Chapter 3

In Vivo Incorporation of Multiple Unnatural Amino Acids Through Nonsense and Frameshift Suppression

This chapter is reproduced, with modification, from *In vivo incorporation of multiple unnatural amino acids through nonsense and frameshift suppression*, by E. A. Rodriguez, H. A. Lester, and D. A. Dougherty, (2006) *Proc. Natl. Acad. Sci. USA*, **103(23)**, 8650–8655.

3.1 Introduction

The site-specific incorporation of unnatural amino acids (UAAs) into proteins biosynthetically is a powerful methodology that is seeing increasing use. The primary approach has been stop codon (nonsense) suppression using a specially designed tRNA with an anticodon recognizing the stop codon. A wide range of *in vitro* translation systems has been employed, along with expression in *E. coli* and, to a lesser extent, yeast. Nonsense suppression in higher eukaryotes has for the most part been limited to the *Xenopus* oocyte (1,2). The incorporation of UAA(s) in *Xenopus* oocytes is shown in Figure 3.1. The mRNA with the suppression site(s) (stop and/or quadruplet codons) (Figure 3.1, 1) (for the mechanism of stop codon and quadruplet codon suppression see Figure 2.2) and the tRNA(s) chemically aminoacylated with the UAA(s) (Figure 3.1, 2) are injected into a *Xenopus* oocyte (Figure 3.1, 3). The oocyte is allowed to incubate for 1–2 days to allow for UAA(s) incorporation by the endogenous translational machinery, protein folding and processing, ion channel assembly, and export to the surface of the cell (Figure 3.1, 4). UAA(s) incorporation is then assayed using the sensitivity of electrophysiology (Figure 3.1, 5). Other experiments in higher eukaryotes have relied on the evolution of a unique tRNA and a complementary aminoacyl-tRNA synthetase (aaRS) to insert an UAA in response to the UAG or UGA stop codon, but currently only 3-iodo-tyrosine (3), p-benzoyl-phenylalanine (4), and 5-hydroxy-tryptophan (5) have been incorporated.

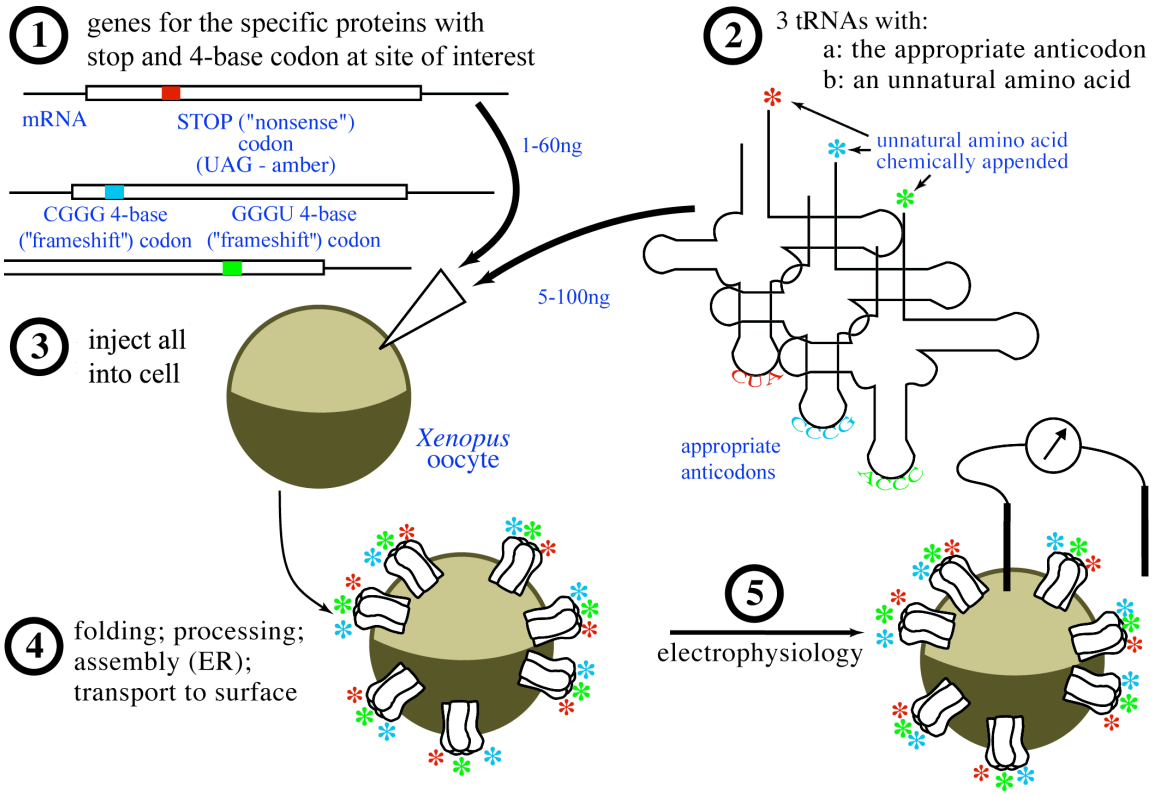

Figure 3.1: Multiple UAA incorporation in ion channels expressed in *Xenopus* oocytes. (1) mRNAs with the appropriate suppression sites are transcribed *in vitro*. (2) Suppressor tRNAs are transcribed *in vitro*. An UAA is synthesized and chemically aminoacylated onto the suppressor tRNAs. (3) mRNAs and tRNAs are micro-injected into the *Xenopus* oocyte. (4) Oocytes are incubated to allow for protein translation with UAAs site-specifically incorporated. The ion channels are folded, processed (signal sequence removal, glycosylation, etc.), assembled into multi-subunit channels, and transported to the plasma membrane. (5) UAA incorporation is detected using the sensitive assay of electrophysiology. Figure adapted from (2).

A remarkable variant of this approach is the use of quadruplet codons, a process termed frameshift suppression, that was pioneered by Sisido and coworkers (6,7). The success of this approach opens up the possibility of developing multiple new codons, and thus incorporating several different UAAs into a protein. This in turn would enable novel biophysical approaches such as incorporating fluorescence resonance energy transfer (FRET) pairs, new structural probes such as novel cross-linking approaches, and more detailed structure-function studies.

To date frameshift suppression *in vivo* has been performed only in *E. coli* by using a unique tRNA/aaRS pair, and homoglutamine is the only UAA incorporated by this method (8). It has yet to be established whether frameshift suppression by chemically aminoacylated tRNA can be effective *in vivo* in general, and in eukaryotic cells such as the *Xenopus* oocyte in particular. In fact, a previous attempt to perform frameshift suppression in *Xenopus* oocytes showed very poor suppression efficiency (9). Here we show that with appropriately designed frameshift suppressor (FS) tRNAs, frameshift suppression is a viable approach to UAA incorporation in eukaryotic cells. Also, the efficiency of frameshift suppression can be substantially improved by "masking" the mRNA of all in-frame quadruplet sequences that match the frameshift suppression site. In particular, we describe two tRNAs with 4-base anticodons that can deliver UAAs in response to the quadruplet codons CGGG and GGGU. When directly compared to an amber suppressor (AS) tRNA (THG73) that has been used extensively in *Xenopus* oocytes, the FS tRNAs are less efficient at delivering UAAs. However, both FS tRNAs are more "orthogonal" than THG73, producing much less incorporation of undesired natural amino acids at promiscuous sites. We also show that suppression by FS tRNAs increases nonlinearly with the amount of injected tRNA. To illustrate the potential of this methodology, we have successfully incorporated two and three different UAAs, simultaneously, into a neuroreceptor expressed in a *Xenopus* oocyte.

3.2 Results

3.2.1 Testing Frameshift Suppression Viability *In Vivo*

To determine whether frameshift suppression is viable in *Xenopus* oocytes, we chose to use a tRNA that can be aminoacylated *in vivo*. We selected the human serine amber suppressor (HSAS), because it is aminoacylated (with serine) in eukaryotic cells, and the seryl-tRNA synthetase does not recognize the anticodon (10–12). The CUA anticodon of HSAS was replaced with CCCG and ACCC to create the human serine frameshift suppressors ($HSFS_{CCCG}$ and $HSFS_{ACCC}$) (cloverleaf structures shown in Figure 3.2, A), which recognize the quadruplet codons CGGG and GGGU, respectively. Prior research showed that these 4-base codons are efficient *in vitro* (7). Injection of wild-type muscle nicotinic acetylcholine receptor (nAChR) mRNA and either $HSFS_{CCCG}$ or $HSFS_{ACCC}$ (2.5 or 10 ng per oocyte; no amino acid ligated to the tRNA) into *Xenopus* oocytes resulted in no detectable channel expression. The addition of the original amber suppressor HSAS with wild type nAChR mRNA did show channel expression with 2.5 ng tRNA per oocyte, but not with 10 ng. These results suggested that the HSFS tRNAs were causing +1 frameshifts, resulting in undesirable truncation of wild-type protein and thus a lack of detectable current. Analysis of the four nAChR subunits revealed four CGGG and one GGGU in-frame quadruplet codons. These were mutated to degenerate codons (see methods) to avoid suppression, and we refer to the resulting mRNAs as the "masked" constructs. Other groups have similarly removed undesired in-frame quadruplets (7,9,13). Injection of 2.5 ng per oocyte of either unligated HSFS plus the masked nAChR mRNAs resulted in functional channels with the same EC_{50} as channels

expressed without tRNA (data not shown). Unless otherwise noted, all subsequent experiments used such masked constructs.

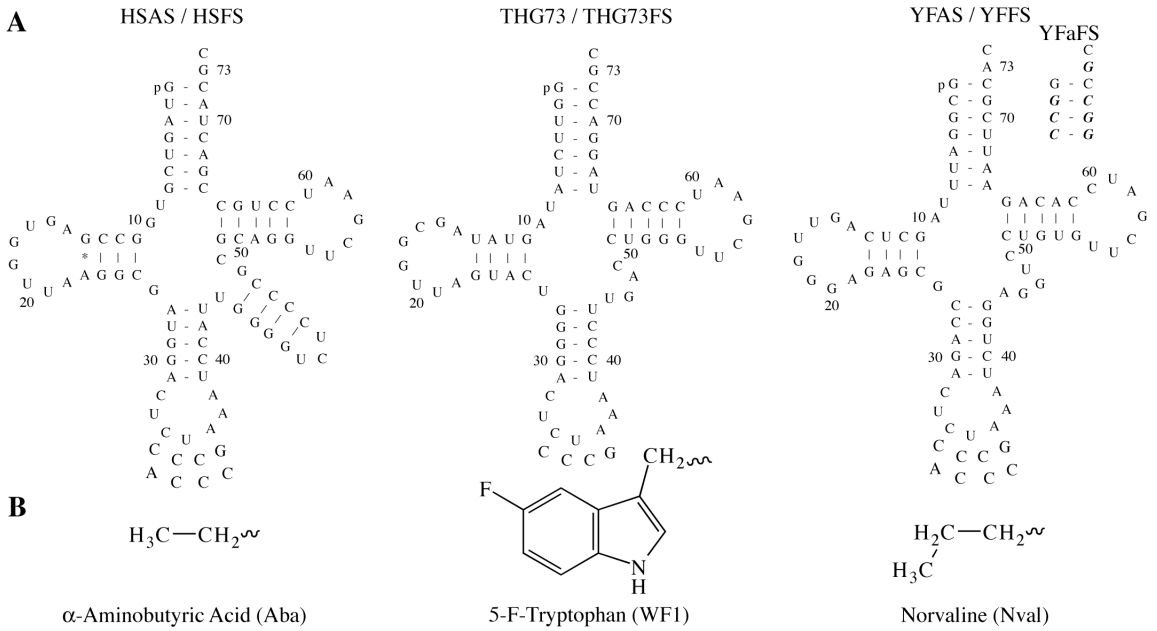

Figure 3.2: tRNAs and UAAs. (A) The AS tRNAs are shown with the CUA anticodon and the FS anticodons used in this study are shown below. YFaFS tRNA acceptor stem mutations are shown next to the YFFS tRNA body (italicized). (B) The three UAAs used in this study.

To test whether a naturally occurring amino acid (serine) could be incorporated in response to a quadruplet codon, we probed a highly conserved leucine of the nAChR M2 domain, a site designated Leu9' (shown in Figure 3.3). This is a promiscuous site in the nicotinic receptor, and replacement of the native leucine with essentially any natural amino acid produces a functional receptor, usually with a quite noticeable shift in EC_{50}. In particular, prior research showed that a leucine-to-serine mutation in the β subunit (β9') resulted in a ≈ 33-fold increased sensitivity to acetylcholine (ACh) (14).

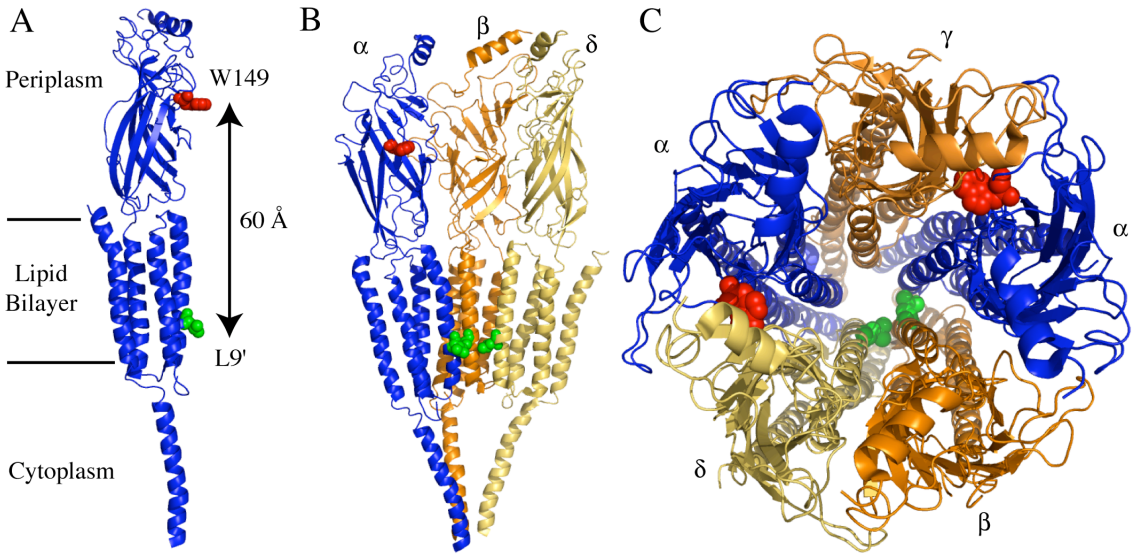

Figure 3.3: nAChR suppression sites used in this research. (A) The α-subunit of the nAChR. αW149 and αL9' are shown in red and green, respectively. (B) The α-, β-, and δ-subunits of the nAChR are shown. αW149 and L9' are shown in red and green, respectively. These were the three subunits used to simultaneously incorporate three UAAs. (C) Top view of the nAChR with αW149 and L9' shown in red and green, respectively. Due to the stoichiometry (2α:β:γ:δ), the simultaneous incorporation of three different UAAs at α149UAG, β9'CGGG, and δ9'GGGU results in four UAAs per ion channel. Figure created from 2BG9.pdb (15).

The β9' site was mutated to UAG, CGGG, or GGGU. When mutant mRNA was injected into *Xenopus* oocytes along with 2.5 ng of unligated HSAS or HSFS tRNA, which should be aminoacylated with serine by the endogenous seryl-tRNA synthetase, significant channel expression was seen. However, the EC_{50} values varied depending on the incubation time (Table 3.1, A). This suggested that natural amino acids other than serine were being placed at the β9' site with two-day incubations, because the conventional mutant, β9'Ser, shows no change in EC_{50} (Table 3.1, A). The variability in EC_{50} between one and two day incubations suggests that the tRNAs are being modified to accept other amino acids. Modification of yeast phenylalanine tRNA in *Xenopus* oocytes has been shown to increase greatly from one-to-two day incubation times (16). Thus, we avoid this complication by incubation for one day. Amber suppression is highly efficient

when the average maximal peak current (I_{max}) is measured at 1.25 ng of tRNA per oocyte and decreases slightly when 2.5 ng is added (Table 3.1, B). CGGG shows lower suppression than GGGU, in agreement with previous *in vitro* studies (7,17). CGGG suppression is highly nonlinear, with a 330% increase in current when twice as much tRNA is injected (Table 3.1, B). GGGU however shows an almost linear relationship, with an increase of 86% in response to doubling (Table 3.1, B). These data suggest that $HSFS_{ACCC}$ is a more efficient tRNA at recognizing its cognate quadruplet codon and/or has less competition with endogenous triplet tRNA in *Xenopus* oocytes than $HSFS_{CCCG}$. These experiments establish that frameshift suppression is viable in *Xenopus* oocytes, and that UAA incorporation should be feasible using the appropriate FS tRNA.

Table 3.1: HSAS and HSFS suppression experiments at the β9' site.

A

β9'X	tRNA (2.5 ng)	EC_{50} (1 day)*	n_H	n	EC_{50} (2 day)*	n_H	n
AGC (Serine)	none	1.5±.04	1.7±.07	5	1.5±.2	1.9±.3	3
UAG	HSAS	1.7±.06	1.7±.09	6	.70±.008	1.9±.07	14
CGGG	$HSFS_{CCCG}$	2.1±.09	1.7±.1	8	1.3±.1	1.9±.3	13
GGGU	$HSFS_{ACCC}$	1.9±.08	1.5±.08	9	.68±.1	1.7±.04	5

B

β9'X	tRNA	I_{max}†±SE (1.25)‡	n	I_{max}†±SE (2.5)‡	n	% HSAS (1.25)‡	% HSAS (2.5)‡	% Change§
UAG	HSAS	-19±2	12	-14±3	11	100%	100%	-26%
CGGG	$HSFS_{CCCG}$	-1.3±.3	10	-5.6±1	12	6.8%	40%	330%
GGGU	$HSFS_{ACCC}$	-8.6±3	10	-16±3	12	45%	110%	86%

* Incubation time. † Avg. I_{max} (μA) recorded at 50 μM ACh. ‡ ng of tRNA. § 1.25 to 2.5 ng of tRNA.

3.2.2 UAA Incorporation By Frameshift Suppression

THG73 is an AS tRNA (cloverleaf structure shown in Figure 3.2, A) (18) used extensively for incorporating UAAs into various ion channels expressed in *Xenopus*

oocytes (2). Initially a FS derived from THG73 recognizing the quadruplet codon CGGG ($THG73FS_{CCCG}$) was tested for UAA incorporation. Attempts to suppress β9'CGGG with $THG73FS_{CCCG}$-L, where Leu was chemically aminoacylated onto the tRNA, showed no current *in vivo*. This is consistent with data from Voss and coworkers, who saw very little UAA incorporation with $THG73FS_{ACCC}$ in *Xenopus* oocytes (9).

Western blots of $THG73FS_{CCCG}$-W suggested that the tRNA may be stuck on the ribosome and stopping translation (Chapter 2). Uhlenbeck and coworkers have shown that nucleotides in the anticodon loop at position 32 and 38 effect ribosome binding of tRNAs (19). $THG73FS_{CCCG}$ and most amber suppressors have C32 and A38 (Figures 3.2 & 3.4), which is the consensus sequence for optimal suppression by amber suppressor tRNAs and is thought to cause tighter binding to the ribosome. Mutations to $tRNA^{Ala}_{GGC}$ at positions 32 and 38 alter ribosome binding where A32-A38 causes tighter binding to the ribosome, while C32-G38 and A32-U38 weaken binding to the ribosome (19). These mutations were placed on $THG73FS_{CCCG}$ and shown in Figure 3.4. Another possibility is that $THG73FS_{CCCG}$ could be recognizing multiple codons or even doublet codons, which would cause frameshifts and truncation of the gene. $tRNA^{Gly}_{(UCC\ \&\ CCC)}$ uses the 32nd position to discriminate the 3rd nucleotide of the triplet codon (20). $tRNA^{Gly}_{CCC}$ (similar anticodon to $THG73FS_{CCCG}$) with C32, which is the same as $THG73FS_{CCCG}$ (Figure 3.4), promotes doublet decoding of GG over the full triplet codon GGG (21). Therefore, mutations at the 32nd position could alter ribosomal binding and/or decoding of $THG73FS_{CCCG}$ to allow for the incorporation of UAAs. $THG73FS_{CCCG}$ was mutated to create the constructs $THA32G73FS_{CCCG}$, $THG38G73FS_{CCCG}$, and $THA32U38G73FS_{CCCG}$ (shown in Figure 3.4). Suppression experiments were performed at α149CGGG with the

frameshift suppressor tRNAs ligated with Trp and compared to suppression at α149UAG by the amber suppressor, THG73-W (Table 3.2). All mutations resulted in little expression and therefore none of the THG73-based fraemshift suppressors are viable for UAA incorporation.

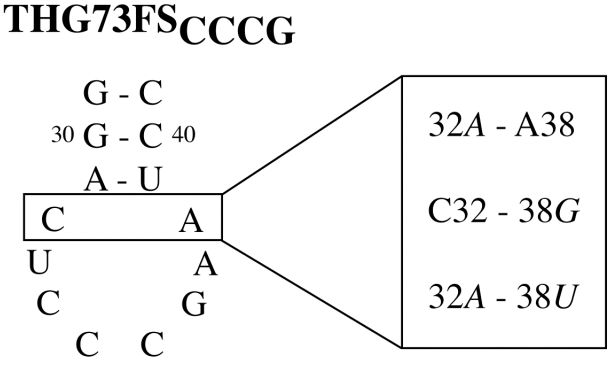

Figure 3.4: THG73FS$_{CCCG}$ anticodon loop mutations. The last three base pairs of the anticodon stem and the anticodon loop are shown. The boxed region corresponds to C32-A38, where mutations were made. The box on the right shows mutations made (italics). On tRNA$^{Ala}_{GGC}$, A32-A38 increases ribosome binding, while C32-38G and A32-U38 decrease ribosome binding (19). C32 on tRNA$^{Gly}_{CCC}$ promotes doublet decoding of GG and therefore mutations at the 32nd position may also promote quadruplet decoding (21).

Table 3.2: THG73FS$_{CCCG}$ and anticodon loop mutations suppression at α149CGGG.

mRNA	tRNA	ng tRNA	n	I_{max}^{*} ± SE
α149UAG	THG73-W	9.4	12	-2.8 ± .6
α149CGGG	THG73FS$_{CCCG}$-W	9.4	12	-.019 ± .002
α149CGGG	THA32G73FS$_{CCCG}$-W	9.4	12	-.013 ± .002
α149CGGG	THG38G73$_{CCCG}$-W	9.4	12	-.014 ± .003
α149CGGG	THA32U38G73FS$_{CCCG}$-W	9.4	12	-.024 ± .002

* Avg. I_{max} (μA) recorded at 1 mM ACh.

We then chose to screen yeast phenylalanine FS (YFFS) tRNAs, which were employed successfully by the Sisido group *in vitro* (7,17). We studied both YFFS$_{CCCG}$ and YFaFS$_{ACCC}$; Figure 3.2, A, shows cloverleaf structures. The latter contains acceptor

stem mutations (denoted by the "a") incorporated to reduce glycyl-tRNA synthetase recognition (7). We first evaluated a non-promiscuous position of the nAChR, α149W, an agonist binding site tryptophan that makes a cation-π interaction with ACh (22) (shown in Figure 3.3). Wild-type recovery, i.e., suppressing the α149 quadruplet codons with $YFFS_{CCCG}$-W or $YFaFS_{ACCC}$-W, resulted in functional, wild-type channels (Table 3.3). To demonstrate UAA incorporation we relied on previous work using the AS THG73 that established that 5-fluoro-tryptophan, WF1 (structure in Figure 3.2, B), incorporated at α149 decreased the cation-π interaction and caused a ≈ 4-fold increase in EC_{50} (22). $YFFS_{CCCG}$-WF1 suppression at α149CGGG resulted in a comparable increase in EC_{50} (Table 3.3), establishing the successful incorporation of the UAA WF1.

Table 3.3: Wild-type recovery and UAA incorporation by frameshift suppression *in vivo*.

mRNA	tRNA	EC_{50} (theo)$^{(ref)\dagger}$	n_H	n
α149CGGG	$YFFS_{CCCG}$-W*	56±2 (50)$^{(14)}$	1.8±.07	8
α149GGGU	$YFaFS_{ACCC}$-W*	53±2 (50)$^{(14)}$	1.6±.03	8
β9'GGGU	$YFaFS_{ACCC}$-Aba	16±.9 (16)$^{(14)}$	1.3±.08	7
δ9'GGGU	$YFaFS_{ACCC}$-Nval	31±2 (36)$^{(14)}$	1.6±.1	6
α149CGGG	$YFFS_{CCCG}$-WF1	190±3 (200)$^{(22)}$	1.6±.03	10

* Rescue of wild type recovery by frameshift suppression.
† EC_{50} values from THG73-UAA incorporation by nonsense suppression.

We next considered the previously mentioned Leu9' residue (shown in Figure 3.3). Suppression at β9'GGGU and δ9'GGGU with $YFaFS_{ACCC}$-Aba and $YFaFS_{ACCC}$-Nval (UAA structures shown in Figure 3.2, B), respectively, resulted in reductions in EC_{50} (Table 3.3) that were consistent with previous studies using the same UAAs and the AS THG73 (14). All frameshift suppression experiments had an I_{max} between -1.6 and

-4.4 μA, which is more than adequate for UAA studies *in vivo* and should allow for the incorporation of multiple UAAs. In all cases, injection of full-length tRNA that had no amino acid attached to the 3' end resulted in no detectable currents in response to added ACh, directly showing a lack of aminoacylation by endogenous, *Xenopus* aaRSs.

3.2.3 Masking Effects on Frameshift Suppression

Experiments with HSFS required the masking of the nAChR subunits in order to avoid protein truncation caused by +1 frameshifts. To demonstrate the effect on UAA incorporation, suppression experiments were performed with wild-type and masked constructs. The quadruplet codon GGGU was chosen because there was only one in-frame quadruplet in the signaling sequence of the nAChR β subunit and none in the α, γ, or δ subunits. Wild-type recovery was performed by suppressing at α149GGGU with $YFaFS_{ACCC}$-W and adding either wild-type or masked β mRNA to the injection mixture. Table 3.4 shows the dramatic effect of masking one position on frameshift suppression. With a 1:1:1:1 ratio of α:β:γ:δ, the masked construct gives a 2.7-fold increase in I_{max} relative to wild type. As the amount of α subunit (which contains the suppression site) is increased, the masking effect decreases to 1.5-fold and 1.2-fold with subunit ratios of 5:1:1:1 and 10:1:1:1, respectively. Calculations that assume two, equally efficient quadruplet codons reproduce this trend (Table 3.4), suggesting that the α149GGGU and the GGGU present in the β subunit have similar suppression efficiencies.

Table 3.4: Masking experiments.

α149GGGU	:	β	I_{max}* ±SE	n	% Difference (theo)†
1	:	1 wild type	-.14±.02	11	63%
1	:	1 masked	-.38±.1	11	(75%)
5	:	1 wild type	-.35±.1	10	32%
5	:	1 masked	-.52±.2	12	(31%)
10	:	1 wild type	-.71±.3	11	15%
10	:	1 masked	-.83±.3	12	(17%)

* Avg. I_{max} (μA) recorded at 1 mM ACh.
† () are theoretical values = 1 – (*P* of α suppression)^2, where both sites are assumed to have the same probability (*P*) and squared because of 2 α subunits per channel.

3.2.4 Comparison of Frameshift and Nonsense Suppression Efficiencies

To compare frameshift and nonsense suppression, the α149 and β9' sites were studied in more detail (shown in Figure 3.3). Suppression of α149CGGG or GGGU with 10 ng of $YFFS_{CCCG}$-W or $YFaFS_{ACCC}$-W resulted in 38% and 48%, respectively, of the current from 10 ng of THG73-W suppression at α149UAG (Table 3.5). Suppression of β9'UAG with 2 ng of THG73-L resulted in the largest I_{max} (Table 3.5). Suppression at β9'CGGG or GGGU with 2 ng of $YFFS_{CCCG}$-L or $YFaFS_{ACCC}$-L resulted in 14% and 36%, respectively, of the current from THG73-L (Table 3.5). We conclude that amber suppression is more efficient than frameshift suppression, in agreement with a trend previously seen in a eukaryotic cell-free translation system (17). In particular, the suppression efficiency observed here follows the order: THG73>$YFaFS_{ACCC}$>$YFFS_{CCCG}$.

Table 3.5: Comparison of suppression efficiency, aminoacylation, and read-through *in vivo*.

mRNA	tRNA	ng tRNA	n	I_{max}* ±SE	% THG73
α149UAG	THG73-W	10	18	-4.8±2	100%
α149CGGG	$YFFS_{CCCG}$-W	10	20	-1.8±.3	38%
α149GGGU	$YFaFS_{ACCC}$-W	10	13	-2.3±.9	48%
β9'UAG	THG73-L†	2	15	-6.1±2	100%
β9'CGGG	$YFFS_{CCCG}$-L†	2	12	-.84±.2	14%
β9'GGGU	$YFaFS_{ACCC}$-L†	2	9	-2.2±.5	36%
β9'CGGG	$YFFS_{CCCG}$-L†	6	13	-8.8±.9	NA
β9'GGGU	$YFaFS_{ACCC}$-L†	6	13	-16±2	NA
β9'UAG	THG73-dCA	2	13	-4.8±1	100%
β9'CGGG	$YFFS_{CCCG}$-dCA	2	13	-.42±.8	8.8%
β9'GGGU	$YFaFS_{ACCC}$-dCA	2	13	-.092±.02	1.9%
β9'UAG	THG73-dCA	6	13	-8.2±1	100%
β9'CGGG	$YFFS_{CCCG}$-dCA	6	12	-1.2±.3	15%
β9'GGGU	$YFaFS_{ACCC}$-dCA	6	11	-.27±.09	3.3%
					% UAG
β9'UAG	-	-	13	-.37±.1	100%
β9'CGGG	-	-	13	-.085±.03	23%
β9'GGGU	-	-	13	-.078±.02	21%

* Avg. I_{max} (μA) recorded at 1 mM ACh.
† Currents in response to 10 μM and 1 mM ACh displayed a ratio of 0.1, as anticipated from the Hill equation fit for one, wild-type receptor.

Interestingly, the yield of receptors from frameshift suppression at the β9' site was substantially improved by increasing the amount of tRNA injected. Suppression with 6 ng of $YFFS_{CCCG}$-L or $YFaFS_{ACCC}$-L gave dramatic increases in I_{max}, with a % change of 950% and 630%, respectively (Table 3.5). This large change in I_{max} in response to a modest increase in tRNA concentration implicates a competition with endogenous triplet tRNA that responds nonlinearly to the amount of injected FS tRNA. A comparable increase in the amount of injected THG73-L led to complications due to reacylation of the tRNA by endogenous aaRSs (undesired) and incorporation of natural

amino acids other than leucine, an issue that is addressed in detail in the following section, in the Discussion, and in Chapters 4 and 5.

3.2.5 Comparison of Aminoacylation of Suppressor tRNA and Read-Through of Suppression Sites

To evaluate aminoacylation *in vivo,* which is undesirable for any tRNA used to incorporate UAAs, the β9' site was again studied, because most amino acids produce functional receptors when substituted at this position (14). In all experiments, tRNAs that had been ligated to dCA but did not contain an amino acid at the 3' end were injected, in order to more closely mimic the biologically active, full-length tRNA. In order to maximize the potential for aminoacylation by endogenous aaRSs, two-day incubations and relatively large mRNA quantities (16.5 ng) were employed. Surprisingly, THG73-dCA, which has been used extensively for UAA incorporation in *Xenopus* oocytes, showed significant aminoacylation *in vivo*, with I_{max} of -4.8 and -8.2 μA for 2 and 6 ng tRNA, respectively (Table 3.5). Note that under other conditions (less mRNA; shorter incubations) previous work has found no complications from aminoacylation using THG73-dCA in *Xenopus* oocytes (9,14,18). Still, the present results establish that THG73 is susceptible to aminoacylation by aaRSs, which is undesired. No aminoacylation was seen with 2 ng of THG73-L, suggesting that aminoacylation by endogenous aaRSs is more likely when non-aminoacylated THG73 is injected, as noted previously (18). Both FS tRNAs show much lower amounts of aminoacylation by aaRSs, as evidenced by the decrease in I_{max} (Table 3.5). $YFFS_{CCCG}$-dCA shows only 8.8% and 15% of the I_{max} of THG73-dCA at 2 and 6 ng, respectively. The most orthogonal suppressor is $YFaFS_{ACCC}$-dCA with 1.9% and 3.3% of the I_{max} of

THG73-dCA at 2 and 6 ng, respectively. The orthogonality trend thus follows the order: $YFaFS_{ACCC}$-dCA>$YFFS_{CCCG}$-dCA>THG73-dCA. $YFaFS_{ACCC}$ is the most orthogonal and efficient FS tRNA, and it therefore offers a viable replacement for THG73, especially when aminoacylation by aaRSs poses a problem *in vivo*.

Read-through at the β9' site was also assessed by injection of mRNA only (Table 3.5). β9'UAG showed the most read-through, presumably because there is only one in-frame stop codon before desired termination. β9'CGGG and β9'GGGU show 23% and 21% read-through relative to the UAG stop codon. This is consistent with the idea that an endogenous triplet tRNA recognizing the first three bases of a quadruplet codon causes a -1 frameshift, which then presents multiple stop codons (frameshift suppression and competition shown in Figure 2.2). Again, we designed this experiment to enhance signals from read-through by injecting large amounts of mRNA (50 ng). No current was detectable after injection of mRNA containing UAG, CGGG, or GGGU at position α149, confirming that this site is much less promiscuous than β9'.

3.2.6 The Effect of Discriminator and Acceptor Stem Mutations on $YFFS_{CCCG}$

Table 3.5 shows that $YFFS_{CCCG}$ is less orthogonal than $YFaFS_{ACCC}$, which contains mutations at the discriminator base (N73) and in the acceptor stem. In previous work the $YFaFS_{ACCC}$ mutations were made to avoid glycyl-tRNA synthetase recognition for *in vitro* reactions, and they significantly reduced aminoacylation (7). In *S. cerevisae* and *H. sapiens* the glycyl-tRNA synthetase recognizes the discriminator base (A73) and acceptor stem recognition includes C2-G71 (23,24). Both are present in $YFFS_{CCCG}$. Therefore, we mutated A73 to create $YFG73FS_{CCCG}$, and we mutated the acceptor stem to create $YFaFS_{CCCG}$. Aminoacylation was determined by injecting 16.5 ng tRNA, not

ligated to dCA, and β9'CGGG. The single A73G mutation resulted in increased aminoacylation *in vivo* as indicated by the increase in I_{max} relative to $YFFS_{CCCG}$, but incorporating the acceptor stem mutations and A73G resulted in slightly less aminoacylation than $YFFS_{CCCG}$ when comparing I_{max} (Table 3.6). The orthogonality trend was as follows: $YFaFS_{CCCG} \approx YFFS_{CCCG} > YFG73FS_{CCCG} > THG73$. These results suggest that glycyl-tRNA synthetase or another aminoacyl-tRNA synthetase is aminoacylating $YFFS_{CCCG}$, and if the glycyl-tRNA synthetase is aminoacylating the $YFFS_{CCCG}$, the recognition of the discriminator base in *Xenopus* oocytes differs from the other eukaryotes studied. There was no significant difference between $YFaFS_{CCCG}$ and $YFFS_{CCCG}$ by a one-way ANOVA, and we continued to use $YFFS_{CCCG}$. However, leaving A73 and only incorporating the acceptor stem mutations may create more orthogonal variants of $YFFS_{CCCG}$ and $YFaFS_{ACCC}$ for the *Xenopus* oocyte system.

Table 3.6: The effect of discriminator base and acceptor stem mutations on $YFFS_{CCCG}$.

mRNA	tRNA	I_{max}* ± SE	n	% THG73
β9'UAG	-	0.026±0.002	5	2.9%
β9'UAG	THG73	0.89±0.4	11	100%
β9'CGGG	-	0.021±0.005	6	2.4%
β9'CGGG	$YFFS_{CCCG}$	0.29±0.1	14	33%
β9'CGGG	$YFG73FS_{CCCG}$	0.71±0.2	12	80%
β9'CGGG	$YFaFS_{CCCG}$	0.23±0.07	10	26%

* Avg. I_{max} (μA) recorded at 400 μM ACh.

3.2.7 Incorporation of Two UAAs

To investigate the simultaneous incorporation of two UAAs, we again built on previous work using THG73 to incorporate UAAs into the nAChR at positions α149, β9' and δ9' (shown in Figure 3.3). Importantly, EC_{50} changes associated with mutations at

these sites are independent of one another (22,25). This allows one to qualitatively anticipate the consequences of multiple mutations. In particular, both β9'Aba and δ9'Nval produce predictable reductions in EC_{50} that should be reproduced when combined with mutations at α149 (14). That is, the previously noted 4-fold increase in EC_{50} seen when the native tryptophan at α149 is replaced by WF1 should persist when in combination with β9'Aba or δ9'Nval.

Successful incorporation of two UAAs to produce large ACh-induced currents could be seen when a 5-fold excess of mutant-to-wild-type mRNA was used. Suppression with α149UAG/THG73-W and β9'CGGG/YFFS$_{CCCG}$-L is a wild-type recovery experiment that gave the expected EC_{50} for ACh of 50 μM (Table 3.7). Maintaining β9'CGGG/YFFS$_{CCCG}$-L, but substituting α149UAG/THG73-WF1 resulted in the anticipated 4-fold increase in EC_{50} (Table 3.7) (22). For incorporation of two UAAs, α149UAG/THG73-W or WF1 was combined with either β9'CGGG/YFFS$_{CCCG}$-Aba or δ9'GGGU/YFaFS$_{ACCC}$-Nval (Table 3.7 and Figure 3.5 show representative traces and fits to the Hill equation). The α149 WF1:W EC_{50} ratios are 4.4 for the both β and δ 9' mutants. These experiments establish that frameshift suppression can be combined with nonsense suppression to incorporate two UAAs in a eukaryotic system.

Table 3.7: Incorporation of two UAAs.

Row	α149	tRNA	β or δ	tRNA	EC_{50} (theo)$^{(ref)*}$	n_H	n
1	UAG	THG73-W	β9'CGGG	$YFFS_{CCCG}$-Aba	14±.4 (16)$^{(14)}$	1.7±.06	9
2	UAG	THG73-W	δ9'GGGU	$YFaFS_{ACCC}$-Nval	41±2 (36)$^{(14)}$	1.9±.1	9
3	UAG	THG73-W	β9'CGGG	$YFFS_{CCCG}$-L	50±3 (50)$^{(22)}$	1.4±.08	20
4	UAG	THG73-WF1	β9'CGGG	$YFFS_{CCCG}$-Aba	61±3	1.5±.08	7
5	UAG	THG73-WF1	δ9'GGGU	$YFaFS_{ACCC}$-Nval	180±7	1.8±.1	6
6	UAG	THG73-WF1	β9'CGGG	$YFFS_{CCCG}$-L	200±7 (200)$^{(22)}$	1.3±.04	9

* EC_{50} values from THG73-UAA incorporation by nonsense suppression.

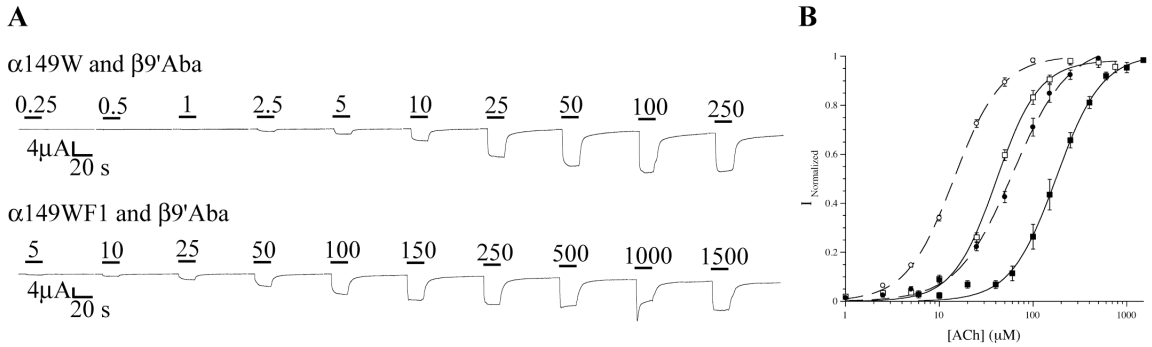

Figure 3.5: Simultaneous incorporation of two UAAs, representative traces and fits to the Hill equation. (A) Representative voltage-clamp current traces from oocytes expressing ion channels with two UAAs simultaneously incorporated. On the top α149UAG/THG73-W and β9'CGGG/$YFFS_{CCCG}$-Aba is shown, which has an EC_{50} of 14 μM ACh. The bottom shows α149UAG/THG73-WF1 and β9'CGGG/$YFFS_{CCCG}$-Aba suppression and represents the incorporation of two UAAs. The EC_{50} is 61 μM ACh and the ratio of the EC_{50}s (WF1:W) is 4.4. (B) Fits to the Hill equation from (left to right) show Row 1 (○), 2 (□), 4 (●), and 5 (■) (Table 3.7). Row 3 and 6 are left out for clarity and have previously been reported (Table 3.3 & (22)).

3.2.8 Incorporation of Three UAAs

To demonstrate the incorporation of three UAAs, we combined the two-UAA incorporation experiments described above, taking advantage of the knowledge that EC_{50} is lowered monotonically by appropriate 9' mutations at multiple subunits (26). Thus one

expects a lower EC_{50} when β9'Aba and δ9'Nval are incorporated simultaneously. Suppression of α149UAG:β9'CGGG:γ:δ9'GGGU using an mRNA ratio of 5:5:1:5 with THG73-W, $YFFS_{CCCG}$-Aba, and $YFaFS_{ACCC}$-Nval resulted in functional channel expression with an EC_{50} of 4.5 μM ACh (Figure 3.6), which is lower than either of the two UAAs (Aba or Nval) incorporated separately. However, the same conditions with THG73-WF1 yielded only small currents. In order to obtain more expression, α149UAG mRNA and THG73-WF1 were initially injected, and 24 hr later β9'CGGG:γ:δ9'GGGU (5:1:5) was injected with $YFFS_{CCCG}$-Aba and $YFaFS_{ACCC}$-Nval (final mRNA ratio 5:5:1:5). This resulted in adequate expression and an EC_{50} of 19 μM ACh (Figure 3.6). The ratio of the EC_{50}s (α149 WF1:W) is 4.2, confirming that three different UAAs were simultaneously incorporated *in vivo*, but this is actually four UAAs per ion channel because WF1 is incorporated in two α-subunits (Figure 3.3).

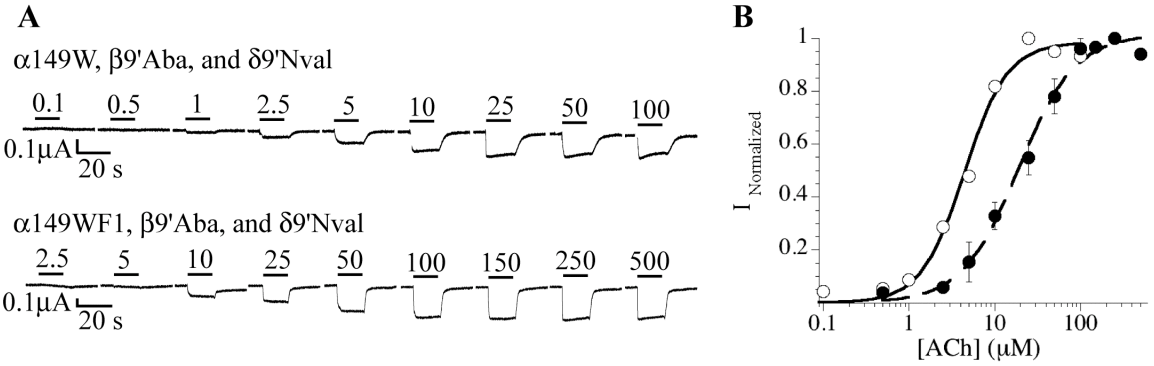

Figure 3.6: Simultaneous incorporation of three UAAs. (A) Representative current traces from oocytes incorporating three UAAs. (B) Dose-response curves showing: α149W, β9'Aba, and δ9'Nval (open circles) and α149WF1, β9'Aba, and δ9'Nval (closed circles). EC_{50} = 4.5±.4, n_H = 1.7±.3 and EC_{50} = 19±2, n_H = 1.3±.1, respectively. The ratio of the EC_{50}s is 4.2.

3.3 Discussion

The present results establish that frameshift suppression is viable in a eukaryotic, vertebrate cell, and that it can be used to incorporate mutiple unnatural amino acids (UAAs) in a single experiment. Previous work in *Xenopus* oocytes found that UAA incorporation using THG73FS$_{ACCC}$ was inefficient, and it was proposed that either the *Xenopus* translational machinery was not compatible with frameshift suppression or that THG73FS$_{ACCC}$ was a poor template for quadruplet recognition (9). Our results support the second rationalization, and a second FS derived from THG73, THG73FS$_{CCCG}$, is also not viable. Mutation of positions 32 and 38 on THG73FS$_{CCCG}$ did not rescue the suppression efficiency (Table 3.2). It thus appears that THG73-derived FS tRNAs are either misfolded, not recognized by EF-Tu, or not accepted by other components of the translational machinery.

Frameshift suppression is viable in the *Xenopus* oocyte, however, using either HSFS or YFFS tRNAs. We find that in *Xenopus* oocytes, the quadruplet GGGU is suppressed more efficiently by both HSFS$_{ACCC}$ and YFaFS$_{ACCC}$ than the corresponding CGGG/tRNA pairs. This is seen despite the fact that in *Xenopus* the GGG triplet is used twice as frequently (12.9 per thousand) as the CGG triplet (27). Frameshift suppression must compete with endogenous triplet-recognizing tRNAs. Codon usage is apparently not a perfect predictor of frameshift suppression efficiency.

We have evaluated three different tRNAs: the amber suppressor THG73, and the frameshift suppressors YFFS$_{CCCG}$ and YFaFS$_{ACCC}$. For UAA incorporation in the *Xenopus* oocyte, both YFFS tRNAs are less efficient than the AS THG73. This finding parallels results from earlier *in vitro* studies (17). Apparently, the competition between

release factors and the AS tRNA is less detrimental than the competition between FS tRNAs and endogenous, triplet-recognizing tRNA. This view is supported by the rapid, nonlinear rise in suppression efficiency when the amount of YFFS tRNA is increased (Table 3.5). CGGG-recognizing tRNAs are more sensitive to the amount injected than GGGU-recognizing tRNAs. Increasing the amount of FS tRNA for UAA incorporation is essential to maximize suppression efficiency.

The incorporation of UAAs site-specifically into proteins requires the suppressor tRNA to be orthogonal to the endogenous, aaRSs. Read-through of the suppression site or aminoacylation of the suppressor tRNA (once the chemically ligated UAA has been removed) can result in the undesired incorporation of natural amino acids at the suppression site. The two YFFS tRNAs studied here exhibit much more orthogonality than THG73 under the extreme conditions (extended incubation time and increased mRNA) used in Table 3.5. However, THG73 is an orthogonal suppressor tRNA to the *Xenopus* oocyte when used properly; THG73 has been used to successfully incoporate over 100 residues at scores of sites in 20 different proteins (1,2). Even promiscous sites, such as the β9'UAG, can be efficiently suppressed by THG73-UAA when using less tRNA, mRNA, and incubation time (14). β9'UAG injected with THG73-dCA shows no greater current than mRNA alone with similar conditions. The small current is less than 1% of typical UAA incorporation experiments and is caused by read-through of the UAG codon (18). Voss and coworkers found that THG73 incorporated 3 UAAs and Phe with efficiencies of 93.5–99.5% (determined by THG73-UAA incorporation relative to natural amino acids placed by read-through or aminoacylation of THG73-dCA) using luciferase expressed in *Xenopus* oocytes (9). The current results show that the YFFS tRNAs are

even more orthogonal and so the efficiency of UAA incorporation (relative to natural amino acids) should be greater than THG73.

An important contributor to our ability to efficiently incorporate two and three UAAs is the masking of undesired quadruplets to prevent loss of UAA. In general, the requirement for masking of mRNA to remove undesirable quadruplet codons does complicate the frameshift suppression approach. The only previous examples of UAA incorporation in higher eukaryotes were performed by nonsense suppression (1–5,10). Frameshift suppression may be limited *in vivo* to cells that are dormant (such as *Xenopus* oocytes), that express large quantities of the target mRNA, or that come from genetically engineered organisms. Also, suppressor tRNAs may be limited to rare codons, because of possible toxicity arising from undesired suppression in other proteins (28).

The combination of nonsense and frameshift suppression allows one to incoporate multiple UAAs site-specifically into proteins expressed in *Xenopus* oocytes. These methods are compatible with our entire library of UAAs (2,29) and will allow for multiple UAAs to be incorporated into other ion channels for novel structure-function studies, cross-linking, and FRET experiments. In principle, further quadruplet codons could be utilized to simultaneously incorporate more than three UAAs.

3.4 Experimental Methods

3.4.1 Materials

All oligonucleotides were synthesized by Caltech Biopolymer Synthesis facility or Integrated DNA Technologies (IDT, Coralville, IA) (Listed in Table 3.8). NotI was purchased from Roche (Indianapolis, IN). BamHI, EcoRI, FokI, T4 DNA ligase, and T4

RNA ligase were purchased from NEB (Beverly, MA). Kinase Max, T7 MEGAshortscript, and T7 mMessage mMachine kits were from Ambion (Austin, TX). dCA and NVOC-protected dCA-UAA were prepared as previously reported (14,22,30). ACh chloride was purchased from Sigma/Aldrich (St. Louis, MO).

Table 3.8: Oligos used in this research.

Masking	
α182CGC-F	GGAAGCTCGCGGCTGGAAGCACTGGG
α182CGC-R	CCCAGTGCTTCCAGCCGCGAGCTTCC
β23AGG-F	CGGTGAGGCCGGCGAGGGAGGTGGGAGACCGCG
β23AGG-R	CGCGGTCTCCCACCTCCCTCGCCGGCCTCACCG
β402AGG-F	CGATGGTCCAACCAGGGCTGTAGGTCTGCCTCAGG
β402AGG-R	CCTGAGGCAGACCTACAGCCCTGGTTGGACCATCG
δ195AGG-F	GGGAGATAGTGCATAGGGCAGCTAAGCTCAATGTGG
δ195AGG-R	CCACATTGAGCTTAGCTGCCCTATGCACTATCTCCC
β1AGC-F	CGCCCCAGGCGCCCGCGGGAGCGAAGCCGAAGGCC
β1AGC-R	GGCCTTCGGCTTCGCTCCCGCGGGCGCCTGGGGCG
Suppression	
α149TAG-F	GCAGCATGAAGCTGGGCACCTAGACCTATGACGGCTCTGTGG
α149TAG-R	CCACAGAGCCGTCATAGGTCTAGGTGCCCAGCTTCATGCTGC
α149CGGG-F	GCAGCATGAAGCTGGGCACCCGGGACCTATGACGGCTCTGTGGTGGCC
α149CGGG-R	GGCCACCACAGAGCCGTCATAGGTCCCGGGTGCCCAGCTTCATGCTGC
α149GGGT-F	GCAGCATGAAGCTGGGCACCGGGTACCTATGACGGCTCTGTGGTGGCC
α149GGGT-R	GGCCACCACAGAGCCGTCATAGGTACCCGGTGCCCAGCTTCATGCTGC
β9'TAG-F	GGGGCTCTCCATCTTTGCCCTGTAGACGCTCACTGTGTTCTTGCTGC
β9'TAG-R	GCAGCAAGAACACAGTGAGCGTCTACAGGGCAAAGATGGAGAGCCCC
β9'CGGG-F	GGGGCTCTCCATCTTTGCCCTGCGGGACGCTCACTGTGTTCTTGCTGCT GTTGGCCG
β9'CGGG-R	CGGCCAACAGCAGCAAGAACACAGTGAGCGTCCCGCAGGGCAAAGAT GGAGAGCCCC
β9'GGGT-F	GGGGCTCTCCATCTTTGCCCTGGGGTACGCTCACTGTGTTCTTGCTGCT GTTGGCCG
β9'GGGT-R	CGGCCAACAGCAGCAAGAACACAGTGAGCGTACCCCAGGGCAAAGAT GGAGAGCCCC
δ9'GGGT-F	CCGTGGCCATCTCAGTGCTCGGGTGCCCAATCTGTCTTCCTGCTGCTTA TCTCCAAGAGGC
δ9'GGGT-R	GCCTCTTGGAGATAAGCAGCAGGAAGACAGATTGGGCACCCGAGCACT GAGATGGCCACGG
tRNA Genes	
$HSFS_{CCCG}$-F	*AATTCGTAATACGACTCACTATA*GTAGTCGTGGCCGAGTGGTTAAGGCGA TGGACTCCCGAATCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGCC GACTACGCCA*TGAGACCCATCCG*
$HSFS_{CCCG}$-R	*GATCCGGATGGGTCTCA*TGGCGTAGTCGGCAGGATTCGAACCTGCGCGG GGAGACCCAATGGATTCGGGAGTCCATCGCCTTAACCACTCGGCCACG

	ACTAC*TATAGTGATGTATTACG*
THG73FS$_{CCCG}$-F	*AATTCGTAATACGACTCACTATA*GGTTCTATAGTATAGCGGTTAGTACTGG GGACTCCCGAATCCCTTGACCTGGGTTCGAATCCCAGTAGGACCGCCA*T GAGACCCATCCG*
THG73FS$_{CCCG}$-R	*GATCCGGATGGGTCTCA*TGGCGGTCCTACTGGGATTCGAACCCAGGTCA AGGGATTCGGGAGTCCCCAGTACTAACCGCTATACTATAGAACC*TATAG TGAGTCGTATTACG*
YFFS$_{CCCG}$-F	*AATTCGTAATACGACTCACTATA*GCGGATTTAGCTCAGTTGGGAGAGCGC CAGACTCCCGAATCTGGAGGTCCTGTGTTCGATCCACAGAATTCGCACC A*TGAGACCCATCCG*
YFFS$_{CCCG}$-R	*GATCCGGATGGGTCTCA*TGGTGCGAATTCTGTGGATCGAACACAGGACC TCCAGATTCGGGAGTCTGGCGCCTCTCCCAACTGAGCTAAATCCGC*TAT AGTGAGTCGTATTACG*
tRNA Primers	
YFG73FS$_{CCCG}$-F	CGATCCACAGAATTCGCGCCATGAGACCCATCCG
YFG73FS$_{CCCG}$-R	CGGATGGGTCTCATGGCGCGAATTCTGTGGATCG
YFa$_1$FS$_{CCCG}$-F	CGTAATACGACTCACTATAGGCCATTTAGCTCAGTTGGGAGAGCGCC
YFa$_1$FS$_{CCCG}$-R	GGCGCTCTCCCAACTGAGCTAAATGGCCTATAGTGAGTCGTATTACG
YFaFS$_{CCCG}$-F	CCTGTGTTCGATCCACAGAATGGCCGCCATGAGACCCATCCGGATCC
YFaFS$_{CCCG}$-R	GGATCCGGATGGGTCTCATGGCGGCCATTCTGTGGATCGAACACAGG
HSFS$_{ACCC}$-F	GGCCGAGTGGTTAAGGCGATGGACTACCCAATCCATTGGGGTCTCCCC GCGC
HSFS$_{ACCC}$-R	GCGCGGGGAGACCCCAATGGATTGGGTAGTCCATCGCCTTAACCACTC GGCC
YFaFS$_{ACCC}$-F	GCTCAGTTGGGAGAGCGCCAGACTACCCAATCTGGAGGTCCTGTGTTC GATCC
YFaFS$_{ACCC}$-R	GGATCGAACACAGGACCTCCAGATTGGGTAGTCTGGCGCTCTCCCAAC TGAGC

All mutated sites are underlined in the oligonucleotide sequence, except for tRNA genes where the underline is the anticodon and the flanking regions are italicized. F—Forward & R—Reverse (written 5' to 3').

3.4.2 Gene Construction and RNA Preparation

The α, β, γ, and δ subunits of the nicotinic acetylcholine receptor (nAChR) were previously subcloned in the pAMV vector (31). All four in-frame CGGG were mutated (underlined) to degenerate codons (α182CGC, β23AGG, β402AGG, and δ195AGG) and one GGGT was mutated at the fourth position (β1AGC), these are known as "masked" constructs. α149TAG, CGGG, GGGT; β9'TAG, CGGG, GGGT; and δ9'GGGT mutations were placed on masked constructs by QuikChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA). Mutations were verified by DNA sequencing (Caltech

Sequencing/Structure Analysis Facility (SAF)). Template DNA was linearized with NotI and mRNA prepared by T7 mMessage mMachine kit. mRNA was purified using RNeasy Mini kit (Qiagen, Valencia, CA) and quantified by absorption at 260 nm.

THG73 and HSAS in pUC19 vector were previously made (10,18). Genes for $HSFS_{CCCG}$, $THG73FS_{CCCG}$, and $YFFS_{CCCG}$ (sequence from (6)) with flanking EcoRI and BamHI overhangs were phosphorylated using Kinase Max kit, annealed, ligated with T4 DNA ligase into EcoRI and BamHI linearized pUC19 vectors, as previously described (30). A73G; C2G,G3C,G4C; and C69G,C70G,G71C mutations (from (7)) were sequentially placed by QuikChange mutagenesis on the $YFFS_{CCCG}$ construct to obtain $YFaFS_{CCCG}$ ("a" refers to acceptor stem mutations). $HSFS_{ACCC}$ and $YFaFS_{ACCC}$ (sequence from (7)) were prepared by replacing the anticodon of $HSFS_{CCCG}$ and $YFaFS_{CCCG}$ with ACCC using QuikChange. All mutations were verified by DNA sequencing (Caltech SAF). Template DNA for tRNA lacking the 3'CA was prepared by FokI digestion and tRNA was transcribed using T7 MEGAshortscript kit. tRNA was desalted using CHROMA SPIN™-30 DEPC-H_2O columns (BD Biosciences, San Jose, CA) and concentration was determined by absorption at 260 nm.

3.4.3 dCA and dCA-UAA Ligation to Suppressor tRNA

dCA and NVOC-protected dCA-UAA were coupled to suppressor tRNA using T4 RNA ligase for 30 min, as previously described (30,32), desalted using CHROMA SPIN™-30 DEPC-H_2O columns, and quantified by absorption at 260 nm. tRNA ligation efficiency was determined by MALDI mass spectrometry (32) and all tRNA dCA or dCA-UAA ligations were greater than 75%.

3.4.4 *In Vivo* Suppression Experiments

Stage VI oocytes of *Xenopus laevis* were prepared as previously described (33). All tRNA were refolded at 65 ˚C for 2 min and tRNA-UAA were deprotected for 5 min by UV irradiation prior to injection (18). Injection volume for all experiments was 50 nl and incubation time was 44–52 hr, unless otherwise noted. Suppression of HSAS and either HSFS 1.25 or 2.5 ng tRNA, with 20 ng mRNA in a subunit ratio 2:5:1:1 α:β9'(UAG, CGGG, or GGGU):γ:δ was recorded after 1 or 2 days. THG73-derived FS comparison was performed with 30 ng of mRNA in the subunit ratio 10:1:1:1 α149(UAG or CGGG):β:γ:δ and 16.5 ng of THG73-W, THG73FS_{CCCG}-W, THA32G73FS_{CCCG}-W, THG38G73FS_{CCCG}-W, or THA32U38G73FS_{CCCG}-W. Single UAA incorporation was performed using 20–30 ng of mRNA in a subunit ratio of 10:1:1:1 α149(CGGG or GGGU):β:γ:δ; 2:5:1:1 α:β9'GGGU:γ:δ; or 2:1:1:5 α:β:γ:δ9'GGGU with 4.8–16.5 ng of YFFS_{CCCG}/YFaFS_{ACCC}-UAA. Comparison of β masked and wild-type suppression contained 25 ng total mRNA injected in the subunit ratio listed in Table 3.4 with 1:1 γ:δ and 10 ng YFaFS_{ACCC}-W. For comparison of suppression efficiency and aminoacylation of tRNA *in vivo*, all mRNA was normalized to the same concentration and 16.5 ng of mRNA was injected in the subunit ratio 10:1:1:1 α149(UAG, CGGG, or GGGU):β:γ:δ or 2:5:1:1 α:β9'(UAG, CGGG, or GGGU):γ:δ with tRNA amounts listed in Table 3.5. For read-through experiments, 50 ng of mRNA in the ratio 2:5:1:1 α:β9'(UAG, CGGG, or GGGU):γ:δ was injected. Comparison of acceptor stem mutations on YFFS_{CCCG} was performed with 20 ng of mRNA in the subunit ratio of α:β9'(UAG or CGGG):γ:δ with 9.4 ng of THG73, YFFS_{CCCG}, YFG73FS_{CCCG}, and YFaFS_{CCCG} (not ligated to dCA). Two UAAs experiments were performed by injection of 20–30 ng mRNA in a subunit ratio

5:5:1:1 α149UAG:β9'CGGG:γ:δ or 5:1:1:5 α149UAG:β:γ:δ9'GGGU with 10–25 ng of each suppressor tRNA-UAA. For three UAAs—α149W, β9'Aba, δ9'Nval—26 ng mRNA in a ratio of 5:5:1:5 α149UAG:β9'CGGG:γ:δ9'GGGU was injected with 20 ng each suppressor tRNA-UAA, and a second injection of 33 ng each tRNA-UAA was done 24 h later. For α149WF1, β9'Aba, and δ9'Nval, 8 ng α149UAG mRNA with 50 ng of THG73-WF1 was injected, and a second injection of 18 ng mRNA with subunit ratio of 5:1:5 β9'CGGG:γ:δ9'GGGU with 25 ng of each $YFFS_{CCCG}$-Aba and $YFaFS_{ACCC}$-Nval was performed 24 h later. Oocytes were recorded three days after first injection.

3.4.5 Electrophysiology

Recordings used two-electrode voltage clamp on the OpusXpress 6000A (Axon Instruments, Union City, CA). ACh was stored at -20 ˚C as a 1 M stock, diluted in Ca^{2+}-free ND96, and delivered to oocytes by computer-controlled perfusion system. For HSAS & HSFS experiments the holding potential was –60 mV, and all UAA experiments were done at either –60 mV or –80 mV. Dose-response data were obtained from at least 9 ACh concentrations and comparisons were tested at one drug concentration, except β9'(UAG, CGGG, or GGGU) with tRNA-L used two concentrations, 10 μM and 1 mM, to check for aminoacylation (Table 3.6). Dose-response relations were fit to the Hill equation to determine EC_{50} and the Hill coefficient (η_H). All reported values are represented as a mean ± SE of the tested oocytes (number (n) listed with each table).

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