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A Possible Approach to Site-Specific Insertion of Two Different Unnatural Amino Acids into Proteins in Mammalian Cells via Nonsense Suppression

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Summary

The site-specific insertion of an unnatural amino acid into proteins in vivo via nonsense suppression has resulted in major advances in recent years. The ability to incorporate two different unnatural amino acids in vivo would greatly increase the scope and impact of unnatural amino acid mutagenesis. Here, we show the concomitant suppression of an amber and an ochre codon in a single mRNA in mammalian cells by importing a mixture of aminoacylated amber and ochre suppressor tRNAs. This result provides a possible approach to site-specific insertion of two different unnatural amino acids into any protein of interest in mammalian cells. To our knowledge, this result also represents the only demonstration of concomitant suppression of two different termination codons in a single gene in vivo.

Introduction

Site-specific insertion of unnatural amino acids into proteins in vitro has been used successfully for a number of applications. Extension of this methodology to an in vivo situation would add a new dimension to the utility of unnatural amino acid mutagenesis. The unnatural amino acids to be used could include those that are photoactivatable or fluorescent; those that carry reactive side chains, such as keto groups; heavy atoms, such as iodine; spectroscopic probes; and those that mimic phosphoamino acids. Besides providing a method for production of proteins with novel chemical and biological properties, proteins carrying such unnatural amino acids could also be used for studies on the folding, structure, stability, and function of proteins, protein-protein interactions, protein localization in vivo, and signal transduction.

The most common approach for site-specific insertion of an unnatural amino acid involves the readthrough of an amber (UAG) stop codon by an amber suppressor tRNA that is aminoacylated with the desired unnatural amino acid. For in vitro work, the suppressor tRNA is chemically aminoacylated with the desired unnatural amino acid and added to an in vitro protein synthesis system [1–4]. For an in vivo approach, the suppressor tRNA is aminoacylated by a mutant aminoacyl-tRNA synthetase (aaRS), which aminoacylates the suppressor tRNA with the desired unnatural amino acid instead of a normal amino acid. The in vivo approach has several key requirements: (1) a suppressor tRNA that is not aminoacylated by any of the endogenous aaRSs in the cell, (2) an aaRS that aminoacylates only the suppressor tRNA but no other tRNA in the cell [5–8], and (3) a mutant aaRS that aminoacylates the suppressor tRNA only with the unnatural amino acid but not with a normal amino acid. Using this approach, Schultz, Yokoyama, and their coworkers have recently described the site-specific insertion of O-methyltyrosine, 2-naphthylalanine, p-azidophenylalanine, and iodotyrosine into proteins in vivo [8–11].

The above approach requires the isolation of aaRS mutants, one at a time, for each specific unnatural amino acid. An alternative system that does not require a mutant aaRS involves the import (by injection, transfection, or electroporation) into cells of a suppressor tRNA already aminoacylated with the unnatural amino acid [12–15]. This approach is quite flexible in that the same suppressor tRNA can be chemically aminoacylated with virtually any unnatural amino acid. The only requirement is that once the suppressor tRNA has delivered the unnatural amino acid into a growing polypeptide chain, it is not aminoacylated by any of the endogenous aaRSs. Otherwise, a suppressor tRNA that has inserted the amino acid analog at a designated site in the target protein will be reaminoacylated with a natural amino acid and insert the natural amino acid instead of the amino acid analog, generating a heterogeneous pool of target protein molecules. We recently described the import of purified suppressor tRNAs into mammalian cells by means of transfection and the identification of an amber suppressor tRNA (*supF*) derived from *E. coli* tyrosine tRNA ($tRNA_1^{Tyr}$) that fulfilled the above requirements for site-specific insertion of unnatural amino acids [13].

As described above, essentially all of the in vitro and in vivo work with unnatural amino acid mutagenesis has, so far, involved the use of an amber suppressor tRNA along with an amber stop codon defining the site of interest in the protein gene. The availability of another suppressor tRNA/nonsense codon pair would greatly add to the versatility of unnatural amino acid mutagenesis and allow site-specific insertion of two different unnatural amino acids into proteins. For example, introduction of two different fluorescent amino acids into a reporter protein would allow the use of fluorescence resonance energy transfer (FRET) to study protein conformation and dynamics in mammalian cells. Similarly, site-specific insertion of phosphoamino acids, such as phosphothreonine and phosphotyrosine, could be used to specifically activate one of the many mitogen-activated protein (MAP) kinases in a cell in the absence of an extracellular or upstream signal.

In this paper, we show that an ochre (UAA) suppressor

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tRNA [16], derived from *E. coli* $tRNA_1^{Tyr}$, is not aminoacylated by any of the mammalian cytoplasmic aaRSs. Import of aminoacylated ochre suppressor tRNA leads to suppression of an ochre codon in a firefly luciferase reporter gene, whereas import of the same tRNA without prior aminoacylation does not. We further show that import of a mixture of aminoacylated ochre and amber suppressor tRNAs leads to concomitant suppression of an ochre and an amber codon in the firefly luciferase gene. This result provides a general approach for site-specific insertion of two different unnatural amino acids into proteins in mammalian cells.

Results

Design of a Dual-Luciferase Reporter System and Isolation of HEK293 Cell Lines for Analysis of Amber and Ochre Suppression in Mammalian Cells

We recently reported that import of an aminoacylated amber suppressor tRNA (*supF* Tyr-tRNA) into mammalian cells by means of transient transfection leads to suppression of an amber codon in the CAT gene. This method provides a general approach to the site-specific incorporation of virtually any unnatural amino acid into a mammalian protein [13]. An important next step would be to expand this approach to site-specific insertion of two different unnatural amino acids by combining in a single mRNA two termination codons, amber and ochre, and importing a mixture of amber and ochre suppressor tRNAs (Figure 1A). For this, a highly sensitive reporter system based on a dual-luciferase fusion protein was designed. The DNA sequences encoding firefly luciferase (*Photinus pyralis*; FLuc) [17] and sea pansy luciferase (*Renilla reniformis*; RLuc) [18] were fused to express a single protein with two bioluminescent activities (Figure 1B). The resulting fusion protein, 865 amino acids long, provides RLuc activity through its N-terminal domain (315 amino acids) and FLuc activity through its C-terminal domain (550 amino acids) [19]. To study the activity of purified suppressor tRNAs imported into mammalian cells, amber and ochre codons were introduced into the FLuc gene to generate plasmids pRLucFLuc (*am70*), pRLucFLuc (*oc70*), and pRLucFLuc (*oc70/am165*) (Figure 1B). These plasmids were in turn used to establish stable HEK293 luciferase cell lines HEK293-E7 (*am70*), HEK293-F22 (*oc70*), and HEK293-D9 (*oc70/am165*). The presence of the upstream RLuc gene allowed screening for stable cell lines, based on resistance to geneticin and high RLuc activity in cell extracts. Stable HEK293 luciferase cell lines produced RLuc activities in the range of 1×10^6 RLU per μg of protein. The RLuc activity could not be used as a common denominator to directly compare the efficiencies of suppression among different cell lines or even different experiments, since the in vivo half-life of the full-length RLucFLuc fusion protein was significantly different from that of the truncated fusion protein consisting of the intact RLuc and 70 amino acids of the FLuc protein [19]. Therefore, results of suppression experiments, in which mixtures of full-length and truncated protein accumulate in the cell, are presented as FLuc activities per μg of total cell protein.

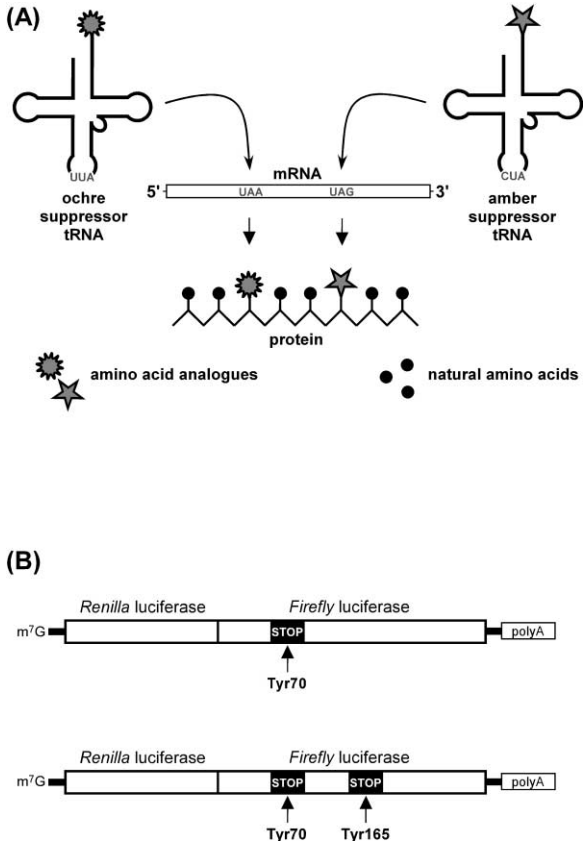

Figure 1. Import of Suppressor tRNAs into Mammalian Cells

(A) Scheme for import of aminoacylated suppressor tRNAs for concomitant suppression of amber and ochre codons in a single mRNA.

(B) Schematic representation of the luciferase reporter mRNA encoding a *Renilla* luciferase/firefly luciferase (RLucFLuc) fusion protein. (Top) RLucFLuc (*am70*) or RLucFLuc (*oc70*); (bottom) RLucFLuc (*oc70/am165*). Stop mutations in the firefly luciferase gene are indicated.

Concomitant Suppression of Amber and Ochre Codons in COS1 Cells Cotransfected with pRLucFLuc Plasmid and Amber and Ochre Suppressor tRNAs Derived from *E. coli* Initiator $tRNA^{fMet}$

We previously showed that amber (*fMam*) and ochre (*fMoc*) suppressor tRNAs derived from the *E. coli* initiator tRNA ($tRNA^{fMet}$) could be imported into mammalian cells and suppressed amber and ochre mutations, respectively, at position 27 of the CAT gene [13]. Both of these tRNAs, *fMam* and *fMoc*, are substrates for yeast and mammalian TyrRS and are aminoacylated with tyrosine by mammalian cell extracts. Here, we have asked whether these two tRNAs can be used for concomitant suppression of two different termination codons located in the FLuc coding region.

COS1 cells were cotransfected with the pRLucFLuc (*oc70/am165*) plasmid and purified *fMam* and *fMoc* suppressor tRNAs. Cells were harvested after 24 hr and extracts assayed for FLuc activity. Cells transfected with a mixture of amber and ochre suppressor tRNAs have substantial amounts of FLuc activity (87.1×10^3 RLU per μg of protein; Table 1, line 1). Cells transfected

Table 1. Concomitant Suppression of Amber and Ochre Codons in COS1 Cells

	fMam (μg)	*fMoc* (μg)	$tRNA^{fMet}$ (μg)[a]	FLuc Activity × 10^3 (RLU/μg)	Relative FLuc Activity
1	2.5	2.5	-	87.1 ± 8.4	100%
2	2.5	-	2.5	0.6 ± 0.1	0.7%
3	-	2.5	2.5	3.2 ± 0.2	3.7%
4	-	-	5.0	0.4 ± 0.1	0.5%

COS1 cells were transfected with a mixture of 2.5 μg of pRLucFLuc (*oc70/am165*) plasmid DNA and *fMam* and *fMoc* suppressor tRNAs as indicated.

[a] *E. coli* initiator $tRNA^{fMet}$ was added to keep the amount of total tRNA constant at 5 μg. In control experiments that were performed in parallel, transient transfection of 2.5 μg of plasmid carrying the wild-type RLucFLuc fusion gene yielded FLuc activities of 1.1–1.2 × 10^6 RLU/μg. FLuc activities obtained in line 1 reflecting the combined suppression of both the amber and ochre codon thereby correspond to a suppression level of ~8%. This would indicate that the amber and ochre codons are each suppressed to the level of ~28%.

with *fMam* tRNA alone have essentially no FLuc activity, indicating that this tRNA is unable to translate the ochre codon at position 70 of the reporter mRNA (Table 1, line 2). Cells transfected with *fMoc* tRNA alone display a low level of FLuc activity (Table 1, compare lines 1 and 3), suggesting that the tRNA also reads the amber codon at position 165 but only weakly. Extracts from cells transfected with pRLucFLuc (*oc70/am165*) and a non-suppressing control tRNA ($tRNA^{fMet}$; Table 1, line 4) yield background of less than 0.5% of maximum FLuc activity.

Previously, we showed that the *fMoc* suppressor tRNA did not suppress the amber codon in the CAT*am27* reporter gene [13]. Our current finding that this tRNA can suppress an amber codon in the FLuc mRNA, albeit weakly, is most likely due to the superior sensitivity of the firefly luciferase assay compared to assay for CAT activity.

Concomitant Suppression of Amber and Ochre Codons in the Stable HEK293-D9 Luciferase Cell Line by *fMam* and *fMoc* Suppressor tRNAs

The use of the HEK293-D9 (*oc70/am165*) luciferase cell line allowed, for the first time, to monitor directly the uptake into mammalian cells of suppressor tRNAs instead of mixtures of reporter plasmid DNA and tRNA, thereby facilitating optimization of transfection conditions for importing mixtures of amber and ochre suppressor tRNAs. Initially, the ratio of *fMam* and *fMoc* tRNA was kept at 1:1 and the total amount of suppressor tRNA at 5 μg. FLuc activity from 2.5 μg each of amber and ochre suppressor tRNA is 255 × 10^3 RLU per μg of protein (Table 2, line 1). Consistent with previous experiments, *fMam* suppressor tRNA is highly specific for suppressing amber codons (Table 2, line 2), whereas *fMoc* suppressor tRNA shows a low level (3.7%) of nonspecific readthrough activity of the amber codon (Table 2, line 3).

Keeping the amount of *fMoc* tRNA constant at 2.5 μg and increasing the amount of *fMam* to 5 μg increases FLuc activity from 210 to 305 × 10^3 RLU/μg of protein while maintaining high specificity (Table 2, compare line 5 to lines 6 and 7). This increase in FLuc activity suggests that the amount of aminoacylated amber suppressor tRNA is limiting when added at a 1:1 ratio of amber:ochre suppressor tRNA. Consequently, adjusting the ratio of amber and ochre suppressor tRNAs used for transfection allows optimal protein expression with minimal nonspecific readthrough of amber codons by ochre suppressor tRNAs.

In a similar experiment, *fMam* tRNA was kept at 2.5 μg and the amount of *fMoc* tRNA was increased to 5 μg (Table 2, compare line 5 to lines 8 and 9). This results in only a small increase in FLuc activity from 210 to 231 RLU/μg of protein, suggesting that the ochre suppressor tRNA is not limiting. At higher concentrations of *fMoc* tRNA and in the absence of *fMam* tRNA, there is increased readthrough of the amber codon from 3.7% to 12.6% (Table 2, compare lines 3 and 9).

Identification, Purification, and Import of an Ochre Suppressor tRNA that Is Not Aminoacylated by Mammalian aaRSs

While the amber and ochre suppressor tRNAs described above were important for establishing the feasibility of concomitant suppression of two different termination

Table 2. Concomitant Suppression of Amber and Ochre Codons in a Stable HEK293 Luciferase Cell Line

	fMam (μg)	*fMoc* (μg)	$tRNA^{fMet}$ (μg)[a]	FLuc Activity × 10^3 (RLU/μg)	Relative FLuc Activity
1	2.5	2.5	-	255 ± 94.5	100%
2	2.5	-	2.5	0.4 ± 0.11	0.2%
3	-	2.5	2.5	9.4 ± 1.10	3.7%
4	-	-	5.0	0.01 ± 0.01	< 0.1%
5	2.5	2.5	2.5	210 ± 24.5	100%
6	5.0	2.5	-	305 ± 37.2	145%
7	5.0	-	2.5	0.6 ± 0.07	0.3%
8	2.5	5.0	-	231 ± 27.3	110%
9	-	5.0	2.5	26.5 ± 4.05	12.6%
10	-	-	7.5	0.01 ± 0.01	< 0.1%

HEK293-D9 (*oc70/am165*) cells were transfected with a mixture of *fMam* and *fMoc* suppressor tRNA as indicated.

[a] *E. coli* initiator $tRNA^{fMet}$ was added to keep the amount of total tRNA constant at 5 μg (lines 1–4) and 7.5 μg (lines 5–10), respectively.

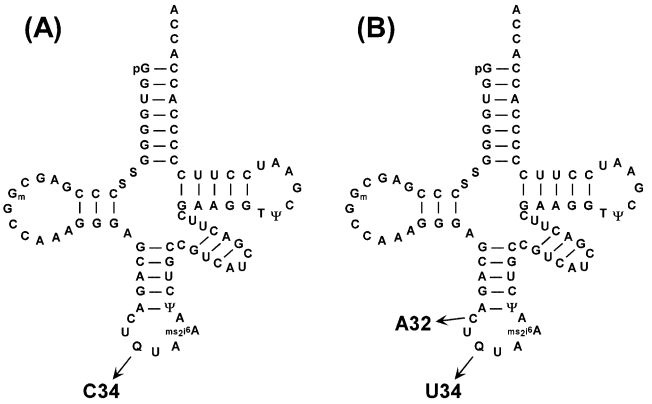

Figure 2. Cloverleaf Structures of the Suppressor tRNAs Derived from the *E. coli* Tyrosine tRNA

(A) *supF* amber suppressor tRNA; (B) *supC*.A32 ochre suppressor tRNA. Arrows indicate the changes in the suppressor tRNAs.

codons in a single mRNA, they are aminoacylated by mammalian TyrRS and therefore unsuitable for site-specific insertion of unnatural amino acids into proteins in mammalian cells. Previously, we demonstrated that the *E. coli supF* tRNA (Figure 2A) is not a substrate for any of the mammalian aaRSs and fulfills all of the requirements for its use in site-specific insertion of unnatural amino acids [13]. Here, we asked whether an ochre suppressor (*supC*) derived from the same tRNA would also not be a substrate for mammalian aaRSs and whether it would specifically suppress ochre codons in mammalian cells. To generate the *supC* ochre suppressor tRNA [16], the anticodon sequence of *supF* tRNA was mutagenized to U34U35A36. Attempts to isolate *supC* tRNA by site-specific mutagenesis of the *supF* tRNA gene only yielded *supC* tRNA mutants that carried additional mutations in the anticodon stem-loop region, likely due to toxicity caused by overexpression of ochre suppressor tRNAs in *E. coli* [16, 20]. One of the mutants, *supC*.A32 (Figure 2B), was selected based on its ability to suppress the ochre codon in a CAT reporter gene and on the level of the suppressor tRNA overproduction in *E. coli* (data not shown).

The *supF* and *supC*.A32 suppressor tRNAs were expressed in *E. coli*, purified (see Experimental Procedures), and *E. coli* TyrRS was used to aminoacylate tRNAs with tyrosine in vitro (Figure 3). The *supF* tRNA or *supF* Tyr-tRNA and *supC* tRNA or *supC* Tyr-tRNA were then transfected into HEK293-E7 (*am70*) and HEK293-F22 (*oc70*) cells, which carry a single termination codon at position 70 of the FLuc coding region. Extracts of cells transfected with suppressor tRNA without prior aminoacylation have essentially no FLuc activity (Table 3, lines 1, 3, 6, and 8). In contrast, extracts from HEK293-E7 (*am70*) cells transfected with *supF* Tyr-tRNA (line 2) and HEK293-F22 (*oc70*) cells transfected with *supC*.A32 Tyr-tRNA (line 7) yield FLuc activities of 52.2×10^3 and 50.9×10^3 RLU/μg of protein, respectively. These results demonstrate that the *supC*.A32 ochre suppressor tRNA is also not aminoacylated by any of the mammalian aaRSs. Thus, *supC*.A32 tRNA represents the first "orthogonal" ochre suppressor tRNA that has been described.

The specificity of these amber and ochre suppressor tRNAs was analyzed by transfecting HEK293-E7 (*am70*) cells with *supC*.A32 Tyr-tRNA and HEK293-F22 (*oc70*) cells with *supF* Tyr-tRNA. Consistent with previous results, *supC*.A32 tRNA also translates the amber codon to a certain extent (11%; Table 3, line 4), whereas *supF* tRNA is highly specific for amber codons (Table 3, line 9).

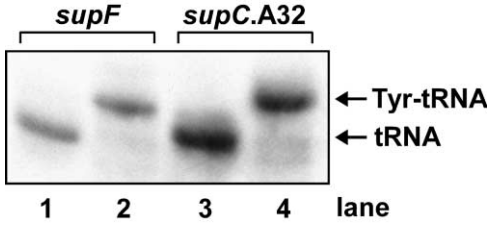

Figure 3. Acid Urea Gel Analysis of Suppressor tRNAs before and after In Vitro Aminoacylation with Tyrosine

Lanes 1 and 2, *supF* amber suppressor tRNA; lanes 3 and 4, *supC*.A32 ochre suppressor tRNA. Radiolabeled oligonucleotides specific for the anticodon stem-loop regions of *supF* and *supC*.A32 tRNA, respectively, were used to visualize suppressor tRNAs by Northern hybridization.

Table 3. Import of *supF* and *supC*.A32 tRNA into Stable HEK293 Luciferase Cell Lines

	Suppressor tRNA[a]	FLuc Activity $\times 10^3$ (RLU/μg)	Relative FLuc Activity
HEK293-E7 (*am70*)			
1	*supF*[b]	0.8 ± 0.34	1.5%
2	Tyr-*supF*	52.2 ± 5.35	100%
3	*supC*.A32[b]	0.8 ± 0.01	1.5%
4	Tyr-*supC*.A32	5.8 ± 0.32	11.1%
5	mock	0.4 ± 0.03	0.8%
HEK293-F22 (*oc70*)			
6	*supC*.A32[b]	0.4 ± 0.02	0.8%
7	Tyr-*supC*.A32	50.9 ± 2.93	100%
8	*supF*[b]	0.01 ± 0.02	<0.1%
9	Tyr-*supF*	0.01 ± 0.02	<0.1%
10	mock	0.01 ± 0.01	<0.1%

HEK293-E7 (*am70*) and HEK293-F22 (*oc70*) cells were transfected with *supF* and *supC*.A32 tRNA with and without prior aminoacylation as indicated.

[a] HEK293 cells were transfected with 3.75 μg of active suppressor tRNA as indicated. $tRNA^{fMet}$ was added to keep the amount of total tRNA constant at 10 μg.

[b] All experiments were carried out in triplicates, except those marked with '[b],' which were done in duplicates.

Table 4. Import of *supF* Tyr-tRNA and *supC*.A32 Tyr-tRNA Leads to Concomitant Suppression of Amber and Ochre Codons in a Stable HEK293 Luciferase Cell Line

	Tyr-*supF*[a] (μg)	Tyr-*supC*.A32[a] (μg)	FLuc Activity × 10^3 (RLU/μg)	Relative FLuc Activity
1	5	2.5	47.8 ± 4.55	100%
2	5	-	0.1 ± 0.00	0.2%
3	-	2.5	1.6 ± 0.40	3.4%
4	-	-	0.05 ± 0.05	0.1%

HEK293-D9 (*oc70/am165*) cells were transfected with 5 μg of *supF* Tyr-tRNA (Tyr-*supF*) and 2.5 μg of *supC*.A32 Tyr-tRNA (Tyr-*supC*.A32) as indicated.
[a] HEK293 cells were transfected with active suppressor tRNA as indicated. $tRNA^{fMet}$ was added to keep the amount of total tRNA constant at 10 μg.

Concomitant Suppression of Amber and Ochre Codons in HEK293-D9 Cells by Import of Aminoacylated *supF* Amber and *supC.A32* Ochre Suppressor tRNAs

HEK293-D9 (*oc70/am165*) cells were transfected with a mixture of aminoacylated *supF* Tyr-tRNA and *supC*.A32 Tyr-tRNA. To ensure high specificity of *supF* and *supC*.A32 tRNA for their respective termination codons, the ratio of amber:ochre suppressor tRNA was adjusted to 2:1. Cells transfected with both of the suppressor tRNAs produce significant amounts of FLuc activity, 47.8 × 10^3 RLU per μg of protein (Table 4, line 1). Cells transfected with *supF* Tyr-tRNA alone have essentially no FLuc activity (Table 4, line 2), whereas cells transfected with *supC*.A32 Tyr-tRNA alone had 3.4% of the maximum FLuc activity obtained with both tRNAs (Table 4, line 3). No FLuc activity is detected upon import of *supF* and *supC*.A32 tRNA without prior aminoacylation (data not shown).

These results clearly illustrate that both *supF* and *supC*.A32 tRNA fulfill the basic requirements for site-specific incorporation of unnatural amino acids into proteins in a mammalian system. They also provide the first indication that the import of a mixture of amber and ochre suppressor tRNAs into mammalian cells followed by the concomitant suppression of amber and ochre codons could form the basis of a general approach to site-specific insertion of two different unnatural amino acids into the same protein or into different proteins.

Discussion

Site-specific incorporation of unnatural amino acids into proteins involving nonsense suppressor tRNAs has so far been limited—both in vitro and in vivo—to the use of amber suppressor tRNAs in conjunction with an amber codon (UAG) at the site of interest in the protein. Introduction of two different unnatural amino acids into a protein requires further expansion of the genetic code and the availability of another suppressor tRNA/nonsense codon pair. Attempts to identify such suppressor tRNAs decoding ochre (UAA) or opal (UGA) codons have so far been unsuccessful [21]. Alternative strategies have used codon-anticodon pairs based on unnatural nucleoside bases [22, 23]. Taking advantage of the fact that some codons are rarely used in bacteria and eukaryotes, Sisido and coworkers have used four- and five-base codons decoded by frameshift suppressor tRNAs containing the complementary four- or five-base anticodons for the site-specific insertion of one or two different unnatural amino acids into proteins in vitro [24–26]. Hecht and coworkers have used an amber suppressor tRNA along with a frameshift suppressor tRNA to synthesize a protein containing two different unnatural amino acids [27]. All of the above strategies are limited, so far, for use in vitro.

The "import" of aminoacylated suppressor tRNAs by transfection offers a new and versatile approach to site-specific insertion of unnatural amino acids into proteins in mammalian cells [13]. Recent work has successfully used electroporation and microinjection for the same purpose [14, 15]. The only requirement for these experiments is that the suppressor tRNA should not be a substrate for any of the mammalian aaRSs. We showed previously that the *E. coli supF* amber suppressor tRNA fulfilled this requirement. In this work, we have demonstrated that the *E. coli supC*.A32 ochre suppressor tRNA also fulfills this requirement. We have further shown that the import of a mixture of aminoacylated *supF* amber and *supC*.A32 ochre suppressor tRNAs leads to concomitant suppression of an amber and an ochre codon in a single mRNA. This result opens up the possibility, for the first time, of incorporating two different amino acid analogs into a mammalian protein. To our knowledge, this result also represents the first report of successful suppression in vivo of two different termination codons in a single mRNA.

The ability to introduce two different unnatural amino acids in proteins would greatly increase the scope of potential applications of unnatural amino acid mutagenesis in biology. For example, the insertion of two different analogs containing fluorescent moieties would allow the use of FRET to study protein conformation and dynamics in cells. In combination with imaging and fluorescence microscopy of cells, such fluorescence reporters could be used as biosensors. In pioneering work, Tsien, Taylor, and collaborators used mutants of *Aequorea victoria* green fluorescent protein (GFP) as FRET-pairs and as biosensors of protein kinases in mammalian cells [28, 29]. The reporter proteins contained cyan fluorescent protein (CFP) at one end and yellow fluorescent protein (YFP) at the other end, with a linker consisting of an SH2 phosphotyrosine binding domain and a consensus substrate sequence -PYAQP- for the tyrosine kinase being probed. Phosphorylation of tyrosine within the consensus substrate sequence led to intramolecular binding of the SH2 domain to the phosphorylated pep-

tide segment and to a change in distance between CFP and YFP, as detected by a change in FRET. While the results obtained were striking, it is desirable to also investigate the use of small molecules as FRET-pairs in vivo, instead of large molecules, such as GFPs. For example, in vitro work using cCrkII as a biosensor of Abl (Abelson leukemia virus) tyrosine kinase yielded different results depending upon the use of CFP/YFP versus fluorescein/rhodamine as FRET-pairs [30, 31].

Introduction of two different phosphorylated amino acid analogs into a MAP kinase may also provide a general method for activating a specific signal transduction pathway in the absence of upstream or extracellular signals. MAP kinases, which are multifunctional serine-threonine kinases, are activated by a cascade of phosphorylations leading to phosphorylation of threonine and tyrosine in the sequence -TXY- in the MAP kinase [32]. Activated MAP kinases enter the nucleus where they phosphorylate and activate transcription factors. In mammals, at least 20 different MAP kinases are known [33]. The existence of such a large number of MAP kinases along with several hundreds of transcription factors in the nucleus has made it difficult to identify the relationship between an individual MAP kinase and its downstream targets [34]. Because of the central role played by phosphorylated amino acids, site-specific insertion of phosphoamino acids or phosphonoamino acids, which are more stable derivatives in vivo and excellent mimics of phosphoamino acids [35], represents a method for generating a constitutively activated MAP kinase without altering the protein sequence. Such constitutively activated MAP kinases could be used for a variety of analyses, including comparison of gene expression profiles with the use of DNA microarrays. Data generated from such studies would provide significant amounts of information on the patterns of downstream gene activation brought about by activation of specific MAP kinases.

While the *supF* amber and *supC*.A32 ochre suppressor tRNAs suppress, respectively, amber and ochre codons and *supF* tRNA is specific for the amber codon, the *supC*.A32 tRNA like other ochre suppressor tRNAs in *E. coli* [20, 36] also reads the amber codon, although to a limited extent (11% in Table 3; 3.4% in Table 4). As in all known cases in translation, this nonspecific readthrough of the amber codon is likely to be much less in the presence of the cognate amber suppressor tRNA. For example, Tirrell and coworkers have shown that in the presence of an orthologous $tRNA^{Phe}_{AAA}$ that is expressed and aminoacylated with an amino acid analog in *E. coli*, the UUU phenylalanine codon, normally translated by the resident $tRNA^{Phe}$ with the GAA anticodon, is now almost exclusively translated by the $tRNA^{Phe}_{AAA}$ [37]. Even if the nonspecific readthrough of the amber codon by the ochre codon remains at a level of 11%, which is extremely unlikely, this should not affect the potential applications of the double-suppression approach for the synthesis and the uses, described above, of proteins carrying two different fluorescent amino acids or two different phosphonoamino acids. For example, if the ochre suppressor tRNA delivers the same fluorescent amino acid to the site of the amber codon and the ochre codon, a small fraction of the protein will have the same fluorescent amino acid at two positions in the reporter protein. This should not interfere in any way on intramolecular FRET between two different fluorescent amino acids on the rest of the reporter protein.

Finally, in contrast to bacterial ochre suppressor tRNAs, eukaryotic ochre suppressor tRNAs are specific for the ochre codon [38–40]. Therefore, in addition to the *supC*.A32 ochre suppressor tRNA used here, eukaryotic ochre suppressor tRNAs that are not aminoacylated by mammalian aaRSs will constitute an excellent source of ochre suppressor tRNAs for the site-specific insertion of two different unnatural amino acids into proteins in mammalian cells. Work along these lines is in progress.

Significance

In the present work, we demonstrate—for the first time—concomitant suppression of two different termination codons in a single mRNA in mammalian cells by import of an amber (UAG) and an ochre (UAA) suppressor tRNA. Both tRNAs are derived from *Escherichia coli* tyrosine tRNA. Neither of the suppressor tRNAs are substrates for mammalian aminoacyl-tRNA synthetases, as demonstrated by the finding that the import of a mixture of aminoacylated amber and ochre suppressor tRNAs leads to the concomitant suppression of amber and ochre codons, whereas import of the same tRNAs without prior aminoacylation does not. The ochre suppressor described here represents the first example of an "orthogonal" ochre suppressor.

These results provide a possible approach to site-specific insertion of two different unnatural amino acids into any protein of interest in mammalian cells via nonsense suppression. The ability to introduce two different unnatural amino acids in vivo greatly increases the scope of unnatural amino acid mutagenesis in biology. Potential applications include the generation of proteins carrying two different fluorescent amino acids for use as biological sensors and for studying protein structure and dynamics in cells by fluorescence resonance energy transfer analysis. Another application would be the insertion of two different phosphoamino acids for activating specific components of a signal transduction pathway, e.g., a mitogen-activated protein kinase, in the absence of an extracellular or upstream signal.

Experimental Procedures

Reporter System Based on a Dual-Luciferase Fusion Protein

A dual-luciferase reporter system was developed based on firefly luciferase (FLuc) and *Renilla* luciferase (RLuc). The 1.65 kb FLuc gene from pSP-luc + NF (Promega) and the SV40 late poly(A) signal from pGL3-Basic (Promega) were inserted into pBluescript II (SK+) (Stratagene). The 0.95 kb RLuc gene was amplified from pRL null (Promega) by PCR with primers designed to introduce a BstEII site in place of the termination codon. This modified RLuc gene was then inserted upstream of the FLuc gene to form the 2.6 kb RLuc-FLuc fusion [19]. Site-directed mutagenesis was used to replace the codon for tyrosine 70 of the wild-type FLuc gene with an amber or ochre termination codon to generate RLucFLuc (*am70*) and RLuc-FLuc (*oc70*), respectively. In addition, tyrosine 165 in the RLucFLuc (*oc70*) gene was replaced by an amber codon to generate RLucFLuc (*oc70/am165*). The mutant RLucFLuc genes were cloned into the

retroviral expression vector pLNCX (Clontech) to generate plasmids pRLucFLuc (*oc70*), pRLucFLuc (*am70*), and pRLucFLuc (*oc70/am165*). These plasmids were then used to establish the following stable HEK293 luciferase cell lines: HEK293-E7 (*am70*), HEK293-F22 (*oc70*), and HEK293-D9 (*oc70/am165*). The stable cell lines were selected on the basis of resistance to geneticin and confirmed by expression of RLuc activity [19].

Plasmids Carrying Suppressor tRNA Genes

Plasmids pRSVCAT/trnfM U2:A71/U35A36/G72 [41] and pRSVCAT/trnfM U2:A71/U34U35A36/G72 [13] contain the genes for amber (*fMam*) and ochre (*fMoc*) suppressor tRNAs derived from the *E. coli* $tRNA_2^{fMet}$. The plasmid pCDNA1 (Invitrogen) contains the gene for the *supF* amber suppressor derived from *E. coli* $tRNA_1^{Tyr}$ [42]. A 329 bp fragment carrying the gene for *supF* tRNA, including its original promoter and transcription termination signals, was amplified by PCR and inserted into the BamHI site of pRSVCAT*am27* [38], which carries the gene for chloramphenicol acetyltransferase (CAT) with an amber mutation at position 27, to generate pRSVCAT*am27/supF*. In attempts to construct the ochre suppressor *supC*, the *supF* gene was mutagenized to introduce a C34 to U34 change in the anticodon of the tRNA using site-directed mutagenesis. No clones carrying the wild-type *supC* tRNA could be isolated, likely due to toxicity of overexpression of *supC* tRNA in *E. coli* [16]. Instead, a *supC* tRNA mutant with a C32 to A32 mutation (*supC*.A32), which was found to be active as an ochre suppressor in *E. coli*, was isolated. Position 27 of the CAT reporter gene was changed from an amber to an ochre codon to generate pRSVCAT*oc27/supC*.A32.

Purification of Suppressor tRNAs

Overexpression and purification of the *fMam, fMoc*, and the *supF* suppressor tRNAs have been described previously [13]. The *supC*.A32 ochre suppressor tRNA was isolated from *E. coli* strain CA274 [*lacZ125(am) trp49(am) relA1 spoT1*] carrying the plasmid pRSVCAT*oc27/supC*.A32 and purified by benzoylated-naphthoylated DEAE-cellulose column chromatography. Separation of *supC*.A32 tRNA from wild-type $tRNA^{Tyr}$ was monitored by acid urea gel electrophoresis of column fractions followed by RNA blot hybridization using 5′-^{32}P-labeled oligonucleotides [43]. Fractions containing *supC*.A32 tRNA free of $tRNA^{Tyr}$ were pooled. The purity of *fMam, fMoc*, and the *supF* suppressor tRNAs was greater than 90% based on amino acid acceptor activity and polyacrylamide gel electrophoresis; the purity of *supC*.A32 tRNA was 45%–50%.

In Vitro Aminoacylation and Isolation of Aminoacyl-tRNAs

Aminoacylation of *supF* and *supC*.A32 tRNA was carried out as described previously [13] on 1 A_{260} unit of tRNAs with purified *E. coli* tyrosyl-tRNA synthetase (TyrRS). Aminoacylation of tRNAs was essentially quantitative as analyzed by acid urea gel electrophoresis followed by RNA blot hybridization [43].

Transfection of Mammalian Cells

COS1 cells were cultured in DMEM (with 4500 mg/L of glucose and 4 mM glutamine; Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc.), 50 units/ml of penicillin, and 50 μg/ml of streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere. HEK293 cell lines were maintained in the medium described above supplemented with 250 μg/ml of geneticin (Invitrogen). Eighteen to twenty hours before transfection, cells were subcultured into twelve-well dishes. Transfection of COS1 and HEK293 cells with tRNA and/or plasmid DNA via Effectene (Qiagen) was as described before [13]. The amount of suppressor tRNA used per transfection was adjusted according to tyrosine acceptance which reflects the amount of "active" suppressor tRNA present per sample. A nonsuppressing tRNA ($tRNA^{fMet}$) was used to keep the amount of total tRNA constant throughout the transfection experiments.

Assay for Luciferase Activity

The Dual-Luciferase Reporter System (DLR; Promega) was used to measure FLuc and RLuc activities in mammalian cell extracts. Fifteen to twenty-four hours posttransfection, the medium was removed and cells were washed twice with PBS. Passive lysis buffer (PLB; supplied by the manufacturer; 200 μl of 1×) was added per well, and cells were lysed for 15 min at room temperature with gentle shaking. Lysates were clarified by centrifugation, and the supernatants were immediately analyzed as follows. Luciferase assay reagent II (LAR II; 20 μl) was added to lysate (2–4 μl), and firefly luciferase activity was read. Quenching of the FLuc signal and concomitant activation of RLuc were performed by adding 20 μl of Stop & Glo Reagent. Measurement of luciferase activities was carried out on a Sirius tube luminometer (Berthold Detection Systems). For standard DLR assays, a 10 s premeasurement delay and a 15 s measurement period were programmed. Luciferase activities are given as relative luminescence units (RLU) per μg of total cell protein; the values shown in the tables represent the averages of at least three independent experiments. The protein concentration of cell lysates was determined with a BCA protein assay (Pierce), using BSA as standard.

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