# Chapter 2

Developing a Mammalian Cell Expression System for Unnatural Amino Acid Incorporation: Screening Suppressor tRNAs and Transfection Methods

# 2.1 Introduction

Unnatural amino acid incorporation into proteins by nonsense suppression has proven to be a valuable tool for structure-function studies [1-5]. Using the *in vivo* nonsense suppression methodology [6], information on ligand binding and ion channel gating mechanisms has been obtained on a variety of ion channels including the nicotinic ACh receptor (nAChR) [7-12], 5-HT3<sub>A</sub> receptor [13], and the Shaker [14] and Kir2.1 [15] potassium channels. To date, such studies have been limited to the *Xenopus* oocyte heterologous expression system. There would be clear benefits to expanding the technology to a mammalian cell expression system. This would provide a more relevant environment for many proteins of mammalian origin and would allow for studies of cellspecific signal transduction pathways.

When developing a new expression system for unnatural amino acid incorporation, there are many variables to be considered. Importantly, a suppressor tRNA needs to be identified. This tRNA must be orthogonal to the endogenous tRNA synthetases, yet still be recognized by the translational machinery of the cell. For the *Xenopus* oocyte expression system, a tRNA derived from *Tetrahymena thermophilus* has been optimized for unnatural amino acid delivery, THG73 [16]. This tRNA is an obvious candidate to test in mammalian cells, but is not necessarily the best suppressor tRNA. For this reason, several suppressor tRNAs - including tRNAs specifically designed for mammalian expression systems - must be tested. These include the human serine amber suppressor (HSAS) [17], the *E. coli* alanine amber suppressor (EAAS) [18], *E. coli* glutamine amber suppressor (EQAS) [19, 20] and the *E. coli* tyrosine amber suppressor (EYAS) [21].

A variety of methods to deliver tRNA to mammalian cells must also be explored. One needs to deliver enough aminoacyl-tRNA to each cell in order to generate a detectable amount of protein. One of the greatest challenges in developing a mammalian cell expression system for unnatural amino acid incorporation arises from the fact that the aminoacyl-tRNA is a stoichiometric reagent. The amount of protein made containing the unnatural amino acid is limited by the amount of aminoacyl-tRNA that can be delivered to the cell. Because of the relatively small size of mammalian cells (10 - 30  $\mu$ M in width) in comparison to *Xenopus* oocytes (diameter ~ 1 mm), much less aminoacyl-tRNA can be delivered to the former, hence less protein can be made.

Initial experiments by Dr. Pamela England involved microinjection into mammalian cells. These experiments were unfortunately unsuccessful. Several factors may have accounted for this, one being clogging of the pipette due to high concentrations of aminoacyl-tRNA used. Microinjection is a tedious transfection method, so rather than pursuing this method further we set out to develop a transfection technique that would be efficient, easy and general. We explored a variety of techniques including electroporation, peptide-mediated transfection, lipofection, and biolistics.

# 2.2 Results and Discussion

# 2.2.1 Screening suppressor tRNAs in vitro

For the *Xenopus* oocyte expression system, the suppressor tRNA THG73 was optimized to maximize protein expression while maintaining orthogonality to the endogenous synthetases. THG73 is an obvious candidate to test for a mammalian

expression system, however there are other amber suppressor tRNAs that have been used successfully in mammalian expression systems (Figure 2.1). The human serine amber suppressor HSAS is a tRNA that is recognized and aminoacylated by endogenous mammalian serine synthetases, and is therefore not suitable for unnatural amino acid delivery [17]. However, HSAS is a useful tool for optimizing DNA delivery to mammalian cells (discussed in detail in Chapter 3). Importantly, there have been examples of orthogonal amber suppressor tRNAs used for nonsense suppression in mammalian cells. For example, Vogel and co-workers recently described successful EGFP fluorescence recovery by nonsense suppression by microinjection of aminoacyl-EAAS (E. coli alanine amber suppressor tRNA) into CHO cells [18]. RajBhandary demonstrated suppression of chloramphenical acetyl-transferase (CAT) using the transfection reagent Effectene (Qiagen) to deliver aminoacyl-EYAS (E. coli tyrosine amber suppressor tRNA) [21]. An older example of nonsense suppression in mammalian cells also comes from the labs of RajBhandary. They found that the EQAS (E. coli glutamine amber suppressor) tRNA was orthogonal to COS1 and CV1 cells. Expression of the CAT gene by nonsense suppression was dependent on the co-expression of the E. coli glutamine amber suppressor tRNA gene and the E. coli glutamine synthetase [19, 20]. Although in this latter example the EQAS was expressed and aminoacylated by the cells, rather than being delivered after in vitro transcription, these experiments still demonstrate that EQAS is a functional and orthogonal amber suppressor tRNA that may be useful for unnatural amino acid incorporation. Because of their success in various expression systems, all of these tRNAs were tested for unnatural amino acid delivery into mammalian cells.



**Figure 2.1.** Amber suppressor tRNAs tested in developing a mammalian cell expression system for unnatural amino acids. THG73 (derived from *Tetrahymena thermophilus*); HSAS (human serine amber suppressor); EAAS (*E. coli* alanine amber suppressor); EYAS (*E. coli* tyrosine amber suppressor); EQAS (*E. coli* glutamine amber suppressor). In vitro transcribed tRNAs do not contain modified bases.

# 2.2.1.1 Results

An in vitro transcription system was used to initially screen the amber suppressor tRNAs of interest. Rabbit reticulocyte lysate was used to approximate a mammalian cell expression system. mRNA encoding for the  $\alpha$  subunit of the muscle-type nAChR was used in all of the in vitro translation reactions, because it contained an HA epitope for use

in Western blots. As can be seen in Figure 2.2 and 2.3, both aminoacyl-THG73 and HSAS suppress several TAG mutants very efficiently, when compared to wild-type expression. In the case of THG73, efficient suppression is seen for both an amino acid (5,7-difluorotryptophan  $F_2W$ ) as well as an alpha-hydroxy acid (valine hydroxy acid ValOH). Importantly, no read-through is observed in the absence of aminoacyl-THG73.



- 1. wt alphaHA
- 2. Rainbow ladder
- 3. Ala122TAG + HSAS
- 4. Leu146TAG + HSAS
- 5. Tyr127TAG + HSAS
- 6. Ala122TAG + THG73 (74mer)
- 7. Ala122TAG + THG-ValOH
- 8. Tyr127TAG + THG73 (74mer)
- 9. Tyr127TAG + THG-ValOH
- 10. Blank (rabbit reticulocyte only)

**Figure 2.2.** Western blot demonstrating nonsense suppression by THG73-ValOH and HSAS in rabbit reticulocyte lysate. The major band observed in each lane is the  $\alpha$  subunit of the muscle-type nAChR, and contains an HA epitope. Suppression by aminoacyl-THG73 was achieved using the value alpha-hydroxy acid (ValOH).



- 1. wt alphaHA
- 2. Rainbow ladder
- 3. Blank (rabbit reticulocyte only)
- 4. Leu146TAG
- 5. Leu146TAG + HSAS
- 6. Leu146TAG + THG73 (74mer)
- 7. Leu146TAG + THG- $F_2W$

**Figure 2.3.** Western blot demonstrating nonsense suppression by THG73- $F_2W$  and HSAS in rabbit reticulocyte lysate. The major band observed in each lane is the  $\alpha$  subunit of the muscle-type nAChR, and contains an HA epitope. Suppression by aminoacyl-THG73 was achieved using the amino acid 5,7-difluorotryptophan ( $F_2W$ ).

The suppressor tRNAs EQAS, EAAS and EYAS were also tested in vitro. As shown

in Figure 2.4, expression levels above background by EQAS suppression were not

observed. EAAS also did not show any sign of suppression at all, and EYAS only

resulted in a band that was slightly stronger than background (Figure 2.5).

1 2 3 4 5 6 7 8 9 10

- 1. wt alphaHA
- 2. rainbow ladder
- 3. Ala122TAG
- 4. Leu146TAG
- 5. Tyr127TAG
- 6. Ala122TAG + EQAS
- 7. Ala122TAG + EQAS-ValOH
- 8. Tyr127TAG + EQAS
- 9. Tyr127TAG + EQAS-ValOH
- 10. Blank (rabbit reticulocyte only)

**Figure 2.4.** Western blot demonstrating that EQAS is not an efficient suppressor tRNA in rabbit reticulocyte lysate. The major band observed in lane 1 is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope. Suppression was tested using EQAS aminoacylated with value alpha-hydroxy acid (ValOH).



- 1. wt alphaHA
- 2. Rainbow ladder
- 3. Blank (rabbit reticulocyte only)
- 4. Leu146TAG
- 5. Leu146TAG + EAAS
- 6. Leu146TAG + EAAS-Ala
- 7. Leu146TAG + EAAS-Ala
- 8. Leu146TAG + EYAS
- 9. Leu146TAG + EYAS-Ala
- 10. Leu146TAG + EYAS-Ala

**Figure 2.5.** Western blot demonstrating that EAAS and EYAS are not efficient suppressor tRNAs in rabbit reticulocyte lysate. The major band observed in lane 1 is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope. Suppression was tested using EAAS or EYAS aminoacylated with alanine.

### 2.2.1.2 Discussion

From the data shown, it is clear that in rabbit reticulocyte THG73 and HSAS are the most efficient suppressor tRNAs tested. Furthermore, it is demonstrated that in this expression system THG73 is fully orthogonal to the endogenous synthetases and efficiently delivers amino acids and alpha-hydroxy acids. It is somewhat surprising that EQAS, EAAS and EYAS all functioned so poorly in our hands, since they have been used successfully in other labs. In the cases of EQAS and EYAS, RajBhandary and coworkers used the highly sensitive CAT assay, and this may explain the difference. It is more difficult to explain why EAAS did not work at all for us, since the assay used by Vogel and co-workers was to rescue wild-type EGFP in CHO cells, certainly not significantly more sensitive than our in vitro assay. We speculated that rabbit reticulocyte may not be an appropriate mimic of a mammalian cell, and decided that it was still worth testing these amber suppressor tRNAs in mammalian cells.

### 2.2.2 Electroporation of tRNA

Electroporation is a common method used to deliver many different types of macromolecules to cells, and is generally a favoured DNA transfection technique [22, 23]. It is operationally easy, reproducible, and works for many cell types including cultured neurons. Furthermore, cells that are either attached or in suspension can be transfected by electroporation. In essence, the application of an electric field to a population of cells results in reversible membrane breakdown, and the formation of large pores that allow the passage of macromolecules. The basic steps of electroporation include (i) electric field generation, (ii) polarization of the outer membrane, (iii) pore

formation, (iv) transmembrane transport and (v) electropore resealing. The mechanism of pore formation and resealing is not known. Another advantage to this technique is that there are many parameters to control for optimization, including electric field strength, pulse duration and the number of pulses applied. A disadvantage is that there is no standard protocol, so optimization must be done for each individual system studied. Finally, electroporation has been used to successfully deliver aminoacyl-tRNA (rabbit liver tRNA) to CHO cells, although in this report the tRNA was found to not be functional within the cells [24]. Because of its generality, electroporation was tested as a method to deliver tRNA to adherent mammalian cells.

#### 2.2.2.1 Microelectroporation

It can be imagined that electroporation of a large population of cells would require very large quantities of aminoacyl-tRNA. This can be circumvented using a "microporator" designed by Teruel and Meyer (Figure 2.6) [25, 26]. This electroporation device allows for the transfection of a small area of attached cells (10 mm<sup>2</sup>), using only a small volume of solution ( $\geq 1 \mu$ l). They have demonstrated successful transfection using both DNA and mRNA, with over 90% cell survivability and over 50% transfection efficiency in rat basophilic leukemia cells, neocortical neuroblastoma cells and hippocampal neurons.



**Figure 2.6.** The microelectroporator used for transfecting adherent mammalian cells. A 35 mm dish is shown for scale. The power supply (left) can deliver up to 150 V, using 25, 50, 100 or 200 ms square pulses. The electrode holder (right) is lowered into a 35 mm dish of cultured cells, placing the electrodes 300  $\mu$ M above the adherent cells.

# 2.2.2.1.1 Results

Electroporation of wild-type EGFP DNA and mRNA led to successful EGFP expression in CHO-K1 cells as shown in Figures 2.7 and 2.8. Transfections with DNA typically led to higher expression of EGFP, and expression could be seen as soon as 2 hours after transfection.

In order to determine if tRNA can be successfully delivered to cells using the microelectroporator, a fluorescently labeled tRNA was made. This was done by including rhodamine green-UTP (RhG-UTP) in the THG73 tRNA in vitro transcription reaction, at a ratio of 3 UTP to 1 RhG-UTP. The RhG-labeled tRNA was dialyzed to ensure that no free RhG-UTP was left when electroporating the cells. As can be seen in Figure 2.9, fluorescent tRNA was successfully delivered to CHO-K1 cells. Because it

was not possible to determine how many fluorophores were incorporated per tRNA molecule, the amount of tRNA delivered to each cell could not be quantified.



**Figure 2.7.** Wild-type EGFP expression in CHO-K1 cells by transfection with pCS2gapEGFP DNA. The cells were transfected with a 5  $\mu$ l solution containing 2.5  $\mu$ g/ $\mu$ l DNA, applying four 120 V, 50 ms pulses. The bright field (left) and fluorescence images (right) were taken 2 hours after transfection.



**Figure 2.8**. Wild-type EGFP expression in CHO-K1 cells by transfection with pCS2gapEGFP mRNA. The cells were transfected with a 10  $\mu$ l solution containing 3  $\mu g/\mu l$  DNA, applying four 120 V, 50 ms pulses. The bright field (left) and fluorescence images (right) were taken 3 hours after transfection



**Figure 2.9.** Electroporation of Rhodamine-Green tRNA into CHO-K1 cells. The cells were transfected with a 4  $\mu$ l solution of 1  $\mu$ g/ $\mu$ l RhG-tRNA (dialyzed), applying four 120 V, 50 ms pulses. The bright field (left) and fluorescence images (right) were taken immediately after transfection.

Given that the above results demonstrate that DNA, mRNA and tRNA can all be efficiently delivered to mammalian cells by electroporation, we wanted to next test the delivery of aminoacyl-tRNA (see Chapter 3 for detailed description of HSAS suppression of EGFP by electroporation). Toward this goal, we designed an EGFP assay. We made the EGFP mutants Ala37TAG, and Tyr66TAG to be co-electroporated with tRNA-Ala, or tRNA-Tyr, respectively. It was hoped that wild-type EGFP recovery would be observed in these experiments, while no EGFP expression would be observed in the absence of aminoacyl-tRNA. The mutant Val55TAG was also made for co-electroporation with tRNA-ValOH (valine alpha-hydroxy acid). The aminoacyl-linkage of alpha-hydroxy acids on tRNA is less susceptible to hydrolysis than for amino acids, so they can be easier to work with. Finally, we obtained the pT7EGFP Leu64TAG construct from the labs of Horst Vogel. They observed wild-type EGFP recovery using EAAS-Leu(4PO) for nonsense suppression by microinjection, therefore we wanted to test the electroporation method using these same constructs.

The results from these experiments are summarized in Table 2.1. In essence, EGFP recovery by nonsense suppression was not observed for any of the aminoacyltRNAs tested. Only HSAS suppression of Ser29TAG ever worked using EGFP recovery as an assay (see Chapter 3).

tRNA Species						
Amino Acid	THG73	HSAS	EQAS	EAAS	EYAS	EGFP
						mutant
Ala(NVOC)	×		×	×	×	A37TAG
ValOH	×		×			V55TAG
Tyr(NVOC)	×	×				Y66TAG
Leu(4PO)				×		L64TAG*
		$\checkmark$				S29TAG

**Table 2.1** Attempts to recover EGFP using various tRNA-aa for nonsense suppression. The tRNAs THG73, HSAS, EQAS, EAAS and EYAS were aminoacylated with wild-type Ala or Tyr (NVOC protected), wild-type Leu (4PO protected) or with the valine alpha hydroxy acid (ValOH), and were tested for nonsense suppression using the various EGFP mutants shown. An " $\star$ " represents failed experiments, i.e., no EGFP expression by nonsense suppression was observed using the corresponding tRNA-aa and EGFP mutant. A " $\checkmark$ " represents successful EGFP expression by nonsense suppression. An empty box represents combinations that were not attempted. \* pT7EGFP Leu64TAG was obtained from the Horst Vogel lab [18].

Because nonsense suppression was not observed for any tRNAs studied, including

THG73 which has been shown to work very well in Rabbit Reticulocyte, we next sought

out to ensure that amino acid wasn't hydrolyzing off of the tRNA during the

electroporation protocol. Mammalian cells must be electroporated at physiological pH,

but the aminacyl-ester bond between the tRNA and the amino acid is more labile at

higher pHs (we normally keep tRNA-aa at pH 4.5 for the Xenopus oocyte methodology).

A solution of tRNA-aa at pH 4.5 or 7.4 was allowed to sit at room temperature for 10

minutes (Figure 2.10), or  $\geq$  1 hour (Figures 2.11 and 2.12), and added to an in vitro transcription reaction. This was done using both protected (NVOC) and photodeprotected THG73-Ala, as well the valine alpha-hydroxy acid ValOH. It can be seen in Figure 2.10 that a 10-minute incubation of tRNA-aa at room temperature, pH 7.4, has minimal impact on suppression, implying that the aminoacyl-ester bond between the amino acid and tRNA is stable under these conditions. Only for longer incubations of  $\geq$  1 hour does the alphaHA band appear weaker, presumably due to reduced amounts of functional aminoacyl-tRNA. This is observed for both THG73-Ala (Figure 2.11) and THG73-ValOH (Figure 2.12).



- 1. wt alphaHA
- 2. Rainbow ladder
- 3. Ala122TAG
- 4. Ala122TAG + THG73 74mer
- 5. Ala122TAG + THG73-Ala(NVOC) pH 7.4, photo-deprotected 10 minute, at room temperature, prior to translation reaction assembly
- 6. Ala122TAG + THG73-Ala(NVOC) pH 4.5, translation reaction assembled immediately
- 7. Ala122TAG + THG73-Ala(NVOC) pH 4.5, translation reaction assembled immediately
- 8. Ala122TAG + THG73-ValOH pH 7.4, sat for 10 minutes prior to assembly of translation reaction
- 9. Ala122TAG + THG73-ValOH pH 4.5, sat for 10 minutes prior to assembly of translation reaction

**Figure 2.10**. Testing the stability of tRNA-aa at pH 7.4 (10 minute incubation) by in vitro translation. The major band observed in all lanes is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope.



- 1. wt alphaHA
- 2. Ala122TAG + HSAS
- 3. Ala122TAG
- 4. Ala122TAG + THG73-Ala(NVOC), remained protected pH 7.4,  $\geq$  1 hour, deprotected immediately immediately prior to translation reaction assembly
- 5. Ala122TAG + THG73-Ala(NVOC), pH 7.4, photo-deprotected ≥ 1 hour prior to translation reaction assembly

**Figure 2.11**. Testing the stability of THG73-Ala at pH 7.4 ( $\geq 1$  hour incubation) by in vitro translation. The major band observed in all lanes is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope.



- 1. Ala122TAG + THG73-Ala(NVOC)
- 2. Rainbow ladder
- 3. Ala123TAG + HSAS
- 4. Blank
- 5. Ala122TAG
- 6. Ala122TAG + THG73 76mer (dCA)
- 7. Ala122TAG + THG73 74mer
- 8. Ala122TAG + THG73-ValOH, translation reaction assembled immediately
- 9. Ala122TAG + THG73-ValOH, sat at pH  $7.4 \ge 1$  hour prior to assembly of translation reaction

**Figure 2.12**. Testing the stability of THG73-ValOH at pH 7.4 ( $\geq$  1 hour incubation) by in vitro translation. The major band observed in all lanes is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope.

Lastly, we wanted to ensure that there were no components in the  $CO_2$ 

independent electroporation buffer that may cause hydrolysis of the amino acid. The

recipe for this buffer is proprietary, so we simply added the buffer to an in vitro

transcription reaction to ensure that the tRNA-aa was still functional. As can be seen in

Figure 2.13, the presence of the electorporation buffer did not appear to compromise the integrity of the aminoacyl-tRNA.



- 1. wt alphaHA
- 2. Rainbow ladder
- 3. Ala122TAG
- 4. Ala122TAG + THG73 74mer
- 5. Ala122TAG + THG73 76mer (dCA)
- 6. Ala122TAG + THG73-ValOH
- 7. Ala122TAG + THG73-Ala(NVOC)
- 8. Ala122TAG + THG73-ValOH in  $CO_2$  independent electroporation buffer
- 9. Ala122TAG + THG73-Ala(NVOC) in CO<sub>2</sub> independent electroporation buffer

**Figure 2.13**. Testing the stability of THG73-aa in the presence of the CO<sub>2</sub> independent electroporation buffer by in vitro translation. The major band observed in all lanes is the  $\alpha$  subunit of the muscle-type nAChR, containing an HA epitope.

# 2.2.2.1.2 Discussion

It was somewhat discouraging that no sign of EGFP expression by nonsense suppression was observed for any of the aminoacyl-tRNAs tested, given the HSAS (Chapter 3) and RhG-tRNA experiments demonstrating that tRNA is efficiently delivered to cells by electroporation. Particularly surprising was that EAAS failed in our hands, since Vogel and co-workers found this to be a functional suppressor tRNA in CHO cells. The main difference between their experiments and ours was that they microinjected EAAS-Leu, rather than using electroporation. Perhaps microinjection simply delivers more material than microelectroporation. When the in vitro experiments are considered (2.2.1) it may not be surprising that HSAS works in mammalian cells while EQAS, EAAS and EYAS do not, since this was true of rabbit reticulocyte, which is also a mammalian expression system. From all the data obtained, we therefore felt that EQAS, EAAS and EYAS would not be efficient suppressor tRNAs for unnatural amino acid incorporation using our methodology.

The fact that THG73 worked so well in vitro and seemingly not at all in cells led us to do a series of experiments examining the stability of the tRNA-aminoacyl linkage. We wanted to ensure that the amino acid was not hydrolyzing off of the tRNA at any point during the electroporation protocol. These experiments included incubating aminoacyl-tRNA at room temperature, pH 7.4, for 10 minutes to  $\geq$  1 hour. Although the ~ 1-hour room temperature incubations at pH 7.4 did lead to less efficient nonsense suppression by the aminoacyl-tRNA, the 10-minute incubations had minimal effect. Experimentally, it takes less than 10 minutes from the time the aminoacyl-tRNA is photodeprotected to the time that it is electroporated into the mammalian cells. Therefore, it seems unlikely that aminoacyl-tRNA hydrolysis is a concern. Furthermore, the CO<sub>2</sub> independent electroporation buffer also did not seem to interfere with the aminoacyl-tRNA.

From the data presented above, it seemed that functional aminoacyl-tRNA should be getting into adherent mammalian cells by electroporation, and that THG73 should be a functional amber suppressor tRNA in a mammalian cell expression system. By deduction, we were left with the possibility that not enough aminoacyl-tRNA was getting into the cells for us to actually observe suppression, and that we were operating below the EGFP detection limit. For this reason we abandoned the EGFP assay and moved on to a more sensitive assay, electrophysiology (see Chapter 4). 2.2.2.2 Epizap

The Epizap is a commercially available electroporator, designed for transfecting adherent cells [27]. The cells are grown on optically transparent indium-tin oxide (ITO) coated glass slides. This electrically conductive surface acts as the anode of the electroporator. One advantage we thought that Epizap would have over our microelectroporator is that prior to electroporation, all of the growth media is removed from the cells (on the glass slide) and replaced with a minimum volume of 17  $\mu$ l of electroporation solution. Although the microelectroporator uses smaller volumes, we were concerned that the media remaining in the tissue culture dish was diluting the electroporation solution. We therefore hoped that a higher concentration of electroporation solution could be delivered using Epizap.

### 2.2.2.2.1 Results

Electroporation of both CHO-K1 cells and HEK cells was tested using the Epizap, using either wild-type EGFP DNA or Ser29TAG DNA and HSAS tRNA (Figures 2.14 and 2.15). In general, it was found that the electroporation efficiency was on occassion similar to, but usually worse than, the microelectroporator. Furthermore, it took longer for EGFP expression to be observed when compared to the microelectroporator. One complication that arose was that the cell health seemed to be compromised when grown on the ITO surface. At least half of the experiments failed because of massive cell death, even after several attempts to optimize the electroporation conditions by modifying the voltage and capacitance. Basically there was a lot of variability and it was difficult to reproduce the experiments that actually did work well.



**Figure 2.14**. Electroporation of CHO-K1 cells using Epizap. The bright field (upper) and fluorescence images were taken 7 hours after transfection. (A) Cells were electroporated with a 17  $\mu$ l solution of wild-type pCS2gapEGFP DNA (2  $\mu g/\mu$ l). (B) Cells were electroporated with a 17  $\mu$ l solution of HSAS tRNA (4  $\mu g/\mu$ l) and pCS2EGFP Ser29TAG DNA (2  $\mu g/\mu$ l). (C) Cells were electroporated with a 17  $\mu$ l solution of pCS2EGFP Ser29TAG DNA (2  $\mu g/\mu$ l). For all cases, four 45 V, 1.0  $\mu$ F pulses were delivered to the cells. The fluorescent image in (C) was taken with four times the exposure length as in (A) and (B).



**Figure 2.15**. Electroporation of HEK cells using Epizap. The bright field (upper) and fluorescence images were taken 7 hours after transfection. (A) Cells were electroporated with a 17  $\mu$ l solution of wild-type pCS2EGFP DNA (2  $\mu$ g/ $\mu$ l). (B) Cells were electroporated with a 17  $\mu$ l solution of HSAS tRNA (4  $\mu$ g/ $\mu$ l) and pCS2gapEGFP Ser29TAG DNA (2  $\mu$ g/ $\mu$ l). For all cases, four 30 V, 10  $\mu$ F pulses were delivered to the cells. The fluorescent images were taken with the same exposure length.

# 2.2.2.2.2 Discussion

It can be concluded that the Epizap did not work well in our hands. Most of the time there was so much cell death that the experiments were a total loss. There was also a great deal of variability, and it was therefore very difficult to reproduce any experiments that worked well. These issues, along with the fact that the Epizap consumed much more material (aminoacyl-tRNA in particular) than the microelectroporator, brought us to the conclusion that it wasn't really worth pursuing further.

2.2.3 Peptide-mediated delivery of tRNA

Another method to incorporate tRNA into mammalian cells that was explored took advantage of a class of peptides that have the unique ability to drag cargo across cell membranes, protein transduction domains (PTD). The most efficient of these are the ANTP peptide (antenopedia, isolated from Drosophila, also known as Penetratin), the Tat peptide (isolated from HIV-1) and VP22 (isolated from herpes simplex virus). These peptides can transport cargo across a cell membrane when fused to proteins (up to 120 kDa) or oligonucleotides (up to 55mer) at either their C or N terminus. Transport occurs with equal efficiency at 4°C as it does at 37°C suggesting that it is not receptor or transporter mediated, and nonendocytotic. This phenomenon is independent of the cell line used, and works for neuronal cultures, suggesting that transport is due to a direct interaction with the cellular membrane. In fact, the ANTP peptide was shown to cross a synthetic bilayer [28]. Maximal intracellular concentrations are achieved on the order of minutes and are dependent upon the extracellular PTD concentration. These peptides have been reviewed in detail [29-39].

The third helix of the ANTP homeoprotein contains the minimal domain required for transport across a biological membrane. This domain consists of 16 amino acids, (RQIKIWFQNRRMKWKK) and has been shown to successfully transport oligonucleotide cargos [40-43]. Therefore, we attempted to couple this peptide to tRNA for transport into mammalian cells.

2.2.3.1 Covalent coupling between the ANTP peptide and tRNA

Initial experiments were designed to covalently couple the ANTP peptide to tRNA. Although this may render the tRNA nonfunctional in an expression system, we were more interested in seeing if these peptides could transport tRNA across a cell membrane. Therefore, our primary strategy focussed on reacting maleimide functionalized ANTP peptides with phosphorothioate or thiol modified RNAs.

## 2.2.3.1.1 Results

Our initial coupling strategy consisted of labeling the 5'-end of the tRNA with a phosphorothiate group, which would be reactive toward a maleimide-functionalized ANTP peptide. Phosphorothioate labeling can be achieved using the KinaseMax kit (Ambion). When tRNA is combined with ATP- $\gamma$ -phosophorothioate (ATP- $\gamma$ -S), a T4 kinase-catalyzed exchange reaction occurs, replacing the 5'-phosphate with a phosphorothioate (tRNA- PO<sub>3</sub>S). We performed both 16-hour and 20-hour exchange reactions, and then monitored the reaction by coupling to maleimide-functionalized tetramethyl-rhodamine (TMR-MI).

The results from the TMR-MI coupling experiments are shown in Figure 2.16. The coupling reactions between tRNA- PO<sub>3</sub>S and TMR-MI are in lanes 5, 6, 9 and 10, and the rest of the lanes are controls. The white arrow indicates a unique band observed for the experimental coupling reactions, which may be the coupled product. The two major bands below this (observed in lanes 3 - 13) are THG73 tRNA, which runs in multiple conformations in agarose. Identification of the coupled product is not clear because of the result seen in lane 15. When ATP- $\gamma$ -S is reacted with TMR-MI and run on the same agarose gel, it can be seen that there is a band that runs at the same position as the putative tRNA-TMR coupled product, in addition to a stronger band that runs faster (lane 15). However, when this ATP- $\gamma$ -S/TMR-MI reaction mixture is run through a nucleotide removal column, both bands disappear (lane 14). Since all the tRNA coupling reactions were run through nucleotide removal columns, then one would presume the free ATP- $\gamma$ -S to be gone, and not available to react with TMR-MI. So although it is suspicious that the band thought to be coupled tRNA product runs the same as one of the bands seen in lane 15, we presumed to have successfully made tRNA- PO<sub>3</sub>S, and went ahead and tried to couple it to the ANTP peptide.



- 1. ATP- $\gamma$ -S + TMR-MI, 2 hr
- 2. 100 base-pair ladder
- 3. ATP, 20 hr exchange + TMR-MI, 2 hr
- 4. ATP, 20 hr exchange + TMR-MI, 1 hr
- 5. ATP-γ-S, 20 hr exchange + TMR-MI, 2 hr
- 6. ATP-γ-S, 20 hr exchange + TMR-MI, 1 hr
- 7. ATP, 16 hr exchange + TMR-MI, 2 hr
- 8. ATP, 16 hr exchange + TMR-MI, 1 hr
- 9. ATP-γ-S, 16 hr exchange + TMR-MI, 2 hr
- 10. ATP-γ-S, 16 hr exchange + TMR-MI, 1 hr
- 11. 74mer THG73 + TMR-MI, 2 hr
- 12. 74mer THG73 + TMR-MI, 1hr
- 13. 74mer
- 14. ATP- $\gamma$ -S + TMR-MI, run through nucleotide removal column
- 15. ATP- $\gamma$ -S + TMR-MI, 1 hr

**Figue 2.16**. Agarose gel showing the results of TMR-MI coupling reactions with 5'phosphorothioate labeled THG73 tRNA (tRNA- PO<sub>3</sub>S). Coupling reactions to TMR-MI were done with tRNA- PO<sub>3</sub>S that had undergone 16-hour (lanes 9, 10) or 20-hour (lanes 5, 6) exchange reactions with ATP- $\gamma$ -S. Controls included reacting TMR-MI with tRNA that had undergone exhange reactions with ATP (lanes 3, 4, 7, 8); reacting TMR-MI with 74mer tRNA (no exchange reaction, lanes 11, 12); reacting ATP- $\gamma$ -S with TMR-MI (lanes 1, 15); and reacting ATP- $\gamma$ -S with TMR-MI, then running the reaction through a nucleotide removal column (lane 14). Also shown is a 100-base pair ladder (lane 2) and 74mer tRNA only (lane 13). The white arrow indicates the band suspected to be tRNA-TMR coupled product. A 100  $\mu$ l reaction mixture contained 5  $\mu$ g tRNA, 20-fold excess TMR-MI, with 10% DMSO in a HEPES buffered solution (pH 7.5), incubated for 2 hours at room temperature.

In order to covalently couple the ANTP peptide to tRNA, we obtained an Nterminus maleimide-functionalized ANTP peptide (PTD-mal). A variety of conditions were tested for coupling tRNA-  $PO_3S$  to the ANTP peptide. In most cases when tRNA was combined with ANTP and loaded onto a gel, it was observed that nothing in the reaction mixture entered the gel; everything just remained in the wells. There was also some precipitation observed in the reaction mixtures. To reduce any nonspecific electrostatic interactions between these two species, the reaction was done under high salt conditions (1 M NaCl). This is shown in Figure 2.17. Unfortunately, it can be seen that there is no difference between the experimental tRNA- PO<sub>3</sub>S and PTD-mal coupling reaction and any of the control reactions. Interestingly, whenever PTD was present (with or without the maleimide) the tRNA ran slower on the gel than in the absence of PTD. This suggests that even in high salt conditions, there is still a nonspecific electrostatic interaction between the ANTP peptide and tRNA. Therefore it is impossible to say whether or not the coupling reaction was successful, based on the data shown.



- 1. HSAS-PO<sub>4</sub>
- 2. HSAS-PO<sub>4</sub> + TMR-MI
- 3.  $HSAS-PO_4 + PTD$
- 4.  $HSAS-PO_4 + PTD-mal$
- 5. HSAS-PO<sub>3</sub>S
- 6. HSAS-PO<sub>3</sub>S + TMR-MI
- 7.  $HSAS-PO_3S + PTD$
- 8.  $HSAS-PO_3S + PTD-mal$

**Figure 2.17**. PAGE gel showing attempts to couple tRNA- PO<sub>3</sub>S to the ANTP peptide. Lanes 1 - 4 are reactions between unlabeled HSAS tRNA (HSAS-PO<sub>4</sub>) and TMR-MI (lane 2), non-functionalized PTD (lane 3) and PTD-mal (lane 4). Lanes 5 - 8 are reactions between HSAS- PO<sub>3</sub>S and TMR-MI (lane 6), non-functionalized PTD (lane 7) and PTD-mal (lane 8). A 20  $\mu$ l reaction contained 2  $\mu$ g tRNA, 10-100 fold excess PTD, 1 M NaCl, 10% DMSO, was phosphate buffered (pH 7.5), and incubated for 2 hours at room temperature.

ANTP is thought to be an alpha helix that interacts with the minor-groove of

DNA helices. Therefore, the ANTP peptide may have been binding to double-helical

structures of the tRNA, leading to the nonspecific interactions observed. For this reason,

an alternate strategy for coupling the ANTP peptide to tRNA was pursued. A 14mer

RNA molecule was made, that corresponds to the 14 nucleotides found at the 5'-end of

the tRNA. Likewise, a new tRNA construct was made that lacked these 5' nucleotides (Figure 2.18). Coupling of the 14mer to the ANTP peptide would be followed by annealing and ligation to the new truncated tRNA species, thereby circumventing the nonspecific electrostatic interactions. These experiments were designed using the HSAS tRNA, so that delivery to mammalian cells could be tested without having to chemically aminoacylate the tRNA.



**Figure 2.18**. ANTP-tRNA coupling strategy. A 14mer RNA (HSAS 5'14mer) molecule was made for coupling to the ANTP peptide (Penetratin). This could then be annealed and ligated to the corresponding truncated tRNA (HSAS 3'71mer). The tRNA shown is HSAS.

Coupling of the 14mer RNA to the PTD was attempted using a variety of conditions [44-49]. In almost all cases, there was some precipitation observed when tRNA-PO<sub>3</sub>S and PTD-mal were combined. The best examples are shown in Figures 2.19 and 2.20. The major bands observed are the 14mer RNAs, but a unique band can be seen for coupling reactions between tRNA-PO<sub>3</sub>S + PTD-mal (indicated by the arrows). The best reaction conditions seem to be the high salt conditions, with 100-fold excess of the PTD-mal, based on the intensity of this band (Figure 2.20). However, it can be seen that

the reactions are far from completion since the unreacted RNA band is much more intense.



- 1. HSAS + PTD (20  $\mu$ g)
- 2. HSAS-PO<sub>3</sub>S + PTD (20  $\mu$ g)
- 3. HSAS + PTD-mal (20  $\mu$ g)
- 4. HSAS-PO<sub>3</sub>S + PTD-mal (20  $\mu$ g)
- 5. HSAS + PTD  $(2 \mu g)$
- 6. HSAS-PO<sub>3</sub>S + PTD (2  $\mu$ g)
- 7. HSAS + PTD-mal  $(2 \mu g)$
- 8. HSAS-PO<sub>3</sub>S + PTD-mal (2  $\mu$ g)

**Figure 2.19**. PAGE gel showing the coupling reactions of HSAS 5'14mer to the ANTP peptide (PTD). 14mer RNA was labeled with phosphorothioate using ATP- $\gamma$ -S and T4 kinase (KinaseMax kit, as described for tRNA labeling). A 100-fold excess (lanes 1 - 4) or 10-fold excess (lanes 5 - 8) of PTD was included. Reactions between HSAS-PO<sub>3</sub>S + PTD-mal are shown in lanes 4 and 8. Controls included reactions with unlabeled RNA (lanes 1, 3, 5, 7) and nonfunctionalized PTD (lanes 1, 2, 5, 6). A 10  $\mu$ l reaction contained 5  $\mu$ g RNA, 2 or 20  $\mu$ g PTD, 20 mM MgCl<sub>2</sub>, was buffered by PBS (pH 7.4), and was included for 2 hours at 37°C.



- 1. HSAS + PTD (20  $\mu$ g)
- 2. HSAS-PO<sub>3</sub>S + PTD (20  $\mu$ g)
- 3. HSAS + PTD-mal (20  $\mu$ g)
- 4. HSAS-PO<sub>3</sub>S + PTD-mal (20  $\mu$ g)
- 5. HSAS + PTD  $(2 \mu g)$
- 6. HSAS-PO<sub>3</sub>S + PTD  $(2 \mu g)$
- 7. HSAS + PTD-mal (2  $\mu$ g)
- 8. HSAS-PO<sub>3</sub>S + PTD-mal  $(2 \mu g)$

**Figure 2.20**. PAGE gel showing the coupling reactions of HSAS 5'14mer to the ANTP peptide (PTD), high salt conditions. 14mer RNA was labeled with phosphorothioate using ATP- $\gamma$ -S and T4 kinase (KinaseMax kit, as described for tRNA labeling). A 100-fold excess (lanes 1 - 4) or 10-fold excess (lanes 5 - 8) of PTD was included. Reactions between HSAS-PO<sub>3</sub>S + PTD-mal are shown in lanes 4 and 8. Controls included reactions with unlabeled RNA (lanes 1, 3, 5, 7) and nonfunctionalized PTD (lanes 1, 2, 5, 6). A 10  $\mu$ l reaction contained 5  $\mu$ g RNA, 2 or 20  $\mu$ g PTD, 20 mM MgCl<sub>2</sub>, 1 M NaCl, was buffered by PBS (pH 7.4), and incubated for 2 hours at 37°C.

The ATP- $\gamma$ -S exchange reaction with oligonucleotides usually does not go to completion. This would explain why the reactions between HSAS-PO<sub>3</sub>S 14mer RNAs and PTD-mal also did not go to completion. To remedy this, 5'thiol-modified 14mer RNAs were synthesized. Therefore all of the RNA molecules should be reactive toward the PTD-mal, and should also be more reactive than the phosphorothioate group.

These experiments were done when we thought that the EQAS tRNA would still be useful for unnatural amino acid incorporation, so we designed an EQAS 5'thiol modified 14mer RNA. Coupling reactions were done using a variety of conditions [44-49], however none of these gave promising results (Figure 2.21). The gels for these reactions were very messy, and difficult to interpret. It is possible that the deprotection steps for the RNA 2'OH and the 5'thiol compromised the integrity of the RNA. The arrows in Figure 2.21 indicate bands that were unique to the coupling reactions, not observed in the control reactions, but it is certainly difficult to identify these bands as coupled product with any certainty.



- 1. Water only
- 2. 2 M NaCl, HEPES
- 3. 50% acetonitrile, TEAA
- 4. TEAA
- A. EQAS + PTD
- B. EQAS-SH + PTD
- C. EQAS + PTD-mal
- D. EQAS-SH + PTD-mal

**Figure 2.21**. PAGE gel showing the coupling reactions between EQAS 14mer RNA, 5'thiol modified, with the ANTP peptide (PTD). The coupling reactions were done in (1) water, (2) 2 M NaCl, HEPES buffered, (3) 50% acetonitrile, TEAA buffered (4) TEAA buffered solutions. The control reactions are shown (A - C), and the EQAS-SH/PTD-mal coupling reactions are shown in D. A 10  $\mu$ l reaction contained 4  $\mu$ g RNA, 20  $\mu$ g PTD, and was incubated at 37°C for 4 hours. Arrows indicate bands that may be the coupled RNA-peptide product.

While the ANTP-RNA coupling reactions were being worked out, so was the

ligation reaction between the HSAS 5'14mer and HSAS 3'71mer RNA. Several failed

attempts of this ligation using a variety of conditions [50-53] were done before realizing

that the 5'-end of the HSAS 3'71mer tRNA was not a monophosphate. In vitro

transcription yields a 5'-triphosphate, which needs to be phosphatased off, followed by reaction with T4 kinase to put on a single phosphate group. This tRNA could then be successfully ligated to the 14mer RNA, as seen in Figure 2.22, although under the conditions used the reaction did not go to completion.



- 1. HSAS 83mer
- 2. HSAS 71mer + 14mer ligation reaction
- 3. HSAS 71mer
- 4. HSAS 71mer, alkaline phosphatase
- 5. HSAS 71mer

**Figure 2.22.** PAGE gel showing the tRNA ligation reaction between HSAS 3'71mer and HSAS 5'14mer. The HSAS 3'71mer tRNA had to be first reacted with alkaline phosphatase, followed by T4 kinase, before ligation to the HSAS 5'14mer RNA. The 83mer indicated by the arrow is full length HSAS, and the 71mer is the truncated HSAS tRNA.

### 2.2.3.1.2 Discussion

At the time these experiments were in progress we decided to focus our attention on the electroporation protocol, which was proving to be more promising. However, the data suggest that the ANTP coupling to the 14mer RNAs was working, as well as the HSAS 3'71mer/5'14mer ligations. Although some optimization of these reactions is required, the next step would be to ligate the 14mer-ANTP coupled product to the 3'71mer tRNA, and see if it goes into mammalian cells. The easiest way to do this would be to make rhodamine green-labeled tRNA, since the tRNA may not be functional with the ANTP peptide coupled to it. It would also be interesting to design a system whereby the ANTP was coupled to the tRNA via a disulphide bond. Upon entry into a cell, the ANTP would be released from the tRNA by reduction of the disulphide bond, rendering a functional tRNA. Using HSAS tRNA would be the most convenient tRNA for optimizing such experiments since it is aminoacylated by the cell, and hence does not require chemical aminoacylation.

#### 2.2.3.2 Non-covalent coupling between the ANTP peptide and tRNA

It was discovered when trying to covalently couple ANTP to tRNA that there was a very strong non-covalent interaction between these two species, most likely due to electrostatics (Figure 2.17). Although there are no examples of ANTP transporting cargo across a biological membrane via a non-specific, non-covalent complex, we thought it would be worth exploring all the same.

## 2.2.3.2.1 Results

The ANTP peptide was mixed as a 1 to 1 ratio with rhodamine green-labeled tRNA (RhG-tRNA), and applied to adherent CHO-K1 cells. As can be see in Figures 2.23 and 2.24, only cells that were treated with both ANTP and RhG-tRNA are fluorescent. This was true 30 minutes after transfection (Figure 2.23), as well as 24 hours after transfection (Figure 2.24).



**Figure 2.23.** ANTP delivery of tRNA to CHO-K1 cells by non-covalent coupling, imaged 30 minutes after transfection (upper images bright field, lower fluorescent). (A) A 1:1 ratio of tRNA an ANTP (5  $\mu$ g and 30  $\mu$ g, respectively) were mixed in 50  $\mu$ l of Ham's F12 growth media, added to CHO-K1 cells, and incubated for 30 minutes at 37°C. The media was then swapped to L15 for imaging. (B) ANTP peptide only. (C) RhG-tRNA only. The fluorescent images were taken with the same exposure length.



**Figure 2.24**. ANTP delivery of tRNA to CHO-K1 cells by non-covalent coupling, imaged 24 hours after transfection (upper images bright field, lower fluorescent). (A) A 1:1 ratio of tRNA an ANTP (5  $\mu$ g and 30  $\mu$ g repectively) were mixed in 50  $\mu$ l of Ham's F12 growth media, added to CHO-K1 cells, and incubated for 30 minutes at 37°C. The media was swapped to L15 for imaging. (B) ANTP peptide only. (C) RhG-tRNA only. The fluorescent images were taken with the same exposure length.

Although these data were promising, it is possible that the ANTP-tRNA complex was simply stuck to the surface of the cells (possibly through electrostatics) rather than having been transported inside the cells. To test for tRNA transport, the Ser29TAG EGFP DNA was electroporated into CHO-K1 cells 30 minutes prior to application of an ANTP/HSAS mixture. The results from these experiments are shown in Figure 2.25. It can be seen that there is a fair amount of background expression in the negative controls (C - E), which is typical when imaging 24 hours after transfection. Cells transfected with Ser29TAG followed by ANTP/HSAS application demonstrated slightly higher expression than the negative controls (F), but not nearly as high expression as cells that were co-electroporated with both DNA and tRNA (B).



**Figure 2.25**. Non-covalent delivery of HSAS tRNA to CHO-K1 cells by ANTP. CHO-K1 cells were electroporated first with DNA, either wild-type EGFP or Ser29TAG (4  $\mu$ l, 2.5  $\mu$ g/ $\mu$ l). The cells were then incubated for 1 hour at 37°C. A 500  $\mu$ l solution of ANTP (300  $\mu$ g) and/or HSAS tRNA (50  $\mu$ g) was then added, and the cells were incubated for a further 30 minutes. 1.5 ml of fresh media was added, and the media was totally replaced after 5 hours. (A) wt EGFP DNA electroporated, flu exposure 1 s. (B) Ser29TAG DNA (2.5  $\mu$ g/ $\mu$ l) + HSAS (4  $\mu$ g/ $\mu$ l), co-electroporated, flu exposure 0.3 s. (C) Ser29TAG DNA electroporated, flu exposure 4 s. (D) Ser29TAG DNA electroporated, flu exposure 7 s. (E) Ser29TAG DNA electroporated, HSAS added to media 30 minutes later, flu exposure 4 s. (F) Ser29TAG DNA electroporated, HSAS + ANTP added to media 30 minutes later, flu exposure 4 s. the bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.

## 2.2.3.2.2 Discussion

Delivery of tRNA by non-covalent coupling to the ANTP peptide does not seem to be nearly as efficient as microelectroporation. Although these are only preliminary results, it is not obvious how one would optimize this method. Also, it could be tricky to optimize this for delivery of aminoacyl-tRNA. Incubation of aminoacyl-tRNA in cell growth media at 37°C may hydrolyze the amino acid off of the tRNA. Electroporation delivers aa-tRNA in seconds, whereas the time scale of ANTP delivery by non-covalent coupling is uncertain. Although the above results are interesting, microelectroporation seems superior to ANTP-mediated delivery of tRNA to cells, therefore the latter may not be worth pursuing further.

# 2.2.4 Lipofection

Lipid-mediated transfection was also tested for aminoacyl-tRNA delivery to mammalian cells. This method of transfection traditionally involves the loading of DNA into phospholipid vesicles that fuse with the cellular plasma membrane. Transfection with the use of synthetic cationic lipids is termed lipofection. There are many commercially available lipofection reagents that exhibit efficient transfection using DNA. One disadvantage of using lipofection is that the transfection protocol varies significantly between cell types. Another drawback is that the cationic lipids used are usually toxic to cells, and therefore compromise cell health. Furthermore, lipofection has not traditionally been used for RNA delivery to cells [25]. However, RajBhandary and coworkers have recently demonstrated delivery of aminoacyl-tRNA (EYAS-Tyr) using the lipofection reagent Effectene (Qiagen) [21]. They observed suppression of chloramphenicol acetyl transferase (CAT) in COS1 cells. Although their protein expression levels were very low (they did not see expression at the single cell level, but rather had to harvest many cells for their assay) we felt it was worth exploring this method all the same, to compare it to microelectroporation. We decided to test the lipofection reagents Effectene (Qiagen), Polyfect (Qiagen), GeneJammer (Stratagene) and Lipofectamine 2000 (Invitrogen).

#### 2.2.4.1 Results

Because we want to observe protein expression at a single cell level, we used our EGFP assay rather than the CAT assay used by RajBhandary. A new EGFP construct was used for some of these experiments, pEGFP-N1-G2. This EGFP had an N-terminal handle added to the EGFP gene consisting of 40 amino acids, including a 6-His tag. This construct was made to introduce TAG stop codons in the N-terminal handle, such that any unnatural amino acid could be incorporated. This would allow us to test a variety of unnatural amino acids (such as alpha-hydroxy acids which are more stable than amino acids) without compromising the EGFP structure itself. Therefore the TAG mutant Leu28TAG (within the N-terminal handle) was made for this purpose.

Effectene was used to test delivery of aminoacyl-tRNA to CHO-K1, HEK and COS1 cells (Figures 2.26, 2.27 and 2.28). Figures 2.26 and 2.27 show that there is a high level of background expression for the pEGFP-N1-G2 Leu28TAG mutant, much higher than that observed for the pCS2gapEGFP Ser29TAG transfection in COS1 cells (Figure 2.28). However, wild-type expression was significantly higher than read-through expression. Unfortunately, HSAS suppression, which is usually comparable to wild-type

expression, did not lead to expression higher than background. Finally, poor cell health was observed in most cases, when compared to electroporation.



**Figure 2.26**. Effectene transfection of CHO-K1 cells with EGFP DNA and HSAS tRNA. (A) Wild-type pEGFP-N1-G2, fluorescence exposure 1 s. (B) Leu28TAG DNA only, fluorescence exposure 10 s. (C) Leu28TAG DNA and HSAS tRNA, fluorescence exposure 2 s. The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.



**Figure 2.27**. Effectene transfection of HEK cells with EGFP DNA and HSAS tRNA. (A) Wild-type EGFP-N1-G2 DNA, fluorescence exposure 0.1 s. (B) Leu28TAG DNA only, fluorescence exposure 1 s. (C) Leu28TAG DNA + HSAS, fluorescence exposure 1 s. The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.



**Figure 2.28**. Effectene transfection of COS1 cells with EGFP DNA and HSAS tRNA. (A) Wild-type pCS2gapEGFP, fluorescence exposure 5 s. (B) Ser29TAG DNA only, fluorescence exposure 5 s. (C) Ser29TAG DNA + HSAS, fluorescence exposure 5 s. The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.

Polyfect was also tested for tRNA delivery to CHO-K1 cells. It can be seen in Figure 2.29 that there is again read-through of the Leu28TAG mutant. In this case HSAS suppression resulted in higher expression than background, much closer to wild-type expression levels than were observed when using Effectene. However, like Effectene a lot of cell death was apparent after Polyfect transfection.



**Figure 2.29**. Polyfect transfection of CHO-K1 cells using EGFP DNA and HSAS tRNA. (A) Wild-type pEGFP-N1-G2, fluorescence exposure 0.5 s. (B) Leu28TAG DNA only, fluorescence exposure 5 s. (C) Leu28TAG + HSAS, fluorescence exposure 1 s. The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.

We next tested the lipofection reagent GeneJammer. In these experiments we also co-electroporated a dish with TAG mutant DNA and HSAS tRNA to compare the efficiency of lipofection to electroporation. As can be seen in Figure 2.30, there is little background expression of the pCS2gapEGFP mutant. When electroporation of HSAS and Ser29TAG is compared to GeneJammer transfection, the expression levels were

comparable in some cases (Figure 2.30 C and D). However, in most cases electorporation was still more efficient, and expression was observed within a few hours where as expression with GeneJammer transfection was not observed until the next day. There was also a lot of cell death with this lipofection reagent, as observed for the others examined.



**Figure 2.30.** GeneJammer transfection of CHO-K1 cells using EGFP DNA and HSAS tRNA. (A) Wild-type pCS2EGFP DNA, fluorescence exposure 5 s. (B) Ser29TAG DNA only, fluorescence exposure 7 s. (C) Ser29TAG + HSAS tRNA, co-electroporation control, fluorescence exposure 7 s (four 120 V, 50 ms pulses, 4  $\mu$ l of 2  $\mu$ g/ $\mu$ l each DNA and tRNA). (D) Ser29TAG DNA + HSAS tRNA (GeneJammer), fluorescence exposure 7 s. The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.

Lastly, we tested the lipofection reagent Lipofectamine 2000. These experiments were a bit different, in that we first transfected the cells with DNA using Lipofectamine 2000, followed by electroporation of HSAS tRNA. The rational was to see if the cells would tolerate a combination of transfection techniques, so that we could increase the concentration of tRNA in the electroporation solution. The results from these experiments are shown in Figure 2.31. Electroporation of empty buffer into cells

transfected with wild-type EGFP DNA did compromise cell health to some extent, as a lot of cell death was apparent. Electroporation of HSAS tRNA into cells transfected with Ser29TAG DNA did lead to expression, although it was lower than wild-type expression and the cells were unhealthy.



**Figure 2.31.** Lipofectamine 2000 transfection of CHO-K1 cells using HSAS tRNA, following electroporation of EGFP DNA. (A) Wild-type pCS2gapEGFP, delivered using lipofectamine 2000, fluorescence exposure 4 s. (B) Wild-type pCS2gapEGFP DNA, delivered using lipofectamine 2000 followed by electroporation of empty buffer (four 120 V, 50 ms pulses), fluorescence exposure 4 s. (C) Ser29TAG DNA only, delivered with lipofectamine 2000, fluorescence exposure 7 s. (D) Ser29TAG DNA delivered with lipofectamine, followed by electroporation of a 4  $\mu$ l solution of HSAS (2  $\mu$ g/ $\mu$ l, four 120 V, 50 ms pulses). The bright field (upper) and fluorescence (lower) images were taken 24 hours after transfection.

## 2.2.4.2 Discussion

In general, it can be summarized that lipofection offers no real advantage over

microelectroporation. The cells were typically quite unhealthy after transfection,

apparent from the amount of cellular detachment and debris in the tissue culture dishes.

Furthermore, EGFP expression was not observed until the day after transfection whereas

electroporation leads to expression within a few hours. These observations were true for all of the reagents tested, and were important factors to consider for future experiments. Lipofection reagents exhibit a lot of variability between cell types and do not work for transfection of neurons, a limitation we want to avoid. It was our ultimate goal to study ion channel expression in a variety of mammalian cells by electrophysiology, and the cells must be healthy enough for whole-cell patch clamping. Given that most of the cells transfected with lipofection reagents were detached the next day, this is most likely not the best transfection method for our purposes.

## 2.2.5 Biolistics

A final method that we explored for tRNA delivery to mammalian cells was biolistics [21]. The main reason we wanted to try this method is because it is a fairly general transfection technique that works on many cell types including neuronal cultures. Furthermore, RNA has been successfully delivered to cells using biolistics [54, 55], one of the few transfection techniques to do so.

# 2.2.5.1 Results

Precipitation of wild-type EGFP DNA, or co-precipitation of EGFP TAG mutants and tRNA onto gold microcarriers was done prior to transfection. The EGFP mutants included Val55TAG DNA with THG73-ValOH (alpha-hydroxy acid), and Ala37TAG DNA with THG73-Ala. The TAG mutants were also delivered to cells alone, or with 74mer tRNA. These experiments were done before developing the HSAS assay, so HSAS suppression was unfortunately not examined. Figure 2.32 shows CHO-K1 cells transfected with wild-type DNA. There was good transfection efficiency, and the cells appeared quite healthy after transfection. Unfortunately, no expression was observed for any of the other experiments.



**Figure 2.32**. Transfection of CHO-K1 cells using biolistics. Wild-type EGFP DNA was precipitated onto gold microcarriers for transfection. The bright field (left) and fluorescence (right) images were taken 24 hours after transfection.

# 2.2.5.2 Discussion

The biolistics experiments were not explored to their full potential. Transfection with wild-type EGFP DNA gave good expression, and the cells were in good health. It is not surprising that suppression of the EGFP TAG mutants was not observed, similar to electroporation. These experiments were unfortunately done before the HSAS assay was developed, and it would be interesting compare biolistics to electroporation using this assay. However, biolistics is an expensive transfection technique, in that it requires not only a gene-gun, but also fresh gold microcarriers for each experiment. So although this method may be a general mammalian cell transfection method, it may not be the most practical.

# 2.3 Conclusions

Of all the transfection methods tested, the microelectroporator is by far the most efficient. It is the most general (also see Chapter 3), leads to the least amount of cell death, and protein expression can be observed as soon as two hours after transfection. One disadvantage of this method is that it does consume large quantities of aminoacytRNA, in comparison to the *Xenopus* oocyte method. Only a small percentage of the tRNA applied to the cells actually goes inside the cells, and the vast majority is thrown away. This is an area that needs optimization. As an alternative electroporation strategy, single-cell electroporation is now being explored by Dr. Rigo Pantoja. This method has the advantage of requiring significantly less material than the microelectroporator. A micropipette is filled with a small volume (hundreds of nanoliters) of transfection solution, and this can be used to transfect hundreds of cells in multiple dishes. In summary, electroporation is an optimal way to deliver DNA, mRNA and tRNA to mammalian cells for unnatural amino acid incorporation.

# **2.4** Experimental Methods and Materials

### 2.4.1 Materials

Synthetic DNA oligonucleotides were synthesized on an ABI 394 DNA Synthesizer on site. Peptides were synthesized on an ABI 433A Peptide Synthesizer and stored as a lyophilized powder. Restriction enzymes and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). Rhodamine Green-UTP and tetramethylrhodamine-maleimide (TMR-MI) were purchased from Molecular Probes (Eugene, OR). Slide-A-Lyzer was purchased from Pierce (Rochford, IL). ATP- $\gamma$ -S was purchased from Boehringer Mannheim (Inndianapolis, IN). Polyfect and Effectene were purchased from Qiagen (Valencia, CA), GeneJammer from Stratagene (La Jolla, CA), and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). Gold microcarriers and tubing for biolistics were purchased from Bio-Rad (Hercules, CA). Stains-All was purchased from Aldrich (Milwauke, WI). The monoclonal antibody mouse anti-HA was purchased from Covance Research Products (Denver, PA), and the monoclonal antibody anti-mouse was purchased from Jackson Immunoresearch (West Grove, PA). The ECL reagents for chemiluminescent visualization of western blots were purchased from Amersham Biosciences (Piscataway, NJ). The GFP mammalian expression vectors pCS2gapEGFP (membrane-localized) and pCS2EGFP were obtained from Jack Horne (Caltech). pEGFP-F (membrane-localized) and pEGFP-N1 (soluble EGFP construct) were purchased from BD Biosciences Clontech (Palo Alto, CA). The KinaseMax kit, mMessage mMachine and MegaShortScript in vitro transcription kits were purchased from Ambion (Austin, TX). Maxiprep kits used for plasmid isolation, and nucleotide

removal kits were purchased from Qiagen (Valencia, CA). The Rabbit Reticulocyte in vitro protein translation system was purchased from Promega (Madison, WI). Ham's F12 tissue culture media and DMEM were purchased from Irvine Scientific (Santa Ana, CA), and CO<sub>2</sub> independent media was purchased from GIBCO Invitrogen Corporation (Carlsbad, CA). The microelectroporator was built on site. Epizap and indium-tin oxide coated plates were purchased from ASK Science Products (Kingston, ON). The Helios GeneGun system from Bio-Rad (Hercules, CA) was used for biolistics.

#### 2.4.2 Mutagenesis, mRNA and tRNA synthesis

The tRNA genes EQAS, EAAS, EYAS, HSAS 3'71 and EQAS 3'61 were constructed as follows: Two complementary synthetic oligonucleotides encoding for the T7 promoter, the tRNA gene, and the Fok I restriction site were synthesized, annealed and ligated into the EcoR I and BamH I restriction sites of pUC19. After linearlization of the DNA with Fok I, in vitro transcription with the MegShortScript kit (Ambion) yields the corresponding tRNA (Figure ). All mRNA was synthesized using the mMessage mMachine kit (Ambion) with linearized template DNA.

 oligo 1:
 5'-AATTCGTAATACGACTCACTATA-tRNAgene-TGAGACCCATCCG-3'

 oligo 2:
 3'-GCATTATGCTGAGTGATAT-tRNAgene-ACTCTGGGTAGGCCTAG-5'

**Figure 2.33**. Oligo design for tRNA gene construction. The tRNA sequence is inserted where indicated. The T7 promoter is shown in bold on the sense-strand. The sticky ends AATT and CTAG correspond to Eco RI and Bam HI respectively, for insertion into pUC19.

To synthesize Rhodamine green labeled tRNA, the Ambion protocol was modified as follows. A three-to-one mixture of UTP and rhodamine green-UTP (RhG- UTP), respectively, was added to the transcription reaction, with a final total concentration of 2.4 mM. The other nucleotides had a final concentration of 3 mM (total volume of 25  $\mu$ l). After a 2 hour incubation at 37°C, tRNA was isolated by PCI extraction and NH<sub>4</sub>OH/isopropanol precipitation. The tRNA was resuspended in 100  $\mu$ l of RNase free water, and dialyzed (Slide-A-Lyzer, molecular weight cut off of 3500) in 1800 ml of millipore purified water at 4°C for 16 hours to eliminate any unincorporated RhG-UTP. For a RhG-UTP control, 20  $\mu$ g of THG73 tRNA was mixed with an equivalent amount of RhG-UTP as used in the transcription reaction to a total volume of 25  $\mu$ l, and worked up as described.

All EGFP mutants (TAG 29, TAG37, TAG55, TAG66) were made following the Quickchange mutagenesis protocol (Stratagene). The pEGFP-N1-G2 construct (wild-type and Leu28TAG) was made by inserting an N-terminal handle on the EGFP-N1 construct (Clontech). This construct was made to introduce TAG stop codons in the N-terminal handle, such that any unnatural amino acid could be incorporated. This would allow us to test a variety of unnatural amino acids (such as alpha-hydroxy acids which are more stable than amino acids) without compromising the EGFP structure itself. The DNA sequence inserted

(atgcgcggcagccaccaccaccaccaccaccggcatggccggatccagctcatgaccggcggccagcagatgggccgcg acctgtacgacgacgacgacgacaaggaccccccgccgagttc) corresponds to the peptide sequence MRGSHHHHHHGMAGSSSMTGGQQMGRDLYDDDDKDPPAEF. To remove the original kozak sequence and initiator methionine (which was causing a lot of readthrough of the TAG28 mutant), the EGFP gene with the N-terminal handle was cut out of the original vector (using Not I and Eco RI) and subcloned into the corresponding sites of the pGEM-11Zf(+) vector.

### 2.4.3 Tissue culture

CHO cells were grown in Ham's F12 media, and HEK and COS1 cells were grown in DMEM, at 37°C and 5%  $CO_2$ , enriched with glutamine, fetal bovine serum (FBS, 10 %), penicillin, and streptomycin. One to two days prior to electroporation, the cells were passaged onto 35 mm tissue culture dishes such that confluency was typically 50% or less at the time of transfection.

# 2.4.4 Microscopy

Cells were visualized with an inverted microscope (Olympus IMT2), a 250 W Hg/Xe lamp operating at 150 W, a GFP filter set (Chroma, model 41017) with an excitation band pass of 450 to 490 nm and an emission band pass of 500 to 550 nm, 10x/0.25NA or 40x/1.3NA lens, and a Photometrix Quantix CCD camera running Axon Imaging Workbench 4.0.

### 2.4.5 Electroporation

### 2.4.5.1 Microelectroporation

The DNA, mRNA or tRNA to be electorporated into mammalian cells was precipitated alone or as co-precipitates in ethanol and ammonium acetate, and left at -20°C for at least 1 hour. For tRNA-aa, the amino acids have an onitroveratryloxycarbonyl (NVOC), or a 4-pentenoyl (4-PO) protecting group at the N-

terminus. Deprotection of NVOC consisted of irradiating a 15  $\mu$ l solution of tRNA-aa (1  $\mu g/\mu l$ ) in 1 mM NaOAc (pH 4.5) for 6 minutes, using a 1000 W Hg/Xe arc lamp (Oriel, Stratford, CT) operating at 400 W, equipped with WG355 and UG11 filters (Schott, Duryea, PA). Deprotection of the 4-PO consisted of mixing a 15  $\mu$ l solution of tRNA-aa with 15  $\mu$ l of a saturated iodine solution, and allowing it to sit at room temperature for 10 minutes. Reporter EGFP DNA and the AChR subunit mRNA or NMDA subunit DNA were then combined with this and precipitated with ammonium acetate and ethanol. This was then microcentrifuged at 15,000 rpm, 4°C for 15 minutes, vacuum dried for 5 minutes, and resuspended in  $CO_2$  independent medium to the desired final concentration. Immediately prior to electroporation, the cell tissue culture media was swapped to CO<sub>2</sub> independent media (with no glutamine, FBS or antibiotics). Approximately 5  $\mu$ l of the electroporation solution was applied to the cells, followed by application of electrical pulses. This was typically four 120 V pulses of 50 ms duration. The CO<sub>2</sub> independent media was immediately replaced with fresh growth media, and the cells were placed back into the 37°C incubator.

# 2.4.5.2 Epizap

The day prior to electroporation, cells were passaged onto indium-tin oxide coated glass slides (ASK Science Products) such that confluency was typically 50% or less at the time of transfection. The DNA or tRNA to be electorporated was precipitated alone or as coprecipitates in ethanol and ammonium acetate, and left at -20°C for at least 1 hour. This was then microcentrifuged at 15,000 rpm, 4°C for 15 minutes, vacuum dried for 5 minutes, and resuspended in  $CO_2$  independent medium to the desired final concentration.

The cells were typically transfected with EGFP DNA at a concentration of 2.5  $\mu g/\mu l$ , with or without HSAS tRNA at a concentration of 4  $\mu g/\mu l$ . Immediately prior to electroporation, the cell tissue culture media was removed from the glass slides. Approximately 17  $\mu$ l of the electroporation solution was applied to the cells, followed by application of electrical pulses. This was typically four 30 - 50 V pulses, 1.0 - 10  $\mu$ F. Fresh growth media was applied, and the cells were placed back into the 37°C incubator. Imaging of EGFP was done as soon as 2 hours after transfection.

#### 2.4.6 In vitro protein synthesis

Various amber suppressor tRNAs were tested using a Rabbit Reticulocyte expression system (Promega), according to the manufacture's instructions. Typically 1  $\mu$ g of mRNA and 1 - 3  $\mu$ g of tRNA would be added to the lysate mixture along with the amino acid mix. This sat at 30°C for 90 minutes, and was then run on a 10% Tris-Cl polyacrylamide gel in an SDS/Tris/Glycine running buffer. All of the mRNAs used (alpha subunit of the muscle-type nAChR receptor) contained a HA-epitope. The bands were transferred to nitrocellulose, and labeled with monoclonal mouse anti-HA (Covance), followed by monoclonal anti-mouse antibodies coupled to horseradish peroxidase (Jackson) for visualization by chemiluminescence (ECL reagents, Amersham Biosciences).

### 2.4.7 Peptide-oligonucleotide coupling reactions

5'phosphorothioate labeling of tRNA was achieved by an in vitro T4 kinase exchange reaction, according to the Ambion KinaseMax kit protocol. Briefly, a typical reaction consisted of 10  $\mu$ g of tRNA, 80-fold excess of ATP- $\gamma$ -S and 2  $\mu$ l of T4 kinase (20 units) with a total reaction volume of 10  $\mu$ l. Reactions were incubated for 16 or 20 hours at 37°C, and purified with a Qiagen nucleotide removal kit.

tRNA-PO<sub>3</sub>S was assayed by reaction with tetramethylrhodamine-5-maleimide (TMR-MI). A typical reaction (100  $\mu$ l) consisted of combining 5  $\mu$ g of tRNA-PO<sub>3</sub>S with a 20-fold excess of TMR-MI in 10% DMSO, pH 7.5 (HEPES). The reaction was allowed to proceed for 2 hours at room temperature and was then loaded directly onto a 3% agarose gel, run at 50 V for 3-4 hours. A band shift was used to identify tRNA-PO<sub>3</sub>S, as interpreted by a reaction with TMR-MI (ethidium bromide stained gel). Control reactions using unlabeled tRNA were also done.

Reactions of tRNA-PO<sub>3</sub>S with N-terminal maleimide functionalized ANTP peptides (PTD-mal) were also attempted. A typical reaction consisted of combining 2  $\mu$ g of tRNA-PO<sub>3</sub>S with ~ 10-100-fold excess PTD-mal in a 1 M NaCl, 10% DMSO, phosphate buffered solution (pH 7.5) to a total volume of 20  $\mu$ l. Control reactions with unlabeled tRNA, and with the PTD lacking the reactive maleimide were also performed. After reacting for 2 hours at room temperature, the reaction mixture was loaded onto a 12% PAGE gel (19:1, 7M urea, 1 x TBE, running buffer 1 x TBE and 0.1 % SDS), and run at 170 V for 30 minutes. Bands were visualized by treatment with Stains-All.

Synthetic 14mer RNAs had to undergo 2'OH deprotection (silyl) before use. Typically, 1  $\mu$ mol of RNA was mixed with 300  $\mu$ l of 1 M TBAF for 24 hours. The volume was reduced by vacuum, and brought up to 500  $\mu$ l using a 50 % acetonitrile solution. This was purified using a NAP-25 column (Amersham), following the manufacturer's instructions. The eluant was reduced in volume by vacuum, then precipitated using a 1/10 volume of 1 M NH<sub>4</sub>OH, 1 mM spermidine, and 1 volume of isopropanol.

The 5'thiol modified 14mer RNAs had to undergo deprotection of the thiol (trityl) before use. This was done using the Glen Research protocol. Briefly, 15  $\mu$ l of a 1 M AgNO<sub>3</sub> solution was added to a 50  $\mu$ l solution of 14mer in 0.1 M TEAA. After sitting at room temperature for 30 minutes, 20  $\mu$ l of 1 M DTT was added. This was centrifuged, the pellet washed with 100  $\mu$ l of 0.1 M TEAA, the supernatents combined, and brought up to 500  $\mu$ l with 50% acetonitrile. This was desalted on a NAP-25 column (Amersham) according to the manufacturer's instructions. The final eluant was lyophilized to dryness, and stored as a powder.

Coupling reactions between 5'thiol-modified 14mer, 5'phosphorothioate labeled 14mer RNA and PTD-mal were done under a variety of conditions. A typical reaction was done using 5  $\mu$ g of 14mer RNA, with 2 - 20  $\mu$ g of PTD-mal, in 10  $\mu$ l total volume. These were done at room temperature, typically for ~ 2 hours. A variety of buffers and salt concentrations were tested (see results section).

Ligation of the HSAS 3'71mer to the HSAS 5'14mer was done using the following protocol. The HSAS 3'71 was reacted with alkaline phosphatase, cleaned up by PCI extraction, and then reacted with polynucleotide kinase (PNK) and again cleaned up by PCI extraction. 50  $\mu$ g of this tRNA was mixed with 50  $\mu$ g of the 14mer RNA in 180  $\mu$ l, and heated to 90°C for 2 minutes. Separately, a solution of 200 mM MgCl<sub>2</sub> and 400 mM HEPES were heated to 90°C. 40  $\mu$ l of each of these solutions was added to the hot RNA solution. This was cooled to room temperature, and 80  $\mu$ l of a 5x ligation buffer was added, as well as 40  $\mu$ l DMSO and 20  $\mu$ l of RNA ligase. This was then incubated at 15°C overnight. The 5x ligation buffer consisted of 20  $\mu$ l 100mM DTT, 7.5  $\mu$ l 10 mM ATP, 20  $\mu$ l 20mg/ml BSA-Ac, 50.5  $\mu$ l water and 2  $\mu$ l RNase inhibitor.

### 2.4.8 Lipofection

#### 2.4.8.1 Effectene

Cells were transfected following the manufacturer's instructions (Qiagen). Briefly, 1.7  $\mu$ g of DNA, with or without 3.5  $\mu$ g of HSAS tRNA, was combined with 85  $\mu$ l of the provided EC buffer and incubated for 5 minutes. 5.1  $\mu$ l of Enhancer was added and incubated for a further 5 minutes. 10.2  $\mu$ l of Effectene was added to this, incubated for 10 minutes and diluted to 600  $\mu$ l with prewarmed DMEM (10% FBS, including antibiotics). This was added to the cells, followed by 1.5 ml of fresh media and incubated overnight.

# 2.4.8.2 Polyfect

Cells were transfected following the manufacturer's instructions (Qiagen). Briefly, 3  $\mu$ g of DNA with or without 7  $\mu$ g of HSAS tRNA was diluted to 150  $\mu$ l using serum free media. 15  $\mu$ l of the Polyfect reagent was added to this, and incubated for 10 minutes. Fresh media (1 ml) containing serum and antibiotics was added to the cells, and 1 ml of fresh media was added to the Polyfect complex, the latter of which was also applied to the cells. This was incubated overnight.

### 2.4.8.3 GeneJammer

Cells were transfected following the manufacturer's instructions (Stratagene). Briefly, 6  $\mu$ l of the GeneJammer reagent was added to 100  $\mu$ l of serum-free media, and incubated for 10 minutes. 1  $\mu$ g of DNA with or without 5  $\mu$ g of HSAS tRNA was added to this and incubated for a further 10 minutes. 900 ml of fresh serum containing media was added to the cells, and the transfection mix was added to this drop-wise. The cells were incubated for 3 hours, 1 ml of fresh media was then added, and the cells were further incubated overnight.

### 2.4.8.4 Lipofectamine 2000

Cells were transfected following the manufacturer's instructions (Invitrogen). Briefly, 1  $\mu$ g of DNA was added to 250  $\mu$ l of serum-free media. Separately, 3  $\mu$ l of the Lipofectamine 2000 reagent was added to 250  $\mu$ l of serum-free media and incubated for 5 minutes. The DNA and Lipofectamine solutions were combined and incubated for 20 minutes. 2 ml of fresh serum containing media was added to the cells, followed by addition of the 500  $\mu$ l DNA/Lipofectamine complex solution. This was incubated for 4 hours, at which point the media was replaced with fresh growth media.

## 2.4.9 Biolistics

Preparation of the DNA/tRNA coated gold microcarriers was done according to the Bio-Rad protocol. Briefly, 17.5 mg of 1.6  $\mu$ m gold beads were weighed out. 50  $\mu$ l of a 50 mM spermidine solution was added to the beads, and sonicated for 5 minutes. 25  $\mu$ g of DNA with or without 50  $\mu$ g of tRNA was added, and the mixture was vortexed. While vortexing, 50  $\mu$ l of a 1 M CaCl<sub>2</sub> solution was added drop-wise. The suspension was incubated at room temperature for 10 minutes, with vortexing every 2 minutes. This was then centrifuged, washed three times with 100% ethanol, and resuspended in 300  $\mu$ l PVP/ethanol solution (0.02 mg/ml PVP in ethanol) with vortexing. This was transferred to a 15 ml conical tube, and diluted to 3.5 ml with the PVP/ethanol solution. The gold-coat tubing was then prepared as described by the Bio-Rad Helios Gene Gun protocol.

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