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Factors limiting autogene-based cytoplasmic expression systems

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SPECIFIC AIMS

The purpose of this study was to determine the factors limiting the autogene-based cytoplasmic expression system and determine ways in which levels of gene expression can be increased.

1) The CMV promoter was replaced with the RSV or SV40 promoter and its effect on nuclear and cytoplasmic expression was examined;

2) The T7 phage RNA polymerase (RNAP) gene was replaced and compared with SP6 or T3 RNAP genes;

3) The EMCV internal ribosome entry site (IRES) currently used to allow for the translation of uncapped cytoplasmic transcripts was replaced and compared with eIF4G or Gtx IRES elements;

4) Translation efficiency of capped, uncapped, or EMCV IRES containing mRNA transcripts was compared to determine the efficiency of the EMCV IRES at driving translation;

5) Levels of transgene mRNA levels were quantitated after transfection with the cytoplasmic expression system.

PRINCIPAL FINDINGS

1. Replacing the CMV promoter with the RSV or SV40 nuclear promoter has little effect on autogene expression

The enhanced dual promoter autogene system relied on the CMV promoter for the first round of nuclear transcription, "triggering" the cytoplasmic expression system. The effect of other commonly used promoters was explored. Autogenes and their nuclear controls containing RSV or SV40 promoters were constructed. The different promoters had little effect on the maximum levels of autogene activity, but a dramatic effect on levels of nuclear gene expression. In the case of the SV40 promoter, the autogene system demonstrated a >200-fold increase in expression over the nuclear system. Even though the promoters resulted in dramatically different amounts of nuclear expression (consistent with published data), they had little effect on maximum levels of cytoplasmic expression. This is consistent with only a small catalytic amount of nuclear expression being required to drive the cytoplasmic expression system and that cytoplasmic expression was reaching an apparent saturation level.

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

The T7 RNAP protein was compared with two other RNAP proteins commonly used for in vitro transcription: SP6 and T3 RNAPs. The T7 RNAP gene in the cytoplasmic system (R011) was replaced with the SP6 or T3 RNAP gene. Using the T3 or SP6 RNAP in place of the T7 RNAP did not yield a substantial increase in gene expression. T3 RNAP showed an almost 2-fold increase in gene expression, which was not found to be significant ($P>0.05$).

3. Substitution of the EMC IRES by Gtx or eIF4G IRES elements reduced autogene expression

Higher expression levels have been observed using IRES elements other than the EMC, so luciferase reporter plasmids were constructed containing EMCV, eIF4G, Gtx, or no IRES element at all. These plasmids were tested in an in vitro transfection system, cotransfecting with a nuclear plasmid encoding for the T7 RNAP. The data show that only EMC IRES was efficient at driving cap-independent translation of cytoplasmic transcripts. 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. The results indicate that EMC IRES was the most effective IRES in the cytoplasmic expression system and

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that no advantage was gained by using either of the other two classes of IRES sequences.

4. Nuclear mRNA transcripts are translated 20-fold more efficiently than cytoplasmic autogene transcripts

To understand other factors that may limit the expression levels, translation efficiency of the cytoplasmic transcripts (IRES-luciferase) was compared with nuclear transcripts (5′ cap-luciferase). mRNA was synthesized in vitro and translation efficiency of these transcripts was determined via in vitro mRNA transfection. As can be seen in **Fig. 1**, whereas inclusion of the EMCV IRES results in an increase in expression over no IRES at all, the cytoplasmic transcripts are translated ~20-fold less efficiently than the capped nuclear transcripts. Inclusion of the EMCV IRES into a capped transcript inhibited the expression by ~5-fold, consistent with published data. Though 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 (compared with a capped mRNA) is playing a major role in lower-than-expected levels of gene expression. However, the low translation may not be the primary factor limiting autogene expression. That a 20-fold increase in luciferase protein expression is obtained with the cytoplasmic expression system whereas mRNA transcripts are being translated 20-fold less efficiently than the capped nuclear transcripts suggested the possibility that a factor responsible for limiting gene expression was at the level of mRNA production.

5. mRNA production is saturated during autogene expression

Given that the cytoplasmic transcripts are translated much less efficiently than nuclear transcripts, it was

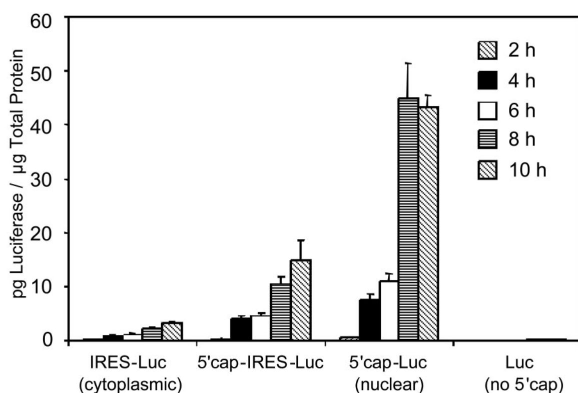

Figure 1. Comparison of translation efficiency of nuclear vs. cytoplasmic mRNA transcripts. BHK cells were transfected with a total of 1 µg/well of in vitro synthesized mRNA using Transmessenger reagent. All transcripts were of similar size. Error bars indicate standard error. Capped transcripts (nuclear) were translated 20-fold more efficiently than uncapped transcripts containing the EMC IRES (cytoplasmic). Insertion of the IRES into a capped transcript decreased the translation by 5-fold. The lack of any IRES sequence or cap resulted in background levels of expression.

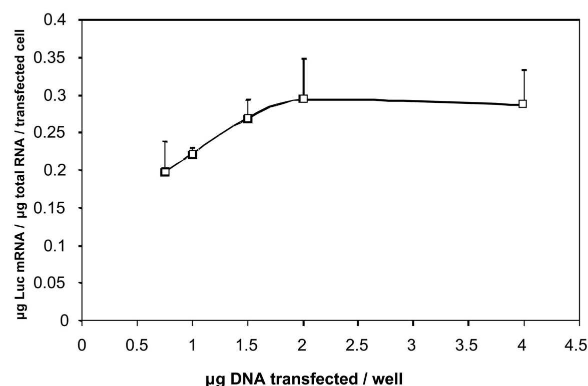

Figure 2. Autogene dose-response study in BHK cells. Values for mRNA levels and % of transfected cells were obtained. The amount of luciferase mRNA produced/transfected cell increases with increasing amount of plasmid transfected up to a point, when it appears to reach a saturation level (~30% of total RNA levels), and when the addition of more plasmid has no subsequent increase on luciferase mRNA levels.

clear that RNA output of the cytoplasmic system must be much higher than that of the nuclear system to achieve the marked increase in luciferase expression. A quantitative RNase protection assay (RPA) was performed and it was found that the amount of luciferase mRNA in the autogene transfection peaks at ~24 h post-transfection. Using the standard curve and determining the percentage of cells transfected using immunofluorescence, it was determined that at 30 h, ~12 (±0.4)% of 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-fold 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, a dose-response study was performed; RPA and immunofluorescence were used to determine the amount of luciferase transcript/transfected cell. If mRNA production was a limiting factor, we would expect to see a point where the addition of additional plasmid has no effect on mRNA production. Cells were harvested 24 h post-transfection to reduce cytotoxic effects possibly due to the large amount of foreign mRNA production. The amount of luciferase transcript produced reaches a saturation point at ~30 (±5) % of the total RNA (**Fig. 2**). 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; these large mRNA species were not present in RNA from nontransfected cells. This is consistent with the cells being able to produce only a finite amount of luciferase mRNA; adding more plasmid cannot increase this level. This indicates that the amount of transgene mRNA production (in addition to its poor translation) is the

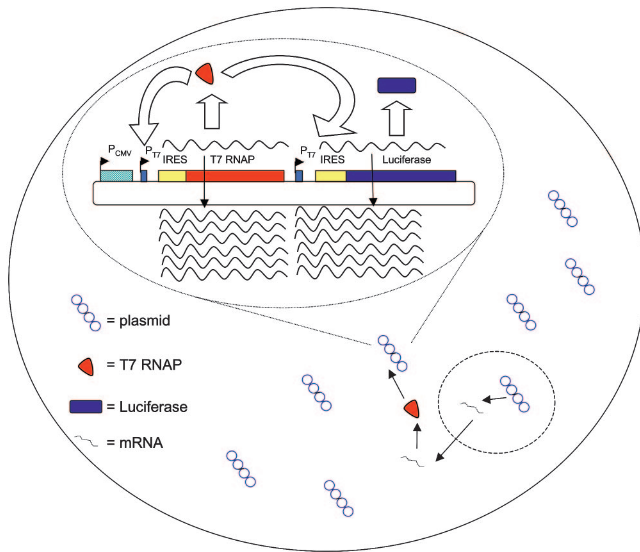

Figure 3. Schematic diagram.

major factor limiting autogene based cytoplasmic expression.

CONCLUSIONS AND SIGNIFICANCE

The studies performed indicate that the major factors limiting autogene expression were 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 10-fold the sum of every other transcript in the cell). This significant allocation of cellular resources (such as NTPs) would be expected to have a detrimental effect on cell growth, consistent with the observation that the autogene mRNA expression decreases rapidly after 24 h. This may arise because of the short half-life of the cytoplasmic transcripts, high levels of foreign mRNA production having a cytotoxic effect, or 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 translation of mRNA is the limiting factor. There most likely exists an equilibrium between mRNA levels and translation of the cytoplasmic transcripts; increasing 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 number of a suicide gene (e.g., thymidine kinase) is required, 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 to produce high levels of RNA in transfected cells.

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. Factors responsible for limiting autogene expression are most likely at the level of transgene mRNA saturation and 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 uncapped cytoplasmic transcripts could lead to a significant increase in the utility of such systems. FJ