of any destabilizing element, with liposomes of a bilayer-forming cationic lipid, and

(b) transfecting said target cells with the obtained complex.

The new method, providing for the introduction of in vitro synthesized mRNA into the cytoplasm of target cells, opens up entirely new possibilities for the practical exploitation of modern, high-efficiency in vitro mRNA synthesis techniques. The new RNA transfection technique of the present invention is simple, efficient and highly reproducible, and appears to give substantially higher transfection efficiency than the established DEAE dextran technique.

It has further been found that, following transfection, the mRNA efficiently translates to the corresponding polypeptide.

The invention also encompasses a process for polypeptide production, by growing the target cells, transfected with a mRNA containing sequences encoding a polypeptide, cap and 3'- and 5'-untranslated regions as hereinabove described, in a suitable culture medium, harvesting the transfected cells, and recovering the polypeptide encoded by said mRNA sequences.

The possible practical applications of this RNA transfection and translation technique may include, for example

- the short-term bulk production of protein,
- the short-term rescue of mutant phenotypes or characterization of new mutations in cell lines refractory to DNA transfection,
- the analysis of sized cellular mRNA fractions for generation of specific phenotypes as a prelude to cDNA cloning,
- the generation of viable retroviruses in a rapid manner, by introduction of <u>in vitro</u> synthesized retroviral RNA into packaging cell lines,
- the short-term treatment of genetic or virally induced disease of the blood, based on experiments focusing on conferring coding or antisense RNA sequences, etc.

If techniques can be developed to substantially increase the half life of polynucleotide translation templates, this approach also raises the possibility that the use of DOTMA-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.

In another aspect, the invention is based on the discovery that by using the cationic lipid-mediated nucleic acid transfection protocol, nucleic acids (including both RNA and DNA) can be introduced not only into cultured cells

but also into whole tissues and, specifically, into germ cells.

Therefore, the invention also relates to a process for the introduction of DNA into the nuclei of tissue cells, comprising:

(a) complexing the DNA with a liposomes of a cationic lipid and

(b) transfecting said tissue cells with the obtained complexes.

The cationic lipid preferably is a synthetic, bilayer-forming lipid, such as a non-toxic salt of racemic or optically active (N-[1-(2,3-dioleyloxy)propyl]-N,N-dimethylamine) incorporated into liposomes.

The tissue cells may, for example, be germ cells, such as cells of pre-implantation embryos or oocytes.

The cationic lipid-mediated DNA and RNA transfection substantially simplifies the procedure of manipulation of pre-implantation embryos. It requires quick cultivation of the pre-implantation embryos with the required DNA or mRNA and then retransplantation of the embryo. This method does not require any physical manipulation of the zygote itself.

In a still further aspect, the invention provides for a fast and efficient procedure for generating transgenic animals.

According to this aspect, the invention relates to a method of generating transgenic animals, comprising:

(a) complexing an exogenous DNA sequence with liposomes of a cationic lipid,

(b) providing a fertilized mammalian egg, or a pre-implantation embryo developed therefrom,

(c) transfecting said fertilized mammalian egg or pre-implantation embryo with the obtained complex, such that it is introduced into the nuclei thereof, and

(d) implanting the transfected fertilized egg or embryo into a host female animal of the same species from which said fertilized mammalian egg has been obtained, and

permitting said female animal to gestate, so that said host female animal gives birth to said transgenic animal incorporating said DNA in its genome.

The cationic lipid preferably is a synthetic, bilayer-forming lipid, such as a non-toxic salt of racemic or optically active (N-[1-(2,3-dioleyloxy)propyl]-N,N-dimethylamine) incorporated into liposomes.

The invention further relates to the transgenic animals generated by the above method.

The present invention is directed to the above aspects and all associated methods and means for accomplishing such. For example, the invention includes techniques requisite to the preparation of DNA templates, RNA synthesis and purification, cloning procedures, DOTMA synthesis and liposome preparation, protein production, isolation, purification and analysis, cell cultivation procedures, implantation techniques, etc.

Brief Description of the Drawings

Figure 1 (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 TBE gel showing the integrity of the six RNA transcripts used for the comparative analysis summarized in Figure 6 and Table 2. In vitro transcription conditions were as described, with 2 μg of purified mRMA loaded per lane. The variation in transcript size of 1.7 to 1.9 kD is not resolved under these conditions.

Figure 2 illustrates the luciferase specific activity as a function of Lipofectin to RNA ratio. [Lipofectin is a 1:1 by weight mixture of DOTMA and dioleyl phosphatidylethanolamine (DOPE).] Subconfluent 10 cm plates of NIH 3T3 cells were transfected with 20 μg RNA (5μg cap - ßg Luc ßg An + 15 μg carrier) complexed with varying quantities of Lipofectin liposomes indicated as per

Methods. Thirty μg of total cellular lysate protein from each transfection was then analyzed for luciferase light emission activity with integration over a 30 second counting interval. Data shown represents the average specific activity of 2 transfections after correction for background counts.

Figure 3 illustrates luciferase specific activity versus the quantity of mRNA transfected. (A) Subconfluent 10 cm dishes of NIH 3T3 cells were transfected with varying quantities of mRNA (cap - ßg Luc ßg An) with the addition of carrier RNA to a total of 20 μg/transfection under the standard conditions. Lysates were prepared 8 hours after addition of RNA/Lipofectin complex to cells and analyzed for luciferase specific activity as described. All transfections were performed in duplicate as shown. (B) Transfections were performed as above with 5 μg mRNA and no carrier. Addition of non-specific RNA increases the overall luciferase protein production (about 2-fold) presumably by altering stability of mRNA. Lysates were prepared at the time indicated, and analyzed for luciferase activity as described in Methods and Figure 2.

Figure 4 illustrates the efficiency of RNA transfection in different cell types. Fifty μg of lipofection liposomes were used to transfect various cell lines either with (+) or without (-) 20 μg of mRNA (cap - ßg Luc ßg An) as per Methods. Lysates were prepared 8 hours after addition of Lipofectin, and analyzed for luciferase specific activity as before.

Figure 5 illustrates the effect of various untranslated sequences on mRNA translation. The various RNA preparations seen in Figure 1 were transfected into NIH 3T3 cells and analyzed for luciferase specific activity 8 hours after RNA/liposome complex addition. A total of 20 μg of RNA was used for each transfection, with the specific activity indicated as a function of the mass of mRNA transfected using the cricket graph software package.

Figure 6 illustrates luciferase activity after transfecting DNA into frog embryos by lipofection.

Figure 7 depicts luciferase activity after transfecting RNA into Xenopus laevis embryos being in neurula and gastrula states of development by lipofection.

Figure 8 shows the bases 223-381 of linearized plasmid pIBI31.

Figure 9 illustrates the construction of plasmid T7 LUCFR An.

Figure 10 illustrates the construction of plasmid del5' T7 LUCFR An from plasmid pSP64 An (Promega).

Figure 11 shows the results of time course analysis of the mRNA preparations illustrated in Figure 1, transfected into NIH 3T3 cells.

Detailed Description of the Invention

1. Definitions

A transfection system suitable for the transfection of nucleic acids, including RNA and DNA into target cells is provided.

The term "transfection" as used throughout the specification and in the claims refers to the introduction of nucleic acids into target cells. To effect DNA transfection, the DNA molecules are introduced into the nuclei of target cells, while RNA is introduced into the cytoplasm of cells, where translation takes place.

"Target cells" may be cultured cells, e.g. cells of any desired cell line, or may be cells organized into tissues, including cells organized into germ cells being in different stages of development, e.g. oocytes or neurula stage or pre-implantation embryos.

The term "nucleic acid" includes both single and double stranded RNA and single and double stranded DNA sequences. Both these sequences and the sub-sequences encoding heterologous proteins may be isolated from a natural source, may be synthesized or produced by restriction digest.

The term "cationic lipid" refers to positively charged lipid compounds. Such compounds include non-toxic salts of racemic or optically active (N-1-(2,3-dioleyloxy)propyl]-N,N-dimethylamine) in particular (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA), but the invention is not restricted to the use of these chemical compounds. The use of various, bilayer-forming cationic lipids that, either alone or in combination with neutral phospholipids, give rise to physically stable liposomes is also contemplated. Such derivatives are, for example disclosed in the Published European Patent Application No. 0,187,702, and include racemic and optically active

N-(2,3-di-octadecyloxy)-prop-1-yl-N,N,N-trimethylammonium chloride;

N-(2,3-di-(4-(Z)-decenyloxy))-prop-1-yl-N,N,N-trimethylammonium chloride;

N-(2,3-di-hexadecyloxy)-prop-1-yl-N,N,N-trimethylammonium chloride;

N-(2,3-di-decyloxy)-prop-1-yl-N,N,N-trimethylammonium chloride;

N-(2-hexadecyloxy-3-decyloxy)-prop-1-yl-N,N,N-trimethylammonium chloride;

N-(2-hexadecyloxy-3-decyloxy)-prop-1-yl-N,N-dimethylamine hydrochloride;

N-(9,10-di-decyloxy)-dec-1-yl-N,N,N-trimethylammonium chloride;

N-(5,6-di-(9-(Z)-octadecenyloxy))-hex-1-yl-N,N,N-trimethylammonium chloride; and

N-(3,4-di-(9-(Z)-octadecenyloxy))-but-1-yl-N,N,N-trimethylammonium chloride.

Another structurally similar compound is the cationic surfactant didodecyldimethylammonium bromide (DDAB).

These and other structurally similar compounds would necessarily be required to function equivalently as DOTMA.

The term "non-toxic salt" relates to salts that, when employed in amounts required to effect RNA or DNA transection have no toxic effect on cells or tissues with which they become associated. Such salts may be obtained by reacting the amine compounds of the invention with suitable organic or inorganic acids. Typical representatives of non-toxic salts include halides, such as fluoride, chloride, bromide, and iodide; sulfates, sulfites, phosphates, hydrochlorides, oxalates, tartarates, citrates, p-toluene-sulfonates, etc.

In a preferred embodiment, the present invention is based on the use of liposomes containing DOTMA and related compounds incorporated into liposomes along with other, secondary surfactants, preferably neutral phospholipids, e.g., dioleyl phosphatidylethanolamine (DOPA). Secondary surfactants suitable for incorporation into the liposomes used in practicing the present invention can, for example, be found in the above-referenced European Patent Application and in the publications cited therein.

The terms "cap" or "capping" are used to refer to the 7meGppp group attached to the 5' terminal end of a mRNA obtained by transcription from a DNA template.

The terms "translationaly efficient 5'-untranslated region" and "3'-untranslated region devoid of any destabilizing element" are used to refer to 5'- and 3'-untranslated elements that facilitate mRNA translation and stability. These sequences are not limited to those specifically described in the Examples but will include 5'-untranslated sequences from viral or cellular origin which have been shown to efficiently mediate translation, 3'-sequences or chemical blocking of the 3'-end of the mRNA which increase RNA half life, and will require incorporation of Cap in most cases on the 5'-terminus of the mRNA. As a source for translationally efficient 5'-untranslated sequence elements, we refer to McGarry et al., Supra; Aziz et al., Supra; Darveau et al., Proc. Natl. Acad. Sci. USA 82, 2315 (1985); Hentze et al., Proc. Natl.

Acad, Sci. USA 84, 6730 (1987); Klemenz et al., The EMBO J. 4, 2053 (1985); Rao et al., Mol. and Cell. Biol. 8, 284 (1988); Kozak, Nucl. Acids Res. 15, 8125 (1987); Dolph et al., J. of Virology 62, 2059 (1988). Appropriate 3'-untranslated regions devoid of every destabilizing element may, for example, be chosen using the teachings of the following publications: Lee et al., Proc. Natl. Acad. Sci. USA 85, 1204 (1988); Shaw et al., Cell, August 29, 1986, pp. 659-667; Kabnick et al., Mol. and Cell. Biol. 8, 3244 (1988), Raghow, TIBS 12, 358, September 1987; Paek et al., Mol. and Cell. Biol. 7 1496 (1987); Mullner et al., Cell 53, 815 (1988); Guyette et al., Cell 17, 1013 (1979); Brawerman et al., Cell 48, 5 (1987); Hunt, Nature 334, 567 (1988); Dean et al., The J. of Cell. Biol. 106, 2159 (1988); Graves et al., Cell 48, 615 (1987); McCrae et al., Eur. J. Biochem. 116, 467 (1981); Caput et al., Proc. Natl. Acad. Sci. 83, 1670 (1986).

The most frequently used abbreviations are as follows:

DMEM = Dulbeccos Modified Eagles Medium
CS = calf serum
FCS = fetal calf serum
CEF = chick embryo fibroplast
ATP = adenosine triphosphate
UTP = uracyl triphosophate
CTP = cytosine triphosphate
GTP = guanosine triphosphate
TBE = Tris borate EDTA
MR = Modified Ringers Solution
cap = 7meGppp5'-mRNA
PBS = phosphate buffered saline

In the method of generating transgenic animals the heterologous protein-encoding DNA subsequences are generally operably linked to sequences operative for their expression.

Preferably, the promoter sequence of such linked sequences includes or is linked to a regulatory sequence by which expression of the gene in the transgenic animal may be controlled by exogenous agents, such as a metal or hormone which is fed to or inoculated into the animal. Promoter/regulator DNA sequences suitable for use in practice of the invention are derived from avian and mammalian cells and include: the iron and steroid hormone-responsive promoter/regulator sequence naturally associated with the transferrin (conalbumin) gene of chickens; the steroid hormone-responsive promoter/regulator sequence associated with ovalbumin gene in chickens; and the metal and steroid hormone-responsive promoter/regulator sequence of the mouse metallothionein-I or metallothionein-II genes.

The invention, however, is not limited to promoters with associated regulator DNA sequences. Examples of neither metal nor steroid- or hormone-responsive promoter DNA sequences which may be employed include: liver promoters, i.e. albumin, glycolytic enzymes, transferrin, ceruloplasmin and alpha-2-microglobulin; histocompatibility gene promoters, immunoglobulin gene promoters, interferon gene promoters, heat shock gene promoters and retroviral gene promoters.

2. Materials and General Methods

Conventional manipulations of recombinant DNA technology were essentially carried out as described in Maniatis et al., Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Publishing, New York (1982).

Cells and Media

The materials the source of which is not specifically indicated hereinbelow are commercially available or widely distributed among scientists, and are therefore readily available; and/or are described in standard textbooks, e.g. Maniatis et al., supra., or Methods in Enzymology 152, Guide to Molecular Cloning, S. L. Belger and A. R. Kimmel, eds. (1987).

Murine fibroblast NIH 3T3 clone 2B cells were obtained from Marguerite Vogt (The Salk Institute) and are in wide circulation (see e.g. Methods in Enzymology op. cit.) These cells were maintained in DMEM + 10% CS prior to transfection.

Human histiocytic lymphoma cell line U937 (ATCC CRL 1593), human chronic myelogenous leukemia K562 cells (ATCC CCL 243), rat adrenal pheochromocytoma PC-12 cells (ATCC CRL 1721), human erythroleukemia HEL cells (ATCC TIB 180), and human lymphoblastic leukemia CCRF-CEM cells (ATCC CCL 119) were cultured in RPMI 1640 (GIBCO) + 10% FCS prior to use. S194 non-secreting mouse myeloma cells (ATCC TIB 19), mouse embryonal teratorarcinoma F9 cells (ATCC CRL 1720), FEG human choriocorcinoma cells (ATCC HTB36) and human epitheloid cervical carcinoma HeLa cells were maintained in DMEM + 10% FCS. Xenopus laevis kidney A6 cell line (ATCC CCL 102) was maintained in 50% Liebowitz L-15 medium (GIBCO) + 10% CS. Drosophila KC cells were obtained from Dr. Michael McKeown (The Salk Institute) and maintained in D22 medium (GIBCO).

3T3-derived retroviral packaging lines PA 317 (ATCC CRL 9078) and psi-2 (from Richard Mulligan; the Whitehead Institute) were maintained in DMEM + 10% CS.

Primary chick embryo fibroplasts (CEF) were prepared from white leghorn embryos and cultured in 211 medium (2% tryptose phosphate, 1% calf serum, 1% heat inactivated chick serum in DMEM).

RNA Synthesis and Purification

DNA templates were prepared essentially as described in Maniatis et al., Supra, with two CsCl purifications and no RNAse treatment.

Plasmid transcription templates were linearized by 5-fold overdigestion with the appropriate restriction enzyme, followed by phenol/chloroform extraction and ethanol precipitation in 0.3M sodium acetate. Linear DNA templates were resuspended in diethylpyrocarbonate-treated water, and concentration determined by optical density.

Following this, capped RNAs were transcribed in a reaction medium containing 40mM Tris-HCl pH 8.0, 8mM $MgCl_2$, 5mM dithiothreitol, 4mM spermidine, 1mM rATP, UTP and rCTP, 0.5mM rGTP, 0.5mM m7G(5')ppp(5')G (New England Biolabs), 4000μ/ml T7 RNA polymerase (New England Biolabs), 2000μ/ml RNAsin (Pharmacia), and 0.5 mg/ml linearized DNA template for 60 minutes at 37°C. Transcription reaction mixtures were treated with RQ1 DNAse (Pharmacia, 2μ/μg template) for 15 minutes at 37°C. The reaction mixture was extracted with phenol/chloroform and precipitated with ethanol/NaOAc. The products were resuspended in water treated with diethylpyrocarbonate, and passed over an RNAse-free Sephadex G50 spin column (Boehringer Mannheim). The eluate was analyzed for concentration, purity and quality by optical density determination and TBE/agarose non-denaturing gel electrophoresis.

Uncapped RNAs were prepared in a similar fashion, except that m7G(5')ppp(5')g was excluded and rGTP concentration was raised to 1mM.

Radioactive RNA was prepared without capping as described above, adding 4μCi 32P-UTP/μg template DNA.

All RNA species used for the data disclosed herein were prepared in bulk using reactions yielding from 100μg to 1mg of purified DNA.

Plasmid Constructions

Cloning procedures were carried out essentially as described in Maniatis et al., Supra.

Linkers were obtained from New England Biolabs, enzymes from Pharmacia and New England Biolabs.

T7 RNA polymerase transcription templates as well as various mRNAs produced from them are outlined in Figure 1.

Xenopus laevis beta globin sequences were derived from the plasmid pSP64 T [Krieg et al., Nucleic Acids Research 12, 7057 (1984)], with the 5' beta globin sequences obtained as the HindIII to BglII fragment and the 3' beta globin sequences released as the BglII to EcoRI

fragment. These 3' sequences include a terminal polynucleotide tract of $A_{23}C_{30}$.

The P. pyralis luciferase sequences were obtained as the HindIII to BamHI fragment of pJD 206 [de Wet et al., Molecular and Cellular Biology 7, 725 (1987)], and include 22 bases of luciferase cDNA sequence which precedes the open reading frame, as well as 45 bases of cDNA sequence downstream of the termination codon, but are devoid of the luciferase polyadenylation signal.

T7 LUCFR An was constructed from plasmids pIBI31 (Figure 8) and pSP64 T as shown in Figure 7.

The 30 base poly A tail of the plasmid del5' T7 LUCFR An was obtained from pSP64 An (Promega). The construction of this plasmid is shown in Figure 8.

All transcripts were generated from the T7 RNA polymerase promoter [Dunn et al., Journal of Molecular Biology 166, 477 (1983)].

DOTMA Synthesis and Liposome Preparation

(N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA) was prepared by reaction of 3-(dimethylamino)-1,2-propanediol with oleyl-p-toluenesulfonate and subsequent quaternization, and was incorporated into liposomes with dioleyl phosphatidylethanolamine (DOPE) as described in Felgner et al., Supra. The preparation of this and structurally related compound is disclosed in the published European Patent Application No. 0,187,702.

DOTMA Mediated RNA Transfection of Cells

Unless otherwise indicated, synthetic mRNA was mixed with uncapped carrier RNA to yield a total of 20μg RNA/transfection. The RNA was then added to 4ml Opti-Mem Medium (Gibco) containing 50μg of Lipofectin (1:1 DOTMA:DOPE). Cells to be transfected were then washed with Opti-Mem, and incubated with the RNA/Lipofectin/medium mixture for the indicated period (8 hours in most cases). Transfections of adherent cells were performed using 10-cm tissue culture plate monolayers which were about to reach

confluency. Non-adherent cells were counted before transfection, and 10^7 cells were used with the above conditions for each transfection. Harvesting of transfected cells was performed by scraping at 8 hours post transfection, unless otherwise indicated.

Freeze thaw cytoplasmic protein extraction, Bradford determination of protein yield, and P. pyralis luciferase activity assays were performed as described in de Wet et al., Supra, and Bradford, Analytical Biochemistry 72, 248 (1976), with a total of 30μg of total protein extract used to perform each luciferase assay in a total volume of 150μl of reaction mixture. Assays were performed in a Monolight 2001 luminometer (Analytical Luminescence Laboratories) with automatic injection of substrate and integration of counts over a thirty second interval.

Mock transfections were performed using carrier RNA, Lipofectin, and Opti-Mem, and analyzed in parallel with corresponding experimental samples. Results are expressed as the difference of experimental and mock transfected cell light emission specific activity (60 second light emission counts/30 micrograms total extract protein).

Analysis of DOTMA Mediated RNA Association with Cells

In order to determine the efficiency of association of the Lipofectin/RNA mixture with its cellular target, uncapped ^{32}P-labeled RNA was mixed with Opti-Mem in the presence or absence of Lipofectin, as described above, and placed on NIH 3T3 cells. At various time points, transfected cell supernatant was aspirated and the cells were harvested by scraping in PBS. Cells were washed in PBS, and the wash pooled with the media supernatant. Cells were then lysed in Tris buffered saline, containing 1% NP-40 (Shell Oil Co.), and the lysate Phenol/$CHCl_3$ extracted. The organic phase was re-extracted with H_2O and the aqueous cell lysate phases pooled. Aliquots were counted in Eco-Lite scintillation cocktail, and the fraction of cell associated RNA expressed as a ratio of the cell lysate

counts to the total recovered counts. RNAse resistant counts were determined as above, with the addition of a 30 minute, 37°C treatment of the harvested cells with 100μg/ml RNAse A in PBS, prior to lysing.

in vitro Reticulocyte Translations

Nuclease-treated rabbit reticulocyte lysate was obtained from Promega, and all reticulocyte translations were performed in a 50μl reaction mixture, with 1mCi/ml ^{35}S-methionine and 2μg mRNA, using the protocol provided. Samples were removed at the indicated times, and stopped by placing on ice. Total translation mixture was diluted 1:100 prior to luciferase analysis, and 2μl of the dilution analyzed for light emission activity as hereinabove described.

3. Examples

The invention is further illustrated by the following non-limiting Examples.

Example 1

DOTMA-mediated transfection of mRNA coding for P. pyralis luciferase

a. Optimization of Lipofectin to RNA ratio

Because the DOTMA-RNA complex involves a high affinity interaction between the nucleic acid phosphate backbone and the DOTMA quaternary amine, as well as an ionic interaction between the complex and the charged outer membrane of the target cell, it is necessary to optimize the ratio of RNA to DOTMA.

Addition of nucleic acid will gradually titrate out the charge present on the liposomes, reducing the affinity of the complex for other negative charges such as that of the target and hence reducing transfection efficiency. However, loading cells with positively charged DOTMA lipid is associated with cytotoxicity (see Felgner et al., Supra). Optimization of the Lipofectin:RNA ratio was thus performed for NIH 3T3 cells by varying the amount of Lipofectin used to transfect 20μg of RNA (15μg carrier + 5μg capped T7 LUCFR An runoff mRNA) and then scoring for

luciferase specific activity, after 8 hours of transfection. The results shown in Figure 2 indicate that the specific activity of luciferase protein is optimized at 50μg of Lipofectin under these conditions. This ratio (2.5μg of Lipofectin:1μg of RNA) was then used for all further transfections.

b. Efficacy of RNA Transfection

In order to determine how efficiently the DOTMA/RNA complex associates with cells, labeled RNA was prepared without capping or polyadenylation from EcoRV linearized pIBI 31 (Figure 8) ($2x10^5$ cpm/μg RNA) and 10^6 Cpm was transfected into NIH 3T3 cells and then analyzed or association with the cells as described in the previous section. Results are summarized in Table 1, and indicate that within one hour, at least 60% of the counts are tightly cell associated, and at least 20% of the radioactivity is RNAse resistant. Moreover, this association appears to be very rapid, consistent with an ionic interaction between cell membrane and the DOTMA/RNA complex.

Table 1

Hours Post Transfection	% RNA Adherent to Cells	% RNAse Resistant RNA
1	71	32
2	75	18
5	60	N.D.
8.2	N.D.	23
9	57	N.D.
15	N.D.	37

N.D. = not determined

c. Kinetics of mRNA Transfection

The relationship between the quantity of mRNA transfected and the resulting protein translation was investigated. A total of 20μg of RNA containing varying

amounts of cap-βg Luc βg An RNA was used to transfect NIH 3T3 cells. Following transfection for 8 h, all luciferase specific activity values varied less than 2-fold over this range, and a linear relationship was observed between specific activity and quantity of transfected mRNA (Fig. 3A). It is worth noting that the assay is sensitive enough to detect protein synthesized following transfection of as little as 10ng of RNA.

Figure 3B 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 minutes following transfection and then the synthesis of the luciferase protein increases with time following transfection. Thus it appears that some fraction of the transfected RNA is functional in vivo for at least 5 hours (Fig. 3B).

d. 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 20μg of cap-βg Luc βg An RNA. We empirically used 50μg of Lipofectin in 4 ml OPTI-MEM (optimal for NIH 3T3 cells - Fig. 2) and incubated the cells for 8 h. Figure 4 shows that nearly all cell types, regardless of whether they were adherent (HeLa) or suspension cells (U937), undifferentiated stem cells (F9), neuronal cells (PC12), amphibian (A6) or insect cells (KC) exhibited luciferase activity. The varying efficiencies of RNA transfection in different cell types could be due to differential transfectability, suboptimal lipofectin:RNA ratios, translational efficiency or stability of mRNA.

e. Role of Cap, 5' and 3' UT Sequences

The influence of various untranslated (UT) sequences present in an mRNA on its ability to translate proteins has been studied by either transfecting DNA constructs into cells or microinjection of RNA into Xenopus oocytes. The RNA transfection technique developed here could be used to investigate these parameters by directly

transfecting mRNA transcripts generated in vitro from DNA constructs containing specific sequences. The following types of transcripts shown in Figure 1A were generated: (i) capped or uncapped 5' untranslated β-globin-luciferase coding region and 3' untranslated β-globin region containing an $A_{23}C_{30}$ track (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) track of ≈30 A residues (Luc An). About 0.2, 1, 2.5 and 5μg of each mRNA were transfected in a total of 20μg RNA (with appropriate amounts of the carrier RNA) into NIH 3T3 cells for 8 h under the optimal conditions and analyzed for luciferase specific activity. Figure 5 shows that maximal protein synthesis was obtained with the mRNA transcript which contains the cap and both the β-globin 5' and 3' untranslated regions. Results obtained with all the 6 transcripts were analyzed by linear regression and are tabulated in Table 2.

Table 2

Comparison of in Vivo and in vitro Translation

mRNA	Slope of Line	Correlation Coefficient	in vitro Translation (60s Light Emission)
Cap-βg Luc βg An	44798	0.99	85,280
βg Luc βg An	1172	1.00	185,110
Cap-Luc βg An	5124	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 Figure 1 were translated following transfection into NIH 3T3 cells or using rabbit reticulocyte lysate as described in the Materials and General Methods Section. Transfection data diagrammed in Figure 5 is expressed as a linear slope and regression correlation coefficient. Reticulocyte lysate translation is expressed as luciferase activity of the total translation preparation as indicated in the Materials and General Methods Section.

The data indicated that the capped mRNA is nearly 40-fold more efficiently translated than the uncapped identical mRNA. It should be noted that the exact extent of the capping of the mRNA remains uncertain. The data in Table 2 also shows that the presence of 5'-untranslated region of β-globin mRNA imparts nearly 9-fold greater translational efficiency. Finally the presence of β-globin 3' untranslated region plus an $A_{23}C_{30}$ polynucleotide track confers at least 6-fold advantage over the transcripts containing only a poly(A) stretch of 30 residues.

f. Efficiency of Translation in Reticulocyte Extracts

Table 2 also shows that when the mRNA transcripts used for transfection were translated in vitro in a rabbit reticulocyte cell extract, the relative levels of the translated protein are different from that observed in NIH 3T3 cells. All mRNA transcripts regardless of the presence of the cap or 5' or 3' UT sequences are translated efficiently (within 2 to 3 fold) suggesting a basic difference between these in vitro and in vivo systems to study translational machinery. In addition, the reticulocyte data indicate that all mRNA samples were capable of generating high levels of luciferase protein.

g. Time Course of Protein Synthesis

In order to determine the relative effect of translational efficiency and message stability on the overall levels of luciferase protein produced by the various message contexts, a time course analysis was performed for each mRNA. For this analysis, 20 μg of each mRNA shown in Figure 1 were complexed with 50 μg Lipofectin in 4 ml Opti-MEM medium and transfected into NIH 3T3 cells, as hereinabove described. Cells were harvested and luciferase freeze-thaw lysates were determined using the method of de Witt et al., Supra. The time coruse results are shown in Figure 11.

Discussion of the results

The above experimental results show that a simple, efficient and highly reproducible technique has been developed for the transfection of mRNA synthesized in vitro, using DOTMA-containing liposomes. The transfection efficiency appears to be substantially higher than the efficiency of the established DEAE dextran technique. The translational efficiency and RNA stability are facilitated by the presence of capping and 3'- and 5'-untranslated RNA elements.

The availability of reporter gene sequences such as the P. pyralis luciferase, coupled with the ability to efficiently co-transfect RNA allow the cellular analysis of many cis acting aspects of RNA metabolism by providing the reproducability and internal controls necessary for quantitative analysis of translatability, cytoplasmic stability, cytoplasmic processing, and protein binding.

These experiments have defined conditions for transfection which allow relatively rigorous analysis for various mRNA species. By performing multiple independent transfections of a given mRNA at various concentrations, and then harvesting and analyzing cellular protein from each at a given time after transfection, the overall "activity" of an mRNA, i.e. the ability to produce protein upon transfection, can be reliably assessed. The results clearly indicate that 5' untranslated sequences, 3' untranslated sequences, and capping affect the overall level of (transfected message specific) protein production.

Ability to generate large amounts of RNA by in vitro transcription using SP6 or T7 polymerase has alleviated the major obstacle to developing RNA transfection systems. Furthermore, it has been shown that the translation of in vitro synthesized mRNA in NIH 3T3 cells continues for at least 5 h (Fig. 3B). It is thus feasible to study the parameters of translation machinery by direct RNA transfection, rather than introducing DNA constructs. Using this method it was found that 70% of the

transfected RNA is associated with the cells, a large fraction of which 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 like 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 methodology. 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:Lipofectin ratio for the desired cell type.

Example 2

Introduction of DNA into frog embryos

As a test system,we have used Xenopus laevis (frogs) oocytes to study DOTMA-mediated DNA transfection. The DNA contained the luciferase gene expressed from the RSV LTR (de Wet et al.,Supra), as a marker DNA. Xenopus laevis embryos were dejellied with 2% cysteine in 100% Modified Ringers Solution (MR), and the vitelline membranes were removed with 1 mg/ml pronase in 100% MR. 12 neural plate xenopus embryos were treated with 100µg DOTMA in 1.5 ml 100% MR, with or without 50µg PRSVL [structure] (luciferase plasmid DNA). Embryos were incubated for 8 hours, then shifted to 33% MR. 48 hours after initial treatment with DOTMA, the embryos were disrupted, freeze/thaw lysates were prepared, and luciferase activity was determined. The results are illustrated in Figure 6.

This experiment established the basis that foreign DNA can be introduced into the nucleus of frog oocytes via the procedure of DOTMA-mediated transfection (Lipofectin).

Example 3

Introduction of RNA into the neurula and gastrula stages of frog development

Xenopus laevis embryos were dejellied with 2% cysteine in 100% MR, and the vitelline membrane was stripped with 1 mg/ml pronase in 100% MR. 12 embryos per group were treated with 50μg DOTMA with or without 20μg capped, polyadenylated, in vitro transcribed luciferase RNA in a total of 2 ml 100% MR for 8 hours. Embryos were then disrupted by aspiration and lysed by the freeze/thaw method. Luciferase activity was determined by the standard light emission assay. The results are shown in Figure 7.

Example 4

Introduction of genes into mouse preimplantation embryos

200 mouse eggs following fertilization are removed, and stripped of their external zona pellucida by treatment with proteases and then put into a petri dish containing DNA complexed with DOTMA. The specific DNA used is the previously described rat growth hormone gene. [see e.g. Palmiter et al., Nature 300, 611 (1982)] The embryos are allowed to be in the petri dish for 8 to 12 hours following which they are implanted in the fallopian tube. Of the 20 mice born after carrying out this procedure, a large proportion appear to contain the gene.

Example 5

Introduction of genes into chick preimplantation embryos

The apparatus used for transfection consists of a sterile support cup, a sterile support sling for the embryo, and a cover. In this apparatus, 3-day chick embryos were overlayered with 0.5 ml of a solution containing 200 μg Lipofectin, and 100 μg PRSVL DNA in deionized water. They were then incubated at 37 °C for three days to allow further development. The living embryos were dissected away from the yolk sack analyzed in

a solution of 0.1M mono-potassium phosphate (pH 7.4), 1 mM DTT and 1% NP40, and then assayed for luciferase activity. Using sham injection embryos, background luciferase counts were found to be 483 and 566 per 30s. A viable embryo recovered from 6 treated with the Lipofectin/DNA mixture, gave 427562 emissions in a 30s counting interval.