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RESEARCH ARTICLE

An enhanced autogene-based dual-promoter cytoplasmic expression system yields increased gene expression

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The relatively low levels of transfection that can be achieved by current gene-delivery systems have limited the therapeutic utility of gene transfer. This is especially true for nonviral gene-delivery systems, where the levels of gene expression achieved are usually below the levels achieved by viral gene transfer systems. One strategy for increasing gene expression is to design a cytoplasmic expression system that does not require nuclear delivery for gene expression to occur. This can be achieved through the use of an autocatalytic cytoplasmic expression system using phage RNA polymerases. Here we describe cytoplasmic expression systems that yield increased levels of gene expression following in vitro transfection. We demonstrate direct evidence for an exponential, autocatalytic increase in gene expression using autogenes, as well as levels of reporter gene expression that are 20-fold higher than standard CMV-based nuclear expression systems. The development of a high-efficiency plasmid-based expression system could significantly improve the gene expression properties of nonviral gene-delivery systems, thereby increasing their clinical utility.
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Keywords: *cytoplasmic expression; gene expression system; T7 RNA polymerase; bicistronic; plasmid design; autogene*

Introduction

A major limitation of gene-delivery systems is the relatively low level of gene expression in transfected tissues. One strategy to increase levels of gene expression following transfection employing a nonviral vector involves improving the plasmid design. The incorporation of a cytoplasmic expression system represents one such approach.[1–3] Cytoplasmic expression systems bypass the requirement for nuclear delivery of plasmid DNA, a major obstacle in present day gene therapy.[4,5–7] In addition, they take advantage of the large number of plasmids found in the cytoplasm of the cell following transfection with nonviral vectors.[8] Cytoplasmic expression systems can be designed to utilize the unique properties of the bacteriophage RNA polymerases (RNAPs). Phage RNAPs are moderately sized (~100 kDa), single subunit proteins capable of synthesizing RNA from DNA templates. They require no additional cofactors and have demonstrated efficient cytoplasmic transcriptional activity.[9,10] These features make phage RNAPs attractive candidates for the development of autocatalytic cytoplasmic expression systems using autogenes. Phage RNAP autogenes consist of an RNAP gene, driven by its own cognate promoter.[3] In order to evade the requirement for exogenous RNAP to initiate the expression system, a nuclear promoter can be added upstream of the RNAP promoter.[11] Although the first round of RNAP expression must occur via the nuclear promoter, the resulting RNAP in the cytoplasm drives the cytoplasmic expression system, producing RNA from plasmid DNA template in the cytoplasm.

RNA produced in the cytoplasm lacks the 5′ cap that stabilizes nuclear transcripts and assists in ribosomal recruitment.[12,13] Viral Internal Ribosome Entry Site (IRES) elements are sequences that have been shown to enhance the recruitment of the cytoplasmic translational machinery in the absence of 5′ capping.[14] Early dual-promoter cytoplasmic expression systems did not contain IRES elements and, as a result, the vast majority of the mRNA produced was not translated.[11] Although an autogene based on the T7 bacteriophage RNAP that contained an EMCV IRES has been previously described,[15] it did not contain a eukaryotic promoter and required the cotransfection of RNAP protein or mRNA, thereby limiting its utility.

In this study, we have characterized a novel autogene construct containing both the EMCV IRES element (to allow cap-independent translation) and the CMV promoter (to bypass the need for additional exogenous RNAP protein or mRNA during transfection). In addition, we have combined the autogene and reporter gene cassettes on a single plasmid, whereas other systems have utilized separate plasmids. We also describe a cell-free system for the evaluation of RNAP-based transfection reagents and use it to demonstrate the potential of the autogene system to elicit high levels of gene expression.

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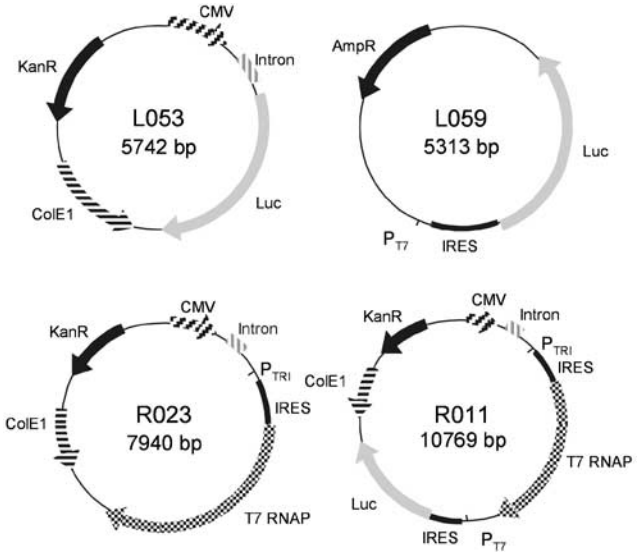

Figure 1 *Plasmid diagrams of major constructs used. R023 is the basic autogene construct, containing the T7 RNAP gene driven by the T7, T3, and SP6 promoters (P_{TRI}). L059 is the luciferase reporter gene cassette. R011 is the bicistronic autogene construct (R023 + L059). L053 is the CMV-driven nuclear expression construct.*

Materials and methods

Plasmids and primers

Plasmid R023 consists (Figure 1) of a basic autogene cassette driven by a CMV promoter and intron. The autogene cassette was derived from the plasmid T7-G1, a gift of Dr Jon Wolff (Waisman Center, WI, USA). T7-G1 contains the basic autogene cassette, consisting of the T7 promoter, EMCV IRES, and T7 RNAP gene. The nuclear localization sequence was removed from the T7 RNAP via PCR prior to subcloning into R023. L059 consists of a pTRI-Amp (Ambion) backbone with EMCV IRES, *Photinus pyralis* luciferase and beta-globin poly-adenylation site derived from EMC-Luc (Jon Wolff). L053 consists of the CMV promoter (with intron) from NGVL3 and the *Photinus pyralis* luciferase gene. L069 and L070 consist of L053 containing one or two irrelevant 2.5 kb spacer fragments, respectively. R037 consists of R023 without the T7 and T3 promoters. R011 is a bicistronic plasmid consisting of R023 with a downstream luciferase reporter gene cassette from L059 (bicistronic). PT7-Luc (Promega) consists of the *Photinus pyralis* luciferase gene driven by a T7 RNAP promoter. RPA-RNAP consists of a 350 bp *Kpn*I–*Afl*II T7 RNAP fragment blunted and ligated into the *Sma*I site of pTRI-Amp in reverse orientation. RPA-Luc consists of a 250 bp *Xcm*1–*BsrG* 1 luciferase fragment blunted and ligated into the *Sma*1 site of pTRI-Amp in the reverse orientation.

The NVSC1 primer sequence is 5′-TCCTGCAGCCC GGGGGATCCTCTAG-3′.

Transcription and translation assay

A 25 μl reaction was set up using a Promega (Wisconsin) Coupled *In Vitro* Transcription and Translation kit, as per the manufacturer's instructions. A measure of 250 ng of PT7-Luc was added to all reactions. Then, 250 ng of either R023 (containing a T7 RNAP gene driven by the T7, SP6, and T3 promoters) or R037 (containing a T7 RNAP gene driven only by the SP6 promoter) was added to the reactions. SP6 RNAP (0.5 U; Promega) was added and each reaction was incubated at 30°C. At the time points indicated, 2 μl of reaction mixture was removed and assayed for luciferase expression, as described below. All reactions were performed in triplicate.

Transfections

Lipoplexes were formed by mixing plasmid DNA with large unilamellar vesicles (LUVs) composed of equimolar amounts of DOPE:DODAC (50:50) on ice and incubated for 20 min prior to use. All transfections were performed at a cationic lipid to plasmid DNA charge ratio of 3:1. Lipoplexes were diluted with serum-containing media before addition directly to the cell media. BHK cells were plated at 25 000 cells per well in 24-well plates. Neuro2A cells were plated at 30 000 cells per well in 24-well plates. The total mass of plasmid added was identical in all transfections. Equimolar transfections using plasmids of different sizes were achieved through the addition of an empty vector (pBlueScript) to normalize the total mass of DNA in each transfection. All transfections were performed in triplicate. Data are presented as mean values ± standard error.

Luciferase and BCA assays

Cells were washed twice with 1 ml PBS, followed by the addition of 0.2 ml lysis buffer (PBS with 0.1% Triton X-100) before being stored at −70°C. Cells were thawed and 5–20 μl of sample was assayed in duplicate on a 96-well plate. Samples were assayed using a Berthhold Centro LB960 Microplate Luminometer and Luciferase Assay System (Promega). Standard luciferase assays were performed and transfection data are reported as mass quantities of luciferase protein, using a standard curve obtained from serial 10-fold dilutions of a 20 mg/ml *Photinus pyralis* luciferase standard (Promega). Cell-free luciferase assays are reported in RLUs. Total protein was quantified using a Pierce BCA assay kit, as per the manufacturer's instructions.

Immunofluorescence

BHK cells were plated on glass coverslips in six-well plates (150 000 cells/well) and transfected with 1.5 μg of plasmid DNA. At 24 h post-transfection, cells were washed once with 2 ml PBS-IF (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4) prior to fixation for 10 min with 2 ml 2% paraformaldehyde. Cells were subjected to three 30 s washes before permeabilization with 0.25% Triton X-100 in PBS-IF for 5 min. After washing three times for 30 s with PBS-IF, cells were incubated with blocking buffer (10% BSA in PBS-IF) for 1 h, shaking gently at room temperature. Cells were washed three times for 10 min with PBS-IF, followed by addition of a primary antibody solution consisting of a 1:1000 dilution of goat anti-T7 RNAP antibody (a gift from Dr Paul Fisher at the Department of Pharmacological Sciences, State University of New York at Stoney Brook) or 1:1000 dilution of mouse antiluciferase monoclonal antibody (Abcam) in 2% BSA in PBS-IF. Cells were incubated with primary antibody solution for 2 h while shaking at room temperature. Cells were washed three times for 10 min in PBS-IF, followed by the addition

of secondary antibody (rabbit anti-goat IgG, FITC labelled (QED Bioscience Inc) or rabbit anti-mouse Texas Red labelled (Abcam), 1:200 dilution in 2% BSA-PBS-IF) and incubation for 2 h while shaking at room temperature. Cells were washed four times for 10 min with PBS-IF before being mounted and photographed on a Zeiss Axiovert S100 fluorescence microscope. The percentage of cells transfected was determined by counting transfected and nontransfected cells under the microscope. Data indicate the average of six separate counts from three different experiments.

RNase protection assay

RNAP and luciferase probes were prepared from *EcoR*1-digested RPA-RNAP or RPA-Luc plasmid, respectively. GAPDH probe was purchased from Pharmingen. Probes were labelled following the manufacturer's protocol using ^{32}P-αUTP (3000 Ci/mmol, 10 mCi/ml) (NEN).

BHK cells were plated on six-well plates (150 000 cells/well) and transfected with 1.5 μg of R011 or L053 in triplicate. After 24 h, cells were treated with 20 μg/ml Actinomycin D. At 0, 2, 4, 6 or 8 h after actinomycin D treatments, cells were washed once with PBS and recovered by trypsinization. Cells from triplicate wells were pooled before harvesting total RNA (RNeasy miniprep kit, Qiagen). A measure of 10, 5 or 2.5 μg of total RNA was subjected to RNase protection analysis using the RiboQuant RPA system (Pharmingen), according to the manufacturer's protocol. All values shown are the average ± standard deviation of two independent experiments. Data were collected using a Typhoon Phosphoimager (Amersham Biosciences) and analysis was performed using ImageQuant software (Amersham Biosciences).

Primer extension

Primer extension analysis using ^{32}P-labelled primer NVSC1 and 100 μg of RNA isolated from R011-transfected BHK cells (24 h post transfection) was performed using a Primer Extension System (Promega). The ladder was prepared by end-labelling ΦX174 *Hinf*I DNA markers with ^{32}P. All values shown are the average ± standard deviation of two independent experiments. Data were collected as described for RNase Protection assay above.

Results

Autocatalytic gene expression results in an exponential time-dependent increase in gene expression

A hallmark of an autocatalytic, self-amplifying system is an exponential, time-dependent increase in the product being amplified. This exponential relationship would be limited only by the amount of substrate available (ie charged tRNA, GTP, etc), and would continue as long as the template plasmid is in excess. In order to verify the autocatalytic nature of the autogene, a cell-free transcription and translation assay was performed. R023 plasmid DNA (consisting of T7 RNAP driven by both SP6 and T7 RNAP promoters) was incubated with a PT7-Luc reporter gene plasmid (consisting of luciferase driven by only the T7-promoter) in the presence of rabbit reticulocyte lysate and SP6 RNAP. SP6 RNAP transcribes T7 RNAP RNA from the R023 plasmid, leading to the production of T7 RNAP protein that is then able to drive the expression of both the T7 RNAP gene from R023 in an autocatalytic fashion, as well as expression of the luciferase gene from PT7-Luc. Figure 2 shows a dramatic increase in luciferase expression over time, indicating an exponential, autocatalytic increase in T7 RNAP protein. This increase is not observed when a control plasmid (R037, consisting of T7 RNAP driven only by the SP6 promoter) lacking the T7 promoter needed for autocatalytic amplification is used. The reason for the lack of expression from R037 is that, without the autocatalytic amplification, the amount of T7 RNAP produced is not enough to give rise to detectable levels of luciferase expression.

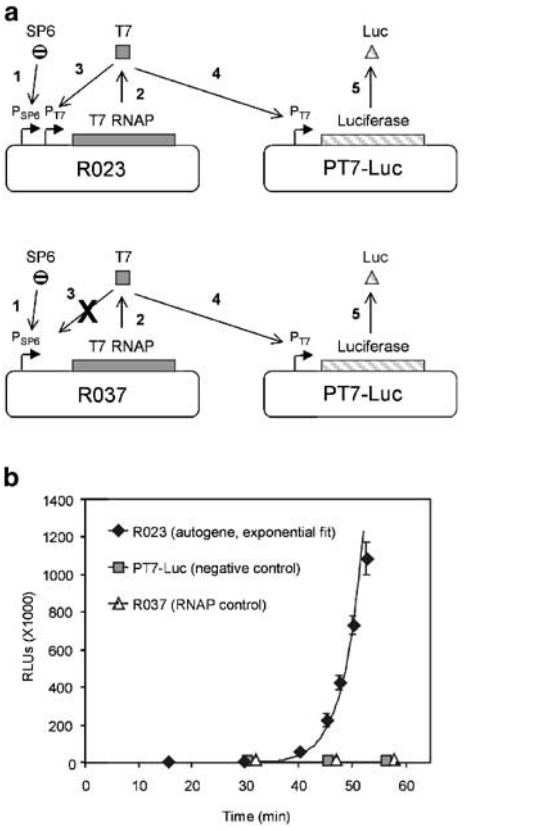

Figure 2 *(a) Schematic diagram of the transcription and translation assay. SP6 RNAP binds to the SP6 promoter (P_{SP6}) on R023 (T7 RNAP driven by SP6 and T7 promoters) (1) transcribing T7 RNAP mRNA, which is (2) translated into the T7 RNAP protein. The T7 RNAP protein then binds the T7 promoter (P_{T7}) on R023 (3), resulting in more T7 RNAP protein (2) and initiating the autocatalytic cycle and an exponential increase in T7 RNAP production. T7 RNAP also transcribes luciferase mRNA from PT7-Luc (4), resulting in an increase in luciferase expression proportional to the amount of T7 RNAP present. In the control reaction (below), the lack of P_{T7} in R037 (T7 RNAP gene driven by only SP6 promoter) prevents any autocatalytic production of T7 RNAP (3). (b) An in vitro coupled transcription and translation (Promega) assay. A measure of 250 ng of PT7-Luc was combined with 250 ng of either R023 or R037 in a total reaction volume of 15 μl, and 0.5 U of SP6 RNAP (Promega) was added and incubated at 30°C. The aliquots (2 μl) were removed at the time points indicated and subjected to luciferase analysis, as described in Materials and methods. After an initial lag phase, the R023 reaction resulted in an exponential increase in luciferase expression, verifying the autocatalytic nature of the system.*

A bicistronic construct results in higher levels of gene expression than a dual-plasmid transfection

Previously published work on cytoplasmic expression systems employed an autogene cassette and a reporter gene cassette on separate plasmids. It was of interest to compare the expression resulting from a dual-plasmid transfection system with a single-plasmid bicistronic system in which the autogene and reporter gene were on one large plasmid. When equimolar amounts of autogene and reporter gene constructs were used to transfect BHK cells, it was found that the bicistronic construct yielded two- to four-fold higher levels of gene expression than the analogous dual plasmid transfection (Figure 3). This result was unexpected because previous results suggest that transfection (delivery to the nucleus and subsequent expression) would be more efficient for the smaller autogene plasmid than the larger bicistronic construct.[16] For the dual-plasmid transfection, this would result in a greater number of cells expressing RNAP via the CMV promoter in the nucleus, and accordingly greater levels of luciferase via the RNAP promoter in the cytoplasm. In order to understand this phenomenon, a series of luciferase plasmids of increasing size were prepared to determine the effect of plasmid size on transfection efficiency in BHK cells. It was found that L053 (5.8 kb), L069 (8.3 kb), and L070 (10.8 kb) yielded similar levels of gene expression when transfected in equimolar amounts (Figure 4). This suggests that, for the system described here, larger plasmids are not at a disadvantage compared to the smaller plasmids. In addition, immunofluorescence studies using anti-T7 RNAP and antiluciferase antibodies showed that the same percentage of cells are being transfected with the bicistronic construct as with the dual-plasmid transfection (data not shown), further supporting this finding.

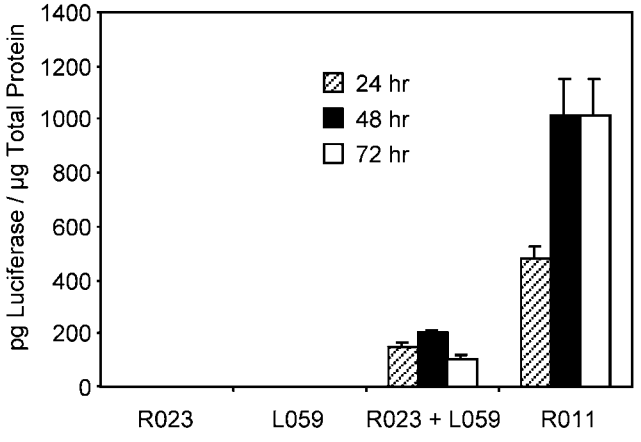

Figure 3 *Comparison of bicistronic construct versus a dual plasmid transfection. BHK cells were transfected with 1 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Transfections and luciferase assays were performed as described in Materials and methods. Error bars indicate the standard error. Transfection with the bicistronic autogene construct (R011) resulted in expression levels that were two- to four-fold higher than the dual-plasmid transfection (autogene and reporter gene on separate plasmids). There is no luciferase expression in the absence of the autogene cassette.*

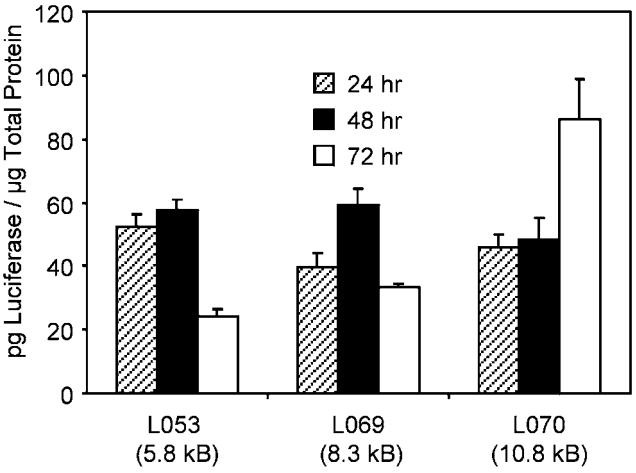

Figure 4 *Plasmid size does not effect the transfection of BHK cells. BHK cells were transfected with a total of 1 µg/well. Equimolar amounts of plasmid were added, and the total mass of DNA per transfection was normalized by adding an unrelated plasmid (pBlueScript). Error bars indicate the standard error. The size of plasmid, ranging from 5.8 to 10.8 kb, does not have an effect on transfection in BHK cells.*

Cytoplasmic expression system results in a 20-fold increase in gene expression per cell, compared to a nuclear expression system

In order to compare the relative efficiency of nuclear *versus* cytoplasmic expression, BHK cells were transfected with equimolar amounts of a CMV-Luciferase (L053) and a bicistronic autogene plasmid containing both the autogene cassette, as well as the luciferase reporter gene cassette (R011). As shown in Figure 5, the autogene system yielded a 20-fold increase in luciferase expression when compared with the CMV-mediated nuclear expression system.

It was of interest to determine whether the increase in luciferase expression was the result of greater levels of luciferase production in each transfected cell or due to an increase in the total number of cells being transfected. The number of cells transfected with the autogene system was experimentally determined and compared with the number of cells transfected with the standard nuclear expression system. Transfected cells were quantified using immunofluorescence with both anti-T7-RNAP and antiluciferase antibodies, and BHK cells transfected with either the autogene or nuclear expression construct. As seen in Figure 6, the autogene and nuclear expression constructs both result in transfection of approximately the same number of cells (autogene $11.4 \pm 3.5\%$, nuclear $10.7 \pm 2.9\%$). The increase in reporter gene expression from the bicistronic autogene construct can therefore be attributed to an increase in the level of gene expression in transfected cells, as opposed to an increase in the number of cells being transfected.

It was important to make sure that the 20-fold increase in expression was not limited to BHK cells. As can be seen in Figure 7, a similar autogene-mediated improvement in gene expression was also observed in the Neuro-2a murine neuroblastoma cell line.

The system described here is initially dependent on the nuclear transcription of T7 RNAP. It was of interest to determine the proportion of nuclear transcripts derived

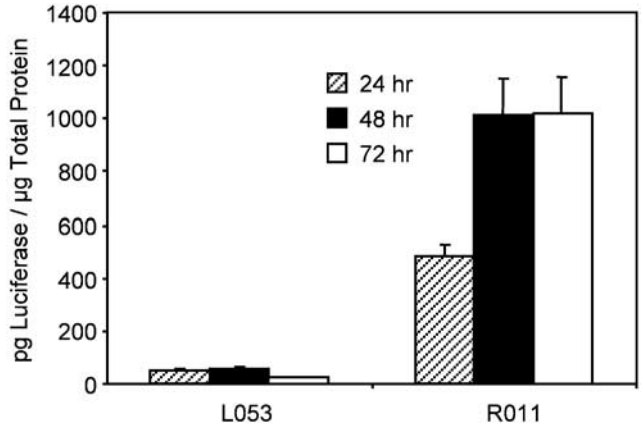

Figure 5 *A comparison of autogene and nuclear expression. BHK cells were transfected with a total of 1 µg/well. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Error bars indicate the standard error. Transfection with the autogene (R011) yielded a 20-fold increase in expression over the standard nuclear expression plasmid (L053).*

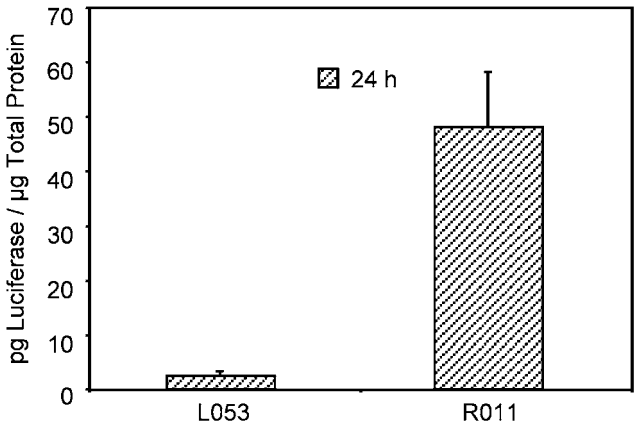

Figure 7 *Increased autogene expression is also seen in Neuro-2a cells. Neuro-2a cells were transfected with a total of 2 µg/well. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Error bars indicate the standard error. Transfection with the autogene (R011) yielded a 20-fold increase in expression over the standard nuclear expression plasmid (L053).*

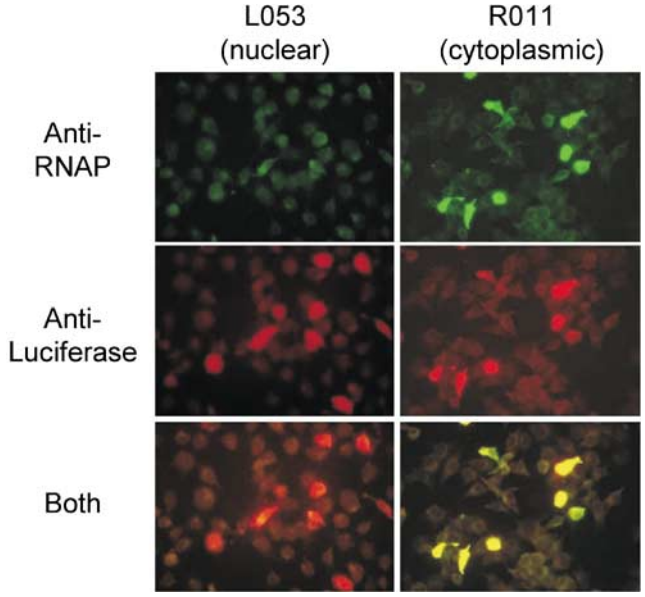

Figure 6 *Immunofluorescence of BHK cells transfected with cytoplasmic or nuclear expression constructs. BHK cells were transfected with equimolar amounts of R011 (autogene) or L053 (nuclear) plasmids. Cells were fixed 24 h post-transfection and subjected to immunofluorescence using anti-T7 RNAP antibodies (with a FITC conjugated secondary) and antiluciferase (with a Texas Red-conjugated secondary). A similar number of cells were transfected when using either the autogene or nuclear expression plasmids. In the case of the autogene transfection, every cell that is expressing RNAP is also expressing luciferase.*

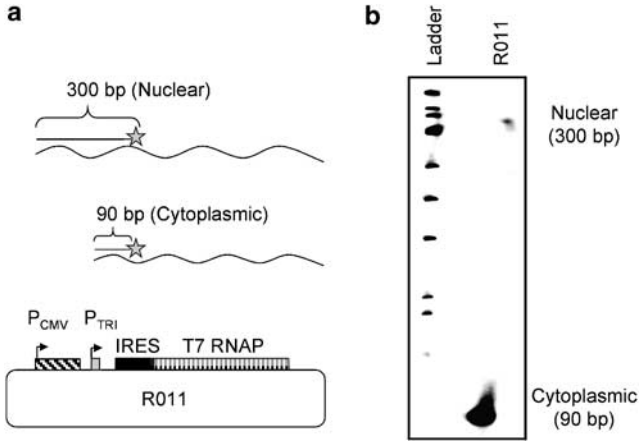

Figure 8 *Primer extension assay performed on BHK cells transfected with the bicistronic autogene construct (R011). (a) The transcripts initiated at the nuclear CMV promoter were predicted to have a longer 5′ UTR, resulting in larger fragments, ~300 bp in size, while transcripts initiated at the T7 promoter were predicted to have a shorter 5′ UTR, ~90 bp in size. (b) Transfection of BHK cells with the bicistronic autogene construct (R011) yielded approximately 57±11-fold more short cytoplasmic transcripts as long nuclear transcripts, indicating that the majority of transcription is initiated by the T7 RNAP in the cytoplasm.*

from the CMV promoter *versus* cytoplasmic transcripts derived from the T7 promoter. As the two promoters have different transcription start sites, the two transcripts will have different length 5′-untranslated regions (UTRs). Therefore, a primer extension assay was performed using a primer that binds downstream of the two promoters, 90 bp downstream from P_{T7} and 300 bp downstream of the P_{CMV}. Figure 8 shows that a much higher proportion of mRNA is transcribed from the T7 promoter than from the CMV promoter (~57±11 fold). This is consistent with previous work that found that the large majority of transcripts in the cell were transcribed by the T7 RNAP in the cytoplasm.[11] This further demonstrates that only a catalytic amount of RNAP needs to be expressed in the nucleus for large amounts of cytoplasmic mRNA to be produced.

Cytoplasmic mRNA transcripts have a shorter half-life than nuclear transcripts

It was of interest to determine the half-life of the cytoplasmic transcripts, as the lack of 5′ cap structure on the cytoplasmic transcripts would be expected to result in a decrease in mRNA stability.[13,17–19] An RNase

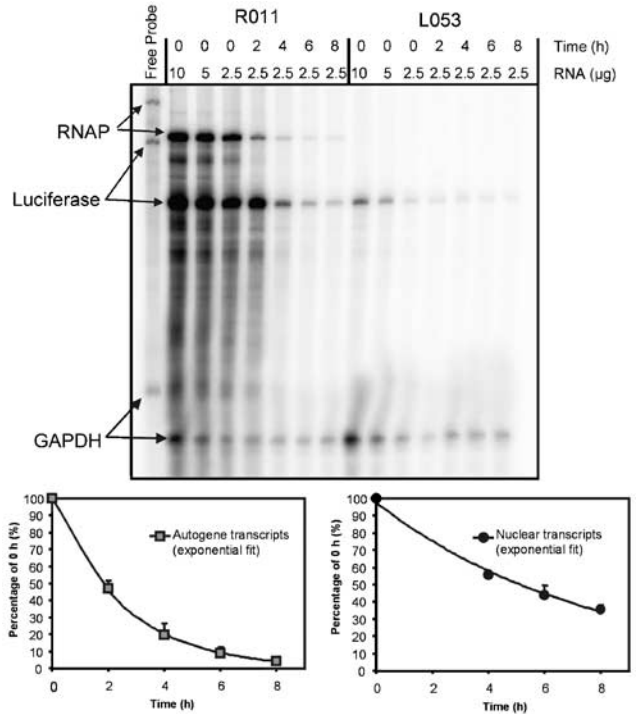

Figure 9 *Ribonuclease Protection Assay of RNA derived from BHK cells transfected with bicistronic autogene construct (R011) or nuclear construct (L053). BHK cells were treated with actinomycin D 24 h post transfection. Total RNA was harvested at 2-h intervals following treatment. In total, 10, 5 or 2.5 µg of total RNA was subjected to an RNase Protection Assay using ^{32}P-labelled probes against T7 RNAP (RNAP) and Luciferase (Luc) transcripts (see Materials and methods). All values were standardized against the GAPDH control. Approximately 20 times as many luciferase transcripts were detected in the autogene-transfected cells as the nuclear-transfected cells. The half-life of the autogene transcripts is approx 103 min, approximately three-fold shorter than the half-life of the nuclear transcripts, 317 min. This is likely because the cytoplasmic transcripts do not contain a 5′ cap structure, known to be important for mRNA stability.*

Protection Assay (RPA) was used to measure both the half-life of the mRNA as well as the relative amounts of RNA present. BHK cells were transfected with equimolar amounts of R011 (autogene) and L053 (nuclear) plasmids. At 24 h post-transfection, 20 µg/ml actinomycin D was added to inhibit all *de novo* RNA synthesis. Previous work had demonstrated that this amount of actinomycin D was sufficient to inhibit $>99\%$ of RNA synthesis (data not shown). Cells were harvested at 2 h intervals and total RNA was isolated. As calculated from Figure 9, the half-life of the autogene transcripts average 103 ± 6 min (88 ± 3 min calculated using the RNAP probe, 115 ± 5 min calculated using the Luciferase probe). The half-life of the nuclear transcripts was 317 ± 6 min. By this analysis, we determined that the cytoplasmic transcripts are not as stable as the nuclear transcripts. Comparing the intensity of the luciferase transcript band from the nuclear and cytoplasmic transfections, there are approximately 20-fold more autogene-derived luciferase transcripts as there are nuclear luciferase transcripts. Given that the half-life of the autogene transcripts is three times shorter than the nuclear transcripts, this suggests that the total output of the autogene system is at least 60-fold higher than the standard nuclear system.

Discussion

Here we describe a novel bicistronic autogene-based cytoplasmic expression system that demonstrates a 20-fold increase in gene expression over a standard CMV-based nuclear expression system. We will discuss the advantages of this system with respect to previous systems, the mechanism of action, and the potential utility of this system.

The system described here has a number of advantages over previously published systems. Previous work with an autogene-based cytoplasmic expression system describes a dual-promoter autogene, consisting of an autogene cassette driven by a CMV promoter.[11] In this system, the initial supply of T7 RNAP was derived from nuclear transcripts via the CMV promoter, obviating the need to add exogenous RNAP protein or mRNA during transfection. Although this system showed increased expression when compared with a standard CMV-based nuclear expression system (approximately two- to three-fold increase), the authors found that the majority of the cytoplasmic transcripts were not being translated, likely due to the lack of 5′ cap required for efficient translation in eukaryotic cells. Other work described the creation of a T7 RNAP autogene containing an EMCV IRES sequence.[15] This IRES element facilitated translation of the uncapped cytoplasmic transcripts. It was also noted that the inclusion of the IRES element attenuated the toxicity of the autogene plasmid in host *E. coli* cells, a major problem with earlier autogene plasmids.[3] We observed some evidence of autogene toxicity in *E. coli*, but isolation of plasmid DNA was easily achieved from bacterial culture. The yield of autogene containing plasmids was approximately 0.5 mg of DNA/l of bacterial culture. This is a marked decrease from the 5–10 mg/l we normally expect from plasmids that share the same vector backbone, but do not contain the autogene sequence. We are currently investigating methods by which the toxicity of autogene plasmids may be further alleviated.

The bicistronic autogene system described here is distinguished from previous systems by two key properties. First, it contains both a CMV promoter, bypassing the need for addition of exogenous RNAP protein during transfection, as well as an autogene containing an EMCV IRES sequence, allowing for cap-independent translation of the autogene transcripts. In addition, our system has the autogene cassette and reporter gene cassette on the same plasmid, further simplifying the transfection process and resulting in increased transgene expression.

When we compared the expression levels from our cytoplasmic expression system and a standard nuclear expression system, the cytoplasmic system yielded 20-fold higher expression than the nuclear system. This is in contrast with previous systems that demonstrated a maximum of three-fold increase over a nuclear expression system control.[11] The improvement in performance is most likely due to increased translation of cytoplasmic transcripts generated from our modified expression system. The inclusion of an EMCV IRES element in the autogene cassette described here appears to enhance translation, overcoming the lack of a 5′ cap on cytoplasmic transcripts and resulting in increased transgene expression levels.

We tested our autogene system in Neuro-2a cells and also observed a 20-fold increase in expression with the autogene, as compared with the CMV-based system. This indicates that the autogene system is not limited to BHK cells. However, it should be noted that variable gene expression was seen when using other cell lines and we are currently investigating this phenomenon.

The mechanism whereby the bicistronic autogene system results in increased gene expression is of obvious interest. It was important to verify that the T7 autogene, which has been the basis for all cytoplasmic expression systems to date, truly does exhibit an autocatalytic expression profile. For the results described in Figure 2, assuming that the amounts of substrates and PT7-Luc are not limiting, it is straightforward to show that if an autocatalytic process is occurring, then $N_L(t)=ce^{t/\tau}$, where $N_L(t)$ indicates the number of luciferase molecules at time t and c and τ are constants. The close fit ($R^2=0.94$) of an exponential profile to the luciferase expression observed in Figure 2 thus supports an autocatalytic mechanism. Any deviation from exponential characteristics at longer times can be attributed to either saturation effects as the amount of PT7-Luc becomes limiting, or the system running out of substrate (eg charged tRNA, GTP, etc).

The primer extension and RPA data in Figures 8 and 9 provide further evidence of a cytoplasmic autocatalytic process. As demonstrated in the results presented in Figure 9, there is at least a 20-fold increase in transgene mRNA levels with the cytoplasmic expression system as compared to the standard nuclear expression system. These transcripts had a much shorter half-life than their nuclear counterparts, which is consistent with the lack of a 5′ cap, an important determinant of mRNA stability. When combined with the primer extension data showing that the majority of the transcripts are being made by the T7 RNAP, these data suggest that the increase in gene expression is due to an increase in mRNA levels in the cytoplasm of transfected cells, consistent with the autocatalytic process.

There are many possible explanations for why the bicistronic construct is more effective than a dual plasmid transfection. One possible reason is that the T7 RNAP is able to transcribe RNA from either the first P_{T7}, driving T7 RNAP expression, or the second P_{T7}, driving luciferase expression in the bicistronic construct. Due to the lack of terminator sequence between these two genes, both transcripts will encode for the luciferase gene. Therefore, the cells transfected with the bicistronic plasmid should have more mRNA encoding luciferase than the cells in the dual transfection. Upon examination of the RPA data (Figure 9), it is clear that there are at least twice as many luciferase transcripts than RNAP transcripts following bicistronic transfection, lending support to this hypothesis. In addition, it was found that the luciferase transcripts had a slightly longer half-life than the T7 RNAP transcripts (115 *versus* 88 min). This increased half-life may be attributed to the fact that the luciferase transcripts being made from the first P_{T7} in effect had a much longer 5′ UTR. This would most likely add some stability to the transcript, therefore increasing its half-life and subsequent luciferase expression.

The potential applications of an autogene-based cytoplasmic expression system are many. Aside from increasing the levels of gene expression in plasmid-based nonviral gene-delivery systems, this system could also be used as a tool to express high levels of transgene *in vitro* for characterization or purification purposes.

In summary, the studies described here demonstrate a novel, bicistronic autogene-based cytoplasmic expression system that shows 20-fold higher levels of gene expression compared with a nuclear expression system. This system has been shown to exhibit an exponential autocatalytic gene expression profile, and result in an increase in reporter gene expression per transfected cell, as opposed to an increase in the number of cells transfected. Furthermore, the bicistronic system has been demonstrated to be more effective than a cytoplasmic expression system carried on two plasmids. This system has a wide range of applications, not the least of which is increasing the therapeutic utility of plasmid-based gene-delivery systems.

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