Factors limiting autogene-based cytoplasmic expression systems

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ABSTRACT

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 non-viral gene delivery systems, where the levels of gene expression achieved are usually well below the levels achieved by viral gene transfer systems. Previous work from our laboratory describes an enhanced dual promoter autogene-based cytoplasmic expression system that gives rise to levels of gene expression 20-fold higher than that of a CMV nuclear expression plasmid control. Here various strategies are described to increase the levels of autogene-based gene expression by changing variables such as the type of nuclear promoter, phage RNAP gene, and IRES element. Although insights into the function of various IRES elements were gained, none of these changes demonstrated a significant increase in gene expression. However, determination of the mRNA levels achieved using quantitative RNase protection assays and immunofluorescence experiments revealed that transgene mRNA levels were saturated at up to 10 times higher than all other mRNA in the transfected cell combined. It follows that mRNA production, as well as translation, are important factors limiting autogene-based cytoplasmic expression.

Key words: gene therapy • autogene • T7 RNAP • mRNA production • IRES

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 non-viral 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 (1–3). The development of a dual promoter autogene-based cytoplasmic expression system in 1999 (4) was a substantial advance in the field. However, the full promise of this system has yet to be realized. Dual-promoter autogenes consist of an RNAP gene driven by its own cognate promoter. In order to avoid the requirement for pre-

existing RNAP to initiate the expression system, a nuclear promoter can be added upstream of the RNAP promoter. Catalytic levels of RNAP expression then occur via the nuclear promoter, and the resulting RNAP in the cytoplasm drives the cytoplasmic expression system, producing RNA from plasmid DNA templates in the cytoplasm.

Internal ribosome entry sequences (IRES) are elements that have been shown to increase the translation of un-capped transcripts or drive expression of a second gene in a bi-cistronic construct. Various viral IRES elements, such as the EMCV (encephalomyocarditis virus), FMDV (foot and mouth disease virus), and other picornaviruses based IRES elements share similar features. All are approx 450 bp long and share a conservation of secondary structure, as well as a pyrimidine-rich tract that starts ~25 bp before the 3' end of the IRES (5–7). It is thought that the secondary structure is essential for IRES function. The secondary structure is hypothesized to allow for proper alignment of ribosome subunits and other co-factors necessary for translation. Other IRES sequences (e.g., Gtx) are much shorter than picornaviral IRESs and contain sequences that are complementary to rRNA (8). Other mammalian IRES sequences (eIF4G) contain a polypyrimidine tract and some structural similarities to the EMCV IRES but are shorter than picornaviral IRESs (~100 bp) (9). Previous studies (8, 9) have shown that these smaller IRES sequences can give rise to much higher expression levels than the EMCV IRES that we currently use in our cytoplasmic expression system.

In our previous work, a novel cytoplasmic expression system that resulted in a 20-fold increase in the levels of gene expression over a standard CMV-based nuclear expression system was described (10), comparing favorably with the 2–3 fold increase seen with previous similar systems (4). The potential for this system to give even greater increases in expression prompted further examination of this system, specifically to determine the factors limiting the cytoplasmic expression system and ways in which the levels of gene expression can be increased.

Specifically, the effects of changing the nuclear promoter, RNAP gene, and IRES sequence on autogene expression are examined. We find that mRNA production, as well as translation, are most likely the limiting factors for cytoplasmic autogene expression.

MATERIALS AND METHODS

Plasmids and primers

Plasmid R011 is a bi-cistronic plasmid consisting of a basic autogene cassette (driven by the T7, T3, and SP6 RNAP promoters) driven by a CMV promoter and intron, as well as a downstream *Photinus pyralis* luciferase reporter gene cassette (for construction details, see Finn et al, 2003). L053 consists of the CMV promoter (with intron) from NGVL3 and the *Photinus pyralis* luciferase gene. R031 and L071 consist of R011 or L053, respectively, with the CMV promoter replaced with the SV40 promoter. R032 and L072 consist of R011 or L053, respectively, with the CMV promoter replaced with the RSV promoter. R063 and R068 consists of R011 with the T7 RNAP gene replaced with the SP6 RNAP gene from pSR3, or the T3 RNAP gene from pTG100, both of which are a gift from W. T. McAllister (Department of Microbiology and Immunology, State University of New York Health Science Center at Brooklyn). R017 consists of the T7 RNAP gene driven by the CMV promoter (and intron) from NGVL3. L059 consists of a pTRI-Amp (Ambion) backbone with EMCV IRES, *Photinus pyralis* luciferase, and beta-globin

poly-adenylation site derived from EMC-Luc, a gift from Jon Wolff (Waisman Center, WI). L076 consists of L059 with the EMCV IRES replaced with the eIF4G IRES from pCatEIF4GGal, a gift from Caroline Lee (Faculty of Medicine, National University of Singapore). L059+GTX consists of L059 with the EMCV IRES replaced with the 10 tandem GTX sequences from p(Gtx133-141)10(SI)9BRPh, a gift from Vincent Mauro (Department of Neurobiology, Scripps Research Institute). PT7-Luc (Promega, Madison, WI) consists of the *Photinus pyralis* luciferase gene driven by a T7 RNAP promoter. L080 consists of PT7-Luc with the Luciferase gene driven by both the T7 and the SP6 promoter. 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. We have performed sequencing and restriction enzyme digests confirming that the luciferase coding regions in the constructs used in this work correspond to the published sequence for *Photinus pyralis* luciferase.

mRNA Synthesis

mRNA synthesis was performed using a MEGAscript high-yield transcription kit (Ambion). For all transcripts used for in vitro transfection, plasmid template (L059 for IRES-Luc and Cap-IRES-Luc mRNA; L080 for Cap-Luc mRNA; PT7-Luc for Luc mRNA) was linearized using *EcoR* 1, and 1 µg of each plasmid was used per manufacturer's protocols. mRNA was recovered by the LiCl procedure as outlined in manufacturer's protocol. For the synthesis of R011 mRNA (used for RPA standard curve), undigested R011 was used as template for mRNA synthesis, as the undigested plasmid more closely resembles that present in the cytoplasm and would serve as a better control than digested plasmid. After LiCl precipitation, the RNA pellet was washed 3´ with 70% ethanol to completely remove any unincorporated NTPs.

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 heat-inactivated serum-containing media before addition directly to cell media. BHK 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 (pPUC19) to normalize the total mass of DNA in each transfection. For mRNA transfections, TransMessenger reagent (Qiagen) was used to transfect 0.6 µg of various mRNA transcripts into BHK cells (30,000 cells per well in 24-well plates). An RNA to TransMessenger reagent ratio of 1:4 was used for all transfections. Transfections were performed per manufacturer's instructions. Three hours post-transfection, the medium was removed and replaced with fresh serum containing medium. Cells were harvested and subjected to luciferase assays at time points indicated. All transfections were performed in triplicate. Data is 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 ºC. 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 is 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). Total protein was quantified using a Pierce BCA assay kit as per manufacturer's instructions.

Immunofluorescence

BHK cells were plated in sixwell plates (150,000 cells per well) and transfected with various amounts 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 being permeabilized 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 primary antibody solution consisting of a 1:700 dilution of goat anti-T7 RNAP antibody, a gift from Paul Fisher (Department of Pharmacological Sciences, State University of New York at Stony Brook) 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 labeled (QED Bioscience Inc.; 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. Percentage of cells transfected was determined by counting transfected and non-transfected cells under the microscope. Data indicate the average of six separate counts from at least three different experiments.

RNase Protection assay

Luciferase probe was prepared from *EcoR* 1 digested RPA-Luc. GAPDH probe was purchased from Pharmingen. Probes were labeled following the manufacturers protocol using ^{32}P-αUTP (3000 Ci/mmole, 10 mCi/mL; NEN).

BHK cells were plated on six-well plates (150,000 cells per well) and transfected with various amounts of R011. At various time points, cells were washed once with PBS and recovered by treatment with trypsin. Total RNA was harvested from cells using an RNeasy miniprep kit (Qiagen). Various amounts of sample RNA were subjected to RNase protection analysis using the RiboQuant RPA system (Pharmingen) according to the manufacturer's protocol. In all experiments, the total amount of RNA was brought up to 2 μg using untransfected BHK total RNA. Standard curves were prepared from in vitro synthesized mRNA from supercoiled R011 as described above. These mRNA transcripts are similar to those that will be generated from the cytoplasmic system. Serial dilutions of the R011 mRNA were made and various amounts were added to 2 μg untransfected BHK total RNA and subjected to RPA analysis. All values are standardized to the GAPDH control, and are the average +/– standard deviation of at least three independent experiments. Data were collected using a Typhoon Phosphoimager (Amersham Biosciences), and analysis was performed using ImageQuant software (Amersham Biosciences).

Note that when quantitating the GAPDH control, the blots were overexposed to give a sufficient value for GAPDH mRNA levels.

Calculation of amount of transgene mRNA per transfected cell

Using the standard curve from the quantitative RNase protection assay, the amount of luciferase mRNA present in each mRNA sample was calculated. Knowing the total amount of RNA loaded on the gel, the amount of luciferase mRNA per μg of total RNA was determined. Using immunofluorescence to determine the percentage of cells transfected, the amount of luciferase mRNA produced per transfected cell could be determined. For example: for 24 h time point on Fig. 6, 1.7 ng of luciferase mRNA was present in the sample (value derived from standard curve in Fig. 5*C*). 0.5 μg of total RNA was used for that sample, so the total amount of luciferase mRNA per μg of total RNA is 1.7 ng/0.5 μg = 3.4 ng luciferase mRNA/μg total RNA. By examining the immunofluorescence data, we found that ~3% of the cells were transfected with the autogene. Therefore, 3.4 ng luciferase mRNA/μg total RNA X 100/3 (dilution factor) = 113 ng luciferase mRNA/μg total RNA/transfected cell. Therefore, 0.113 μg luciferase mRNA/μg total RNA/transfected cell.

RESULTS

Replacing the CMV promoter with either the RSV or SV40 nuclear

Promoter has little effect on autogene expression

The enhanced dual-promoter autogene system described previously (10) relied on the CMV promoter for the first round of nuclear transcription, effectively "triggering" the cytoplasmic expression system. It was of interest to explore the effect of other commonly used promoters. To this end, autogenes and their nuclear controls containing either the RSV or the SV40 promoters were constructed. As can be seen in Fig. 1*B*, the different promoters had very little effect on the maximum levels of autogene activity. In contrast, the different promoters had a dramatic effect on the levels of nuclear gene expression. In the case of the SV40 promoter, the autogene system demonstrated a more than 200-fold increase in expression over the nuclear system. It is important to note that even though the different promoters resulted in dramatically different amounts of nuclear expression [consistent with previously published data (11)], they had very little effect on the maximum levels of cytoplasmic expression. This is consistent with only a small, catalytic amount of nuclear expression required to drive the cytoplasmic expression system and that the cytoplasmic expression was reaching an apparent saturation level.

Autogene expression is not sensitive to the type of phage RNAP used

It was of interest to compare the T7 RNAP protein with the two other RNAP proteins commonly used for in vitro transcription purposes, the SP6 and T3 RNAPs (12–14). To this end, the T7 RNAP gene in R011 was replaced with either the SP6 or the T3 RNAP gene. The R011 vector plasmid already had all three of the RNAP promoters in tandem, so there was no need to "add" in the promoter fragments. As can be seen in Fig. 2*B*, using either the T3 or the SP6 RNAP in place of the T7 RNAP did not give a substantial increase in gene expression. It is noted that the

T3 RNAP did show almost a twofold increase in gene expression. However, this increase was not found to be significant (*P*>0.05).

Substitution of the EMCV IRES by either the Gtx or eIF4G IRES elements reduced autogene expression

Higher expression levels have been observed using IRES elements other than the EMCV used in the expression system used here (8, 15). Therefore, luciferase reporter plasmids were constructed containing either the EMCV, eIF4G, Gtx, or no IRES element at all, and these plasmids were tested in an in vitro transfection system, co-transfecting with a nuclear plasmid encoding for the T7 RNAP. As can be seen in Fig. 3*B*, only the EMCV IRES was efficient at driving cap-independent translation of cytoplasmic transcripts. The eIF4G and Gtx IRES sequences appeared to have little if any effect on translation over the control that contains no IRES (which demonstrated background levels of gene expression). Similar results were seen using a cell free transcription and translation assay (data not shown). These results indicate that the EMCV IRES was the most effective IRES in the cytoplasmic expression system and that no advantage was to be gained by using either of the other two classes of IRES sequences.

Nuclear mRNA transcripts are translated 20 times more efficiently than cytoplasmic autogene transcripts

In an effort to understand what other factors may be limiting the expression levels, the translation efficiency of the cytoplasmic transcripts (IRES-Luciferase) was compared with the nuclear transcripts (5' cap-Luciferase). mRNA was synthesized in vitro, and the translation efficiency of these transcripts was determined via in vitro mRNA transfection. As can be seen in Fig. 4, although the inclusion of the EMCV IRES does result in an increase in expression over no IRES at all, the cytoplasmic transcripts are translated ~20 times less efficiently than the capped nuclear transcripts. It is also interesting to note that the inclusion of the EMCV IRES into a capped transcript inhibited the expression ~fivefold. This is consistent with previously published data (16). Although it has long been known that IRES elements are not as efficient at recruiting ribosomes as the 5' cap of mRNA transcripts, it is clear that the low translation of the cytoplasmic transcripts (as compared with a capped mRNA) is playing a major role in lower levels of gene expression than were expected. However, the low translation of the cytoplasmic transcripts may not be the primary factor limiting autogene expression. The observation that a 20-fold increase in luciferase protein expression is obtained with the cytoplasmic expression system, while the mRNA transcripts are being translated 20 times less efficiently than the capped nuclear transcripts, suggested the possibility that one of the factors responsible for reaching limiting levels of gene expression was at the level of mRNA production.

mRNA Production is saturated during autogene expression

Given that the cytoplasmic transcripts are translated much less efficiently than the nuclear transcripts, it was clear that the RNA output of the cytoplasmic system must be much higher than that of the nuclear system in order to achieve the marked increase in luciferase expression. Therefore, a quantitative RNase protection assay (RPA) was performed to determine the amount of luciferase mRNA being produced. As can be see in Fig. 5*C*, the amount of luciferase mRNA in the autogene transfection peaks at around 24 h post transfection. Using the standard curve and

determining the percentage of cells transfected using immunofluorescence, we determined that at 30 h, ~12 (+/– 0.4)% of the total RNA in each transfected cell was luciferase mRNA. Noting that in BHK cells only ~3% of the total RNA is mRNA (data not shown), this indicates that ~3.5 times more luciferase transcript was being produced than the sum of all other mRNA transcripts in the cell. Since this represents a significant allocation of cellular resources (such as NTPs, including ATP, the major energy source of the cell), it appeared likely that mRNA production was a major factor limiting autogene expression. To test this hypothesis, a dose response study was performed and RPA and immunofluorescence were used to determine the amount of luciferase transcript per transfected cell. If mRNA production was a limiting factor, we would expect to see a point at which the addition of further plasmid has no effect on mRNA production. Cells were harvested 24 h after transfection to ensure that mRNA levels had not reached a saturating level and had begun to decrease (see Fig. 5), possibly due to either a decrease in the amount of cytoplasmic plasmid or due to cytotoxic effects that might be expected to result from the large amount of foreign mRNA production. As can be seen in Fig. 6, the amount of luciferase transcript produced reaches a saturation point at approximately 30 (+/– 5) % of the total RNA. This observation is supported by the presence of large-molecular-weight mRNA species when total RNA from transfected BHK cells was run on a denaturing agarose gel and subjected to ethidium bromide staining (data not shown). These large mRNA species were not present in RNA from non-transfected cells. This finding is consistent with the cells only being able to produce a finite amount of luciferase mRNA, and adding more plasmid cannot increase this level. This suggests that the amount of transgene mRNA production, in addition to its poor translation, is an important factor limiting autogene-based cytoplasmic expression.

DISCUSSION

Previous work described a dual-promoter autogene-based cytoplasmic expression system that demonstrated a 20-fold increase in gene expression over a standard CMV-based nuclear control. In the present work, attempts were made to increase the levels of cytoplasmic gene expression further, revealing some of the factors limiting gene expression in this system. Here, we discuss the changes made to the autogene system, the finding that mRNA production is a limiting factor in autogene expression, and the implications of this result for future studies.

In an effort to further increase gene expression levels, the effect of changing the nuclear promoter (CMV vs. RSV vs. SV40), the RNAP gene (T7 vs. SP6 vs. T3), or the IRES (EMCV vs. eIF4G vs. Gtx) element was examined. We found that altering any of these three parameters did not increase autogene expression significantly.

For example, replacing the T7 RNA polymerase with either the SP6 or the T3 RNAP (both commonly used in in vitro transcription systems) resulted in very minimal (maximum twofold) increase in autogene expression. In fact, the SP6 RNAP appeared to be slightly less effective at driving autogene expression than either the T7 or the T3 RNAP. It should be noted that the T7 and T3 RNAP share almost 80% amino acid identity, while the SP6 RNAP shares about 25% identity with both the T7 and the T3 RNAP. It is known that the SP6 RNAP does not effectively transcribe linear DNA (17). It may be that cytosolic nucleases convert the supercoiled plasmid into a linear form (18), therefore accounting for the decreased efficiency of the SP6 RNAP when compared with the T7 or T3 RNAP. Regardless of the mechanisms behind the differences, using either the SP6 or the T3 RNAP did not give any appreciable increase in autogene expression.

Although other laboratories have reported increased expression when using other classes of IRES elements [60-fold increase with Gtx IRES (8), 100-fold increase with eIF4G IRES (15)] in a bi-cistronic context, no such enhancement was observed here. The most likely explanation for this is that the eIF4G and Gtx IRES sequences have thus far only been used to drive expression of the second gene in a bi-cistronic transcript. These transcripts are made in the nucleus and therefore contain a 5' cap structure. It is most likely that these small sequences do not recruit ribosomes de novo, but facilitate the re-initialization of translation of the ribosome after the first gene has been translated. The type of bi-cistronic system that these small IRES elements were studied in previously is not the same as the system tested in this study. Here the ability of the IRES elements to directly recruit ribosomes to the 5' end of an uncapped mRNA transcript is assessed. The eIF4G and GTX IRES elements cannot recruit ribosomes to an uncapped mRNA transcript. The larger viral EMCV IRES has a complex secondary structure (19–22), which might be able to recruit ribosomes de novo, thus accounting for its ability to increase the translation of uncapped cytoplasmic transcripts. This hypothesis is consistent with certain controversial aspects of IRES sequences and how they function. Convincing evidence indicates that IRES elements act as true internal ribosome entry sites [indicated by the ability for the EMCV IRES to allow translation of circular transcripts (23)], as well as evidence suggesting that the IRES element only act as rescue translation stimulators (16), allowing the ribosome to re-initiate translation when inserted after the stop codon of the first cistron in a bi-cistronic construct. The evidence presented here suggests that, although the Gtx and eIF4G IRES elements may be effective in driving second gene expression in a bi-cistronic construct, they are ineffective for recruiting ribosomes de novo in the absence of a 5' cap structure.

The studies performed to determine factors that may be limiting the autogene expression indicate that the major factors limiting the autogene expression were both at the level of mRNA production and translation. It is clear that the cytoplasmic expression system is producing a large amount of mRNA (corresponding to transgene mRNA levels that are 10 times the sum of every other transcript in the cell). This significant allocation of cellular resources (such as NTPs) would be expected to eventually have a detrimental effect on cell growth. This is consistent with the results summarized in Fig. 6, where it is demonstrated that the autogene expression rapidly decreases after 24 h. This could arise because of the short half-life of the cytoplasmic transcripts, the high levels of foreign mRNA production having a cytotoxic effect, or because of the loss of cytoplasmic plasmid (due to cytosolic nuclease activity).

Although it cannot be stated conclusively that transgene mRNA saturation is responsible for limiting the autogene expression, it cannot be ruled out as a potential factor. Alternately, the cytoplasmic translation machinery may be saturated, meaning that the translation of the mRNA is the limiting factor. This seems unlikely, since the relatively low levels of protein expression observed (~1% of total cellular protein) are relatively low when compared with a viral system, such as that of vaccinia virus, where up to 10% of the total cellular protein comprises viral proteins (24). Most likely an equilibrium exists between mRNA levels and the translation of the cytoplasmic transcripts, and increasing either the levels of mRNA or the translation of the cytoplasmic transcripts would lead to a further increase in expression.

The cytoplasmic expression system described here has many potential applications. An example is suicide cancer gene therapy where a large amount of a suicide gene (e.g., Thymidine Kinase) is required to be expressed, leading to the death of that cell and a corresponding large bystander

effect. For researchers using siRNA or ribozyme expression, this system could be used for producing high levels of RNA in transfected cells. In addition, future work aimed at increasing the translation efficiency of the cytoplasmic transcripts could lead to a dramatic increase in gene expression, which would improve the utility of non-viral gene therapy based systems.

In conclusion, the results presented here demonstrate that modifications to the dual promoter autogene-based cytoplasmic expression system, including changes in the nuclear promoter, RNAP gene, or IRES element, had a minimal effect on autogene expression. The factors responsible for limiting autogene expression are most likely at both the level of transgene mRNA saturation, and the poor translation of the cytoplasmic transcripts. This work demonstrates the ability of autocatalytic expression systems to produce extremely large amounts of transgene mRNA and that advances in improving the translation efficiency of un-capped, cytoplasmic transcripts could lead to a significant increase in the utility of such systems.

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Fig. 1
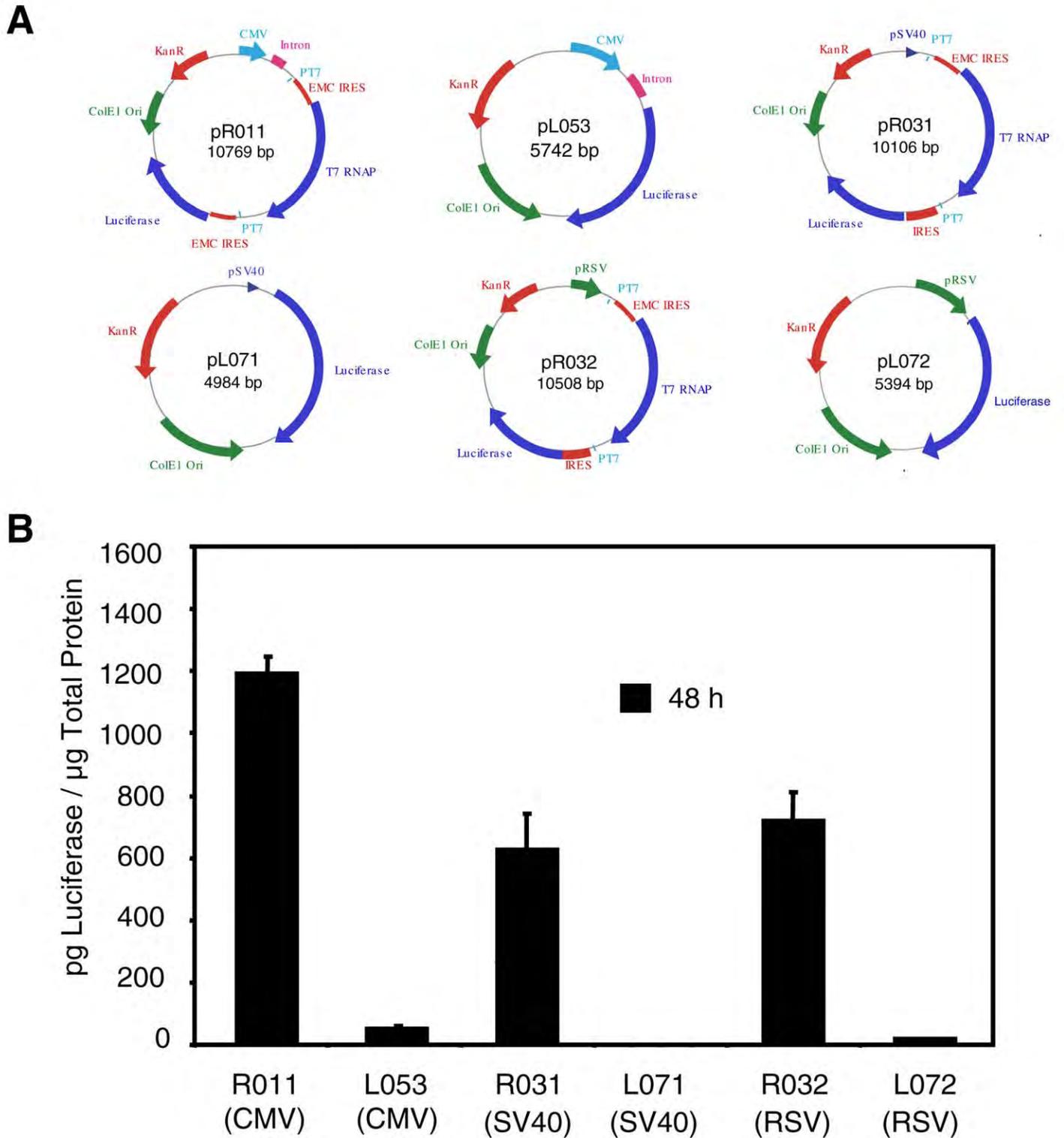
Figure 1. Nuclear promoter comparison. *A*) Diagrams of plasmids used. *B*) Effect of various nuclear promoters on cytoplasmic versus nuclear expression. BHK cells were transfected with 0.75 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pPUC19). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error.
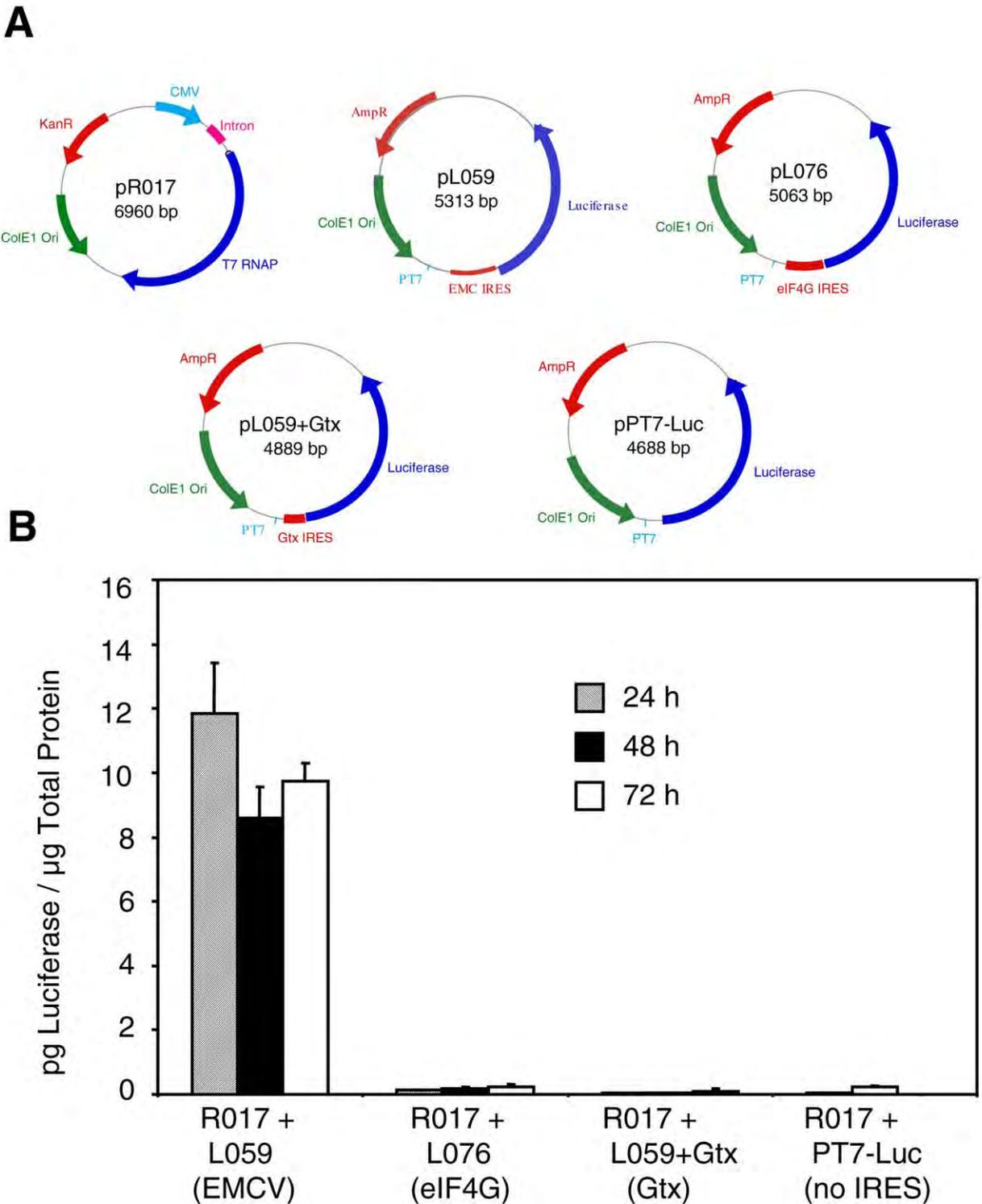
Fig. 2
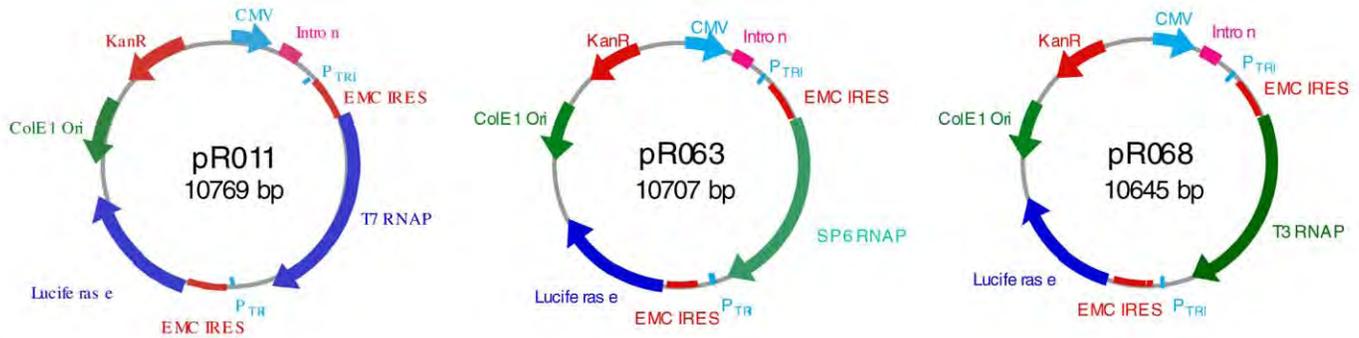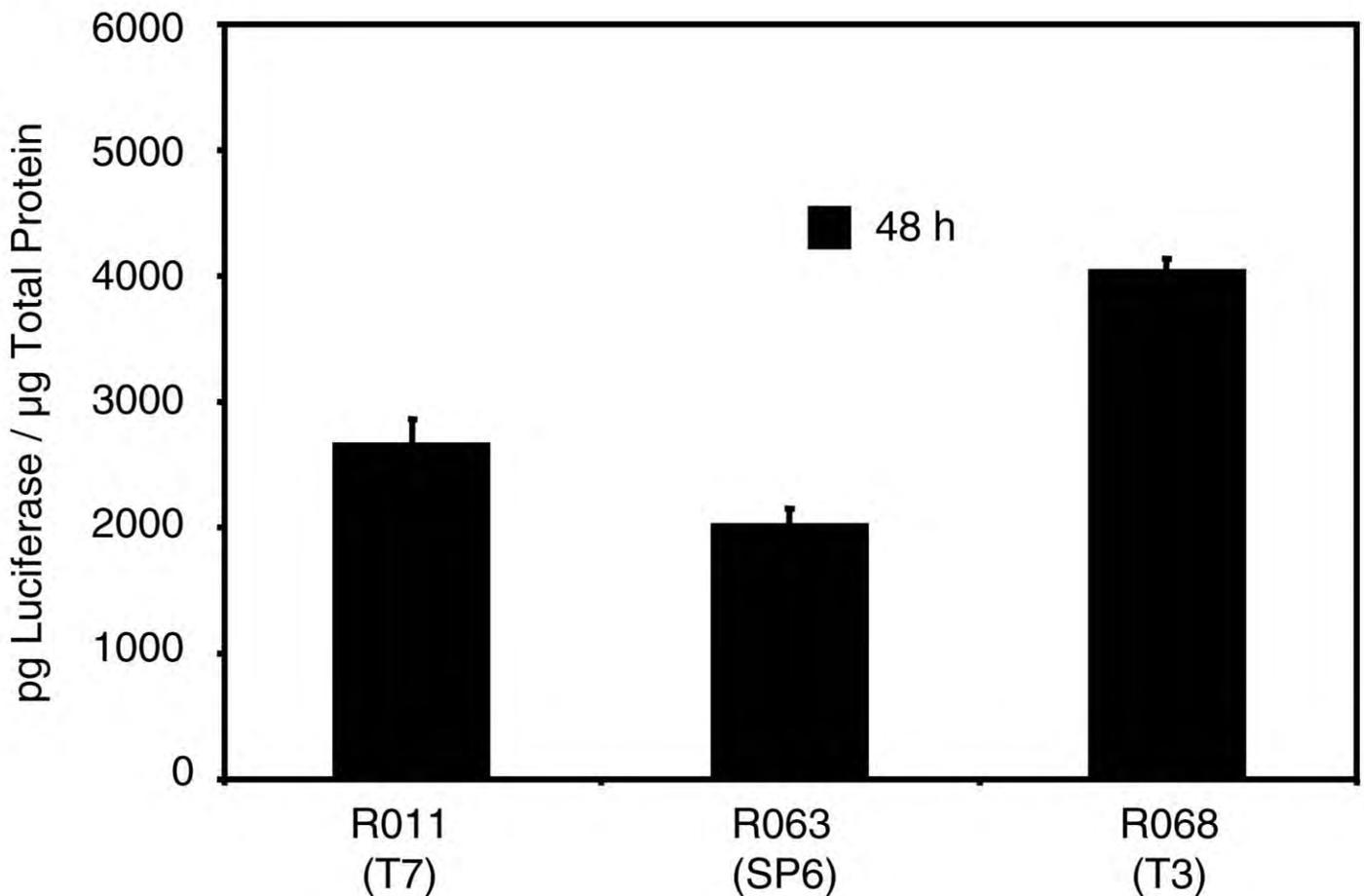
Figure 2. RNAP comparison. *A*) Diagrams of plasmid constructs used. *B*) Comparison of T7, SP6, and T3 RNAP in autogene-based cytoplasmic expressions system. BHK cells were transfected with 0.75 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmids (pPUC19). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error. Transfection with either of the bi-cistronic autogene constructs (R011, R063, or R068) resulted in similar expression levels after 48 h. The T3 RNAP based autogene (R068) resulted in slightly (less than twofold) higher expression than the T7 RNAP based autogene (R011).

Fig. 3

Figure 3. IRES comparison. *A*) Diagrams of plasmid constructs used. *B*) Effect of IRES on cytoplasmic expression. BHK cells were transfected with a total of 0.75 µg/well of plasmid DNA. Half of the amount of DNA was R017 (CMV-T7-RNAP), and the other half was the T7 RNAP based luciferase expression cassette. Equimolar amounts of experimental plasmid were added, and the total mass of DNA per transfection was normalized by adding an unrelated plasmid (pPUC19). Error bars indicate standard error.

Fig. 4
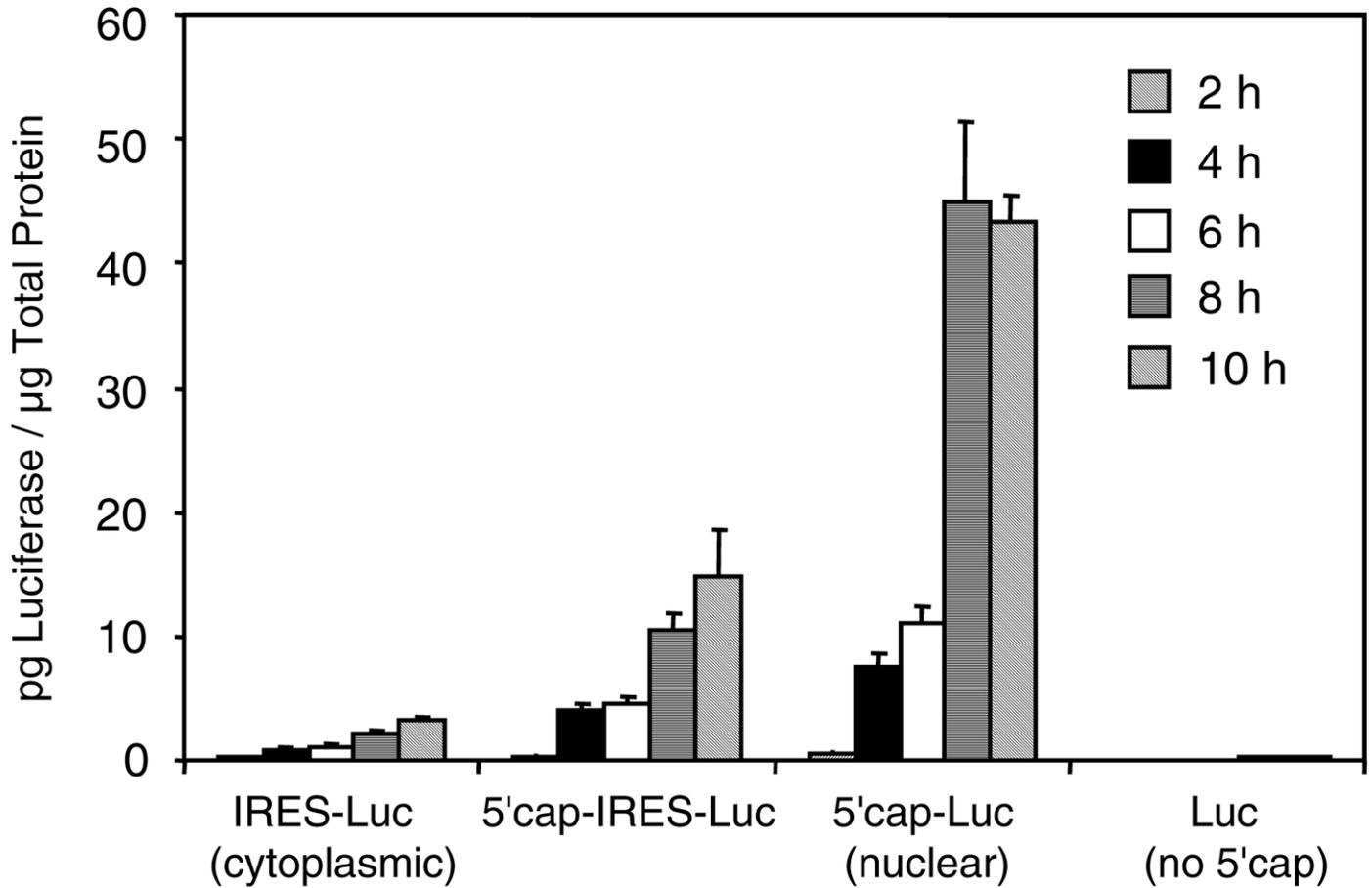
Figure 4. Comparison of translation efficiency of nuclear versus cytoplasmic mRNA transcripts. BHK cells were transfected with a total of 1 µg/well of in vitro synthesized mRNA using Transmessenger reagent as described in Materials and Methods. All transcripts were of a similar size. Error bars indicate standard error. Capped transcripts (nuclear) were translated 20 times more efficiently than uncapped transcripts containing the EMCV IRES (cytoplasmic). The insertion of the IRES into a capped transcript decreased the translation fivefold. The lack of any IRES sequence or cap resulted in background levels of expression.

Fig. 5
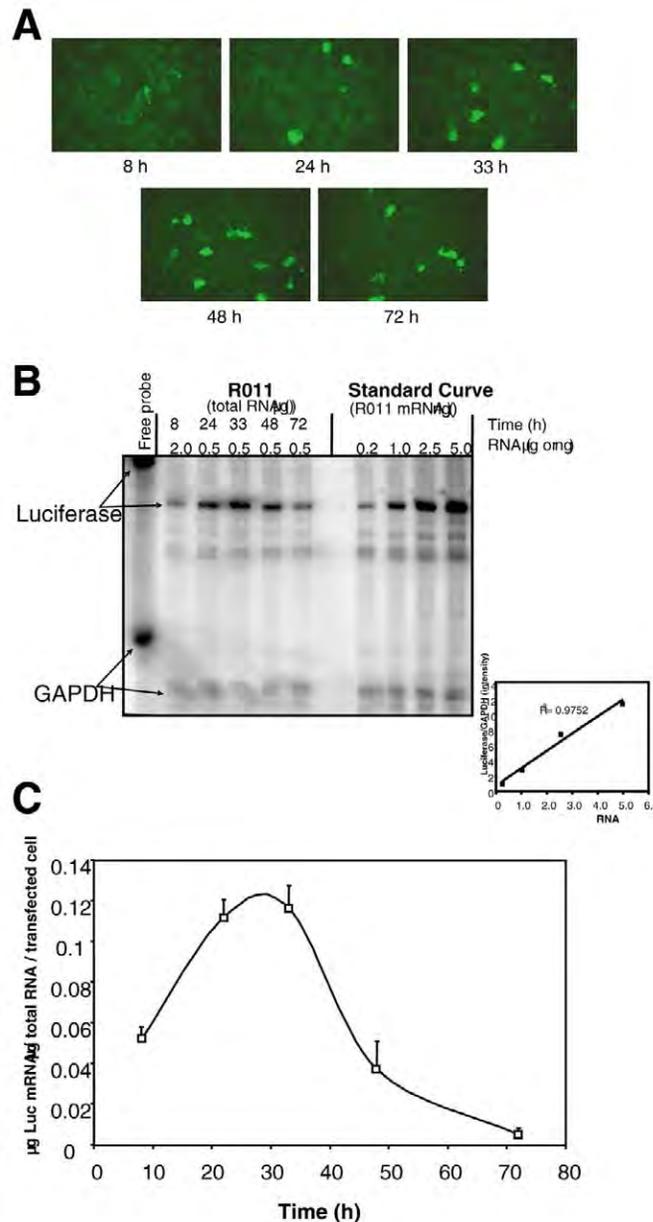
Figure 5. Amount of transgene mRNA per transfected cell. *A*) Immunofluorescence of BHK cells transfected with R011. BHK cells were transfected with 0.75 µg of R011. Cells were fixed at time points indicated and subjected to immunofluorescence using anti-T7 RNAP antibodies and a FITC conjugated secondary antibody. The percentage of cells transfected was used to calculate the amount of transgene mRNA expressed per transfected cell. *B*) RNase Protection Assay (RPA) of BHK cells transfected with R011. BHK cells were transfected with 0.75 µg of R011 and total RNA was harvested at time points indicated as described in Materials and Methods. Various amounts of total RNA (ranging from 0.5 to 2 µg) were subjected to RPA analysis using a ^{32}P-labeled probe against luciferase mRNA as well as GAPDH control mRNA. An R011 standard curve was prepared by spiking 2 µg of untransfected BHK total RNA with various amounts (0.25 to 20 ng) of in vitro synthesized R011 mRNA. The inset is a graph of the standard curve of R011 mRNA. Intensity values were obtained using ImageQuant and luciferase mRNA values were standardized against GAPDH values (to control for lane loading differences). Blots were overexposed to get sufficient values for GAPDH controls. Standardized intensities were plotted against amount of R011 mRNA, and this standard curve was used to determine the amount of luciferase mRNA in experimental samples. *C*) Amount of luciferase mRNA produced per transfected cell over time. BHK cells were transfected with 0.75 µg of R011 as seen in Figure 5. The amount of luciferase mRNA per transfected cell was determined as described in Materials and Methods. The amount of luciferase mRNA peaks at around 30 h at ~12% of the total RNA, and then rapidly decreases after 72 h.

Fig. 6
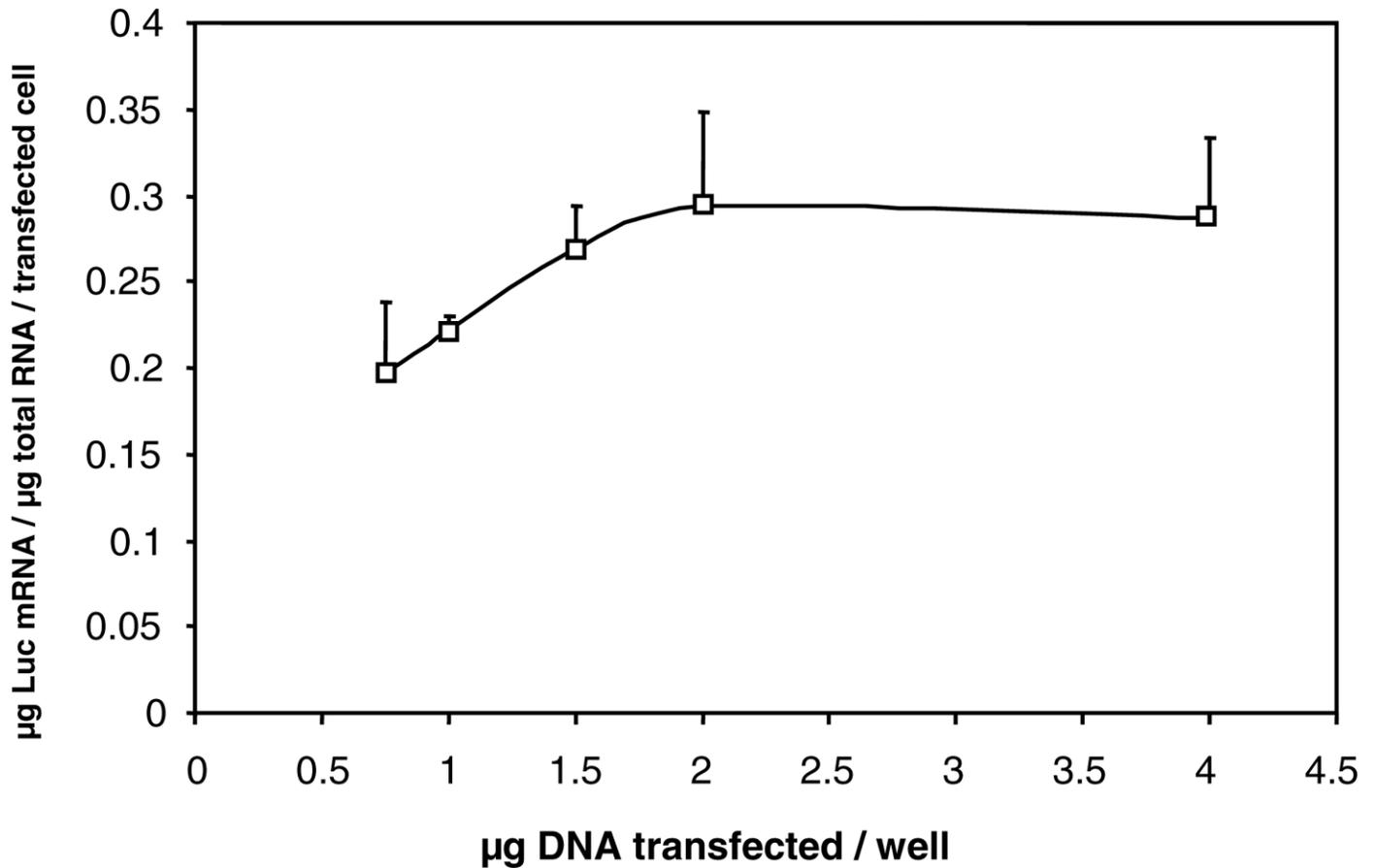
Figure 6. R011 dose-response study in BHK cells. Values for mRNA levels and percentage of cells transfected were obtained as described in Materials and Methods and shown in **Fig. 5**. The amount of luciferase mRNA produced per transfected cell increases with increasing amount of plasmid transfected up to a point, at which it then appears to reach a saturation level (~30% of total RNA levels) at which point the addition of more plasmid has no subsequent increase on luciferase mRNA levels.