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Monitoring mis-acylated tRNA suppression efficiency in mammalian cells via EGFP fluorescence recovery

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ABSTRACT

A reporter assay was developed to detect and quantify nonsense codon suppression by chemically aminoacylated tRNAs in mammalian cells. It is based on the cellular expression of the enhanced green fluorescent protein (EGFP) as a reporter for the site-specific amino acid incorporation in its sequence using an orthogonal suppressor tRNA derived from *Escherichia coli*. Suppression of an engineered amber codon at position 64 in the EGFP run-off transcript could be achieved by the incorporation of a leucine via an *in vitro* aminoacylated suppressor tRNA. Microinjection of defined amounts of mutagenized EGFP mRNA and suppressor tRNA into individual cells allowed us to accurately determine suppression efficiencies by measuring the EGFP fluorescence intensity in individual cells using laser-scanning confocal microscopy. Control experiments showed the absence of natural suppression or aminoacylation of the synthetic tRNA by endogenous aminoacyl-tRNA synthetases. This reporter assay opens the way for the optimization of essential experimental parameters for expanding the scope of the suppressor tRNA technology to different cell types.

INTRODUCTION

The site-specific incorporation of unnatural amino acids into proteins in living cells is of importance to analyze *in vivo* protein structure and function as well as cellular processes using amino acid analogs comprising probes which are photoactivatable, fluorescent or chemically reactive (1–6). This emerging technology relies on the suppression of nonsense codon mutations by chemically acylated tRNAs and has been originally developed as an *in vitro* method (7–12). Meanwhile, several reports for its *in vivo* application in *Xenopus* oocytes (4–6,13,14), *Escherichia coli* (15–18) and COS1 cells (19) appeared in the literature. An expansion of this technology to other cell lines would demand a reporter system permitting the definition of optimal parameters for the site-specific incorporation of amino acid analogs into proteins.

Here, suppressor tRNA technology was applied to Chinese hamster ovary (CHO) cells which, like other mammalian cell types, are generally more suitable for structural and functional studies of human-derived proteins if specific post-translational modifications are important. In addition, certain proteins such as neuro-receptors are optimally expressed only in particular cell lines. We focused on the enhanced green fluorescent protein (EGFP) as a reporter to assess the efficiency of nonsense codon suppression directly in living cells. An amber stop codon mutation was site-specifically introduced in the core position of the EGFP, removing an amino acid essential for the formation of the fluorophore. The transfer of that mutagenized *in vitro* transcript into CHO cells was followed by the expression of an incomplete, non-fluorescent protein. After co-transfer with a cognate synthetic suppressor tRNA, we could monitor the successful re-incorporation of the missing amino acid by recovery of the EGFP fluorescence signal, which could be quantified by using laser-scanning confocal microscopy on living cells.

Unlike other fluorescent reporters such as luciferase or β-galactosidase, EGFP does not require the addition of substrate or cofactors nor cell lysis or fixation. Furthermore, it is stable over a period of several days and, due to its strong fluorescence, allows an accurate and sensitive determination of suppression efficiencies in individual cells. This strategy could be used to find proper conditions for an efficient suppression in a number of different mammalian cell lines.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were purchased at MWG-Biotech AG (Ebersberg, Germany). Kits for plasmid and DNA-fragment purification were obtained from QIAGEN GmbH (Hilden, Germany). Restriction endonucleases (*Bsa*I, *Eco*RI and *Not*I) were provided by New England Biolabs (MA, USA). The MEGAscript kit for *in vitro* transcription and the cap analog $m^7G(5')ppp(5')G$ were from Ambion (TX, USA). Purified rEGFP was purchased at Clontech (CA, USA). Octadecyl rhodamine B (R18) and Alexa Fluor 546 C_5 maleimide were obtained from Molecular Probes (OR, USA). Other chemicals were purchased at Sigma-Aldrich (MO, USA).

Transcription of reporter gene

The coding sequence of the EGFP (pEGFP-N1, Clontech) was modified by the addition of a T7 promoter site and a poly(A) tail using PCR amplification with synthetic oligonucleotides

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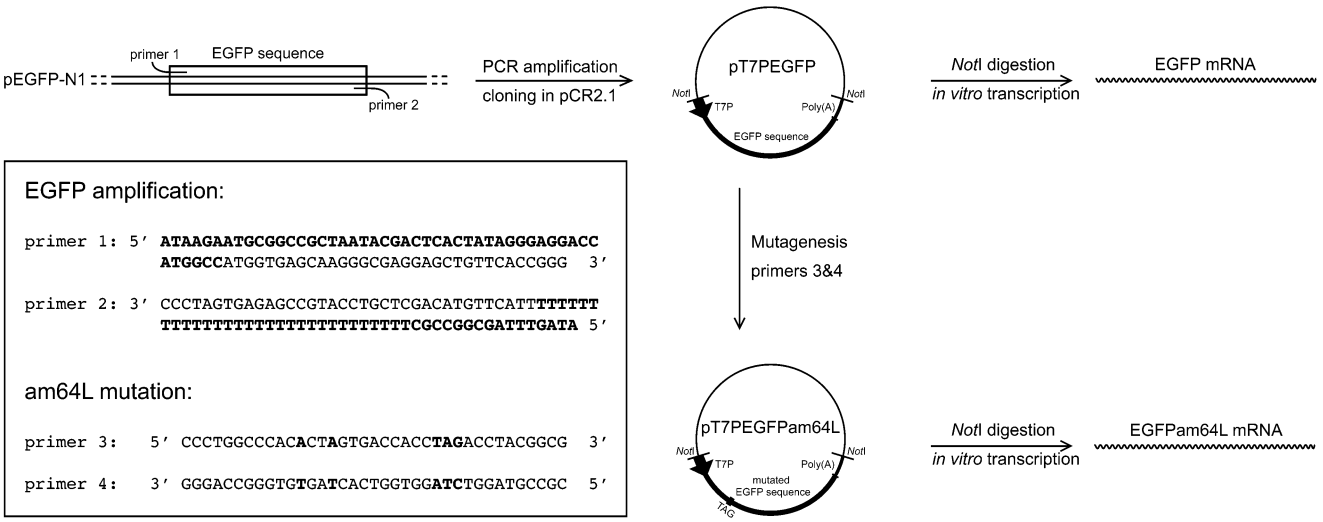

Figure 1. Scheme of the cloning steps for the wild-type (pT7PEGFP) and mutant EGFP (pT7PEGFPam64L) encoding plasmids used for the *in vitro* transcription. PCR amplification with primers 1 and 2 was performed to add a T7 promoter to the 5′ end and a poly(A) tail to the 3′ end of the EGFP coding sequence. Primers 3 and 4 were used for replacing the leucine 64 codon by TAG, and providing silent mutations for clonal selection. All nucleotide sequence modifications are shown in bold letters.

(Fig. 1). The resulting 814 bp fragment was ligated into the pCR2.1 vector using the TA cloning kit (Invitrogen, CA, USA) to obtain the plasmid pT7PEGFP.

The pT7PEGFPam64L is a mutated version of the pT7PEGFP plasmid. The CTG codon at position 64 of the EGFP coding sequence was mutated to a nonsense amber (TAG) codon by site-directed mutagenesis using the Quickchange kit (Stratagene, CA, USA). All plasmid constructs were controlled by restriction mapping and DNA sequencing.

After linearization of the wild-type and mutated plasmids by *Not*I, *in vitro* transcription was performed with T7 RNA polymerase using the MEGAscript kit (Ambion). Capping of mRNA was achieved during transcription by replacing 80% of the GTP level with the cap analog $m^7G(5')ppp(5')G$ (Ambion). After removing the DNA template by DNase I treatment, the resulting mRNAs were purified by successive phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1) extractions, precipitation with an equal volume of isopropanol for 1 h at –20°C, followed by centrifugation at 0°C/20 800 *g* for 15 min. The mRNA pellet was dried and redissolved in sterile DEPC-treated H_2O. The integrity and size of the mRNAs were assayed by agarose gel electrophoresis under denaturing conditions, and the concentration was determined by measuring the optical density at 260 nm.

Transcription of suppressor tRNA gene

A 105 bp synthetic template fragment encoding the *E.coli* suppressor $tRNA^{Ala}_{CUA}$ was prepared by reannealing and ligating two synthetic oligonucletides containing the tRNA gene flanked by a T7 promoter (Fig. 2). This blunt-end DNA fragment was cloned into pCR4 vector using the TOPO cloning kit (Invitrogen), following the instructions of the manufacturer. The resulting plasmid pEcoliAlaCUA was checked by restriction mapping and sequencing.

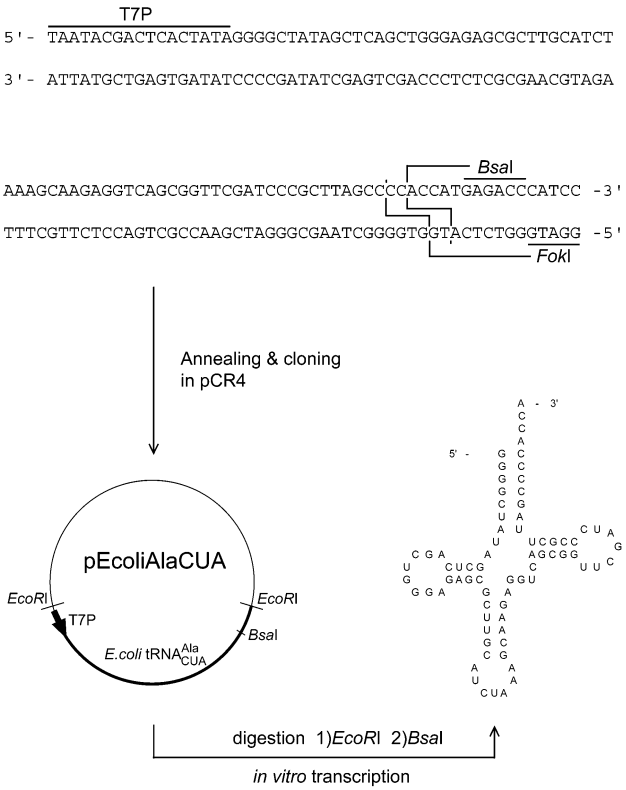

Figure 2. Scheme for the *in vitro* synthesis of the suppressor tRNA from a synthetic DNA template by T7 run-off transcription. Two synthetic complementary oligonucleotides encoding the *E.coli*-derived suppressor $tRNA^{Ala}_{CUA}$ were annealed and cloned into the pCR4 vector, leading to pEcoliAlaCUA. T7 RNA polymerase run-off transcription was performed on the purified *Eco*RI and *Bsa*I fragment giving rise to a 76mer suppressor tRNA.

Plasmid pEcoliAlaCUA was linearized by successive restriction endonuclease digests with *Eco*RI and *Bsa*I. After agarose gel purification this DNA fragment was used for the

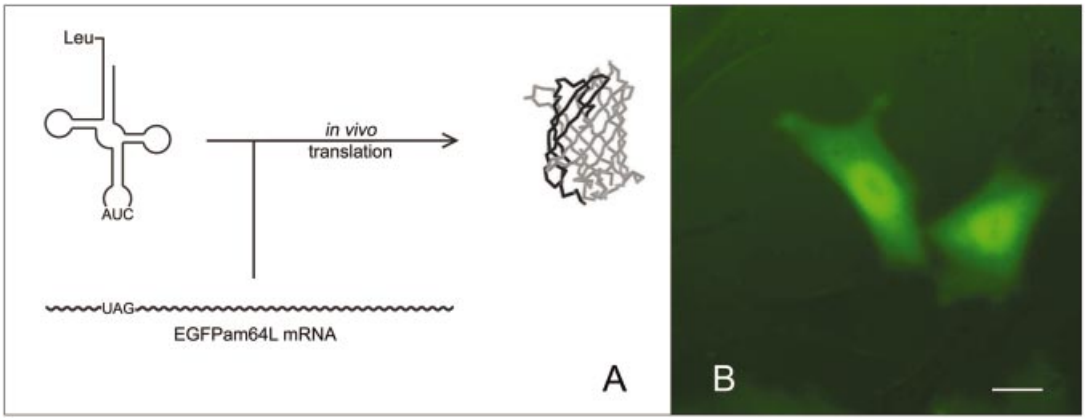

Figure 3. *In vivo* suppression visualized by EGFP fluorescence recovery. (**A**) Schematic view of the assay for the amber suppressor tRNA function: co-injection of a mutated EGFP mRNA containing an amber codon at position 64 with an amber suppressor leucyl-tRNA. A successful suppression leads to complete translation of the mRNA transcript, thus to the appearance of the EGFP molecule. (**B**) Laser-scanning confocal micrograph of CHO cells visualizing EGFP fluorescence 20 h after co-injection of the mutated EGFP-mRNA with the amber suppressor tRNA. Size bar is 10 µm.

run-off transcription with T7 RNA polymerase following the protocol of the MegaScript kit (Ambion). After removing the template DNA by DNase I treatment the tRNA was purified by following the protocol described above for mRNA. The integrity and purity of the tRNA was assayed by polyacrylamide gel electrophoresis, and the concentration was determined by measuring the optical density at 260 nm.

Deprotection of *N*-(4-pentenoyl)-*S*-leucyl-tRNA

Pentenoyl-protected aminoacylated $tRNA^{Ala}_{CUA}$ was synthesized at Cruachem Ltd (Glasgow, Scotland, UK). Ten micrograms of lyophilized protected tRNA were resuspended in 10 µl of H_2O. Deprotection was accomplished by adding 2.5 µl of 25 mM I_2 in 1:1 THF–H_2O and incubating the mixture for 10 min at 25°C. The leucyl-tRNA was precipitated by successive additions of 1.25 µl of 3 M NaOAc pH 5.3 and 31.25 µl of cold ethanol. After centrifugation at 0°C/20 800 *g* for 15 min the pellet was washed once with 70% cold ethanol, dried and dissolved in H_2O to a final concentration of 4 µg/µl.

Cell lines and cell culture

Adherent mammalian cells (CHO) were grown in Dulbecco's modified Eagle's medium (DMEM/F12; GIBCO BRL, Rockville, USA). The medium was supplemented with 2.2% fetal calf serum (GIBCO BRL). The cultures were split, distributed in 35 mm Nunc dishes at a density of 50 000 cells/ml 1 day prior to injection, and kept at 37°C in a humidified atmosphere with 5% CO_2.

Injections

Microinjections of CHO cells with mRNA and tRNA mixtures diluted in sterile DEPC-treated H_2O were performed using an InjectMan controller and a Transjector 5246 system (both from Eppendorf, Hamburg, Germany) mounted on an Axiovert S100TV inverted microscope (Carl Zeiss AG, Oberkochen, Germany). FemtotipsII capillaries (Eppendorf) were used for all injections.

Laser-scanning confocal microscopy

Laser-scanning confocal microscopy was performed using a Zeiss LSM510 (Carl Zeiss AG). Detection and distinction of the fluorescence signals of EGFP (excitation 488 nm/emission 505–530 nm), Alexa546 and R18 (excitation 543 nm/emission >560 nm) was achieved by appropriate filter sets using a multitracking mode. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorophores.

RESULTS

The general scheme used for *in vivo* visualization of misacylated tRNA suppression is presented in Figure 3A. The suppression efficiency was determined by measuring the appearance of EGFP fluorescence in living cells. The EGFP coding sequence was mutated at position 64 by replacing the CTG (leucine) codon with an amber stop codon in the core of the reporter gene, giving rise to the EGFPam64L *in vitro* transcript. A successful suppression by co-injection of this mutated mRNA with a suppressor tRNA carrying the 'removed' amino acid would lead to the completion of the EGFP translation. Twenty hours after co-injection of suppressor leucyl-tRNA with EGFPam64L mRNA, we observed the recovery of the EGFP fluorescence in CHO cells by laser-scanning confocal microscopy (see Fig. 3B), whereas the co-injection of the mutated mRNA together with the non-aminoacylated tRNA did not lead to any detectable fluorescence within the cells (data not shown).

Because the injected volume may slightly vary between cells, mostly depending on their intrinsic viscosity, it was necessary to quantify this parameter in order to validate further comparisons between different experiments. Therefore, the injections of mRNA and tRNA were performed together with a known concentration of Alexa546. This dye is non-toxic for the cell and its excitation and emission wavelengths are well distinguishable from those of EGFP.

Correlation between the fluorescence of the co-loaded dye and the injected volume was obtained by measuring its dilution in the cell and by calculating individual cell volumes.

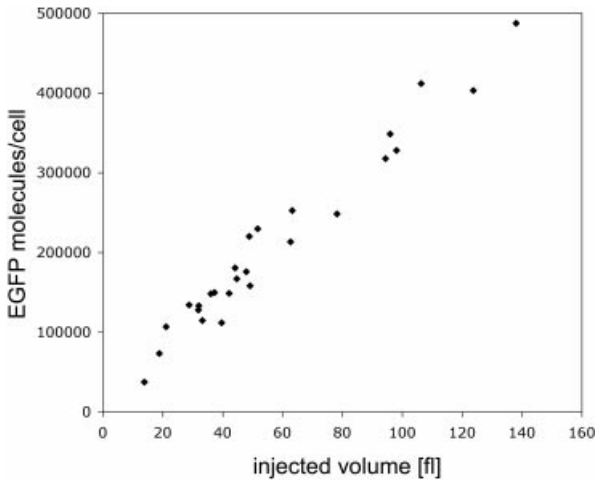

Figure 4. Correlation between injection volume and number of EGFP molecules per cell by microinjection of variable volumes of a solution containing 500 ng/µl rEGFP and 250 ng/µl Alexa546 in CHO cells. The injected volume is calculated on the basis of the known cell volume (see text for detailed description) and the final concentration of Alexa546 dye measured directly in the cell (based on a calibration curve established before).

We first elaborated a fluorescence intensity calibration curve by measurements on droplets containing known concentrations of the Alexa546 dye using laser-scanning confocal microscopy. A linear relation between fluorescence intensity and dye concentration was found. Fluorescence intensity measurements of the co-injected Alexa546 dye on living cells permitted the determination of the dye concentration within the cells on the basis of this calibration curve.

The injection volume of the Alexa dye together with mRNA and tRNA mixtures can then be determined by calculating the cell volume. Therefore, we detached the adherent cells by addition of 1 mM EDTA and incubation at 37°C for 10 min. Under these conditions the cells became spherical. They were then membrane-stained with R18 (5 µM/10 min incubation at 37°C) to measure their diameter by laser-scanning confocal microscopy, leading to a volume of 1.2 ± 0.1 pl (data not shown). The number of EGFP molecules which were expressed during *in vivo* translation in individual cells can be estimated by comparison of cell-derived EGFP fluorescence intensities with EGFP fluorescence intensities measured on droplets of known concentrations of purified rEGFP.

To validate the precision of our injections and concentration calibrations, we also co-injected variable volumes of a solution containing 500 ng/µl rEGFP and 250 ng/µl Alexa546 in CHO cells. As seen in Figure 4, there is a linear correlation between injected volume, given by the Alexa546 fluorescence signal, and the number of EGFP molecules per cell.

On the basis of the presented control experiments we performed all following measurements using concentrations of 250 ng/µl Alexa546, 2 µg/µl mRNA and 2 µg/µl leucyl-$tRNA^{Ala}_{CUA}$. These concentrations gave the best suppression efficiencies: (i) permitting the highest number of RNA molecules delivery without capillary blocking due to the resulting high solution viscosity, and (ii) favoring the suppression against competing endogenous termination factors at an excess of 10:1 of tRNA:mRNA molecules.

The EGFP fluorescence resulting from *in vivo* suppression was stable between 12 and 24 h post-injection. The injected cells were easily identified under the microscope via fluorescence of the co-injected dye. Thereby we could confirm that every injected cell turned green and could be re-identified even after 24 h incubation at 37°C. Using the EGFP and Alexa546 fluorescence intensity calibration curves, we calculated the number of translated EGFP molecules by confocal microscopy 20 h after microinjection. Because the variation between injection volumes was 0.05 ± 0.01 pl, we corrected the number of translated EGFP molecules by normalization to an average injection volume of 0.05 pl (Table 1). We obtained suppression efficiencies of 15 ± 3% by comparing EGFP fluorescence signals resulting from *in vivo* suppression with fluorescence signals derived from the injection of the identical amounts of non-mutated EGFP mRNA.

The microinjection of a non-aminoacylated tRNA together with the mutagenized reporter transcript did not produce any detectable fluorescence at the EGFP emission wavelength of 510 nm in CHO cells when we used the identical parameters of confocal microscopy (laser intensity, sensitivity of detection, pinhole diameter, filter sets, etc.) as for measuring the EGFP fluorescence recovery upon nonsense codon suppression. If we substantially increased the laser power for the excitation at 488 nm and the sensitivity of fluorescence signal detection at 510 nm we could hardly detect unspecific cellular autofluorescence, identical to that of non-injected control cells.

DISCUSSION

A novel assay has been developed using EGFP as a reporter for direct quantification of termination codon suppression efficiencies in living mammalian cells. We have shown by fluorescence recovery that an aminoacylated tRNA derived from *E.coli* can suppress an amber mutation at codon 64 of the EGFP mRNA by *in vivo* translation in CHO cells, whereas the

Table 1. Number of mRNA and tRNA molecules per cell directly calculated after microinjection by comparison of the co-injected Alexa dye with the confocal calibration curve on droplets

0.05 pl injection	mRNA ($\times 10^3$ molecules/cell)	tRNA ($\times 10^3$ molecules/cell)	EGFP ($\times 10^3$ molecules/cell)
EGFPam64L mRNA + $tRNA_{CUA}$	250 ± 20	2500 ± 200	ND
EGFPam64L mRNA + leucyl-$tRNA_{CUA}$	250 ± 20	2500 ± 200	270 ± 30
EGFP mRNA	250 ± 20	–	1800 ± 200

Expression of EGFP molecules per cell determined on 50 individual cells, 20 h after injection of RNA. The values are corrected for a standard injection volume of 0.05 pl. Co-injections were all processed using concentrations of 2 µg/µl mRNA, 2 µg/µl tRNA and 250 ng/µl Alexa546. ND, not detectable.

non-aminoacylated form of the same tRNA cannot. EGFP fluorescence intensities measured in individual cells by laser-scanning confocal microscopy allowed us to quantify suppression efficiencies. Kohrer *et al.* (19) have used a different approach to insert amino acid analogs into the chloramphenicol acetyltransferase (CAT) by importing a suppressor Tyr-tRNA in COS1 cells using lipofection. These authors measured CAT activities in cell extracts to determine average levels of termination codon suppression efficiencies in cell samples but not on individual cells.

The use of EGFP as a suppression reporter offers advantages compared to other detection techniques such as those based on luciferase, β-galactosidase or CAT. In contrast to these destructive methods, EGFP suppression can be followed *in vivo* in a single cell without further chemical or enzymatic treatment, due to the sufficiently high quantum yield of this protein.

To improve the suppression-specific fluorescent signal, we increased the signal-to-noise ratio by lowering the background signal resulting from possible natural suppressor tRNAs. This was fulfilled by choosing an amino acid in the protein sequence that plays an important role for the fluorescence properties of the EGFP. In consequence, a cell line which comprises a natural suppressor tRNA aminoacylated with a different amino acid would provide a background signal reduced to an undetectable value. We selected the leucine at position 64 for its importance in the fluorescence properties of the EGFP, in particular because it greatly improves correct protein folding (20,21). Furthermore, due to the fact that it is neither a charged nor a polar amino acid, it is more quickly accepted and processed by the ribosome, thus better competing with the undesirable effect of termination factors which could prevent the completion of the full-length protein (22–26).

To determine the contribution of the unnatural suppression under investigation, the suppressor tRNA should also not be recognized by any endogeneous aminoacyl-tRNA synthetases. This natural aminoacylation of the suppressor tRNA can be minimized by choosing a tRNA from another organism. In our present study, the tRNA sequence was based on that of *E.coli* $tRNA^{Ala}_{GGC}$ (27) mutated to CUA at the anticodon site and modified by two other mutations: A38U improves amber suppression efficiency (28–30) and C70U renders the non-aminoacylated tRNA a poor substrate for *E.coli* alanyl-tRNA synthetase (31). This tRNA sequence has been shown by Karginov *et al.* (9) to have good suppression properties in *in vitro* translation systems.

Our experiments to determine suppression efficiencies by measuring EGFP fluorescence intensities in cells and to estimate amounts of injected mRNA and tRNA molecules were based on laser-scanning confocal microscopy measurements. Microinjection of defined amounts of fluorescent dyes in CHO cells demonstrated the suitability of this transfection technique to incorporate, in a controlled way, quantifiable molecule amounts, in contrast to saponin permeabilization (32), electroporation (33) or lipofection (19) of mammalian cells. Furthermore, unlike these other techniques, control of the transfected amount does not depend on cell type or density, allowing a more accurate comparison between different cell types. Finally, microinjection requires only low amounts of material, and prevents the RNA degradation that can take place during delivery in other transfection techniques.

We were able to observe amber suppression of an amber mutated EGFP mRNA using mis-acylated tRNA in CHO cells, obtaining 15 ± 3% of the fluorescence signal obtained by injection of the same amount of non-mutated EGFP mRNA. After estimation of the RNA molecules and EGFP protein numbers, we obtained approximately one suppressed translation by mRNA (see Table 1). Referring to a typical injection volume in CHO cells of 0.05 pl, the number of proteins resulting from unnatural suppression was in the range of 270 000 ± 30 000.

The general applicability of the presented EGFP-based reporter assay will allow us to extend the suppressor tRNA technique for many other cell types, and hence permit the selection of suitable suppressor tRNAs for the site-directed modification of proteins. Of particular interest for applying suppressor tRNA technology are investigations of selective molecular interactions in live biological cells by fluorescence techniques with high time, spatial and single molecule resolution, to elucidate the complex cellular biochemical networks (34–36).

ACKNOWLEDGEMENTS

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