Cytoplasmic expression systems triggered by mRNA yield increased gene expression in post-mitotic neurons

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ABSTRACT
Non-viral vectors are promising vehicles for gene therapy but delivery of plasmid DNA to post-mitotic cells is challenging as nuclear entry is particularly inefficient. We have developed and evaluated a hybrid mRNA/DNA system designed to bypass the nuclear barrier to transfection and facilitate cytoplasmic gene expression. This system, based on co-delivery of mRNA(A64) encoding for T7 RNA polymerase (T7 RNAP) with a T7-driven plasmid, produced between 10- and 2200-fold higher gene expression in primary dorsal root ganglion neuronal (DRGN) cultures isolated from Sprague–Dawley rats compared to a cytomegalovirus (CMV)-driven plasmid, and 30-fold greater expression than the enhanced T7-based autogene plasmid pR011. Cell-free assays and in vitro transfections highlighted the versatility of this system with small quantities of T7 RNAP mRNA required to mediate expression at levels that were significantly greater than with the T7-driven plasmid alone or supplemented with T7 RNAP protein. We have also characterized a number of parameters, such as mRNA structure, intracellular stability and persistence of each nucleic acid component that represent important factors in determining the transfection efficiency of this hybrid expression system. The results from this study demonstrate that co-delivery of mRNA is a promising strategy to yield increased expression with plasmid DNA, and represents an important step towards improving the capability of non-viral vectors to mediate efficient gene transfer in cell types, such as in DRGN, where the nuclear membrane is a significant barrier to transfection.

INTRODUCTION
The concept of gene therapy is attractive and nearly two decades of research have demonstrated its potential as either a standalone treatment or an adjuvant therapy for inheritable and acquired diseases. Despite the significant advances made, there has been limited success in the clinical application of gene therapy, which is largely attributable to a lack of safe and efficient gene transfer (1–3). Synthetic vectors based on polycations or cationic lipids are promising vectors for gene delivery as they are relatively safe and can be readily modified by the incorporation of ligands for targeting to specific cell types. However, the levels of gene expression mediated by synthetic vectors are low compared to viral vectors (4). A major factor restricting transgene expression is inefficient transfer of DNA from the cytoplasm to the nucleus with only 1% of polyplex DNA reaching the nucleus following cytoplasmic microinjection (5). The majority of cells in vivo are post-mitotic or quiescent, and transfection rates are particularly poor in these cell types as there is limited breakdown of the nuclear envelope (6,7). Recent improvements in non-viral vectors, such as lipid/peptide vectors (8) and DNA nanoparticles consisting of a single molecule of DNA (9), have enhanced transgene expression levels in non-dividing cells. However, it is clear that further improvements in the design of non-viral vectors are required to achieve the expression levels required for a broad range of therapeutic applications.

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A widely used approach to enhance transgene expression is to modify the nucleic acid payload delivered by the non-viral vector. To promote nuclear uptake of DNA, for instance, nuclear localization sequence (NLS) peptides have been utilized to exploit intracellular transport mechanisms (10). However, the results to date have been largely disappointing. A 3-log increase in luciferase reporter activity was reported in one study following ligation of an oligonucleotide–NLS conjugate to one or both ends of a linear DNA molecule, although no conclusive localization or mechanistic data was given (11). Whereas, we (12) and others (13) found no effect or only modest increases in gene expression following linkage of NLS peptides to DNA.

Non-viral vectors have also been used to deliver mRNA instead of plasmid DNA (14,15), or T7-based autogene plasmids utilizing bacteriophage T7 RNA polymerase (T7 RNAP) to mediate cytoplasmic expression (16–18). The main advantage of delivering mRNA is that upon entry into the cytoplasm it is immediately translated, and so it can be used to successfully express proteins in post-mitotic, quiescent and slowly-dividing cells (14,19,20). The relative instability of mRNA, however, restricts the duration of gene expression and therapeutic applications have so far been limited. By comparison, the efficiency of T7-based autogene plasmids has gradually increased since their initial development over a decade ago. In most contemporary systems the cytomegalovirus (CMV) promoter has been incorporated to enable easier bacterial amplification of the plasmid. This has meant that there is now a requirement for nuclear entry to trigger the autogene, which is likely to compromise the utility of these systems in non-dividing cells (21,22).

Here, we report on the development and evaluation of a hybrid mRNA/DNA system, designed to bypass the nuclear barrier to transfection and facilitate cytoplasmic gene expression. In particular, we demonstrate that a hybrid mRNA/DNA cassette utilizing T7 RNAP mRNA represents a versatile and efficient system for mediating cytoplasmic expression in post-mitotic dorsal root ganglion neuronal (DRGN) cultures, where the nuclear membrane is a significant barrier to transfection.

**MATERIALS AND METHODS**

**Cell lines and primary cells**

Human lung carcinoma cells (A549, ATCC #CCL-185) and human prostate carcinoma cells (PC-3, ATCC #CRL-1435) were maintained in DMEM, without sodium pyruvate, with L-glutamine (PAA Laboratories, Somerset, UK) supplemented with 10% fetal calf serum (FCS, PAA Laboratories, Somerset, UK) and 1% penicillin streptomycin solution. All cells were incubated at 37°C in a 5% CO2 humidified environment. DRGN cultures were prepared from adult Sprague–Dawley rats. Briefly, spinal ganglia were removed aseptically, washed twice in Neurobasal–A media (Invitrogen, Paisley, UK) and digested in 0.125% collagenase/supplemented Neurobasal-A (2% B27 supplement, 0.5 mM L-glutamine and 0.5% gentamicin) for 2 h at 37°C. Disassociated spinal ganglia cells were removed from the collagenase, washed in Neurobasal-A and purified by centrifugation for 10 min at 120 g on a 15% BSA column.

**Sources of nucleic acids**

Plasmid DNA was grown in *Escherichia coli* and purified using endotoxin-free Qiagen maxiprep kits (Crawley, West Sussex, UK). The concentration and purity of DNA was checked on a spectrophotometer at A260 and A230 absorbance wavelengths. The reporter gene construct pCMVLuc1 was a gift from Dr Manfred Ogris (Munich, Germany). The design and construction of cytoplasmic expression plasmids used in this study are shown in Figure 1A and Supplementary Figures 1–2. Briefly, pCMV/T7-T7pol was constructed as described by Brisson et al. (21); pCMV/T7-T7pol(A64) is a modified version of pCMV/T7-T7pol into which an oligonucleotide containing 64 adenylate residues was inserted into the BamHI–NotI restriction sites downstream from the T7 RNAP gene; pT7Luc, also known as pEMCLucβgAn (17), contains the *Photinus pyralis* luciferase gene flanked upstream by the T7 promoter, and pR011 contains the T7 RNAP gene driven by CMV and triple phage promoters (T7, T3 and SP6), and the luciferase gene driven by a T7 promoter (22).

**Production of mRNA**

*In vitro* transcription to produce capped mRNA encoding T7 RNAP protein was performed using the Ribomax™ large scale mRNA production system (Promega, Southampton, UK), with the addition of either a standard m7G(5′)ppp(5′)G cap, or anti-reverse cap analogue (Ambion) substituted for a portion of the rGTP at a 4:1 ratio, as recommended by the manufacturer. A poly(A) tailing kit (Ambion) was used as indicated to add a >200 base poly(A) tail to cell-free transcribed mRNA prior to purification. The synthesis of mRNA encoding enhanced green fluorescent protein (EGFP) and luciferase has been described previously (20). Products were checked by denaturing gel electrophoresis.

**Cell-free translation systems**

The standard rabbit reticulocyte lysate (RRL) cell-free system (Promega) was used to characterize translation products from mRNA encoding T7 RNAP protein. Reactions were incubated at 30°C for 90 min, prior to storage at —80°C and performed following the manufacturer’s instructions. The cell-free RRL translation system was also used in a standard cell-free transcription/translation system to analyse expression of T7 RNAP from mRNA by subsequent expression and detection of luciferase protein from plasmid pT7Luc. T7 RNAP protein was also added with pT7Luc to the RRL cell-free system and levels of luciferase activity detected. Reaction mixtures were incubated at 30°C and 5 μl aliquots removed at specific time points and assayed for luciferase activity with results expressed in relative light units (RLU).

**Quantitative RT–PCR analysis of mRNA**

A quantitative RT–PCR assay was used to quantify intracellular levels of T7 RNAP mRNA at specific time points post-transfection. In brief, total RNA was extracted using the RNeasy mini kit (Qiagen) from A549 cells co-transfected with T7 RNAP mRNA (100 ng) and pT7Luc (400 ng). A total of 150 ng of total RNA was reverse transcribed to cDNA using random hexamer primers and multiscribe...
reverse transcriptase at 48°C for 30 min. Following addition of T7 forward primer (5'-TCACGACTCCTTCTCGGTACCAT-3'), T7 reverse primer (5'-CATAGTTTTCGCGACTGCTTT-3'), T7 probe labelled with 5' FAM and 3' TAMRA (5'-CGGCTGACGCTGACGAACCTTG-3') and qRT–PCR mastermix, 40 cycles of a two step thermal cycling protocol (denature: 95°C, 15 s; anneal/extend: 60°C, 1 min) was performed using the Applied Biosystems 7000 Sequence Detection System. 18S Ribosomal RNA was detected using appropriate primers and probe (Applied Biosystems) to demonstrate that a similar amount of total RNA was used in each qRT–PCR. The amount of T7 RNAP mRNA present in each sample was then calculated from a T7 RNAP mRNA standard curve generated by performing qRT–PCR on lysate samples prepared from mock-transfected cells spiked with a known amount of T7 RNAP mRNA (equivalent to 0.1, 1, 10 and 100 ng mRNA per well).

Transfection studies
Plasmid DNA and/or mRNA were added to a polypropylene microcentrifuge tube at a final concentration of 50 µg/ml in

Figure 1. Characterization of mRNA used in study. (A) Plasmid pCMV/T7-T7pol was constructed as described in Supplementary Figure 1 (21). pCMV/T7-T7pol(A64) is a modified version of pCMV/T7-T7pol into which an oligonucleotide containing 64 adenylate residues was inserted into the BamHI–NotI restriction sites immediately downstream from the T7 RNAP gene. (B) Cell-free transcribed mRNA was denatured at 80°C for 10 min and analysed on an ethidium bromide stained 1% reducing agarose gel (1 µg RNA per lane) and visualized by ultraviolet (UV) illumination. The relative positions of mRNA bands are indicated. (C) Western blot analysis of T7 RNAP protein translated from T7 RNAP mRNA using the RRL system (Promega). T7 RNAP protein was detected using a primary mouse monoclonal antibody raised against T7 RNAP protein (Novagen, CA) and a secondary anti-mouse antibody IgG horseradish peroxidase (HRP) conjugate. Recombinant T7 RNAP protein was used as a positive control.
Cells were lysed in M-PER cell lysis buffer (100 mM HEPES–NaOH (pH 7.4)). When mRNA was used, aliquots were thawed from −80°C, heated at 80°C for 10 min and chilled on ice for 2 min prior to addition. DOTAP was then added to nucleic acids, unless otherwise stated, at a (w/w) ratio of five and mixed by pipetting the solution up and down ×5 prior to use. Lipoplexes were added directly to a 48-well plate containing 3–4 × 10⁴ cells per well in 120 µl DMEM or optimem without FCS or supplement (cells were plated 24 h before transfection). Freshly isolated DRGN cultures were seeded at a density of ~1500 cells per well in supplemented Neurobasal-A. After 4 h, the medium was discarded and replaced with 500 µl of either DMEM containing 10% FCS, or supplemented Neurobasal-A. Cells were cultured for various times prior to analysis of reporter gene expression as indicated. Cell viability was determined using the MTS assay (Promega) after exposure to free DOTAP or lipoplexes as indicated and normalized to values obtained in their absence as described previously (23).

**Assay of reporter genes**

Luciferase expression following transfection was measured by a luminescence assay using cell lysates. The culture medium was discarded and cell lysates harvested after incubation of cells for 30 min at −80°C in 100 µl lysis reagent buffer (Promega) before being thawed at RT. The lysate was mixed well by pipetting and 20 µl was diluted into 25–100 µl of luciferase assay reagent (Promega). The luminescence was integrated over 10 s on a Victor® 1420 Multilabel Counter (Wallac, Bucks, UK) or a Lumat LB9507 (Berthold Instruments, UK) and the results expressed as RLU per mg of cell protein, determined using the Advanced Protein Assay (Cytoskeleton, Denver). Analysis of EGFP expression was carried out on a Coulter Epics XL flow cytometer. Cells were trypsinized at appropriate times after transfection, washed with phosphate-buffered saline (PBS) and then fixed in 2% paraformaldehyde. EGFP was excited using the 488 nm line of an Argon laser and emitted light collected at 520 nm after 390 min. By comparison, plasmid pT7Luc incubated with 1 µg of T7 RNAP mRNA containing the 64 bp poly(A) sequence and down oligonucleotide to prepare pCMV/T7-T7pol(A64) are outlined in Figure 1A and Supplementary Figure 1. We first prepared mRNA encoding T7 RNAP protein from NotI linearized pCMV/T7-T7pol and pCMV/T7-T7pol(A64) to investigate whether it can trigger expression of co-delivered plasmid DNA, and improve the efficiency of T7-based cytoplasmic plasmids. Denaturing electrophoretic analysis showed that pCMV/T7-T7pol(A64) produced mRNA of the expected size (2899 nt) that was slightly longer than the transcript derived from pCMV/T7-T7pol (2880 nt, Figure 1B). To confirm that transcribed mRNA encoded T7 RNAP protein, T7 RNAP mRNA containing the 64 bp poly(A) sequence was translated utilizing the RRL system. Immunoblotting for the T7 RNAP protein using a mouse anti-T7 RNAP monoclonal IgG antibody gave a band of ~100 kDa that was similar in size to recombinant T7 RNAP protein, indicating that T7 RNAP mRNA had been translated successfully (Figure 1C).

A cell-free transcription/translation assay incorporating the reporter plasmid pT7Luc was performed next to confirm that T7 RNAP protein translated from mRNA was biologically functional. Incubation of pT7Luc mRNA containing the 64 bp poly(A) sequence with 100 U of T7 RNAP protein resulted in luciferase expression that was dose-dependent upon the amount of T7 RNAP protein added (Figure 2A). The addition of 100 U of T7 RNAP protein, for instance, generated a maximum of ~1.7 × 10⁶ RLU after 390 min. By comparison, plasmid pT7Luc incubated with 1 µg of T7 RNAP mRNA gave ~100-fold higher level of luciferase expression (1.7 × 10⁶ RLU, Figure 2B). These results demonstrated that T7 RNAP protein translated from T7 RNAP mRNA was functional and mediated luciferase expression at levels up to 100-fold greater than using T7 RNAP protein directly. However, it is interesting to note that after shorter times T7 RNAP protein mediated higher expression levels than T7 RNAP mRNA. For example, after 30 min, expression driven by T7 RNAP protein (2.0 × 10⁵ RLU) was 10-fold greater than T7 RNAP mRNA (2.0 × 10⁴ RLU, Figure 2). This is likely to reflect the requirement for translation of T7 RNAP mRNA to occur prior to transcription of the luciferase gene.

**Western blot analysis**

Cells were lysed in M-PER cell lysis buffer (100 µl, Perbio Science, Northumberland, UK) containing complete protease inhibitor cocktail (Roche, East Sussex, UK). The lysates were normalized for protein concentration using the Advanced Protein Assay (Cytoskeleton) and stored at −70°C until used for western blot analysis. Each sample (20 µg total protein) was heated at 90°C for 5 min and separated on a 9% SDS–polyacrylamide gel (Invitrogen). Proteins were transferred to nitrocellulose membranes overnight and blocked for 90 min at room temperature in PBS containing 5% non-fat milk. Membranes were blotted with the relevant primary antibody for 90 min, washed in PBS containing 0.1% Tween and the secondary antibody added. For detection Lumiglo® chemiluminescent substrate (Upstate) was added and chemiluminescence detected using the MultiImage software programme WinMDI light cabinet and Fluorchem 8800 software (Alpha Innotech, CA).

**RESULTS**

**Production and analysis of T7 RNAP mRNA**

Construction of pCMV/T7-T7pol and insertion of a 64 bp oligonucleotide to prepare pCMV/T7-T7pol(A64) are outlined in Figure 1A and Supplementary Figure 1. We first prepared mRNA encoding T7 RNAP protein from NotI linearized pCMV/T7-T7pol and pCMV/T7-T7pol(A64) to investigate whether it can trigger expression of co-delivered plasmid DNA, and improve the efficiency of T7-based cytoplasmic plasmids. Denaturing electrophoretic analysis showed that pCMV/T7-T7pol(A64) produced mRNA of the expected size (2899 nt) that was slightly longer than the transcript derived from pCMV/T7-T7pol (2880 nt, Figure 1B). To confirm that transcribed mRNA encoded T7 RNAP protein, T7 RNAP mRNA containing the 64 bp poly(A) sequence was translated utilizing the RRL system. Immunoblotting for the T7 RNAP protein using a mouse anti-T7 RNAP monoclonal IgG antibody gave a band of ~100 kDa that was similar in size to recombinant T7 RNAP protein, indicating that T7 RNAP mRNA had been translated successfully (Figure 1C).

A cell-free transcription/translation assay incorporating the reporter plasmid pT7Luc was performed next to confirm that T7 RNAP protein translated from mRNA was biologically functional. Incubation of pT7Luc in A549 and PC-3 cells. Initial transfections showed that the level of luciferase expression
increased with the dose of T7 RNAP mRNA, generating a maximum of $1.0 \times 10^8$ RLU/mg protein in A549 cells and $1.9 \times 10^8$ RLU/mg protein in PC-3 cells (Figure 3A). By comparison, only background levels of luciferase expression ($\sim 1.0 \times 10^6$ RLU/mg protein in A549 cells) were detected in the absence of T7 RNAP mRNA. The amount of pT7Luc used to transfect A549 and PC-3 cells was also an important determinant of luciferase expression. Expression mediated by 125 ng of T7 RNAP mRNA increased with the dose of pT7Luc up to the maximum amount of 500 ng added to A549 (9.4 $\times 10^7$ RLU/mg protein) and PC-3 (2.3 $\times 10^8$ RLU/mg protein) cells (Figure 3B). Doubling the quantity of plasmid pT7Luc from 250 to 500 ng resulted in a 4-fold increase in expression in A549 cells and a 6-fold increase in PC-3 cells.

Transfections were next performed to directly compare the efficiency of T7 RNAP mRNA with T7 RNAP protein to mediate luciferase expression from pT7Luc. Preliminary experiments were indicated to produce maximum expression levels with 500 ng of pT7Luc in A549 ($\sim 3.0 \times 10^6$ RLU/mg protein) and PC-3 ($\sim 2.0 \times 10^7$ RLU/mg protein) cells (Figure 3C). Subsequent transfections to compare T7 RNAP mRNA with T7 RNAP protein in the same experiment showed that co-delivery of 125 ng T7 RNAP mRNA with 500 ng of pT7Luc gave 5.2-fold and 6.7-fold greater luciferase expression compared to T7 RNAP protein at a dose of 100 U in A549 and PC-3 cells, respectively (Supplementary Figure 3). Taken together, these results demonstrate the superior transfection properties of cytoplasmic expression systems triggered by T7 RNAP mRNA compared to T7 RNAP protein.

Parameters influencing the efficiency of the T7 RNAP mRNA/pT7Luc expression system

We next examined several parameters that have been reported to affect the efficiency of nucleic acid-based expression systems, including the structure of mRNA, intracellular stability, time course of delivery and cytotoxicity (4, 24, 25). First, we compared the efficiency of T7 RNAP mRNA containing different structural components to trigger luciferase expression from co-delivered pT7Luc in A549 and PC-3 cells. Figure 4A shows that T7 RNAP mRNA modified with either m7G(5′)ppp(5′)G (Cap), or an anti-reverse cap analogue (ARCA), and a poly(A64) tail produced the highest levels of luciferase gene expression ($\sim 1.2 \times 10^7$ RLU/mg protein in A549 cells). However, reporter gene expression was abolished by $\sim 80\%$ corresponding to a 4.8-5.6-fold decrease using capped T7 RNAP mRNA lacking a poly(A64) tail. The importance of the poly(A) tail was further demonstrated by a 2.2 to 3.0-fold increase in gene expression when a poly(A) tail of $\sim 200$ residues was added to capped T7 RNAP mRNA lacking the A64 tail with a poly(A) tailing kit (Figure 4A).

To evaluate the intracellular stability and persistence of T7 RNAP protein and pT7Luc, we transfected A549 cells with the relevant nucleic acid components at different time points. For instance, expression levels were abolished by $>240$-fold ($4.9 \times 10^4$ RLU/mg protein) when A549 cells were transfected with 500 ng pT7Luc 12 h prior to transfection with 125 ng T7 RNAP mRNA compared to that detected when both T7 RNAP mRNA and pT7Luc were transfected together ($\sim 1.2 \times 10^7$ RLU/mg protein, Figure 4B). In contrast, luciferase expression ($7.7 \times 10^6$ RLU/mg protein) detected when T7 RNAP mRNA transfection preceded pT7Luc transfection was still $\sim 65\%$ of that when both nucleic acids were transfected together, indicating that the half-life of the T7 RNAP protein was at least 12 h (Figure 4B). This is in agreement with published studies that have estimated the half-life of T7 RNAP protein in 293 cells to be $>24$ h (26). A qRT–PCR assay was next developed to evaluate the persistence of T7 RNAP mRNA in A549 cells and used to calculate the quantity of T7 RNAP mRNA present in transfected cultures over a time course of 24 h. Figure 4C shows that the signal from T7 RNAP mRNA decreased over time (exponential decay) with an estimated half-life in A549 cells of 4.79 h. Finally, cell viability assays using
A549 cells showed that transfection with T7 RNAP mRNA (100 ng) complexed with DOTAP or DOTAP alone gave negligible toxicity (Figure 4D). Co-delivery of T7 RNAP mRNA with pT7Luc also gave minimal toxicity with between 88 and 91% cells remaining viable after 24 h, depending on the dose of plasmid DNA, compared to mock-transfected cells. Altogether, these results characterize a number of parameters that can influence the efficiency of the T7 RNAP mRNA/pT7Luc expression system. In particular, the intracellular stability of plasmid DNA appears to be the most important determinant in limiting gene expression, and not the duration of expression or stability of the T7 RNAP protein.

Enhanced transfection efficiency of mRNA triggered systems in DRGN cultures

We next examined whether cytoplasmic expression systems triggered by mRNA can efficiently transfect primary DRGN cultures, where the majority of cells are likely to be post-mitotic or slowly-dividing. In preliminary experiments, fluorescence microscopy and flow cytometry were used to evaluate the ability of mRNA or plasmid DNA encoding EGFP to transfect DRGN cultures freshly isolated from adult Sprague–Dawley rats. Figure 5A and B show that a high proportion (>50%) of phenotypically identifiable DRGN in culture were transfected with EGFP mRNA. In contrast, no EGFP-positive cells were detected using mRNA encoding for luciferase (LUC mRNA) as a control (Figure 5A). Furthermore, we observed <2% EGFP-positive cells in DRGN cultures with the CMV-driven plasmid pEGFPN1 (Figures 5B and 6).

The transfection efficiency of T7 RNAP mRNA with pT7Luc was then compared against the CMV-driven plasmid pCMVLuc1 in DRGN cultures and A549 cells. In primary DRGN cultures, pCMVLuc1 was a particularly inefficient vector with luciferase expression no higher than that seen.
in non-transfected cells (~1.2 × 10^4 RLU/mg protein; Figure 5C). By comparison, transfection of DRGN cultures with the T7 RNAP mRNA triggered system gave >100-fold increase in luciferase activity (1.3 × 10^6 RLU/mg protein; \( P = 0.0185 \)). This level of reporter gene expression was similar to that obtained using the same system in A549 cells (4.2 × 10^6 RLU/mg protein). In contrast, in rapidly dividing A549 cells, where nuclear access was relatively easy the functionality of pCMVLuc1 was evident, with high luciferase activity (7.8 × 10^6 RLU/mg protein) (Figure 5C).

Further analysis using a more sensitive luciferase assay showed that transfection of DRGN cultures with the T7 RNAP mRNA triggered system gave >2200-fold increase in luciferase activity (2.2 × 10^8 RLU/mg) compared to pCMVLuc1 (9.9 × 10^4 RLU/mg, Supplementary Figure 4).

Evaluation of the time course of gene expression in DRGN cultures showed significant luciferase activity with the T7 RNAP mRNA triggered system after 6 h that increased up...
to 24 h (Figure 5D). At all time points examined the T7 RNAP mRNA triggered system produced significantly greater luciferase activity than pCMVLuc1 in the range of 13- to 634-fold ($P < 0.05$, Figure 5D).

Previously, pCMVLuc1 has been shown to be highly active in primary rat oligodendrocytes when delivered using a particle bombardment gene delivery approach, which suggests that in this study the poor activity of the plasmid alone was due to limited access to the nucleus rather than inactivity of the CMV promoter or the pCMVLuc1 vector in rat cells (27).

Hence, these results demonstrate that cytoplasmic expression systems triggered by mRNA are efficient in transfecting postmitotic or slowly-dividing cells, especially in cell types that are resistant to gene transfer using conventional CMV promoter driven vectors requiring nuclear entry.

**Improved efficiency of T7-based autogene systems with T7 RNAP mRNA**

We next investigated whether the efficiency of cytoplasmic expression systems based on autogene plasmids containing...
the T7 RNAP gene, which enable autocatalytic amplification of the T7 RNAP protein, were also improved by co-delivery of T7 RNAP mRNA. For these experiments, we used an enhanced autogene plasmid pR011 (Supplementary Figure 2), which was recently developed by Finn et al. (22). Enhancements to plasmid pR011 included an IRES sequence located between the bacteriophage promoters and the T7 RNAP gene, and the incorporation of a luciferase expression cassette. Using plasmid pR011 in BHK cells, Finn et al. (22) have demonstrated levels of reporter gene expression that were 20-fold higher than standard CMV-based nuclear expression systems. In addition, direct evidence was given for an exponential, autocatalytic increase in gene expression using autogene-based plasmids. However, pR011 is still limited by a requirement for nuclear entry to drive initial T7 RNAP production to trigger the autogene.

The schematic in Figure 7A outlines the principle of co-delivery of T7 RNAP mRNA to trigger expression of the luciferase reporter gene from cytoplasmic expression plasmids pR011 and pT7Luc. The ability of T7 RNAP mRNA to improve the transfection efficiency of pR011 was evident in A549 cells with a 2.7-fold ($P = 0.0026$) and 3.4-fold increase ($P < 0.0001$) in luciferase expression observed after 24 and 48 h, respectively, compared to pR011 alone (Figure 7B). Transfection of primary DRGN cultures with pR011 in the presence of T7 RNAP mRNA also increased luciferase expression after 24 h (4.8-fold) compared to pR011 alone ($P = 0.0131$, Figure 7C). By comparison, transfection of DRGN cultures with pT7Luc and T7 RNAP mRNA gave >30-fold higher level of luciferase expression than pR011 ($P < 0.05$, Figure 7C). These results demonstrate that T7 RNAP mRNA can also be used successfully to trigger T7-based autogene plasmids and increase gene expression in both rapidly dividing cell lines and primary cultures of post-mitotic DRGN. Although in DRGN cultures combining a simple T7-driven expression plasmid with T7 RNAP mRNA gave significantly greater gene expression than the T7-based autogene plasmid, either with or without T7 RNAP mRNA.

**DISCUSSION**

A prerequisite for gene transfer with synthetic vectors is the efficient delivery of exogenous nucleic acids to the nucleus. This goal is challenging as nuclear entry is particularly inefficient in post-mitotic or slowly-dividing cells since there is limited breakdown of the nuclear envelope (6,7). In this study, we used a hybrid mRNA/DNA system to facilitate cytoplasmic gene expression and bypass problems associated with the nuclear barrier to transfection. In primary DRGN cultures, for example, co-delivery of T7 RNAP mRNA with pT7Luc produced between 10- and 2200-fold higher gene expression compared to the CMV-driven plasmid, pCMVLuc1 and >30-fold increase compared to an enhanced T7-autogene plasmid pR011. In addition, only a small quantity of mRNA was required to mediate significant gene expression. In A549 cells, for instance, co-delivery of 10 ng of T7 RNAP mRNA with pT7Luc was sufficient to mediate a 3-log increase in luciferase activity. These properties demonstrate that the combination of T7 RNAP mRNA with a T7-driven DNA cytoplasmic expression plasmid represents a powerful and versatile non-viral approach for mediating gene expression.

The co-delivery of plasmid DNA with T7 RNAP protein is one of the simplest and most widely used systems to achieve cytoplasmic gene expression. This approach, however, has been shown to be relatively inefficient even in cultured cells (28), with low levels of protein transduction likely to be a limiting factor. Another potential problem is immune recognition of the T7 RNAP protein that will hinder repeated administration in vivo (21). Therefore, the major advantages of using the hybrid mRNA/DNA system are that problems of inefficient protein transduction and immune responses associated with repeat administration of the T7 RNAP protein will be diminished as it is a non-viral formulation based solely on nucleic acids. Preliminary results from a cell-free assay demonstrated that co-incubation of T7 RNAP mRNA with pT7Luc was more efficient than using T7 RNAP protein, with up to a 100-fold increase in luciferase expression. This trend was repeated in A549 cells and primary DRGN cultures (data not shown) with up to a 6.7-fold higher level of expression achieved by co-delivery of pT7Luc with T7 RNAP mRNA rather than T7 RNAP protein. It is likely that amplification of the T7 RNAP protein by multiple rounds...
of translation from T7 RNAP mRNA also contributed towards mediating higher gene expression compared to using a static quantity of T7 RNAP protein.

We also used T7 RNAP mRNA instead of plasmid DNA encoding the T7 RNAP gene as it does not require nuclear uptake for expression, and translation occurs almost immediately upon entry into the cytoplasm. This is especially important in post-mitotic or slowly-dividing cells where access of plasmid DNA to the nucleus will be limited. The incorporation of mRNA into the hybrid expression system therefore

Figure 7. Improved efficiency of pR011 mediated by T7 RNAP mRNA. (A) Schematic comparing T7 RNAP mRNA triggered expression of pR011 with pT7Luc. Luciferase protein is produced following binding of T7 RNAP protein to the pT7 promoter and subsequent transcription/translation of the luciferase reporter gene. In pR011, binding of T7 RNAP protein to the pT7 promoter upstream of the T7 RNAP gene leads to autocatalytic amplification of T7 RNAP protein. (B) A549 cells (3.5 x 10^4/well) were transfected with pR011 (375 ng) either alone or with T7 RNAP mRNA (150 ng) as indicated. Cells were lysed after 24 h (white bars) or 48 h (grey bars) and luciferase activity measured. (C) Freshly isolated DRGN cultures (1.5 x 10^3 cells/well) were transfected with the indicated plasmid (375 ng) either in the presence or absence of T7 RNAP mRNA (150 ng). Cells were lysed after 24 h and luciferase activity measured using the Victor^ multilabel counter. Results are shown as a mean and SD from at least three samples. (***, P < 0.0001; **, P < 0.01; *, P = 0.0131).
enables cytoplasmic expression of co-delivered plasmid DNA in a larger proportion of cells compared to a nuclear dependent plasmid. Evidence for this comes from a number of studies where EGFP mRNA was shown to transfect a higher percentage of cells, such as primary HUVEC (20) and PC-3 cells (29), compared to an EGFP expression plasmid. We also demonstrated in this study that plasmid pEGFPN1 gave poor levels of transfection in DRGN cultures, whereas a significantly higher proportion of cells were transfected with EGFP mRNA.

T7-based autogene systems have the capacity to mediate higher levels of gene expression since, after initial triggering of the T7-autogene by T7 RNAP, further expression of T7 RNAP occurs in an autocatalytic positive feedback loop producing high levels of the protein (16). Subsequently, the large quantities of T7 RNAP produced can be used to mediate expression of a co-delivered therapeutic gene. The efficiency of T7-based autogene plasmids has gradually increased since their initial development. Incorporation of the CMV promoter into the autogene plasmid has enabled easier bacterial amplification of the plasmid, and offered a new triggering system, independent of protein transduction (21,22). While this approach was shown to be effective in rapidly dividing 293 cells, there is now a requirement for nuclear entry to trigger the autogene, which is likely to compromise the utility of these plasmids in non-dividing cells.

A significant finding in this study was that transfection of DRGN cultures with pT7Luc and T7 RNAP mRNA gave >30-fold higher gene expression than the enhanced T7-autogene plasmid pR011. We also showed in DRGN cultures that T7 RNAP mRNA can be used to enhance gene expression mediated by pR011 with up to a 4.8-fold increase in luciferase activity. By comparison, the enhancement in expression with T7 RNAP mRNA was slightly lower in A549 cells (3-fold), which may reflect the higher transfection activity of pR011 in these cells due to increased nuclear entry and subsequent triggering of the CMV promoter. The hybrid T7 RNAP mRNA/DNA system contains important features that will have contributed towards the superior transfection profile in primary DRGN cultures. First, the T7 RNAP mRNA was capped and polyadenylated, and so would have been translated more efficiently and have a longer half-life than autogene derived, IRES containing transcripts. Finn et al. (30) recently showed that IRES mediated translation was relatively inefficient compared to the cap dependent form, with luciferase expression from IRES containing mRNA almost 20-fold lower than from an equivalent capped transcript. Furthermore, T7 RNAP protein produced by mRNA translation drives efficient expression from a co-delivered reporter plasmid almost immediately, without the need for mRNA amplification to occur. This may provide a critical time advantage since degradation of plasmid DNA by cytoplasmic nucleases limits cytoplasmic expression (5,25).

The major factor that appeared to limit expression with the hybrid T7 RNAP mRNA/DNA system was the quantity of plasmid DNA available as a cytoplasmic transcription template, rather than T7 RNAP protein produced from T7 RNAP mRNA. Doubling the quantity of pT7Luc transfected from 250 to 500 ng, for instance, resulted in a 6-fold increase in gene expression in PC-3 cells. The inclusion of an autogene expression cassette within the formulation was not required as a small quantity of T7 RNAP mRNA was sufficient to drive optimal expression from co-transfected plasmid DNA. The challenges now lie in improving the duration of transgene expression and persistence of plasmid DNA, and we are currently exploring different strategies to address these issues, including the use of CpG-depleted plasmids to prolong expression in vivo (31–34). It will also be necessary to determine the extent to which intracellular expression of the T7 RNAP protein leads to potential immunogenicity problems or whether the use of a nucleic acid formulation is sufficient to diminish immune responses to the T7 RNAP protein, and so maintain the viability of transfected cells without the use of immunosuppressants (35).

Based on our findings in primary DRGN cultures, potential therapeutic applications for this hybrid expression system include the delivery of neurotrophic factors to promote axonal regeneration, in possibly a range of neurological conditions, from spinal cord injury to chronic progressive neurodegenerative diseases. Recently, we demonstrated that an effective strategy to sustain gene delivery to axotomized neurons was to immobilize plasmid DNA in gene-activated matrices (GAM) that were placed between the proximal and distal stumps of severed rat optic nerves (36) or into a lesioned rat dorsal column (37). Hence, we envisage that GAM-mediated delivery of hybrid expression systems encoding for neurotrophic factors will represent a more effective strategy to enhance DRGN axonal regeneration in vivo than the use of CMV-driven plasmid DNA. Furthermore, the central nervous system has historically been designated as an ‘immunologically privileged’ site, as it lacks normal surveillance by cells and mediators of the immune system. The central nervous system therefore represents an ideal site for delivery of non-viral vectors containing the hybrid mRNA/DNA expression system, especially when potentially immunogenic proteins are expressed.

In summary, the results from this study demonstrate that co-delivery of mRNA is a promising strategy to yield increased expression with plasmid DNA and facilitate cytoplasmic gene expression. Furthermore, this work represents an important step towards the development of non-viral formulations based on a hybrid mRNA/DNA system that should prove useful for the expression of proteins in post-mitotic or slowly-dividing cells, where the nuclear barrier represents a significant barrier to transfection.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES

1. Buckley, R.H. (2002) Gene therapy for SCID—a complication after remarkable progress. Lancet, 360, 1185–1186.

2. European Society of Gene Therapy (2003) French gene therapy group reports on the adverse event in a clinical trial of gene therapy for X-linked severe combined immune deficiency (X-SCID). J. Gene Med., 5, 82–84.

3. Couzin, J. and Kaiser, J. (2005) Gene therapy. As Gelsinger case ends, gene therapy suffers another blow. Science, 307, 1028.

4. Read, M.L., Logan, A. and Seymour, L.W. (2005) Barriers to gene delivery using synthetic vectors. Adv. Genet., 53(PA), 19–46.

5. Pollard, H., Remy, J.S., Lousouarn, G., Demolombe, S., Behr, J.P. and Escande, D. (1998) Polyethyleneimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem., 273, 7507–7511.

6. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M. and Wagner, E. (2001) Recent developments in autogene-based cytoplasmic expression systems. Adv. Genet., 41, 360–369.

7. Zanta, M.A., Belguise-Valladier, P. and Behr, J.P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the nucleus in mammalian cells. J. Biol. Chem., 274, 18997–19007.

8. Meng, C.H., Robinson, D., Jenkins, R.G., McNulty, R.J. and Hart, S.L. (2004) Efficient transfection of non-proliferating human airway epithelial cells with a synthetic vector system. J. Gene Med., 6, 210–221.

9. Liu, G., Li, D., Pasumarthy, M.K., Kowalczyk, T.H., Gedeon, C.R., Hyatt, S.L., Payne, J.M., Miller, T.J., Brunovskis, P., Fink, T.L. et al. (2003) Nanoparticles of compacted DNA transfect postmitotic cells. J. Biol. Chem., 278, 32578–32586.

10. Bremner, K.H., Seymour, L.W. and Pouton, C.W. (2001) Harnessing nuclear localization pathways for transgene delivery. Curr. Opin. Mol. Ther., 3, 170–177.

11. Zanta, M.A., Belgue-Valladier, P. and Behr, J.P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc. Natl Acad. Sci. USA, 96, 91–96.

12. Bremner, K.H., Seymour, L.W., Logan, A. and Read, M.L. (2004) Factors influencing the ability of nuclear localization sequence peptides to enhance nonviral gene delivery. Bioconj. Chem., 15, 152–161.

13. van der Auwera, M., Koning, G., van der Gugten, J., d’Oliveira, C., Oosting, R., Hennink, W.E. and Crommelin, D.J. (2005) Covalent attachment of an NSL-peptide to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems. J. Control Release, 101, 395–397.

14. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Direct gene transfer into mouse muscle in vivo. Science, 247, 1465–1468.

15. Bettinger, T. and Read, M.L. (2000) Recent developments in RNA-based strategies for cancer gene therapy. Curr. Opin. Mol. Ther., 3, 116–124.

16. Dubendorff, J.W. and Studier, F.W. (1991) Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. J. Mol. Biol., 219, 61–68.

17. Deng, H. and Wolff, J.A. (1994) Self-amplifying expression from the T7 promoter in IT3 mouse fibroblasts. Gene, 143, 245–249.

18. Gao, X., Jaffurs, D., Robbins, P.D. and Huang, L. (1994) A sustained, cytoplasmic transgene expression system delivered by cationic liposomes. Biochem. Biophys. Res. Commun., 200, 1201–1206.

19. Ashley, D.M., Faiola, B., Nair, S., Hale, L.P., Bigner, D.D. and Gilboa, E. (1997) Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. J. Exp. Med., 186, 1177–1182.

20. Bettinger, T., Carlisle, R.C., Read, M.L., Ogris, M. and Seymour, L.W. (2001) Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. Nucleic Acids Res., 29, 3882–3891.

21. Brisson, M., He, Y., Li, S., Yang, J.P. and Huang, L. (1999) A novel T7 RNA polymerase autogene for efficient cytoplasmic expression of target genes. Gene Ther., 6, 263–270.

22. Finn, J., Lee, A.C., MacLachlan, I. and Cullis, P. (2004) An enhanced autogene-based dual-promoter cytoplasmic expression system yields increased gene expression. Gene Ther., 11, 276–283.

23. Read, M.L., Bremner, K.H., Oupicky, D., Green, N.K., Searle, P.F. and Seymour, L.W. (2003) Vectors based on reducible polycations facilitate intracellular release of nucleic acids. J. Gene Med., 5, 232–245.

24. Malone, K.W., Felgner, P.L. and Verma, I.M. (1989) Cationic liposome-mediated RNA transfection. Proc. Natl Acad. Sci. USA, 86, 6077–6081.

25. Lechardeur, D., Sohn, K.J., Haardt, M., Joss, P.B., Monck, M., Graham, R.W., Beatty, B., Squire, J., O’Brodovich, H. and Lukacs, G.L. (1999) Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther., 6, 482–497.

26. Brisson, M., Tseng, W.C., Almonte, C., Watkins, S. and Huang, L. (1999) Subcellular trafficking of the cytoplasmic expression system. Hum. Gene Ther., 10, 2601–2613.

27. Guo, Z., Yang, N.S., Jiao, S., Sun, J., Cheng, L., Wolff, J.A. and Duncan, I.D. (1996) Efficient and sustained transgene expression in mature rat oligodendrocytes in primary culture. J. Neurosci. Res., 43, 32–41.

28. Chen, X., Li, Y., Xiong, K., Aizicovici, S., Xie, Y., Zhuo, Q., Sturtz, F., Shulok, J., Snodgrass, R., Wagner, T.E. et al. (1998) Cancer gene therapy by direct tumor injections of a nonviral T7 vector encoding a thymidine kinase gene. Hum. Gene Ther., 9, 729–736.

29. Read, M.L., Singh, S., Ahmed, Z., Stevenson, M., Briggs, S.S., Oupicky, D., Barrett, I.B., Spicer, R. and Kendall, M.L. (2004) A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. Nucleic Acids Res., 33, e86.

30. Finn, J., MacLachlan, I. and Cullis, P. (2005) Factors limiting autogene-based cytoplasmic expression systems. FASEB J., 19, 606–610.

31. Ross, G.F., Braun, M.D., Uyeda, M., Suzuki, K., Nagoa, K., Whissett, J.A. and Korfhagen, T.R. (1998) Enhanced reporter gene expression in cells transfected in the presence of DMI-2, an acid nucleas inhibitor. Gene Ther., 5, 1240–1250.

32. Bigger, B.W., Tolmachev, O., Collombet, J.M., Fragkos, M., Palaszewski, I. and Couteille, C. (2001) An araC-controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy. J. Biol. Chem., 276, 23018–23027.

33. Sperinde, J.J., Choi, S.J. and Szoka, F.C., Jr (2001) Phage display selection of a peptide DNase II inhibitor that enhances gene delivery. J. Gene Med., 3, 101–108.

34. Yew, N.S., Zhao, H., Przybylska, M., Wu, H., Tousignant, J.D., Scheule, R.K. and Cheng, S.H. (2002) Cpg-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. Mol. Ther., 5, 731–738.

35. Lowenstein, P.R. (2005) The case for immunosuppression in clinical gene transfer. Mol. Ther., 12, 185–186.

36. Berry, M., Gonzalez, A.M., Clarke, W., Greenlee, L., Barrett, L., Tsang, W., Seymour, L., Bonadio, J., Logan, A. and Baird, A. (2001) Sustained effects of gene-activated matrices after CNS injury. Mol. Cell Neurosci., 17, 706–716.

37. Gonzalez, A.M., Berry, M., Greenlee, L., Logan, A. and Baird, A. (2006) Matrix mediated gene transfer to brain cortex and dorsal root ganglion neurons by retrograde axonal transport after dorsal column lesion. J. Gen. Med. (in press).