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mRNA Transfection of Cultured Eukaryotic Cells and Embryos Using Cationic Liposomes

Robert W. Malone
Northwestern University Medical School
303 E. Chicago Avenue
Chicago, Illinois 60611

DNA transfection using cationic liposomes is rapidly gaining acceptance as a method for introducing genetic information into cultured eukaryotic cells, largely because of the high level of efficiency, simplicity and reproducibility of this approach (1-5). These characteristics allow the experimenter to focus on the biological question at hand rather than the technology of gene transfer.

A new mRNA-based application of cationic liposome-mediated delivery has been developed that further simplifies the expression of foreign proteins in tissue culture cells (6). In some ways, mRNA transfection is inherently more attractive than DNA transfection for gene expression. It bypasses the complex issues of transcriptional regulation and only requires that the polynucleotide reach the cytoplasm and be translated. In the past, RNA transfection has primarily been used in the study of viruses that have self-replicating infectious RNAs (7-11) because of the difficulty in obtaining pure mRNA populations, the general lack of understanding of sequence elements that enhance mRNA translation and stability, and the inefficiency of existing RNA transfection techniques. By using the liposome-mediated transfection approach with RNA transcripts generated *in vitro*, a very efficient and highly reproducible technique for expressing exogenous proteins in a wide range of cultured cells has been developed (6). Moreover, with only minor modifications, this technology has been adapted for expressing protein in *Xenopus laevis* embryos.

This report is intended as a practical guide for life scientists interested in applying this approach to their own experimental systems. Many of the issues are similar to those faced when optimizing DNA transfection using cationic liposomes, while others are unique to the transient transfection of mRNA. The examples presented here should allow researchers to evaluate the applications and limitations of this new addition to the increasingly diverse reperitoire of eukaryotic gene expression techniques.

Methods

Construction of RNA Synthesis Plasmids. Cloning procedures were performed essentially as described previously (12). pT7CAT contains the T7 RNA polymerase promoter with the chloramphenicol acetyl transferase (CAT) coding sequence derived from pSV2CAT (ATCC 37155) that had been cloned into the *Xenopus laevis* β-globin 5' and 3' untranslated region of pSP64T (13). βg Luc βg A_n is an analogous construction containing the *P. pyralis* luciferase sequence obtained as the *Hin*d III/*Bam*H I fragment of pJD206 (14) flanked by the β-globin sequence of pSP64T and also under the control of the T7 promoter. Luc A_n contains the pJD206 derived sequences under the control of the T7 promoter and followed by the 30 base poly A tail of pSP64(polyA). Plasmids are diagrammed in figure 1.

Purification of Plasmid DNA. Plasmids were transformed into *E. coli* DH5α™ and prepared as described (12) using a scaled-up alkaline lysis mini-prep protocol, followed by purification of the supercoiled plasmid DNA by cesium chloride equilibrium gradi-

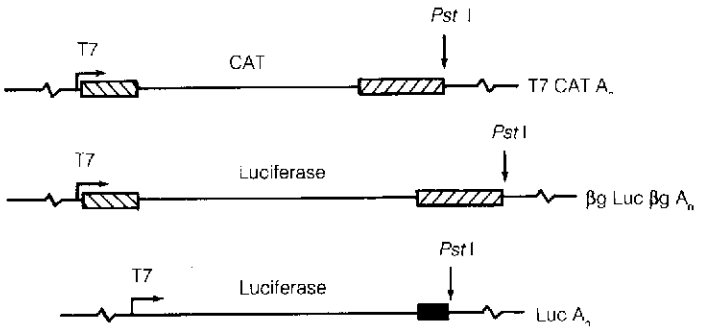

Figure 1. DNA templates for *in vitro* transcription. Schematic representation of the DNA templates used to generate the mRNA preparations used for transfection. Crosshatched boxes correspond to the 5' and 3' untranslated regions of *Xenopus* β-globin that were obtained from the plasmid pSP64T (13). Chloramphenicol acetyl transferase (CAT) coding sequences were derived from pSV2-CAT. Sequences represented as "Luciferase" are derived from pJD206 (14) and code for the *P. pyralis* luciferase protein. The black box at the 3' end of the plasmid construction labeled "Luc A_n" codes for a 30 base poly A tract derived from pSP64(poly A). All plasmids were linearized with *Pst* I prior to mRNA synthesis and the mRNA preparations generated from the templates are identified by the name of the plasmid template.

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IN FOCUS

Note from the Editor

Life Technologies, Inc., provides quality products under the BRL and GIBCO brand names for life science research using molecular biology and cell culture techniques, respectively. In the past year, the company has also expanded its product line for research using immunology techniques. These BRL products and the associated technology have been presented in recent issues of *Focus*.

In this issue, I welcome new GIBCO BRL products for cell biology research to *Focus* (See page 74.). In many ways, modern cell biology research is the application of molecular biology methods to cells in culture. The GIBCO BRL brand name reflects the unique combination of LTI's expertise in molecular biology and cell culture.

Focus will continue to expand its coverage of LTI's research products in 1990 with the addition of information on the use of GIBCO cell culture products. This increased coverage of life science research techniques will not, however, detract from the molecular biology aspect of *Focus* but will lead to an increased number of pages in this quarterly publication in the coming year.

I thank the *Focus* audience for their scientific contributions and for their loyal support. The number of researchers on the subscription list is always growing. With the addition of more LTI research products and information on techniques used with these products, I anticipate that the *Focus* audience will grow even faster. Your articles on molecular biology, cell biology, immunology, and cell culture techniques are invited.

Nancy K. Sasavage

RNA Transfection, *continued*

ent centrifugation. Purity of the final preparation was evaluated by optical density, and preparations with an A_{260}/A_{280} ratio of less than 1.8 were not used.

RNA Synthesis and Purification. DNA templates were linearized downstream of the sequences to be transcribed using a five-fold excess of the appropriate restriction endonuclease. The digests were extracted two times with phenol/chloroform and two times with chloroform, followed by precipitation with 0.3 M sodium acetate and two volumes of ethanol. The DNA was then dissolved at approximately 1 mg/ml in diethylpyrocarbonate (DEPC)-treated water, and the ratio of the absorbance at 260 nm and 280 nm was determined. Preparations with a ratio of less than 1.7 were found to give reduced quality and yield of RNA transcripts in many cases.

Transcription with cap analog incorporation was performed using 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM DTT, 4 mM spermidine, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.5 mM GTP, 0.5 mM cap analog ($^{7me}G^{5}ppp^{5}G$), 0.5 mg/ml DNA template, 2,000 units/ml human placental ribonuclease inhibitor and 4,000 units/ml T7 RNA polymerase. The reactions were incubated at 37°C for 1 h. RNAse-free DNAse was then used to degrade the DNA template.

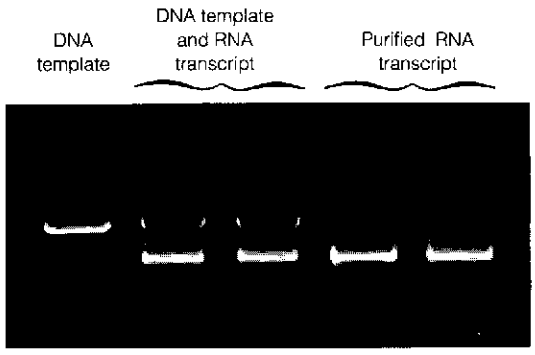

Figure 2. Example of *in vitro* transcription products. T7 CAT mRNA was prepared in two separate reactions and analyzed for degradation by electrophoresis in a 1% (w/v) agarose/Tris-borate EDTA gel containing ethidium bromide as described in Methods. The first lane contains 1 µg of the linearized DNA template. Lanes 2 and 3 show 5 µl of the reaction products after 1 h of incubation. Lanes 4 and 5 show the corresponding mRNA preparations after purification.

The reaction products were extracted two times with phenol/chloroform and two times with chloroform. RNA was precipitated using 0.3 M sodium acetate and two volumes of ethanol, dissolved in DEPC-treated water, and purified over a Sephadex G50 spin column. Sample purity was evaluated using the A_{260}/A_{280} ratio (typically between 2.0 and 2.1), and degradation was assessed by electrophoresis in a 1% (w/v) agarose mini-gel containing 0.25 µg of ethidium bromide/ml agarose (figure 2).

Cells and Media. Murine fibroblast (NIH 3T3, clone 2B) cells were a kind gift from Margurite Voigt and were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% (v/v) calf serum (CS) prior to transfection. Human histiocytic lymphoma cells (U937; ATCC CRL 1593), human chronic myelogenous leukemia cells (K562; ATCC CCL 243), human promyelocytic leukemia cells (HL-60; ATCC CCL 240), rat adrenal pheochromocytoma cells (PC-12; ATCC CRL 1721), human erythroleukemia cells (HEL; ATCC TIB 180), and murine erythroleukemia (MEL) cells were cultured in RPMI 1640 with 10% (v/v) fetal calf serum (FCS) prior to use. 3T3-derived retroviral packaging lines, PA 317 (ATCC CRL 9078) and ψ-2 (from Richard Mulligan) were maintained in DMEM with 10% (v/v) CS. *Xenopus laevis* kidney cell line (A6; ATCC CCL 102) was maintained in 50% (v/v) Liebowitz L-15 medium with 10% (v/v) FCS. Human epitheloid cervical carcinoma cells (HeLa) were maintained in DMEM with 10% (v/v) FCS. Primary chick embryo fibroblasts (CEF) were prepared as per Rein and Rubin (15) from white leghorn embryos and cultured in 211 medium [2% (w/v) tryptose phosphate, 1% (v/v) CS, 1% (v/v) heat-inactivated chick serum in DMEM]. *Xenopus laevis* eggs were obtained from gravid female frogs primed with 200 I.U. of gonatropin 5 to 7 days prior to the induction of egg laying. Eggs were obtained following injection of 500 to 800 I.U. of human chorionic gonadotropin and fertilized *in vitro*. Jelly coats were removed with 2% (w/v) cysteine in 33% (v/v) modified Ringers solution (MR), and vitelline membranes were removed by treatment with 1 mg/ml pronase in 100% MR.

RNA Transfection. Plates (10 cm) of rapidly dividing adherent cells near confluency or 1 x 10^7 suspension cells were transfected as follows unless otherwise noted. Cells were washed once with Opti-MEM® I Reduced Serum Medium and then returned to the incubator covered with the medium during the preparation of the liposome/polynucleotide complexes. Aliquots (4 ml) of Opti-MEM® Medium were placed in 12- x 75- mm polystyrene snap cap tubes, and 50 µg of Lipofectin™ Reagent were added. A mixture of capped mRNA and uncapped carrier RNA (transcribed from *Eco*R V-linearized plBl31) was then added to the media/lipid mixture to a total of 20 µg of RNA. The mixture was immediately vortexed. Cells were removed from the incubator, the medium was aspirated, and the Opti-MEM®/lipid/RNA mixture was added. Cells were returned to the incubator for 8 h, unless otherwise noted, and harvested as described. Unlike other cell lines, A6 cells were maintained at room temperature.

Transfection of *Xenopus* Embryos. Blastula and neurula stage embryos were prepared for transfection as described above. Transfections were performed by adding 50 µg of Lipofectin™ Reagent to 2 ml of 100% MR in a 12- x 75-mm polystyrene snap cap tube, and adding 20 µg of capped mRNA to the mixture followed by vortexing. The mixture was placed into one well of a 32-well plate and 12 dejellied embryos with vitelline membranes removed were added. Disrupted embryo groups were prepared by mincing with a razor blade fragment. The treatment continued for 9 h at room temperature, during which time intact embryos continued to develop normally. Embryos were transferred to microcentrifuge tubes and centrifuged for 30 s at 16,000 x *g*. The transfection medium was removed by aspiration and the embryo tissue was taken up in 100 µl of 0.1 M potassium phosphate (pH 7.8), 1 mM DTT. Cells were lysed by three cycles of freezing and thawing and 37.5 µl of cleared lysate was assayed for luciferase activity as described below.

Analysis of Protein Following Transfection. After incubation with the transfection medium for the indicated amount of time, cells were washed two times in 1 ml of 0.1 M potassium phosphate (pH 7.8), 1 mM DTT (lysis buffer) and scraped off the plate or otherwise resuspended in 1 ml of lysis buffer and placed in a microcentrifuge tube. Cells were centrifuged at 4°C for 5 min at 16,000 x *g*. Cell pellets were resuspended in 100 µl of lysis buffer, frozen in liquid nitrogen, and thawed at 37°C. Three such freeze/thaw cycles were used to lyse the cells, after which the debris was pelleted as above and the supernate was harvested and frozen at -20°C until assayed. Total protein concentration of the lysates was determined by the method of Bradford (16) and 30 µg of total protein was assayed for luciferase or CAT activity. Luciferase assays were performed using either a Monolight 2001 luminometer (Analytical Luminescence Laboratory, Inc., San Diego) or LKB 1251 luminometer (LKB Wallac, Turku, Finland) using an assay mixture comprised of 0.67 mM luciferin, 1.7 mM ATP, 0.1 M potassium phosphate (pH 7.8), and 33 mM $MgCl_2$. Luciferase-catalyzed light emissions were integrated over a 30-s interval but tabulated as light emissions/minute (twice the 30-s value). CAT activity was assayed using thin layer chromatography after incubating 30 µg of cellular protein extract for 2 h at 37°C in a reaction mixture containing 0.58 M Tris-HCl (pH 7.8), 0.67 mM acetyl-CoA, and 50 nCi ^{14}C-chloramphenicol. The acetylated forms were resolved on a silica TLC plate developed in 19:1 (v/v) chloroform:methanol, followed by 24-h exposure of preflashed Kodak XRP film at -70°C.

Results

Optimization of Basic Transfection Conditions

In contrast to cationic liposome-mediated DNA transfection (1-4), the amount of protein produced by a given amount of mRNA is strongly affected by the addition of carrier RNA. Figure 3 demonstrates the difference in CAT activity observed when CAT mRNA is transfected into NIH 3T3 cells using 100 µg of Lipofectin™ Reagent for 8 h with or without 20 µg of a carrier RNA. The results

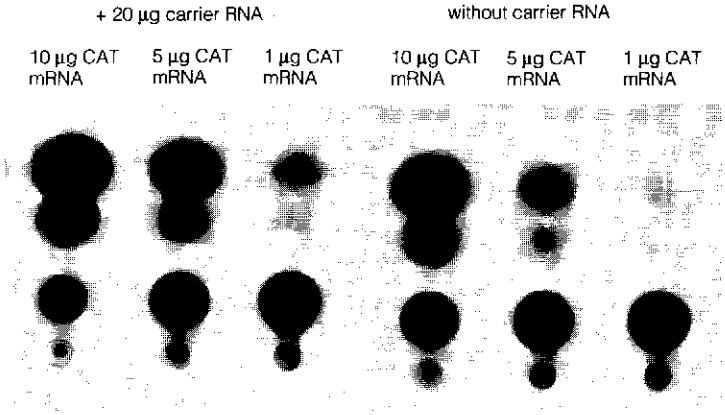

Figure 3. Effect of carrier RNA on synthesis of CAT protein. Purified T7 CAT "A_n" mRNA (1, 5, and 10 µg) was transfected into NIH 3T3 cells with or without nonspecific carrier RNA using 100 µg of Lipofectin™ Reagent. Total protein lysate (30 µg) was prepared and analyzed for CAT activity as described in Methods.

continued on next page

clearly demonstrate that the addition of carrier RNA enhances protein production following RNA transfection, presumably by competing with the mRNA for cytoplasmic RNAse activity. Based on this result, subsequent analysis was performed using a total of 20 µg of RNA, 5 µg of mRNA and 15 µg of a nonspecific carrier RNA.

Since the ratio of polynucleotide to Lipofectin™ Reagent is known to strongly affect the transfection efficiency (1), the ratio of RNA to cationic liposomes was optimized in NIH 3T3 cells. Figure 4 summarizes the specific activity of the cell lysates produced using different ratios of lipid to RNA, as well as total protein yield obtained from each transfection condition. Treatment of cells with high levels of Lipofectin™ Reagent results in cell lysis (1). This lysis may take up to 12 h to occur, although cytotoxicity can be detected soon after transfection by incubating the cells in phosphate-buffered saline containing trypan blue and observing dye uptake or exclusion. Different cell lines show varying degrees of susceptibility (data not shown). The yield of total cellular protein is a somewhat indirect but quantitative measurement of cell lysis and so is useful in optimizing the transfection conditions for a particular application. Panel A demonstrates the effect of cationic lipid dose on total protein yield. While the total yield of luciferase protein was higher using a ratio of 25 µg lipid to 20 µg RNA, the specific activity of luciferase (luciferase activity/30 µg total protein) was higher using a mass ratio of 50:20 lipid:RNA. Based on these results, further transfections were performed using 50 µg Lipofectin™ Reagent and 20 µg RNA.

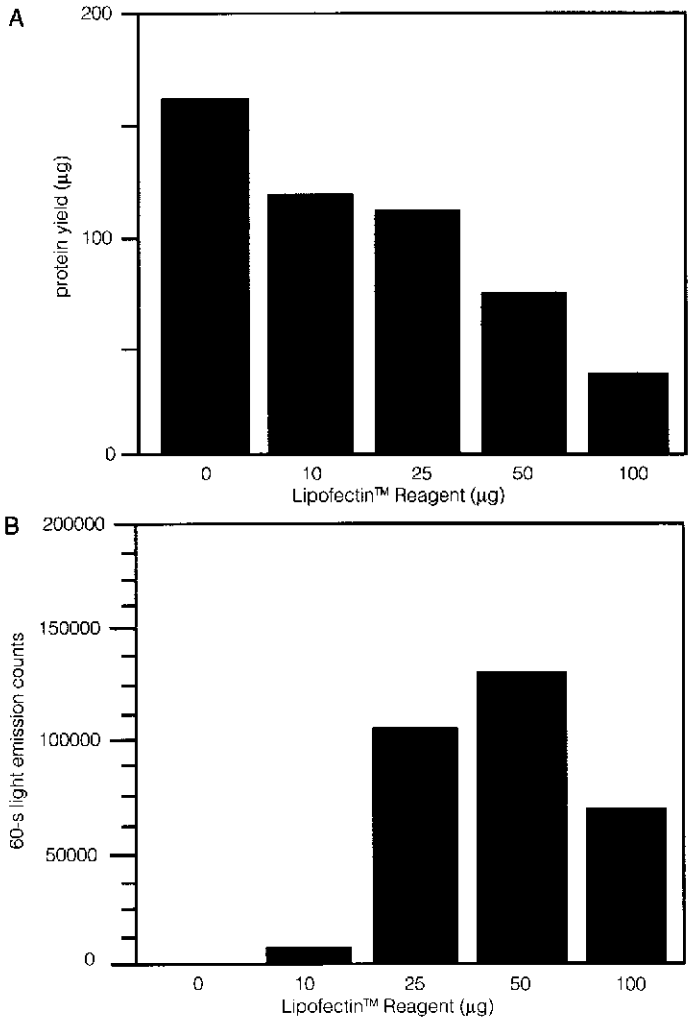

Figure 4. Optimization of ratio of mRNA to Lipofectin™ Reagent in NIH 3T3 cells. Duplicate 10-cm plates of NIH 3T3 cells were transfected with 5 µg of capped "βg Luc βg A_n" mRNA and 15 µg of nonspecific carrier RNA for 8 h using various amounts of Lipofectin™ Reagent as indicated. Cellular lysates were prepared as per Methods. Values shown represent averages of duplicate transfections. Panel A shows the total protein yield from the lysates as determined by the method of Bradford (16). Panel B shows the luciferase activity present in 30 µg of total protein from the corresponding lysates.

Analysis of Efficiency of mRNA Expression

To determine the sensitivity and linearity of this technique, a series of transfections were performed in NIH 3T3 cells using a total of 20 µg RNA containing the indicated amount of luciferase mRNA (figure 5). Luciferase activity above background was detected following transfection of as little as 1 ng of luciferase mRNA. All duplicates varied within two-fold or less in luciferase specific activity over the range of 1 ng to 5 µg of mRNA transfected, and the data fit a linear model with a correlation coefficient (r^2) of 0.96. Similar linear results were obtained with the CAT expression system over this range of mRNA concentrations, although the assay was not sensitive enough to detect protein expression in the nanogram range of transfected mRNA (data not shown).

The actual mass of luciferase protein produced under these conditions was approximated by using a commercially available purified firefly luciferase protein standard (Analytical Luminescence Laboratory, San Diego, CA) to calibrate an LKB 1251 luminometer. Analysis of 30 µg of total protein obtained from the transfection of NIH 3T3 cells with 50 µg of Lipofectin™ Reagent and 20 µg of luciferase mRNA showed a total of 400 pg of luciferase protein were produced in the transfected cells. Based on a luciferase protein molecular weight of 60.7 kDa (11), 6.6 fmoles or 3.9×10^9 molecules of luciferase protein were produced in approximately 1×10^7 cells.

The utility of the conditions developed for transfecting NIH 3T3 fibroblasts was then analyzed by transfecting a panel of immortalized lines and primary cells as described in Methods. These experiments used 5 µg of luciferase mRNA and 15 µg of carrier RNA complexed with 50 µg of Lipofectin™ Reagent. Figure 6 shows the results of this analysis and demonstrates that the specific activity of cell lysates derived from different cell lines varies over a large range. It should be noted, however, that all of the cell lines tested were successfully transfected to some extent. The variability in specific activity may result from the large variation in total protein yield in these various lines (data not shown), differences in translational efficiency, cell type specific variance in the toxicity of the lipofectin reagent, and/or differences in transfection efficiency.

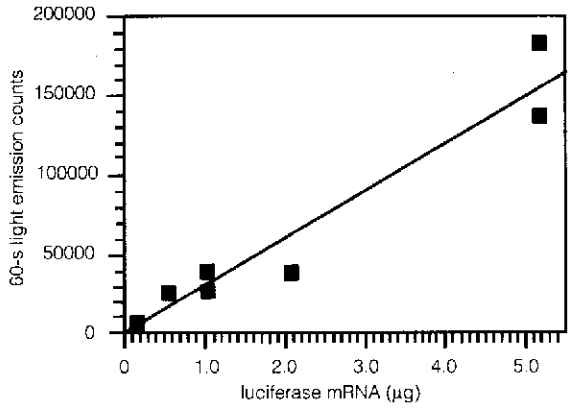

Figure 5. Analysis of sensitivity and linearity of luciferase mRNA transfection. Duplicate 10-cm plates of NIH 3T3 cells were transfected under the standard conditions described in Methods. Following an 8-h transfection, lysates were prepared and 30 µg of total protein were analyzed for luciferase activity.

Effects of Untranslated mRNA Structural and Sequence Elements

Other researchers have experienced difficulty in detecting protein synthesis following cationic liposome-mediated mRNA transfection (17). One hypothesis, which may explain this difficulty, is that specific elements present in the transfected mRNAs greatly enhance the efficiency of protein synthesis and/or the stability of the transfected polyribonucleotides. To test this hypothesis, a series of mRNAs were synthesized that contained different 5′ and 3′ untranslated elements ($^{7me}G^5pppG^5G$ cap and/or specific untranslated sequences) flanking the same *P. pyralis* luciferase coding sequences. Transfection of these mRNAs followed by analysis of luciferase protein production allowed comparison of the effect of the untranslated element as a function of translational efficiency and mRNA stability. The majority of the data derived from the transfection of these RNAs is published elsewhere (6), but the basic outcome of this analysis is shown in figure 7. *In vitro* capping of the luciferase mRNA sequences significantly enhanced the level of protein synthesis following transfection as determined from the time course of luciferase protein activity. Similarly, a comparison of translation of a capped mRNA that only contains a 30 base poly A tail (in addition to the pJD206 derived sequences) to the capped mRNA that contains the β-globin untranslated sequences and the luciferase sequence shows that the untranslated sequence of the open reading frame plays a significant role in permitting efficient protein translation in NIH 3T3 cells. Other mRNA sequences may have altered responses to capping and untranslated elements.

Transfection of *Xenopus laevis* Embryos

Table 1 demonstrates that mRNA transfection with cationic liposomes can be used to generate *in vivo*-synthesized protein in at least one specialized case. Both embryonic fragments, as well as intact embryos, can be transfected with mRNA/Lipofectin™ Reagent complexes to produce luciferase activity. Moreover, such transfected embryos can continue to develop normally (18).

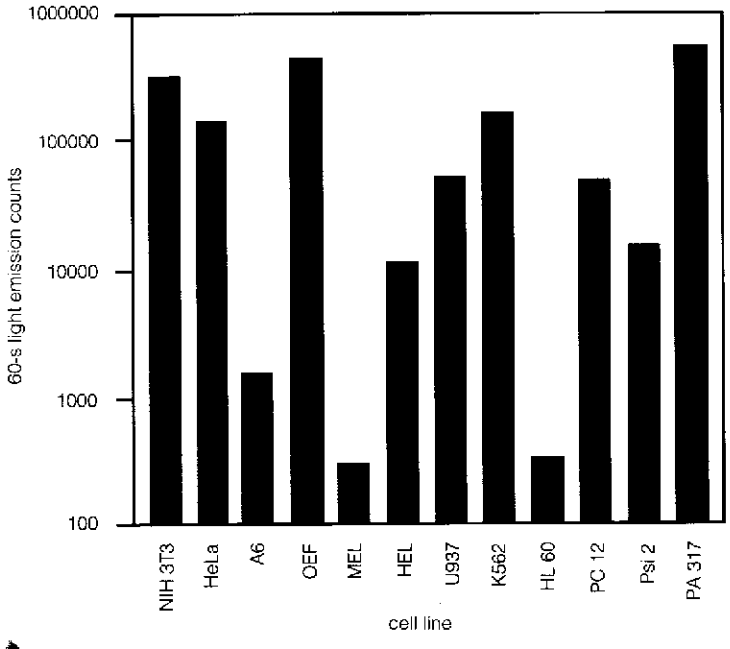

Figure 6. Relative efficiency of mRNA transfection in various cell lines using conditions optimized for NIH 3T3 cells. The standard transfection conditions developed for NIH 3T3 cells were used to transfect a panel of cell lines. Transfections were performed using capped βg Luc βg A_n mRNA as described in Results.

Discussion

In many instances, DNA or virally mediated transfer of genetic information is the only choice for addressing many biological questions. These issues include, but are not limited to, sequence motifs that modulate transcription, affect DNA replication, regulate gene amplification, or facilitate genetic recombination. However, in other instances, transient expression of an exogenous protein may be all that is necessary to answer the question at hand. In these cases, it may be worthwhile for the researcher to consider the mRNA transfection approach because of the relative efficiency of the system.

The ability to bypass the complexities of transcriptional regulation is not a trivial issue in many cell lines. For instance, when using the cell line U937, expression of exogenous proteins in undifferentiated cells is frequently problematic. In one experiment, this cell line was transfected with the DNA expression plasmid pSV2CAT using Lipofectin™ Reagent and then assayed for CAT expression with or without phorbol ester-induced differentiation. CAT expression was difficult or impossible to detect in the undifferentiated cells but clearly detectable in differentiated cells (19). However, mRNA transfection of the same undifferentiated cells yields high levels of protein expression as shown in figure 6. The ability to bypass transcriptional regulation in other cell types may also outweigh the additional difficulty of working with a labile molecule like mRNA.

Table 1. Transfection of *Xenopus laevis* embryos

Treatment	Embryo type: whole blastula	disrupted blastula	whole neurula	disrupted neurula
	(60-s light emission counts)			
negative control	774	801	765	779
+lipid	771	778	748	859
+RNA	786	771	727	774
+lipid +RNA	878	15737	43354	8243

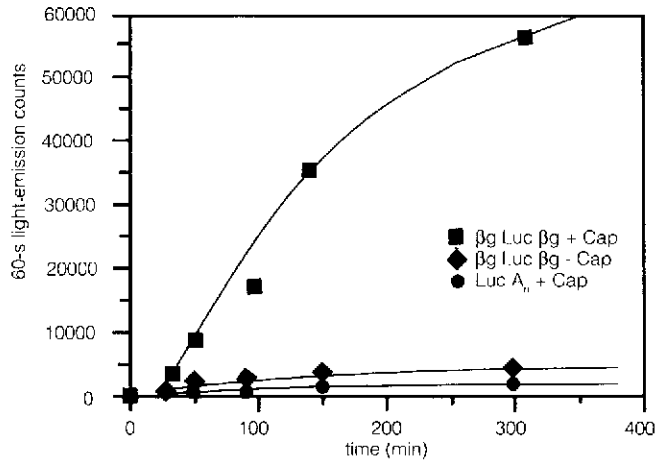

Figure 7. Effect of untranslated regions on luciferase activity following cationic liposome-mediated transfection of luciferase mRNA into NIH 3T3 cells. mRNA preparations (5 μg) were used to transfect NIH 3T3 cells (10-cm plates) that had been plated from pooled cells and prepared for transfection at the same time. Cells were harvested at the indicated time points and analyzed for luciferase activity.

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RNA Transfection, *continued*

In addition to the possible application of this approach to many biological problems that only require efficient protein synthesis, cationic liposome-mediated mRNA transfection may find application in clarifying the role of mRNA stabilizing and destabilizing elements in controlling gene expression. Many reports indicating that the control of mRNA stability directly contributes to the overall level of expression of eukaryotic genes have been somewhat overlooked, perhaps for the lack of a relatively straightforward technology for answering the issues that the existing literature has raised. The recent reviews by Ross (20,21) clearly indicate that there are a large number of intriguing questions in this field that mRNA transfection may help address. The evaluation of the role of 5' untranslated sequences in facilitating or inhibiting translation may also be studied by this method.

Finally, the *Xenopus* embryo transfection data, together with the nature of many of the cell lines that have been successfully transfected, raise the possibility of another application of this approach—mRNA-based *in vivo* expression systems. The idea of using mRNA/liposome complexes as a drug to produce exogenous proteins within the cells of living tissue in a transient, and thus easily controlled, manner is an exciting possibility.

Acknowledgements

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cDNA Synthesis by Cloned Moloney Murine Leukemia Virus Reverse Transcriptase Lacking RNase H Activity

Gary F. Gerard
James M. D'Alessio
Michael L. Kotewicz
Molecular Biology Research and Development
Research Products Division
Life Technologies, Inc.
Gaithersburg, Maryland 20877

Reverse transcriptase (RT) is an essential tool in recombinant DNA technology. It is the only enzyme known that efficiently catalyzes the synthesis of DNA from mRNA. The viral enzyme contains at least two enzymatic activities that reside on a single polypeptide, DNA polymerase and ribonuclease H (RNase H). A major difficulty with cDNA synthesis, caused at least in part by RT RNase H activity, is synthesis of low yields of cDNA transcripts that are less than full-length.

Efforts over a period of years to selectively inactivate RT-associated RNase H or to remove it proteolytically have been unsuccessful (reviewed in 1). Recently, we succeeded in completely eliminating the RNase H activity of cloned Moloney murine leukemia virus (M-MLV) RT by deleting the portion of the RT gene that codes for RNase H (2). Presented here is a description of some of the problems associated with cDNA synthesis that can be alleviated by using RT lacking RNase H activity (H⁻ RT), and a comparison of the performance of RTs that possess and lack RNase H activity.

Materials and Methods

Reverse Transcriptases. Cloned M-MLV H⁺ RT and M-MLV H⁻ RT were prepared as described (2). AMV RT was purified by published procedures (3).

cDNA Synthesis. Unless indicated otherwise, reaction mixtures for carrying out cDNA synthesis with M-MLV RT and AMV RT were incubated at 37°C and contained the components described in references 2 and 4, respectively.

RNAs. Synthetic RNAs, 1.35 kb, 2.3 kb, 4.4 kb, and 6.2 kb, were synthesized *in vitro* with T7 RNA polymerase (2, 5). The 1.35-kb and 4.4-kb RNAs contained a 120-nucleotide poly(A) tail at the 3' end, and the 2.3-kb and 6.2-kb RNAs had a 19-nucleotide poly(A) tail. The RNAs were purified by oligo(dT)-cellulose chromatography (6) to ensure the presence of a poly(A) tail. RNA labeled at the 5′ end was synthesized with [γ-^{32}P]GTP (5). Uniformly labeled RNA was synthesized with all four [α-^{32}P]NTPs (2). RNA was labeled at the 3′ end with [5′-^{32}P]pCp and T4 RNA ligase (7).