In vivo incorporation of multiple unnatural amino acids through nonsense and frameshift suppression

Erik A. Rodriguez*, Henry A. Lester†, and Dennis A. Dougherty**

Divisions of *Chemistry and Chemical Engineering and †Biology, California Institute of Technology, Pasadena, CA 91125

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Site-specific incorporation of unnatural amino acids (UAAs) into proteins is a valuable tool for studying structure–function relationships, incorporating biophysical probes, and elucidating protein–protein interactions. In higher eukaryotic cells, the methodology is currently limited to incorporation of a single UAA in response to a stop codon, which is known as nonsense suppression. Frameshift suppression is a unique methodology for incorporating UAAs in response to quadruplet codons, but currently, it is mostly limited to in vitro protein translation systems. Here, we evaluate the viability of frameshift suppression in Xenopus oocytes. We demonstrate UAA incorporation by using yeast phenylalanine frameshift suppressor (YFFS) tRNAs that recognize two different quadruplet codons (CGGG and GGGU) in vivo. Suppression efficiency of the YFFS tRNAs increases nonlinearly with the amount of injected tRNA, suggesting a significant competition with endogenous, triplet-recognizing tRNA. Both frameshift suppressor tRNAs are less efficient than the amber suppressor tRNA THG73 (Tetrahymena thermophila G73), which has been used extensively for UAA incorporation in Xenopus oocytes. However, the two YFFS tRNAs are more “orthogonal” to the Xenopus system than THG73, and they offer a viable replacement when suppressing at promiscuous sites. To illustrate the potential of combining nonsense and frameshift suppression, we have site-specifically incorporated two and three different UAAs simultaneously into a neuroreceptor expressed in vivo.

nicotinic receptor | tRNA | quadruplet codon | stop codon | protein engineering

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 that recognizes the stop codon. A wide range of in vitro translation systems has been used, along with expression in Escherichia coli and, to a lesser extent, yeast. Nonsense suppression in higher eukaryotes has, for the most part, been limited to the Xenopus oocyte, where microinjection of the required mRNA and aminoacyl tRNA is straightforward and electrophysiology provides a sensitive probe of UAA incorporation (1, 2). Other experiments in higher eukaryotes have relied on the evolution of a unique tRNA and a complementary aminoacyl-tRNA synthetase (aaRS) to insert a 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.

A remarkable variant of this approach is the use of quadruplet codons, a process that is termed frameshift suppression and was pioneered by Sisido and coworkers (6, 7). The success of this approach opens up the possibility of developing multiple additional codons, thus incorporating several different UAAs into a protein. This multiple incorporation, in turn, would enable the use of innovative biophysical approaches such as incorporating FRET pairs, structural probes such as unique 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. Frameshift suppression was used simultaneously with nonsense suppression to incorporate two UAAs in E. coli (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 four-base anticodons that can deliver UAAs in response to the quadruplet codons CGGG and GGGU.

When directly compared with an amber suppressor (AS) tRNA (THG73, Tetrahymena thermophila G73) that has been used extensively in Xenopus oocytes, the FS tRNAs are less efficient at delivering UAs. 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.

Results

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 AS (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 FSs (HSFSCCCG and HSFSACC) (cloverleaf structures shown in Fig. 1A), which recognize the quadruplet codons CGGG and GGGU. Prior research showed that these four-base codons are efficient in vitro (7). Injection of wild-type muscle nicotinic acetylcholine receptor (nACHR) mRNA and either HSFSACC or HSFSCCCG (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 AS HSAS with wild-type nACHR mRNA did show channel expression with 2.5 ng of 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, which were mutated to degenerate codons (see Materials and Methods) to avoid sup-

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Abbreviations: UAA, unnatural amino acid; aaRS, aminoacyl-tRNA synthetase; AS, amber suppressor; FS, frameshift suppressor; HSAS, human serine AS; HSFS, human serine FS; YFFS, yeast phenylalanine FS; WF1, 5-fluoro-tryptophan; Alba, α-amino butyric acid; Nval, norvaline; ACh, acetylcholine; nACHR, nicotinic ACh receptor; dCA, dcinucleotide deoxyCA.

*To whom correspondence should be addressed. E-mail: dodougherty@caltech.edu.

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pression. 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 EC50 as channels expressed without tRNA (data not shown). Unless otherwise noted, all subsequent experiments used such masked constructs.

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 Leu-9/H11032. This site 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 EC50. In particular, prior research showed that a leucine-to-serine mutation in the H9252/H92529/H11032 site resulted in a 33-fold increased sensitivity to acetylcholine (ACh) (14). This 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 EC50 values varied depending on the incubation time (Table 1). This finding suggested that natural amino acids other than serine were being placed at the H9252/H92529/H11032 site with 2-day incubations, because the conventional mutant, H9252/H92529/H11032 Ser, shows no change in EC50 (Table 1). The variability in EC50 between 1- and 2-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 1- to 2-day incubation times (15). Thus, we avoid this complication by incubation for 1 day. Amber suppression is highly efficient when the average maximal peak current (I\text{max}) is measured at 1.25 ng of tRNA per oocyte and decreases slightly when 2.5 ng is added (Table 2). CGGG shows lower suppression than GGGU, in agreement with previous in vitro studies (7, 16). CGGG suppression is highly nonlinear, with a 330% increase in current when twice as much tRNA is injected (Table 2). GGGU, however, shows an almost linear relationship, with an increase of 86% in response to doubling (Table 2). These data suggest that HSFS\textsubscript{ACCC} is a more efficient tRNA at recognizing its cognate quadruplet codon and has less competition with endogenous triplet tRNA in Xenopus oocytes than HSFS\textsubscript{CCCG}. These experiments establish that frameshift suppression is viable in Xenopus oocytes and that UAA incorporation should be feasible when using the appropriate FS tRNA.

UAA Incorporation by Frameshift Suppression. THG73 is an AS tRNA (cloverleaf structure shown in Fig. 1A) (17) that is used extensively for incorporating UAAs into various ion channels expressed in Xenopus oocytes (2). Initially, a FS derived from THG73 that recognizes the quadruplet codon CGGG (THG73\textsubscript{FSCCG}) was tested for UAA incorporation. Attempts to suppress H9252/H92529/H11032 CGGG with THG73\textsubscript{FSCCG}-L, where L was chemically aminoacylated onto the tRNA, showed no current \textit{in vivo}. This result is consistent with data from Voss and coworkers (9), who saw

### Table 1. HSAS and HSFS suppression experiments at the β'9 site

| β'9 X | tRNA, 2.5 ng | EC50, 1 day* | nH | n | EC50, 2 days* | nH | n |
|-------|-------------|-------------|----|---|-------------|----|---|
| AGC (serine) | none | 1.5 ± 0.04 | 1.7 ± 0.07 | 5 | 1.5 ± 0.2 | 1.9 ± 0.3 | 3 |
| UAG | HSAS | 1.7 ± 0.06 | 1.7 ± 0.09 | 6 | 0.70 ± 0.008 | 1.9 ± 0.07 | 14 |
| CGGG | HSFS\textsubscript{CCCG} | 2.1 ± 0.09 | 1.7 ± 0.1 | 8 | 1.3 ± 0.1 | 1.9 ± 0.3 | 13 |
| GGGU | HSFS\textsubscript{ACCC} | 1.9 ± 0.08 | 1.5 ± 0.08 | 9 | 0.68 ± 0.1 | 1.7 ± 0.04 | 5 |

*Incubation time.

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Fig. 1. tRNAs and UAAs. (A) The AS tRNAs are shown, with the CUA anticodon and the FS anticodons shown at the bottom. YFaFS tRNA acceptor stem mutations are shown next to the YFFS tRNA body (italicized). (B) The three UAAs used in this study.
We studied both YFFSCCCG and YFaFSACCC (yeast phenyalanine oocytes. YFaFSACCC-W) resulted in functional, wild-type channels (Table 1). We chose because there was only one in-frame quadrula- truncation caused by frameshift suppression. ACh, directly showing a lack of aminoacylation by endogenous Xenopus aarSSs.

Table 2. HSAS and HSFS suppression experiments at the β9’ site (1-day incubation)

| β9’ X | tRNA | Imax (nA, μM ACh) | n | % HSAS, 1.25 | % HSAS, 2.5 |
|-------|------|-------------------|---|-------------|-------------|
| UAG   | HSAS | 19 ± 2            | 12 | 14 ± 3      | 11           |
| CCGG  | HSFSACCC | 1.3 ± 0.3       | 10 | 5.6 ± 1     | 12           |
| GGUG  | HSFSACCC | 8.6 ± 3         | 10 | 16 ± 3     | 12           |

*Average Imax (in μA) recorded at 50 μM ACh.
†Nanograms of tRNA.
‡Between 1.25 and 2.5 ng.


due little UAA incorporation with THG73FSACCC in Xenopus oocytes. We then chose to screen yeast phenyalanine FS (YFFS) tRNA, which was used successfully by Sisido and colleagues (7, 16) in vitro. We studied both YFFSACCC and YFaFSACCC (yeast phenyalanine containing acceptor stem mutations FS); Fig. 1A shows cloverleaf structures. The latter accepts acceptor stem mutations (denoted by the “a”) incorporated to reduce glycyl-tRNA synthetase recogni- tion (-H11032 GGGU YFaFS ACCC-Nval 31 -H9251 -H9254 > 8652 §Ref. 18. †Rescue of wild-type recovery by frameshift suppression. Table 3. Wild-type recovery and UAA incorporation by frameshift suppression in vivo

| mRNA          | tRNA            | EC50 (theoretical)* | nI  | nNA |
|---------------|-----------------|---------------------|-----|-----|
| α149UGG       | YFFSACCC-W      | 56 ± 2 (50)         | 1.8 ± 0.7 | 8   |
| α149GGGU      | YFaFSACCC-W     | 53 ± 2 (50)         | 1.6 ± 0.03 | 8   |
| β9’ UAG       | THG73-W         | 16 ± 0.9 (16)       | 1.3 ± 0.08 | 7   |
| β9’ CCGG      | YFFSACCC-L1     | 31 ± 2 (36)         | 1.6 ± 0.1 | 6   |
| α149CGGG      | YFFSACCC-W      | 50 ± 3 (200)        | 1.6 ± 0.03 | 10  |

*EC50 values from THG73- UAA incorporation by nonsense suppression.
†Rescue of wild-type recovery by frameshift suppression.
‡Reference 14.
§Reference 18.

wild-type or masked β mRNA to the injection mixture. Table 6, which is published as supporting information on the PNAS web site, shows the dramatic effect of masking one position on frameshift suppression. With a 1:1:1:1 ratio of αβγδ8, the masked construct gives a 2.7-fold increase in Imax relative to wild type. As the amount of a-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 6), suggesting that the α149GGGU and the GGGU present in the β-subunit have similar suppression efficiencies.

Comparison of Frameshift and Nonsense Suppression Efficiencies. To compare frameshift and nonsense suppression, the α149 and β9’ sites were studied in more detail. Suppression of α149GGGU or GGUG with 10 ng of YFFSACCC-W or YFaFSACCC-W resulted in 38% and 48%, respectively, of the current from 10 ng of THG73-W resulted at α149UGG (Table 4). Suppression of β9’ UAG with 2 ng of THG73-L resulted in the largest Imax (Table 4). Suppression at β9’ CCGG or GGUG with 2 ng of YFFSACCC-L or YFaFSACCC-L resulted in 14% and 36%, respectively, of the current from THG73-L (Table 4). 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 (16). In particular, the suppression efficiency observed here follows the order: THG73 > YFaFSACCC > YFFSACCC.

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 YFFSACCC-L or

Table 4. Comparison of suppression efficiency, aminoacylation, and read-through in vivo

| mRNA          | tRNA            | ng | Imax (nA, μM ACh) | % THG73 or % UAG |
|---------------|-----------------|----|-------------------|------------------|
| α149UGG       | THG73-W         | 10 | 18                | -4.8 ± 2         | 100 |
| α149GGGU      | YFFSACCC-W      | 10 | 13                | -2.3 ± 0.9       | 48 |
| β9’ UAG       | THG73-L         | 2  | 15                | -6.1 ± 2         | 100 |
| β9’ CCGG      | YFFSACCC-L1     | 2  | 12                | -0.84 ± 0.2      | 14 |
| β9’ GGUG      | YFaFSACCC-L1    | 2  | 9                 | -2.2 ± 0.5       | 36 |
| β9’ CCGG      | YFFSACCC-L1     | 6  | 13                | -8.8 ± 0.9       | NA |
| β9’ GGUG      | YFaFSACCC-L1    | 6  | 13                | -16.2            | NA |
| β9’ UAG       | THG73-dCA       | 2  | 12                | -4.8 ± 1         | 100 |
| β9’ CCGG      | YFFSACCC-dCA    | 2  | 13                | -0.42 ± 0.8      | 8.8 |
| β9’ GGUG      | YFaFSACCC-dCA   | 2  | 13                | -0.092 ± 0.02    | 1.9 |
| β9’ UAG       | THG73-dCA       | 6  | 13                | -8.2 ± 1         | 100 |
| β9’ CCGG      | YFFSACCC-dCA    | 6  | 12                | -1.2 ± 0.3       | 15 |
| β9’ GGUG      | YFaFSACCC-dCA   | 6  | 11                | -0.27 ± 0.09     | 3.3 |
| β9’ UAG       | —                | 13 | 0.37 ± 0.1        | 100 |
| β9’ CCGG      | —                | 13 | -0.085 ± 0.03     | 23 |
| β9’ GGUG      | —                | 13 | 0.078 ± 0.02      | 22 |

NA, not applicable; —, no tRNA.
*Average Imax (in μA) recorded at 1 mM ACh.
†THG73.
‡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.
§UAG.

Rodriguez et al.
presumably because there is only one in-frame stop codon before
used. Surprisingly, THG73-dCA, which has been used extensively
closely mimic the biologically active, full-length tRNA. To maxi-
did not contain an amino acid at the 3′ receptors when substituted at this position (14). In all experiments,
by aaRSs, as evidenced by the decrease in
(17). Both FS tRNAs show much lower amounts of aminoacylation
when nonaminoacylated THG73 is injected, as noted previously
sired. No aminoacylation was seen with 2 ng of THG73-L, sug-
THG73 is susceptible to aminoacylation by aaRSs, which is unde-
conditions (less mRNA and shorter incubations), previous work has
6 ng of tRNA, respectively (Table 4). Note that under other
aminoacylation by aaRSs poses a problem
therefore offers a viable replacement for THG73, especially when
change of 950% and 630%, respectively (Table 4). This large change
mRNA only (Table 4).

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,
trRNAs that had been ligated to dinucleotide deoxyCA (dCA) but
did not contain an amino acid at the 3′ end were injected to more
closely mimic the biologically active, full-length tRNA. To maxi-
mize the potential for aminoacylation by endogenous aaRSs, 2-day
incubations and relatively large mRNA injections (16.5 ng) were
used. Surprisingly, THG73-dCA, which has been used extensively
for UAA incorporation in Xenopus oocytes, showed significant
aminoacylation in vivo, with an I\textsubscript{max} of −4.8 and −8.2 μA for 2 and
6 ng of tRNA, respectively (Table 4). Note that under other
conditions (less mRNA and shorter incubations), previous work has
found no complications from aminoacylation using THG73-dCA in
Xenopus oocytes (9, 14, 17). Still, the present results establish that
THG73 is susceptible to aminoacylation by aaRSs, which is undes-
sired. No aminoacylation was seen with 2 ng of THG73-L, sug-
gesting that aminoacylation by endogenous aaRSs is more likely
when nonaminoacylated THG73 is injected, as noted previously
(17). Both FS tRNAs show much lower amounts of aminoacylation
by aaRSs, as evidenced by the decrease in I\textsubscript{max} (Table 4).
YFFS\textsubscript{CCCG}dCA shows only 8.8% and 15% of the I\textsubscript{max} of THG73-
dCA at 2 and 6 ng, respectively. The most orthogonal suppressor is
YFaFS\textsubscript{ACCC}dCA, with 1.9% and 3.3% of the I\textsubscript{max} of THG73-dCA
at 2 and 6 ng, respectively. The orthogonality trend thus follows the
order: YFaFS\textsubscript{ACCC}dCA > YFFS\textsubscript{CCCG}dCA > THG73-dCA.
YFaFS\textsubscript{ACCC}dCA 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 4), 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, respectively, relative to the UAG stop codon.
This finding 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.
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′.

Incorporation of Two UAAs. To investigate the simultaneous incor-
poration of two UAAs, we again built on previous work that used
THG73 to incorporate UAAs into the nAChR at positions α149, β9′,
and β9′. Importantly, EC\textsubscript{50} changes associated with mutations
at these sites are independent of one another (18), (19), allowing one
to qualitatively anticipate the consequences of multiple mutations.
In particular, both β9′ Aba and β9′ Nval produce predictable reduc-
tions in EC\textsubscript{50} that should be reproduced when combined with
mutations at α149 (14). That is, the previously noted 4-fold increase
in EC\textsubscript{50} that is seen when the native tryptophan at α149 is replaced
by WF1 should persist when in combination with β9′ Aba or
β9′ 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\textsubscript{CCCG} is a wild-type recovery experiment that
gave the expected EC\textsubscript{50} for ACh of 50 μM (Table 5). Maintaining β9′ CGGG/YFFS\textsubscript{CCCG} but substituting
α149UAG/THG73-WF1 resulted in the anticipated 4-fold increase
in EC\textsubscript{50} (Table 5) (18). For incorporation of two UAAs,
α149UAG/THG73-W or WF1 was combined with either
β9′ CGGG/YFFS\textsubscript{CCCG}-Aba or β9′ GGGU/YFaFS\textsubscript{ACCC}-Nval (Table
5 and Fig. 2; representative traces are shown in Fig. 4, which is
published as supporting information on the PNAS web site). The
α149 WF1:WC50 ratios are 4.4 for the β9′ and β9′ mutants. These
experiments establish that frameshift suppression can be combined
with nonsense suppression to incorporate two UAAs in a eukary-
ocytic system.

Incorporation of Three UAAs. To demonstrate the incorporation
of three UAAs, we combined the two-UAA incorporation experi-
ments described above, taking advantage of the knowledge that

Table 5. Incorporation of two UAAs

| α149 | tRNA   | β or δ | tRNA   | EC\textsubscript{50} (theoretical)\textsuperscript{a} | n\textsubscript{H} | n |
|------|--------|--------|--------|-----------------------------------------------|-----------------|---|
| UAG  | THG73-W | β9′ CGGG | YFFS\textsubscript{CCCG}-Aba | 14 ± 0.4 (16)\textsuperscript{b} | 1.7 ± 0.06 | 9 |
| UAG  | THG73-W | β9′ CGGG | YFFS\textsubscript{CCCG}-Nval | 41 ± 2 (36)\textsuperscript{b} | 1.9 ± 0.1 | 9 |
| UAG  | THG73-W | β9′ CGGG | YFFS\textsubscript{CCCG}-L | 50 ± 3 (50)\textsuperscript{b} | 1.4 ± 0.08 | 20 |
| UAG  | THG73-WF1 | β9′ CGGG | YFFS\textsubscript{CCCG}-Aba | 61 ± 3 | 1.5 ± 0.08 | 7 |
| UAG  | THG73-WF1 | β9′ CGGG | YFFS\textsubscript{CCCG}-Nval | 180 ± 7 | 1.8 ± 0.1 | 6 |
| UAG  | THG73-WF1 | β9′ CGGG | YFFS\textsubscript{CCCG}-L | 200 ± 7 (200)\textsuperscript{b} | 1.3 ± 0.04 | 9 |

\textsuperscript{a}EC\textsubscript{50} values from THG73-UAA incorporation by nonsense suppression.

\textsuperscript{b}Ref. 14.

\textsuperscript{c}Ref. 18.

Fig. 2. Fits to the Hill equation (for incorporation of two UAAs). Data
correspond to the rows in Table 5 as follows: row 1, open circles; row 2, open squares; row 4, filled circles; row 5, filled squares. Rows 3 and 6 are left out for
clarity and have previously been reported (Table 3 and ref. 18).
EC\textsubscript{50} is lowered monotonically by appropriate 9\textsuperscript{th} mutations at multiple subsites (20). Thus, one expects a lower EC\textsubscript{50} when 9\textsuperscript{th} Aba and 89\textsuperscript{th} Nval are incorporated simultaneously. Suppression of \(a\text{149UAG;9}\text{\textsuperscript{th}CGGG;9}\text{\textsuperscript{th}GGGU}\) by using an mRNA ratio of 5:5:1:15 with THG73-W, YFFSCCCG-Aba, and YFaFSACC-Nval resulted in functional channel expression with an EC\textsubscript{50} of 4.5 \(\mu\text{M ACh}\) (Fig. 3), 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. To obtain more expression, \(a\text{149UAG mRNA and THG73-WF1 were initially injected, and, 24 h later, 9\text{\textsuperscript{th}CGGG;9}\text{\textsuperscript{th}GGGU (5:1:5)}\) was injected with YFFSCCCG-Aba and YFaFSACC-Nval (final mRNA ratio of 5:5:1:5). This strategy resulted in adequate expression and an EC\textsubscript{50} of 19 \(\mu\text{M ACh}\) (Fig. 3). The ratio of the EC\textsubscript{50} (\(a\text{149WF1;N}\)) is 4.2, confirming that three UAAs were simultaneously incorporated in vivo.

**Discussion**

The present results establish that frameshift suppression is viable in a eukaryotic, vertebrate cell and that it can be used to incorporate multiple UAAs in a single experiment. Previous work in *Xenopus* oocytes found that UAAs incorporation using THG73FSACC was inefficient, and it was proposed that either the *Xenopus* translational machinery was not compatible with frameshift suppression or that THG73FSACC was a poor template for quadruplet recognition (9). Our results support the second rationalization, and a second FS derived from THG73, THG73FSCCC, was also not viable. It thus appears that THG73-derived FS tRNAs are misfolded, not recognized by the elongation factor EF-Tu, or not accepted by other components of the translational machinery.

However, frameshift suppression is viable in the *Xenopus* oocyte by using either HSFS or YFFS tRNAs. We find that, in *Xenopus* oocytes, the quadruplet GGGU is suppressed more efficiently by both HSFSACC and YFaFSACC than the corresponding CGGG/tRNA pairs. This difference is seen even when the fact in *Xenopus*, the GGG triplet is used twice as frequently (12.9 per 1,000) as the CGG triplet (21). 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: AS THG73 and the FsFs YFFSCCC and YFaFSACC. For UAAs incorporation in the *Xenopus* oocyte, both YFFS tRNAs are less efficient than the AS THG73. This finding parallels results from earlier in vitro studies (16). Apparently, the competition between release factors and the AS mRNA 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 4). CGGG-recognizing tRNAs are more sensitive to the amount injected than GGGU-recognizing tRNAs. Increasing the amount of FS tRNA for UAAs 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 UAAs have 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 4. However, THG73 is an orthogonal suppressor tRNA to the *Xenopus* oocyte when used properly; THG73 has been used to successfully incorporate >100 residues at scores of sites in 20 different proteins (1, 2). Even promiscuous sites, such as the 9\textsuperscript{th} UAG, can be efficiently suppressed by THG73- UAA when using less tRNA, mRNA, and incubation time (14). 9\textsuperscript{th} UAG injected with THG73-dCA shows no greater current than mRNA alone with similar conditions. The small current is <1% of typical UAAs incorporation experiments and is caused by read-through of the UAG codon (17). Voss and coworkers (9) found that THG73 incorporated three 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. 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 with THG73.

An important contributor to our ability to efficiently incorporate two and three UAAs is the masking of undesired quadruplets to prevent loss of UAAs. In general, the requirement for masking of mRNA to remove undesirable quadruplet codons does complicate the frameshift suppression approach. The only previous examples of UAAs 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), express large quantities of the target mRNA, or 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 (22).

The combination of nonsense and frameshift suppression allows one to incorporate multiple UAAs site-specifically into proteins expressed in *Xenopus* oocytes. These methods are compatible with our entire library of UAAs (2, 23) and will allow for multiple UAAs to be incorporated into other ion channels for structure–function studies, cross-linking, and FRET experiments. In principle, further quadruplet codons could be used to simultaneously incorporate more than three UAAs.

**Materials and Methods**

**Materials.** All oligonucleotides were synthesized by the California Institute of Technology Biopolymer Synthesis facility or Integrated DNA Technologies (Coralville, IA) (sequences are listed in Table 7, which is published as supporting information on the PNAS web site). NotI was purchased from Roche Applied Science (Indianapolis). 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 6-nitroveratrylloxycarbonyl-protected dCA-UAA were prepared as reported in refs. 14, 18, and 24. ACh chloride was purchased from Sigma-Aldrich.

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Table 4. Dose–response curves are as follows: \(a\text{149W,9} \text{\textsuperscript{th}Aba, and 89} \text{\textsuperscript{th}Nval}\), open circles; \(a\text{149WF1,9} \text{\textsuperscript{th}Aba, and 89} \text{\textsuperscript{th}Nval}\), filled circles. EC\textsubscript{50} is 4.5 ± 0.4 and \(n\text{\textsubscript{m}} = 1.7 ± 0.3\), and EC\textsubscript{50} = 19 ± 2 and \(n\text{\textsubscript{m}} = 1.3 ± 0.1\), respectively. The ratio of the EC\textsubscript{50} is 4.2.
Gene Construction and RNA Preparation. The α-, β-, γ-, and δ-subunits of nAChR were previously subcloned in the pAMV vector (25). All four in-frame CGGGs were mutated (shown in italics) to degenerate codons (α 182CCG; β 234AGG, β 402AGG, and 6195AGG), and the GGGT was replaced at the fourth position (β 1AGC); these constructs are termed “masked.” α 19TAG, CGGG, GGGT; β 1TAG, CGGG, GGGT; and ɣ 1GGGT mutations were placed on masked constructs by QuikChange site-directed mutagenesis (Stratagene). Mutations were verified by DNA sequencing (at the California Institute of Technology Sequencing/Structure Analysis Facility). Template DNA was linearized with NotI and mRNA prepared with the T7 mMESSAGE mMachine kit. mRNA was purified by using the RNeasy Mini kit (Qiagen, Valencia, CA) and quantified by absorption at 260 nm.

In Vivo Suppression Experiments. Stage VI oocytes of Xenopus laevis were prepared as described in ref. 27. All mRNA were refolded at 65°C for 2 min, and tRNA-UAA were deprotected for 5 min by UV irradiation before injection (17). The injection volume for all experiments was 50 nl, and the incubation time was 44–52 h unless otherwise noted. Suppression of HSAS and HSFS (1.25 or 2.5 ng of each tRNA) with 20 ng of mRNA in a subunit ratio of 2:5:1:1 for α 19UAG (UAG, CGGG, or GGUG) was determined by MALDI mass spectrometry (26), and a second injection of 33 ng of each mRNA was observed in the subunit ratio 10:1:1:1 for α 19UAG (UAG, CGGG, or GGUG) with 10–25 ng of each suppressor tRNA-UAA. For three UAAs, α 149WF1, β 19Ab, and ɣ 19Val, 26 ng of mRNA in a ratio of 5:5:1:1 for α 149WF1 (UAG, CGGG, or GGUG) was injected with 20 ng of each suppressor RNA-UAA, and a second injection of 33 ng of each RNA-UAA was done 24 h later. For α 149WF1, β 19Ab, and ɣ 19Val, 8 ng of α 149UAG mRNA with 50 ng of THG73-WF1 was injected, and a second injection of 18 ng of mRNA with a subunit ratio of 5:1:2 for β 19GGG mRNA (with 25 ng of each YFFSCCCG-Ab and YFASCCCG-Nval was performed 24 h later; oocytes were recorded 3 days after the first injection.

Electrophysiology. Recordings employed 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 Ca2+-free ND96, and delivered to oocytes by computer-controlled perfusion system. For HSAS and HSFS experiments, the holding potential was −60 mV, and all UAA experiments were performed at either −60 or −80 mV. Dose–response data were obtained from at least nine ACh concentrations, and comparisons were tested at one drug concentration, except β 19UAG, CGGG, or GGUG) with tRNA-L, for which two concentrations, 10 μM and 1 mM, were used to check for aminoacylation (Table 4). Dose–response relations were fit to the Hill equation to determine EC50 and the Hill coefficient (nH). All reported values are represented as a mean ± SE of the tested oocytes [number (n) is listed with each table].

Supporting Information. Masking experiments, representative traces for two UAA experiments (α 149WF1 or WF1 and β 19Ab), and oligonucleotides used in this study are detailed in Fig. 4 and Tables 6 and 7.

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