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Downregulation of eRF1 by RNA interference increases mis-acylated tRNA suppression efficiency in human cells

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The site-specific incorporation of non-natural amino acids into proteins by nonsense suppression has been widely used to investigate protein structure and function. Usually this technique exhibits low incorporation efficiencies of non-natural amino acids into proteins. We describe for the first time an approach for achieving an increased level of nonsense codon suppression with synthetic suppressor tRNAs in cultured human cells. We find that the intracellular concentration of the eukaryotic release factor 1 (eRF1) is a critical parameter influencing the efficiency of amino acid incorporation by nonsense suppression. Using RNA interference we were able to lower eRF1 gene expression significantly. We achieved a five times higher level of amino acid incorporation as compared with non-treated control cells, as demonstrated by enhanced green fluorescent protein (EGFP) fluorescence recovery after importing a mutated reporter mRNA together with an artificial amber suppressor tRNA.

Keywords: eukaryotic release factor 1/fluorescent proteins/gene silencing/protein design/suppressor tRNA

Introduction

The site-specific incorporation of non-natural amino acids into proteins by nonsense suppression opens up novel routes for protein engineering (Strømgaard *et al*., 2004). For example, individual proteins can be site-selectively equipped with fluorescent, photo-activatable or other reactive probes suitable for structural and functional investigations. Originally developed as an *in vitro* labeling technique (Kurzchalia *et al*., 1988; Noren *et al*., 1990; Cload *et al*., 1996; Karginov *et al*., 1997; Hohsaka *et al*., 1998; Short *et al*., 1999), nonsense suppression has been progressively adapted to prokaryotic (Liu *et al*., 1997; Liu and Schultz, 1999; Wang *et al*., 2001, 2002) and eukaryotic cells (Turcatti *et al*., 1996, 1997; Chollet *et al*., 1998; Beene *et al*., 2002; Cohen *et al*., 2002). Recently, the scope of this technique was expanded to mammalian live cells (Ilegems *et al*., 2002), yielding suppression efficiencies of the order of 15% and allowing the site-specific modification of proteins with non-natural amino acids (Monahan *et al*., 2003). Here we report on strategies to increase substantially the level of nonsense suppression efficiency in cultured mammalian cells.

The intracellular concentration of the eukaryotic release factor 1 (eRF1) is a parameter that is known to influence natural nonsense suppression efficiency (Carnes *et al*., 2000). Published data point to competitive actions between natural suppressor tRNAs and eRF1 for stop codon recognition based on their structural similarity and on their assumed common binding site to the ribosome (Drugeon *et al*., 1997; Bertram *et al*., 2000, 2001). Whereas a suppressor tRNA is required for incorporation of amino acids and ribosomal read-through, the eRF1 factor triggers the release of the nascent peptide resulting in termination of translation (Carnes *et al*., 2000). Overexpression of eRF1 was shown to increase termination efficiency (Le Goff *et al*., 1997) whereas a decreased eRF1 activity promoted stop codon read-through in eukaryotic cells (Carnes *et al*., 2000). Analogous findings were achieved in prokaryotic cell-free translation systems using partially heat inactivated S-30 extracts that express a temperature sensitive variant of *Escherichia coli* release factor 1 (RF1) (Short *et al*., 1999). Furthermore, it has been shown in recent studies that mRNAs containing a premature stop codon spatially distant from the 3′ end can be degraded by a Upf1 complex in which the eRF1 is involved (Czaplinski *et al*., 1998; Hilleren and Parker, 1999). These observations point to eRF1 as a factor which is potentially influencing nonsense codon suppression also in presence of artificial suppressor tRNAs.

Here we explore the feasibility of post-transcriptional gene silencing of eRF1 to achieve higher efficiencies of amino acid incorporation by nonsense suppression in HEK293 cells. The silencing property of short interfering RNAs (Elbashir *et al*., 2001a,b; Chiu and Rana, 2002; McManus and Sharp, 2002) was used to target eRF1 messenger RNA. Changes in eRF1 gene expression were quantified by real-time RT-PCR. The effects of eRF1 silencing on nonsense suppression efficiency were investigated using an enhanced green fluorescent protein (EGFP) reporter transcript carrying an amber codon suppression site at an amino acid position, which is essential for fluorophore formation, as described previously (Ilegems *et al*., 2002). We found that only the co-injection of the mutated EGFP reporter messenger RNA together with an artificial suppressor tRNA, which can be aminoacylated *in vivo*, gave rise to detectable EGFP fluorescence signals inside HEK293 cells indicating site-specific nonsense suppression events. The level of EGFP fluorescence recovery reflected the suppression efficiency and could be quantified in eRF1 silenced cells compared with non-treated cells using laser-scanning confocal microscopy.

Materials and methods

Materials

Synthetic oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany), kits for plasmid and DNA fragment purification from QIAGEN GmbH (Hilden, Germany), restriction endonucleases (*Bsa*I, *Eco*RI and *Not*I) from New England Biolabs (Beverly, MA), MEGAscript and MEGAshortscript kits for *in vitro* transcription and also the cap analog $m^7G(5')ppp(5')G$ from Ambion (Austin, TX) and

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purified rEGFP from Clontech (Palo Alto, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

Adherent mammalian cells (Human Embryonic Kidney, HEK293) were grown in DMEM/F12 (Dulbecco's modified Eagle medium; GIBCO BRL, Rockville, MD). The medium was supplemented with 2.2% fetal calf serum (GIBCO BRL). One day prior to injection, cells were transferred to 35/12 mm WillCo-dishes (WPI, Stevenage, UK) at a density of 100 000 cells/ml and kept at 37°C in a humidified atmosphere with 5% CO_2.

siRNA synthesis and transfection

The siRNA targeting the eRF1 mRNA was synthesized by annealing *in vitro* transcripts as depicted in Figure 1A: for each RNA fragment, two synthetic DNA oligonucleotides were annealed to obtain templates flanked by a double-stranded T7 promoter (Milligan *et al*., 1987). *In vitro* transcriptions were performed using the MEGAshortscript kit (Ambion). After removing the DNA templates by *DNase*I treatment, the resulting RNAs were purified by successive phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1) extractions. RNA precipitation was achieved by adding an equal volume of isopropanol and by incubation for 1 h at −20°C, followed by centrifugation at 0°C/20 800 *g* for 15 min. The RNA pellets were air dried at room temperature and dissolved in sterile DEPC-treated H_2O. RNA concentrations were determined by measuring the optical density at 260 nm. The two complementary RNA strands were annealed at an equimolar ratio and dissolved in DEPC-treated water finally to obtain the siRNA at a concentration of 20 μM. The integrity and size of the siRNA were assayed by polyacrylamide gel electrophoresis under denaturing conditions.

HEK293 cells were transfected with siRNA using the TransMessenger Kit from Qiagen, following the manufacturer's instructions. Typically, for a 35 mm Nunc dish, 3.4 μl of Enhancer-R, 6 μl of siRNA (20 μM) and 8.5 μl of TransMessenger were used. The medium was changed 4 h after transfection. Control experiments based on non-eRF1 targeting siRNA were carried out using the same transfection protocol and the commercially available negative control siRNA from Qiagen.

Generation of the stable cell line HEK-seRF1

Complementary DNA nucleotides were annealed and ligated into pSIREN-RetroQ (Clontech), as shown in Figure 1B, giving rise to the plasmid pSIRENeRF1. This vector leads to the expression of hairpin RNAs containing a nine-nucleotides loop, as described by Yu *et al*. (2002). After calcium phosphate transfection of HEK293 cells (Jordan *et al*., 1996), the expression was maintained by supplementing 1 μg/ml of puromycin in the growth medium. This selection was made during 3 weeks prior to real-time PCR experiments.

In vitro transcription of reporter genes

The coding sequence of the red fluorescent protein DsRedExpress (pDsRedExpress, Clontech) was modified by the addition of a T7 promoter site and a poly(A) tail using PCR amplification with synthetic oligonucleotides. The resulting fragment was ligated into the pCR2.1 vector using a TA cloning kit (Invitrogen, Carlsbad, CA) to obtain the plasmid pT7PDsRedExpress, which was analysed by restriction mapping and DNA sequencing.

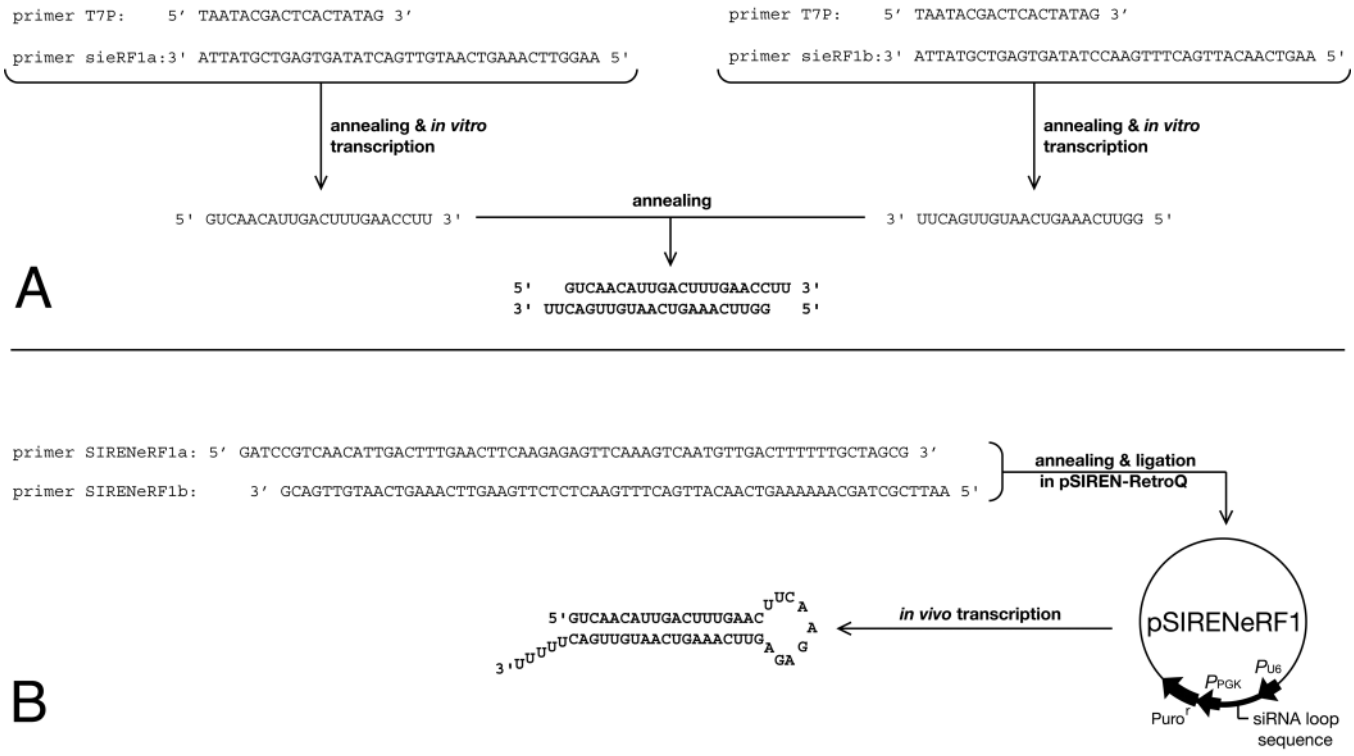

Fig. 1. Scheme of the synthesis of interfering RNAs targeting the eRF1 transcripts. (**A**) siRNA obtained by annealing of complementary, *in vitro*-transcribed RNA strands. (**B**) Hairpin interfering RNA synthesized *in vivo* after transfection of the vector pSIRENeRF1. This vector was obtained by annealing and ligating two synthetic oligonucleotides in the vector pSIREN-RetroQ.

We used the plasmid pT7PEGFPam64L, described elsewhere (Ilegems *et al*., 2002), as the second template. It contains the coding sequence of the EGFP (Clontech) with an amber mutation at an amino acid sequence position important for the correct folding of the fluorophore.

After linearization of the plasmids pT7DsRedExpress and pT7EGFPam64L by *Not*I, *in vitro* transcription was performed with T7 RNA polymerase using the MEGAscript kit. Capping of mRNA was achieved during transcription by replacing 80% of the GTP level with the cap analog $m^7G(5')ppp(5')G$. 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 and 20 800 *g* for 15 min. The mRNA pellet was dried and dissolved in sterile DEPC-treated H_2O. The integrity and size of the mRNAs were analysed 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 116 bp synthetic DNA template encoding the artificial human suppressor $tRNA^{Leu}_{CUA}$ was prepared by annealing two oligonucleotides containing the tRNA gene flanked by a T7 promoter (Figure 2). The resulting blunt-end DNA fragment was ligated into pCR4 vector using a TOPO cloning kit (Invitrogen). The final plasmid (pHumanLeuCUA) was checked by restriction mapping and sequencing. This plasmid

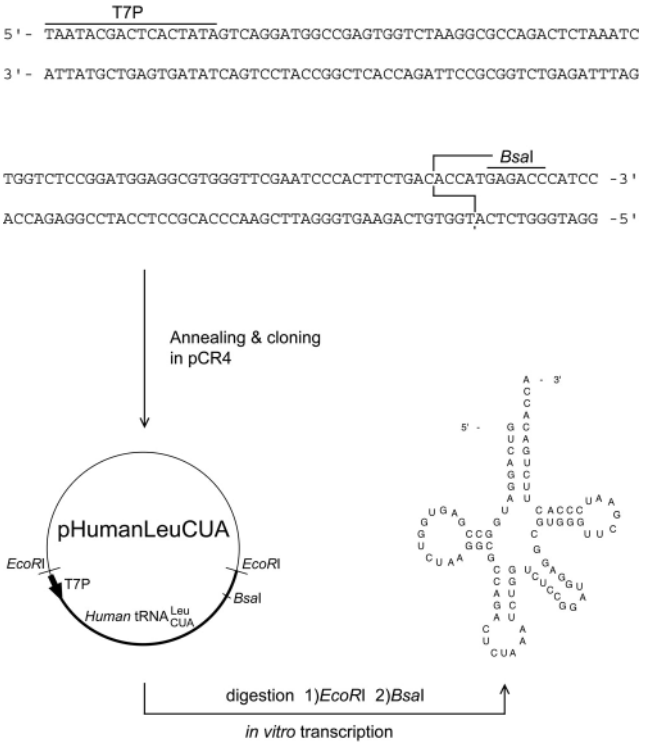

Fig. 2. Scheme for the *in vitro* synthesis of the suppressor tRNA from a synthetic DNA template by T7 run-off transcription. Two synthetic complementary oligonucleotides encoding the artificial human suppressor $tRNA^{Leu}_{CUA}$ were annealed and cloned into the pCR4 vector, leading to pHumanLeuCUA. T7 RNA polymerase run-off transcription was performed on the purified *Eco*RI and *Bsa*I fragment giving rise to a 87-mer suppressor tRNA.

was linearized by successive restriction endonuclease digests with *Eco*RI and *Bsa*I. After agarose gel purification, this DNA fragment was used for the run-off transcription with T7 RNA polymerase following the protocol of the MegaScript Kit. 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 were assayed by polyacrylamide gel electrophoresis and the concentration was determined by measuring the optical density at 260 nm.

cDNA preparation and real-time PCR quantification

Total messenger RNA was extracted from ~2 million cells per sample using TRIZOL Reagent (Invitrogen). The subsequent eRF1 reverse transcription was achieved using a TaqMan Gold RT-PCR Kit from Applied Biosystems (Foster City, CA) and random hexamers. We used the SYBR Green I kit (Eurogentec, Seraing, Belgium) for quantitative PCR, together with the eRF1-specific primers 5′-TCACAGACAAAGAGACCGGACA-3′ and 5′-CGCAAGATACCTCCAATTCCAC-3′. Primers used for the internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied in the TaqMan Gold RT-PCR Kit (Applied Biosystems). The ABI Prism 7700 was used as a sequence detection system and data were treated with the software Sequence Detection Systems 1.9.1, both from Applied Biosystems. The amplification specificities were controlled with dissociation measurements followed by treatment using Dissociation Curves 1.0 software (Applied Biosystems). The efficiencies of the reactions were calculated using LinRegPCR 7.4 (Ramakers *et al*., 2003), with a minimum of five data points for the determination of each amplification slope.

Injections

Microinjections of HEK293 cells with mRNA and tRNA mixtures diluted in sterile DEPC-treated water 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, Oberkochen, Germany). FemtotipsII capillaries (Eppendorf) were used for all injections.

Laser scanning confocal microscopy and fluorescence measurements

Laser scanning confocal microscopy was performed using a Zeiss LSM510 (Carl Zeiss). Detection and distinction of the fluorescence signals of EGFP (excitation at 488 nm) and DsRedExpress (excitation at 543 nm) were achieved by appropriate filter sets using a multitracking mode. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorophores and proper comparison between samples of variable fluorescence intensities.

Results

A schematic view of our strategy to improve mis-acylated tRNA suppression efficiency in human cells is presented in Figure 3. In a first step the intracellular mRNA level of eRF1 is lowered via RNA interference and its effect on nonsense suppression is later evaluated by microinjection of reporter mRNAs. One of the transcripts gives rise to the suppression-dependent expression of a green fluorescent protein. The other non-mutated, red fluorescent reporter is used as an internal

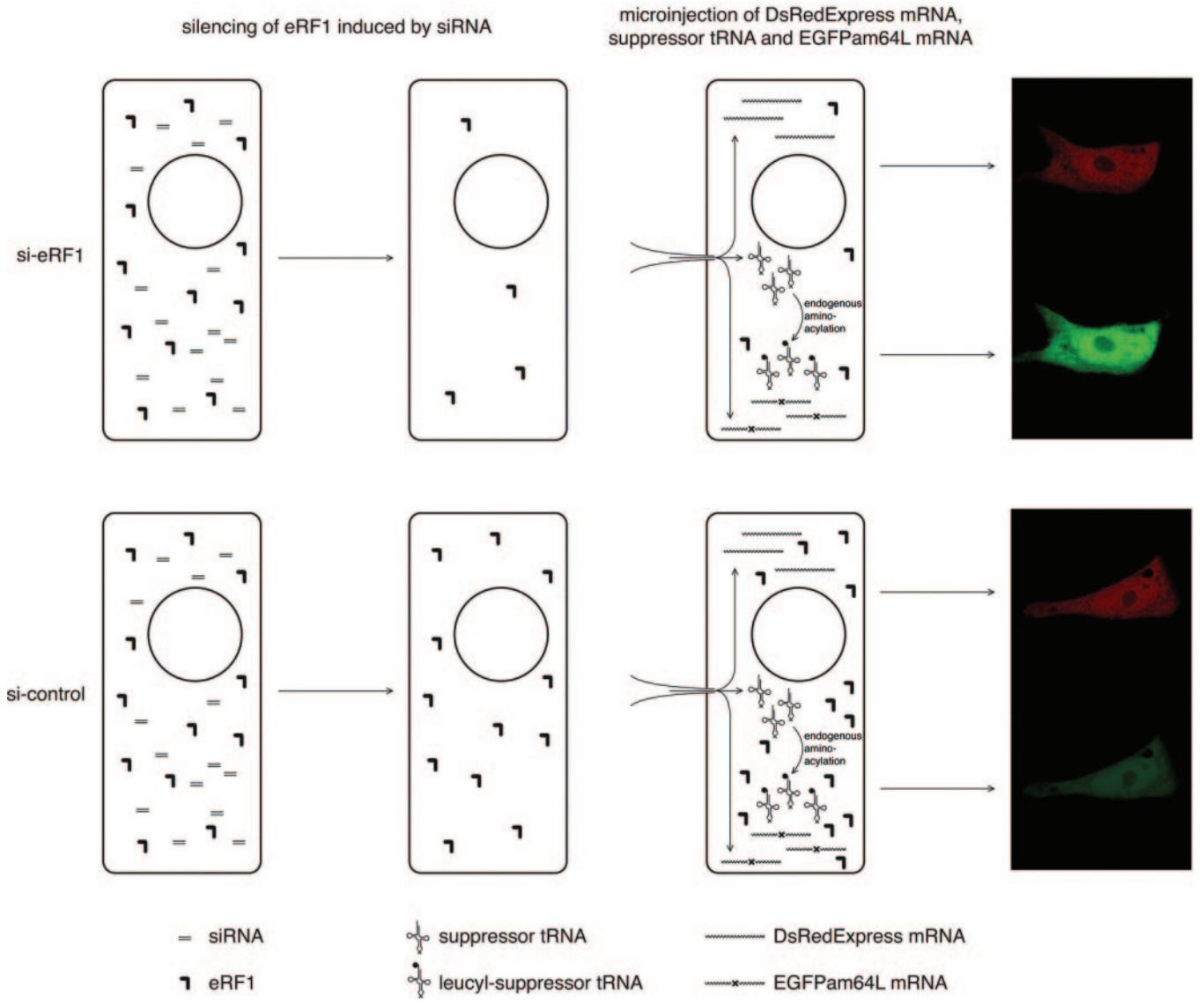

Fig. 3. General representation of the eRF1 silencing effect on the mis-acylated tRNA suppression efficiency. Upper line: interfering RNAs in the cell—obtained by either siRNA or pSIRENeRF1 transfection—lead to a lower intracellular concentration of eRF1. Subsequently, a solution containing DsRedExpress mRNA, suppressor tRNA and EGFPam64L mRNA is micro-injected into the cell. The tRNA is aminoacylated by endogenous AARSs and the expression of EGFP reflects the suppression efficiency, whereas the DsRedExpress expression level serves as a standard for non-suppressed translation (shown by typical confocal images taken 20 h after injection). Lower line: control experiment using non-interfering siRNA, having no effect on the eRF1 level. In the confocal image on the right, the ratio between the EGFP and the DsRedExpress expression levels reflects an increase in the suppression efficiency after silencing of the eRF1.

standard for the amount of injected molecules, allowing the comparison between individual cells (Figure 3).

Lowering of the intrinsic eRF1 messenger concentration has been approached in two different ways using either a transient siRNA transfection or a stable transcription of a hairpin RNA, both targeting eRF1 transcripts (Figure 1). The siRNA sequence was designed following the guidelines described elsewhere (Elbashir *et al*., 2001a,b) and the selection of a specific location on the target eRF1 mRNA was made such that both synthesized RNA strands begin with a guanine (Donze and Picard, 2002).

We studied alterations in eRF1 mRNA concentration by real-time PCR, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an independent reference gene (Figure 4). Transfection of siRNA had substantial effect on the eRF1 mRNA concentration, which decreased to ~40% of its initial value within 2 days after transfection. The cell line HEK-seRF1 stably expressing the eRF1 targeting hairpin RNA exhibited ~60% of the eRF1 mRNA concentration of non-treated cells. No noticeable difference was observed in the eRF1 mRNA level between non-transfected cells and cells pre-transfected with a control (non-eRF1 targeting) siRNA, either 1 or 2 days after transfection (data not shown). A certain variability was observed between experiments carried out on different days, as shown by the relatively large error bars (Figure 4E). However, the same decrease pattern for each of the eRF1-downregulated cell batches was always obtained.

To study the correlation between eRF1 concentration and artificial suppression efficiency, microinjection experiments were performed 2 days after siRNA transfection, which we observed resulted in more homogeneous eRF1 silencing among cells than via DNA transfection, thus permitting precise cell-to-cell comparisons. We co-injected DsRedExpress mRNA, human suppressor $tRNA^{Leu}_{CUA}$ and EGFPam64L

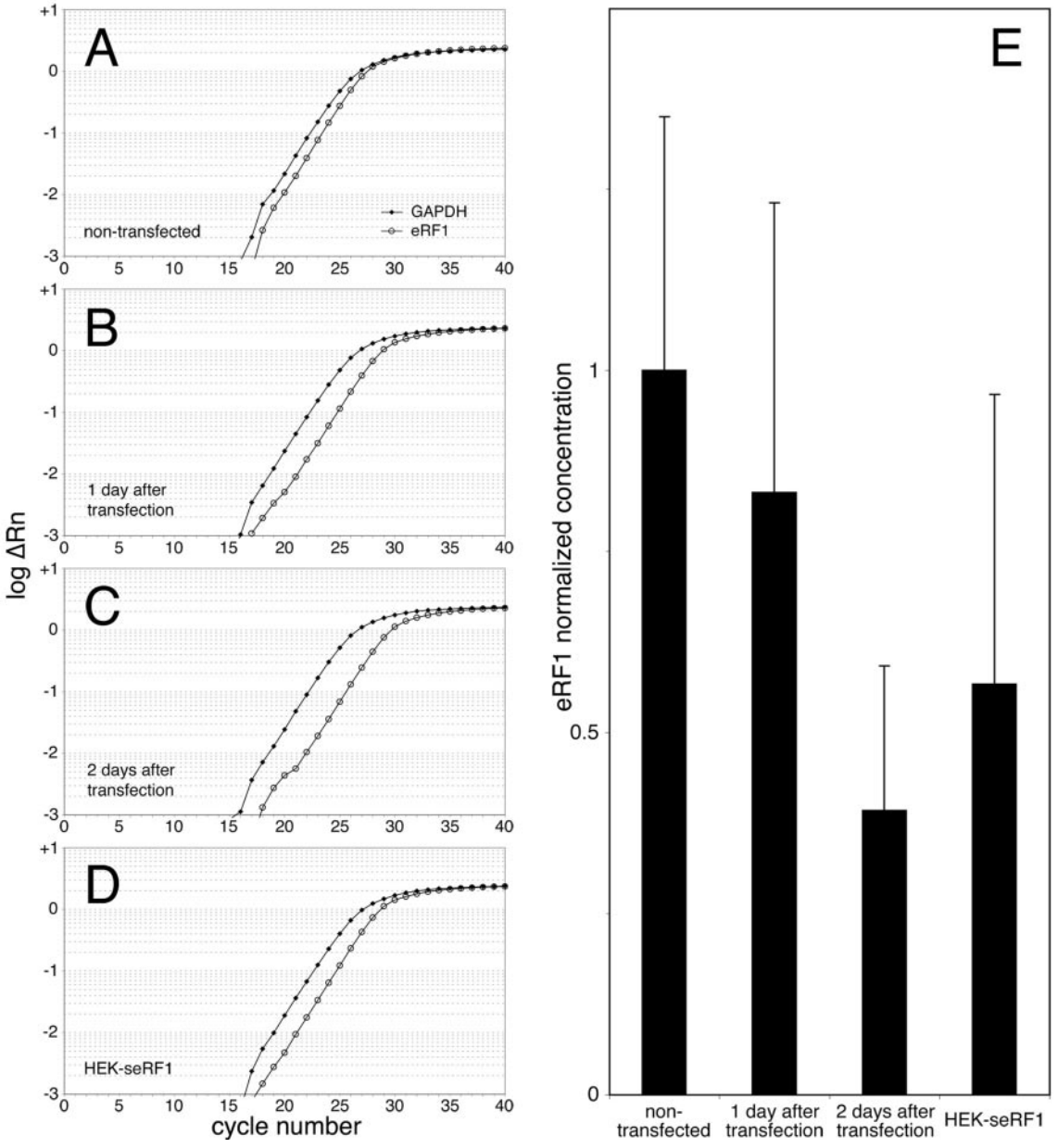

Fig. 4. Real-time PCR experiments. (**A**)–(**D**) PCR profiles of the amplifications of eRF1 and GAPDH (used as an internal standard). The cDNAs were produced from (A) non-transfected cells or cells transfected with a non-interfering siRNA, (B) cells transfected with siRNA 1 day before, (C) cells transfected with siRNA 2 days before and (D) HEK-seRF1 cells (HEK293 cells stably transfected with pSIRENeRF1). Curves are representative of single experiments. (**E**) Comparison of the level of eRF1 mRNA in the different samples, using GAPDH as an internal standard. Error bars take into account experimental uncertainty and standard deviations representative of the values obtained from triplicate experiments carried out on different days.

mRNA at fixed ratios and controlled volumes of about 0.05 ± 0.01 pl (as described by Ilegems *et al*., 2002). About 20 h later, the expression of both reporter proteins was monitored by laser-scanning confocal microscopy. Whereas the DsRed fluorescence intensity was shown to be proportional to the number of injected molecules, the EGFP fluorescence intensity mainly depended on the eRF1-dependent suppression efficiency. Indeed, the mutated EGFPam64L mRNA is only translated in a full-length fluorescent EGFP when its amber codon is suppressed by a specific suppressor tRNA charged with a leucine (for details, see Ilegems *et al*., 2002).

Plotting the fluorescence intensity of the DsRedExpress versus that of EGFP revealed that the suppression efficiency of the siRNA-transfected cells is increased 5-fold compared with the non-treated HEK293 cells (Figure 5, Table I).

The expression level of the DsRedExpress was controlled to be independent of the siRNA transfection by co-injecting treated and non-treated cells with DsRedExpress mRNA together with purified rEGFP as an indicator for injection volume: the DsRedExpress expression level was identical regardless of the eRF1 concentration and depended only on the injection volume reflected by the rEGFP fluorescence intensity (data not shown).

Discussion

We developed a strategy to enhance the yield of artificial nonsense codon suppression in cultured human cells. The eukaryotic release factor eRF1, which is known to compete with suppressor tRNAs for stop codon recognition (Drugeon

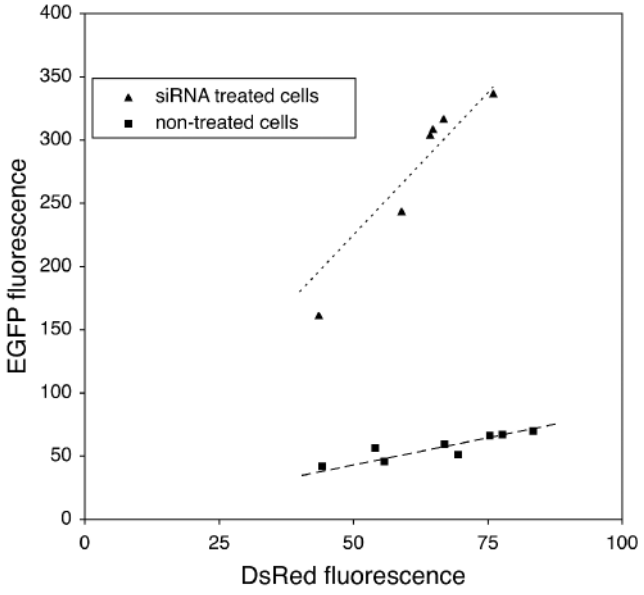

Fig. 5. Fluorescence intensities of DsRedExpress and EGFP 20 h after co-injection of DsRedExpress mRNA, suppressor tRNA and EGFPam64L mRNA, as measured by confocal microscopy. Each data point represents a single cell (squares represent non-treated cells, triangles represent cells pre-transfected with siRNA targeting the eRF1). The discontinuous lines display qualitatively the validity of the DsRed reporter protein for proper cell-to-cell comparison, by their apparent linearity in the particular injection volume range used for this study.

Table I. Concentration of eRF1 mRNA as determined by real-time PCR, and its effect on mis-acylated suppression efficiency as deduced from DsRedExpress/EGFP expression level ratios

	Intracellular eRF1 mRNA concentration	Mis-acylated tRNA suppression efficiency
Non-treated HEK293 cells	100%	100%
HEK transfected with siRNA: 1 day after transfection	83 ± 70%	ND
HEK transfected with siRNA: 2 days after transfection	39 ± 33%	500 ± 100%
HEK-seRF1 cells	57 ± 60%	ND

All samples were microinjected using concentrations of 2 μg/μl DsRedExpress mRNA, 2 μg/μl EGFPam64L mRNA, 2 μg/μl tRNA, and imaged 20 h later. Suppression efficiency was determined by averaging the ratio EGFP/DsRed fluorescence intensity in single injected cells. The error bars represent experimental uncertainty as well as standard deviations between individual experiments. All values are normalized to non-treated HEK293 cells.
ND, not determined.

et al., 1997), was down-regulated on the transcript level. Based on the silencing properties of interfering RNA, a substantial decrease in eRF1 mRNA concentration was achieved. The silencing effect was followed by real-time PCR and increased over time to result in a two-fold reduction of eRF1 transcripts 2 days after transfection. A comparable degree of gene silencing was obtained by stable transfection of HEK293 cells with a vector capable of inducing *in vivo* transcription of a hairpin RNA for targeting eRF1 mRNAs.

The effect of eRF1 gene silencing on nonsense codon suppression efficiency was investigated in individual cells by co-injection of an artificial suppressor tRNA together with *in vitro* transcripts encoding two reporter proteins, DsRedExpress and EGFPam64L. The suppressor tRNA was designed in order to obtain a natural aminoacylation by human cells, required for charging it with a leucine to recover the green fluorescence of the EGFPam64 reporter by amber codon suppression. The tRNA sequence used in this study was based on the human $tRNA^{Leu}_{CAA}$ (Breitschopf *et al*., 1995), which anticodon was mutated to CUA and which was modified by two other mutations A38G/A39 U to improve amber suppression efficiency (Raftery and Yarus, 1987).

Only by co-injecting the amber codon containing EGFP transcript together with the cognate suppressor tRNA was complete translation achieved as indicated by EGFP fluorescence recovery. The injection of the EGFPam64L reporter alone did not result in detectable expression of fluorescent proteins as judged by laser-scanning confocal microscopy (see also Ilegems *et al*., 2002). DsRedExpress mRNA encoding a red fluorescent protein was co-injected as a standard reflecting the amount of injected molecules. Since the two reporter mRNAs were always injected at the same molar ratio, the ratio between the resulting EGFP and the DsRedExpress fluorescence intensities could be used to compare suppression efficiencies in individual cells. Lowering the eRF1 mRNA concentration by a factor of ~2 resulted in a 5-fold increase in suppression efficiency compared with non-treated HEK293 cells. Parallel to our present research, Carnes *et al*. reported a slight enhancement of natural nonsense read-through after 90% silencing of eRF1 transcripts in human cells (Carnes *et al*., 2003). These experiments were done in the absence of artificial suppressor tRNAs. On the basis of our study, we are now able to confirm a positive influence of eRF1 silencing also on artificial nonsense codon suppression.

In general, increased read-through might be problematic for the specific incorporation of non-natural amino acids when using cell lines exhibiting a strong natural suppression; in such a situation, the amount of proteins translated without the desired synthetic amino acid incorporation would increase relative to the labeled protein. However, we determined natural amber codon suppression to be very low in HEK293 cells (see Supplementary data available at *PEDS* Online), making them well suited for labeling experiments. By silencing eRF1 transcripts down to 40% of the original level, we could achieve increased artificial amber suppression without enhancing natural read-through.

In our hands, further silencing of eRF1 by using the strong interfering RNA si1187 (Carnes *et al*., 2003) led to changes in cell morphology as indicated by their detachment from the growth surface, thus impairing microinjection experiments and subsequent subcellular investigations by fluorescence confocal microscopy (not shown).

The prospects for suppression of nonsense codons in prokaryotes and also in eukaryotes might be influenced by the same strategy involving the downregulation or deactivation of intrinsic translation termination factors. This was demonstrated in previous *in vitro* experiments by Hecht's group (Short *et al*., 1999): partial heat deactivation of a thermosensitive mutant of the prokaryotic release factor 1 (RF1) increased amber codon suppression by chemically misacylated suppressor tRNAs in cell-free *E.coli* extracts using dehydropholate reductase (DHFR) as a reporter protein.

Our present approach based on siRNA could be a valuable tool, potentially adaptable to various cell types owing to the generality of the interfering RNA mechanism. Moreover,

although we used microinjection in this present study for quantification reasons, this improvement strategy could be beneficial using other transfection techniques such as electroporation. Finally, our system could also serve for the site-specific incorporation of a wide variety of non-natural amino acids

To conclude, we have provided a general approach for increasing the efficiency of non-natural amino acid mutagenesis since eRF1 is the sole release factor in eukaryotic cells catalysing translation termination at all three stop codons. A homogeneous down-regulation of eRF1 transcripts was achieved in populations of HEK293 cells by transient siRNA transfection, which resulted in similar levels of elevated artificial nonsense suppression in individual cells. This is a significant advance because human cells are most suited for the analysis of proteins of medical importance.

In addition, we have been able to measure low levels of natural amber codon suppression in HEK293 cells (see Supplementary data). This is a key advantage of that cell type since a competition between exogenously added artificial suppressor tRNAs and high levels of natural suppressor tRNAs, which might occur in other cell lines, would impair the desired incorporation of non-natural amino acid at amber codon sites. Stable or transient engineering of mammalian cells to achieve desired biological properties, as presented in our study, is a promising approach to expand the scope of the *in vivo* nonsense suppression methodology.

Our present report on the improvement of the site-selective incorporation of non-natural amino acids makes this method of special interest for the *in vivo* labeling of proteins. Together with complementary orthogonal labeling methods such as FLASH (Adams *et al*., 2002), oligohistidine-NTA chromophores (Guignet *et al*., 2004) or the human alkylguanine transferase procedure (Keppler *et al*., 2003), this approach will offer many applications to investigate selective molecular interactions allowing the elucidation of structure–function relationships in live biological cells.

Acknowledgement

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References

Adams,S.R., Campbell,R.E., Gross,L.A., Martin,B.R., Walkup,G.K., Yao,Y., Llopis,J. and Tsien,R.Y. (2002) *J. Am. Chem. Soc.*, **124**, 6063–6076.

Beene,D.L., Brandt,G.S., Zhong,W., Zacharias,N.M., Lester,H.A. and Dougherty,D.A. (2002) *Biochemistry*, **41**, 10262–10269.

Bertram,G., Bell,H.A., Ritchie,D.W., Fullerton,G. and Stansfield,I. (2000) *RNA*, **6**, 1236–1247.

Bertram,G., Innes,S., Minella,O., Richardson,J. and Stansfield,I. (2001) *Microbiology*, **147**, 255–269.

Breitschopf,K., Achsel,T., Busch,K. and Gross,H.J. (1995) *Nucleic Acids Res*, **23**, 3633–3637.

Carnes,J., Frolova,L., Zinnen,S., Drugeon,G., Phillippe,M., Justesen,J., Haenni,A.L., Leinwand,L., Kisselev,L.L. and Yarus,M. (2000) *RNA*, **6**, 1468–1479.

Carnes,J., Jacobson,M., Leinwand,L. and Yarus,M. (2003) *RNA*, **9**, 648–653.

Chiu,Y.L. and Rana,T.M. (2002) *Mol. Cell*, **10**, 549–561.

Chollet,A., Turcatti,G., Nemeth,K. and Vogel,H. (1998) In Slavik,J. (ed.), *Fluorescence Microscopy and Fluorescent Probes*, Vol. 2. Plenum Press, New York, pp. 87–92.

Cload,S.T., Liu,D.R., Froland,W.A. and Schultz,P.G. (1996) *Chem. Biol.*, **3**, 1033–1038.

Cohen,B.E., McAnaney,T.B., Park,E.S., Jan,Y.N., Boxer,S.G. and Jan,L.Y. (2002) *Science*, **296**, 1700–1703.

Czaplinski,K., Ruiz-Echevarria,M.J., Paushkin,S.V., Han,X., Weng,Y., Perlick,H.A., Dietz,H.C., Ter-Avanesyan,M.D. and Peltz,S.W. (1998) *Genes Dev.*, **12**, 1665–1677.

Donze,O. and Picard,D. (2002) *Nucleic Acids Res.*, **30**, e46.

Drugeon,G., Jean-Jean,O., Frolova,L., Le Goff,X., Philippe,M., Kisselev,L. and Haenni,A.L. (1997) *Nucleic Acids Res.*, **25**, 2254–2258.

Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K. and Tuschl,T. (2001a) *Nature*, **411**, 494–498.

Elbashir,S.M., Lendeckel,W. and Tuschl,T. (2001b) *Genes Dev.*, **15**, 188–200.

Guignet,E.G., Hovius,R. and Vogel,H. (2004) *Nat. Biotechnol.*, **22**, 440–444.

Hilleren,P. and Parker,R. (1999) *Annu. Rev. Genet.*, **33**, 229–260.

Hohsaka,T., Kajihara,D., Ashizuka,Y., Murakami,H. and Sisido,M. (1998) *J. Am. Chem. Soc.*, **121**, 34–40.

Ilegems,E., Pick,H.M. and Vogel,H. (2002) *Nucleic Acids Res.*, **30**, e128.

Jordan,M., Schallhorn,A. and Wurm,F.M. (1996) *Nucleic Acids Res.*, **24**, 596–601.

Karginov,V.A., Mamaev,S.V. and Hecht,S.M. (1997) *Nucleic Acids Res.*, **25**, 3912–3916.

Keppler,A., Gendreizig,S., Gronemeyer,T., Pick,H., Vogel,H. and Johnsson,K. (2003) *Nat. Biotechnol.*, **21**, 86–89.

Kurzchalia,T.V., Wiedmann,M., Breter,H., Zimmermann,W., Bauschke,E. and Rapoport,T.A. (1988) *Eur. J. Biochem.*, **172**, 663–668.

Le Goff,X., Philippe,M. and Jean-Jean,O. (1997) *Mol. Cell. Biol.*, **17**, 3164–3172.

Liu,D.R. and Schultz,P.G. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 4780–4785.

Liu,D.R., Magliery,T.J., Pastrnak,M. and Schultz,P.G. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 10092–10097.

McManus,M.T. and Sharp,P.A. (2002) *Nat. Rev. Genet.*, **3**, 737–747.

Milligan,J.F., Groebe,D.R., Witherell,G.W. and Uhlenbeck,O.C. (1987) *Nucleic Acids Res.*, **15**, 8783–8798.

Monahan,S.L., Lester,H.A. and Dougherty,D.A. (2003) *Chem. Biol.*, **10**, 573–580.

Noren,C.J., Anthony-Cahill,S.J., Suich,D.J., Noren,K.A., Griffith,M.C. and Schultz,P.G. (1990) *Nucleic Acids Res.*, **18**, 83–88.

Raftery,L.A. and Yarus,M. (1987) *EMBO J.*, **6**, 1499–1506.

Ramakers,C., Ruijter,J.M., Deprez,R.H. and Moorman,A.F. (2003) *Neurosci. Lett.*, **339**, 62–66.

Short,G.F., 3rd, Golovine,S.Y. and Hecht,S.M. (1999) *Biochemistry*, **38**, 8808–8819.

Strømgaard,A., Jensen,A.A. and Strømgaard,K. (2004) *Chembiochem*, **5**, 909–916.

Turcatti,G., Nemeth,K., Edgerton,M.D., Meseth,U., Talabot,F., Peitsch,M., Knowles,J., Vogel,H. and Chollet,A. (1996) *J. Biol. Chem.*, **271**, 19991–19998.

Turcatti,G., Nemeth,K., Edgerton,M.D., Knowles,J., Vogel,H. and Chollet,A. (1997) *Receptors Channels*, **5**, 201–207.

Wang,L., Brock,A., Herberich,B. and Schultz,P.G. (2001) *Science*, **292**, 498–500.

Wang,L., Brock,A. and Schultz,P.G. (2002) *J. Am. Chem. Soc.*, **124**, 1836–1837.

Yu,J.Y., DeRuiter,S.L. and Turner,D.L. (2002) *Proc. Natl Acad. Sci. USA*, **99**, 6047–6052.

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