[28] *In Vivo* Incorporation of Unnatural Amino Acids into Ion Channels in *Xenopus* Oocyte Expression System

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Introduction

We have adapted the nonsense codon suppression method[1] for the incorporation of unnatural amino acids into membrane proteins in a *Xenopus* oocyte expression system. Combining this method with electrophysiologic analysis allows us to probe structure–function relationships in ion channels and receptors in ways not possible with conventional mutagenesis.[2–4] In the absence of atomic-scale structural data for membrane proteins, these techniques can provide detailed structural information.

In the nonsense codon suppression method, *Xenopus* oocytes are coinjected with two RNAs: (1) mRNA transcribed *in vitro* from a mutated cDNA containing a TAG nonsense (stop) codon at the position of interest and (2) a suppressor tRNA containing the corresponding anticodon, CUA, and chemically acylated at the 3′ end with an amino acid. During protein synthesis, the aminoacylated suppressor tRNA directs the incorporation of the amino acid into the desired position of the protein (Fig. 1). Because the amino acid is appended synthetically, we are not limited to the natural 20, and many unnatural amino acids have been incorporated into various proteins using this method.

This article describes suppressor tRNA design and synthesis, chemical acylation of the suppressor tRNA, relevant organic synthesis methods, and optimization of mRNA and suppressor tRNA for *Xenopus* oocyte expression.

[1] C. J. Noren, S. J. Anthony-Cahill, M. C. Griffith, and P. G. Schultz, *Science* **244,** 182 (1989).

[2] M. W. Nowak, P. C. Kearney, J. R. Sampson, M. E. Saks, C. G. Labarca, S. K. Silverman, W. Zhong, J. Thorson, J. N. Abelson, N. Davidson, P. G. Schultz, D. A. Dougherty, and H. A. Lester, *Science* **268,** 439 (1995).

[3] P. C. Kearney, M. W. Nowak, W. Zhong, S. K. Silverman, H. A. Lester, and D. A. Dougherty, *Mol. Pharmacol.* **50,** 1401 (1996).

[4] P. C. Kearney, H. Zhang, W. Zhong, D. A. Dougherty, and H. A. Lester, *Neuron* **17,** 1221 (1996).

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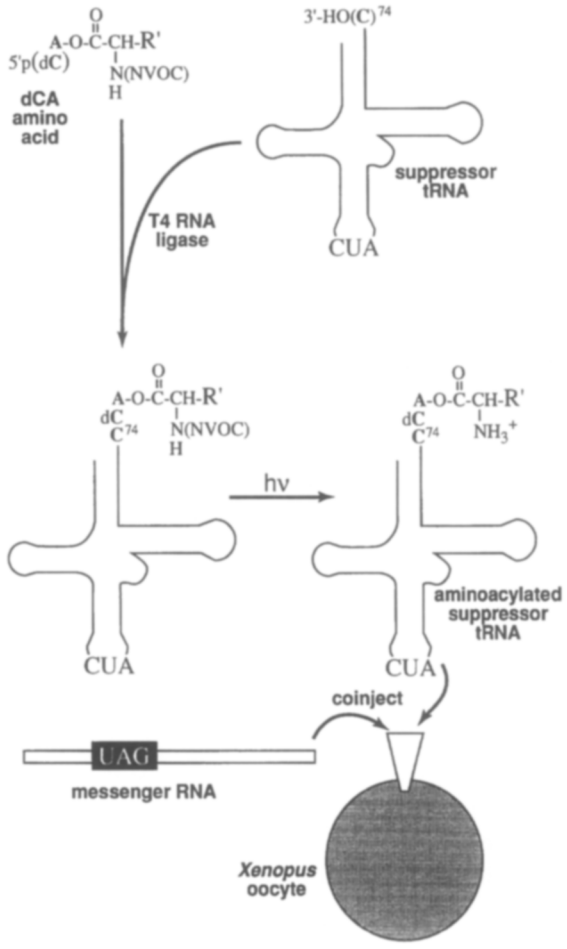


FIG. 1. Scheme for incorporating unnatural amino acids into proteins expressed in *Xenopus* oocytes. (Reprinted with permission from Saks *et al., J. Biol. Chem.* **271,** 23169 (1996).)

Materials

DNA oligonucleotides are synthesized on an Expedite DNA Synthesizer (Perceptive Biosystems, Framingham, MA.). *Fok*I, *Bsa*I, and other restriction endonucleases and T4 RNA ligase are purchased from New England Biolabs (Beverly, MA). T4 polynucleotide kinase, T4 DNA ligase, and RNase inhibitor are purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). [^{35}S]Methionine and ^{14}C-labeled protein molecular weight markers are purchased from Amersham (Arlington Heights, IL).

Inorganic pyrophosphatase is purchased from Sigma (St. Louis, MO). Stains-all is purchased from Aldrich (Milwaukee, WI). T7 RNA polymerase is either purified using the method of Grodberg and Dunn[5] from the overproducing strain *Escherichia coli* BL21 harboring the plasmid pAR1219[6] or purchased from Ambion (Austin, TX). For all buffers described, unless otherwise noted, final adjustment of pH is unnecessary.

Suppressor tRNA Design and Synthesis

Designing a suppressor tRNA presents two major challenges: (1) one must maximize efficiency of the suppressor tRNA at incorporating an unnatural amino acid at the site of interest; and (2) one must prevent editing and/or reacylation of the suppressor tRNA by endogenous tRNA synthetases. Low suppression efficiency may often be overcome by general tactics for overexpressing proteins in the oocyte, because minute signals may easily be detected. In contrast, if reacylation occurs, this must be addressed before usable data can be obtained, because the uncontrolled mixture of amino acids at the site of interest would complicate data interpretation.

Our first suppressor tRNA, tRNA-MN3, was a modification of a yeast $tRNA^{Phe}$(CUA) used previously to incorporate unnatural amino acids in an *in vitro* translation system.[1] The tRNA-MN3 was adequate with respect to efficiency and editing/reacylation in our initial study of conserved tyrosine residues in the putative agonist-binding site of the nicotinic acetylcholine receptor (nAChR).[2] However, we discovered that tRNA-MN3 did not constitute an optimal solution to the *in vivo* suppression problem.[7] By studying positions in the nAChR α subunit that were less conserved and more tolerant with regard to substitution, we found that tRNA-MN3 led to incorporation of *natural* amino acids at the mutation site along with the desired unnatural amino acid. This was probably the result of reacylation and/or editing of the chemically acylated tRNA-MN3 by synthetases endogenous to the *Xenopus* oocyte.

To reduce reacylation and to improve nonsense codon suppression efficiency, we designed a new nonsense suppressor tRNA based on $tRNA^{Gln}$(CUA) from the eukaryote *Tetrahymena thermophila.* In *T. thermophila,* the UAG codon does not signal the termination of protein synthe-

[5] J. Grodberg and J. J. Dunn, *J. Bact.* **170,** 1245 (1988).

[6] P. Davanloo, A. H. Rosenberg, J. J. Dunn, and F. W. Studier, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2035 (1984).

[7] M. E. Saks, J. R. Sampson, M. W. Nowak, P. C. Kearney, F. Du, J. N. Abelson, H. A. Lester, and D. A. Dougherty, *J. Biol. Chem.* **271,** 23169 (1996).

sis but instead codes for the amino acid glutamine. Previous *in vitro* translation experiments showed that when wheat germ[8] or rabbit reticulocyte[9] systems were supplemented with a *T. thermophila* synthetase preparation, $tRNA^{Gln}(CUA)$ efficiently translated normal UAG stop codons in a variety of heterologous mRNAs.

Based on principles derived from studies of translation efficiency and tRNA recognition,[10–12] we modified *T. thermophila* $tRNA^{Gln}(CUA)$ to produce a new suppressor tRNA.[7] We mutated U73 to G to reduce recognition by the oocyte's endogenous glutamine acyltransferase. The T7 RNA promoter is included directly upstream from the first nucleotide of the tRNA gene. To generate a full-length tRNA transcript, or a transcript missing the last two nucleotides (for reasons described later), *Bsa*I and *Fok*I sites are engineered into the 3′ end of the gene (Fig. 2).

T. thermophila $tRNA^{Gln}(CUA)$ G73 Gene Construction

Buffers and Solutions

10× Phosphorylation Buffer

Volume	Reagent	Final concentration
7 ml	1 *M* Tris-Cl, pH 7.6	700 m*M*
0.5 ml	1 *M* Dithiothreitol (DTT)	50 m*M*
1 ml	1 *M* $MgCl_2$	100 m*M*
1.5 ml	H_2O	
10 ml		

10× Annealing Buffer

Volume	Reagent	Final concentration
2 ml	1 *M* Tris-Cl, pH 7.5	200 m*M*
5 ml	1 *M* NaCl	500 m*M*
1 ml	1 *M* $MgCl_2$	100 m*M*
2 ml	H_2O	
10 ml		

[8] C. Schull and H. Beier, *Nucleic Acids Res.* **22,** 1974 (1994).
[9] N. Hanyu, Y. Kuchino, S. Nishimura, and H. Beier, *EMBO J.* **5,** 1307 (1986).
[10] M. E. Saks, J. R. Sampson, and J. N. Abelson, *Science* **263,** 191 (1994).
[11] W. H. McClain, *J. Mol. Biol.* **234,** 257 (1993).
[12] R. Giege, J. D. Puglishi, and C. Florentz, *Prog. Nucleic Acid Res. Mol. Biol.* **45,** 129 (1993).

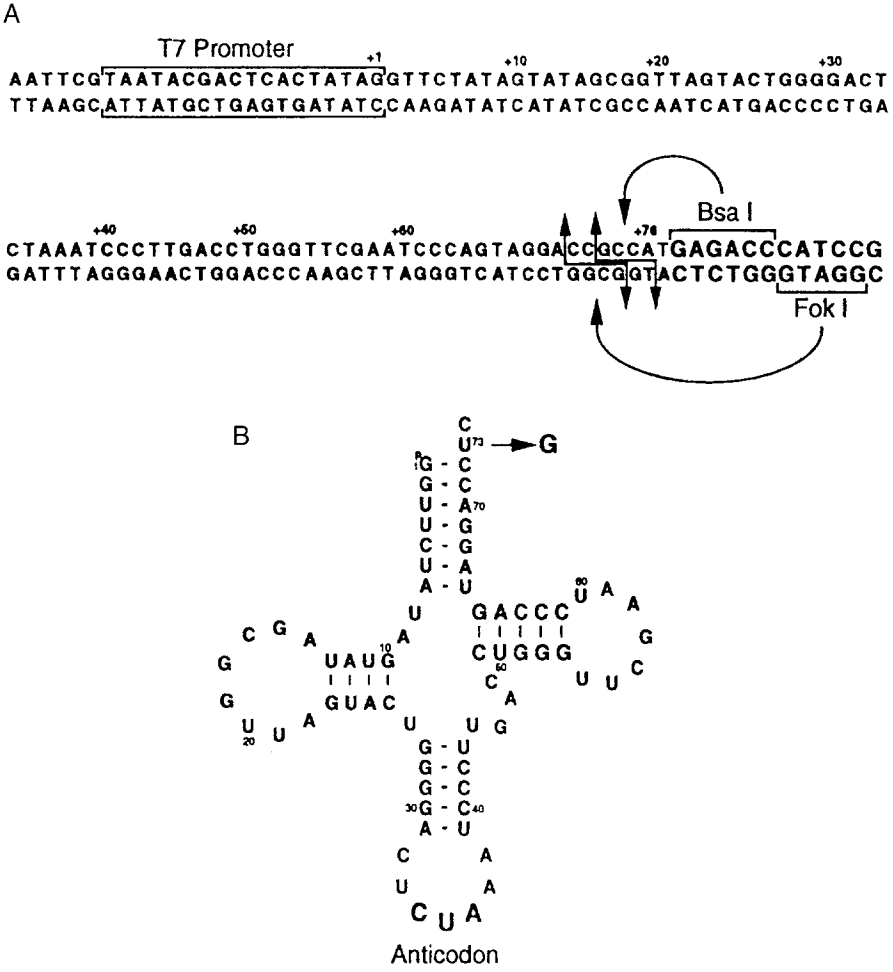


FIG. 2. Design of the engineered *T. thermophila* $tRNA^{GLN}$(CUA) G73 suppressor gene for *in vitro* transcription with T7 RNA polymerase. (A) The DNA sequence for the suppressor tRNA gene, the upstream T7 promoter, and the downstream runoff transcription sites are given. (B) Nucleotide sequence for the suppressor tRNA obtained from runoff transcription of *Fok*I-linearized DNA. (Reprinted with permission from Saks *et al.*, *J. Biol. Chem.* **271,** 23169 (1996).)

10× Ligation Buffer

Volume	Reagent	Final concentration
6.6 ml	1 *M* Tris-Cl, pH 7.5	660 m*M*
0.1 ml	1 *M* DTT	10 m*M*

0.5 ml	1 *M* $MgCl_2$	50 m*M*
1.0 ml	100 m*M* ATP	10 m*M*
1.8 ml	H_2O	
10 ml		

*10× Tris-Acetate-EDTA (TAE) Buffer**

Amount	Reagent	Final concentration
48.4 g	Tris-Cl	400 m*M*
11.4 ml	Glacial acetic acid (99.7%, 2/w)	1.1% (w/w)
20 ml	0.5 *M* EDTA, pH 8.0	10 m*M*
	H_2O (bring final volume to 1000 ml)	

* Adjust pH to 8.0 with NaOH.

The gene for *T. thermophila* $tRNA^{Gln}CUA$ G73[7] is constructed from the eight overlapping synthetic DNA oligonucleotides, four sense and four antisense, shown below.

Oligonucleotide	Sequence
1	5′-AATTCGTAATACGACTCACTATAGGTTCTATAG-3′
2	3′- GCATTATGCTGAGTGATATCCAAGA -5′
3	5′- TATAGCGGTTAGTACTGGGGACTCTAAA -3′
4	3′-TATCATATCGCCAATCATGACCCCTGAG -5′
5	5′- TCCCTTGACCTGGGTTCG-3′ -3′
6	3′-ATTTAGGGAACTGGACCC -5′
7	5′- AATCCCAGTAGGACCGCCATGAGACCCATCCG -3′
8	3′-AGCTTAGGGTCATCCTGGCGGTACTCTGGGTAGGCCTAG-5′

Oligonucleotides 2 through 7 are phosphorylated as follows:

Phosphorylation Reaction

Volume	Reagent	Final concentration
5 μl	10× phosphorylation buffer	1×
5 μl	10 m*M* ATP	1 m*M*
5 μl	1 m*M* spermidine	0.1 m*M*
10 μl	10 μg/μl oligonucleotide	2 μg/μl
1 μl	T4 polynucleotide kinase (10 U/μl)	0.2 U/μl
24 μl	H_2O	
50 μl		

After incubation at 37° for 2 hr, the reaction mixture is extracted once with an equal volume of phenol (saturated with 1 *M* Tris-Cl, pH

8.0) : $CHCl_3$: isoamyl alcohol (25 : 24 : 1) and once with an equal volume of $CHCl_3$: isoamyl alcohol (24 : 1). The aqueous phase is treated with 10 volumes of *n*-butanol, vortexed for 1 min, and centrifuged at 14,000 rpm for 10 min. The pellet is washed with 70% (v/v) ethanol, dried under vacuum, and reconstituted in 20 μl H_2O. The DNA concentration is determined from UV absorption (A_{260}), assuming that an OD of 1 at 260 nm corresponds to 37 μg/ml of DNA.

Oligonucleotides are annealed to each other in pairs (P designates phosphorylated): 1 : 2P, 3P : 4P, 5P : 6P and 7P : 8. The annealing procedure is as follows:

Annealing Reaction

Volume	Reagent	Final concentration
5 μl	10× annealing buffer	1×
	2 nmol sense oligonucleotide	40 μ*M*
	2 nmol antisense oligonucleotide	40 μ*M*
	H_2O (bring final volume to 50 μl)	

Annealing reactions are heated at 95° for 3 min then cooled slowly to room temperature. Reactions are treated with 10 volumes of *n*-butanol, vortexed for 1 min, and centrifuged at 14,000 rpm for 10 min. The pellets are washed with 70% ethanol, dried under vacuum, and reconstituted in 25 μl H_2O. The annealed oligonucleotides are ligated as follows:

Ligation Reaction

Volume	Reagent	Final concentration
2.5 μl	10× ligation buffer	1×
5 μl	Annealed oligonucleotides 1 : 2	
5 μl	Annealed oligonucleotides 3 : 4	
5 μl	Annealed oligonucleotides 5 : 6	
5 μl	Annealed oligonucleotides 7 : 8	
1 μl	T4 DNA ligase (10 U/μl)	0.4 U/μl
1.5 μl	H_2O	
25 μl		

Ligation reactions are incubated overnight at 16°. To test if the ligation is successful, a 5-μl aliquot of the reaction is run on a 1% agarose gel with 1 μg/ml ethidium bromide in 1× TAE buffer. The 110 base pair tRNA gene is purified on a 1% low melting point agarose gel and subcloned into the *Eco*RI/*Bam*HI sites of the pUC19 vector, giving the plasmid pTHG73.

In vitro Transcription and Purification of *T. thermophila* $tRNA^{Gln}$(CUA) G73 (THG73)

Buffers and Solutions

10× Transcription Buffer

Volume	Reagent	Final concentration
4 ml	1 *M* Tris-Cl, pH 8.3	400 m*M*
1 ml	100 m*M* spermidine	10 m*M*
1 ml	5 mg/ml acetylated-BSA	0.5 mg/ml
4 ml	H_2O	
10 ml		

*10× Tris-Borate-EDTA (TBE)**

Amount	Reagent	Final concentration
108 g	Tris-Cl	890 m*M*
55 g	Sodium borate	890 m*M*
40 ml	0.5 *M* EDTA, pH 8.0	20 m*M*
	H_2O (bring final volume to 1000 ml)	

* pH should be 8.0 without further adjustment.

*8% Acrylamide:bisacrylamide (19:1)/7 M Urea in 1X TBE**

Amount	Reagent	Final concentration
7.6 g	Acrylamide	7.6%
0.4 g	Bisacrylamide	0.4%
42 g	Urea	7 *M*
10 ml	10× TBE	1×
20 μl	TEMED	
200 μl	10% ammonium persulfate	
	H_2O (bring final volume to 100 ml)	

* The TEMED and ammonium persulfate are added just prior to pouring the gel.

Bromphenol Blue/Xylene Cyanol (BPB/XC) Dye Solution (Final Volume: 10 ml)

Amount	Reagent	Final concentration
25 mg	Bromphenol blue	0.25%
25 mg	Xylene cyanol	0.25%

4.2 g	Urea	7 *M*
1 ml	10× TBE	1×
3 ml	Glycerol	30%
	H_2O (bring final volume to 10 ml)	

Stains-All Solution (0.005%)*

Volume	Reagent	Final concentration
25 ml	0.1% Stains-all (100 mg solid Stains-all in 100 ml formamide)	0.005%
225 ml	Formamide	50%
250 ml	H_2O	
500 ml		

* Stains-all is extremely light sensitive.

Template DNA for *in vitro* transcription of tRNA lacking the 3′-terminal bases C75 and A76 is prepared by linearizing pTHG73 with *Fok*I. *In vitro* transcription[13] reactions (1 ml) are set up as follows:

In vitro Transcription Reaction

Volume	Reagent	Final concentration
100 μl	10× transcription buffer	1×
50 μl	100 m*M* dithiothreitol (DTT)	5 m*M*
200 μl	Solution with 20 m*M* each of ATP, UTP, GTP, and CTP	4 m*M* each NTP
100 μl	100 m*M* GMP	10 m*M*
100 μl	200 m*M* $MgCl_2$	20 m*M*
100 μl	1.0 μg/μl linearized pTHG73	100 μg/μl
2 μl	RNase inhibitor (40 U/μl)	0.08 U/μl
4 μl	Pyrophosphatase (0.5 U/μl)	0.002 U/μl
130 μl	T7 RNA polymerase (200 U/μl)	26 U/μl
214 μl	H_2O	
1000 μl		

The transcription reaction is incubated at 42° for 3 hr. The tRNA transcripts are visualized on an analytical gel [8% acrylamide : bisacrylamide (19 : 1) in 7 *M* urea, 1× TBE; 0.4 mm thick, 40 cm long]. Before loading the samples, the gel is run at 1000 V for 30 min. Samples are prepared by adding 5 μl of BPB/XC dye solution to 5 μl of reaction followed by heating

[13] J. R. Sampson and O. C. Uhlenbeck, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 1033 (1988).

at 65° for 2 min. Gels are run at 1000 V until the xylene cyanol band migrates 25–30 cm. The bromphenol blue and xylene cyanol dye bands correspond approximately to 10-mer and 55-mer oligonucleotides, respectively. The gel is stained overnight in 0.005% Stains-all solution (protected from light), destained in water with exposure to light, and dried under vacuum. We typically observe two tRNA products: the desired 74-mer and a 75-mer in a ratio of 2:1. The desired 74-mer tRNA transcript will be referred to as THG73.

THG73 is purified either (1) by polyacrylamide gel electrophoresis or (2) by using the Qiagen Total RNA kit (Chatsworth, CA) with modifications. With method 1, the THG73 can be purified away from the 75-mer, while with method 2, the products are obtained as a mixture. We have successfully used both tRNA preparations in suppression experiments. The second method provides greater amounts of material than the first method and takes less time to perform. Both methods are outlined next.

Purification of tRNA-THG73 Transcript by Polyacrylamide Electrophoresis (Method 1)

Buffers/Solutions

Tris-EDTA Buffer (TE): Prepared with Diethyl Pyrocarbonate (DEPC)-Treated H_2O

Volume	Reagent	Final concentration
1 ml	1 *M* Tris-Cl, pH 8.0	10 m*M*
0.2 ml	0.5 *M* EDTA, pH 8.0	1 m*M*
98.8 ml	H_2O	
100 ml		

*Elution Buffer (Prepared with DEPC-Treated H_2O)**

Volume	Reagent	Final concentration
20 ml	1 *M* Potassium acetate, pH 5.0	200 m*M*
2 ml	0.5 *M* EDTA, pH 8.0	10 m*M*
78 ml	H_2O	
100 ml		

* Adjust final pH to 5.0 with HCl.

tRNA Buffer (Prepared with DEPC Treated H_2O)

Volume	Reagent	Final concentration
0.4 ml	250 m*M* Sodium acetate, pH 5.0	10 m*M*
9.6 ml	H_2O	
10 ml		

For the gel-purification procedure (method 1), transcription reactions are terminated by adding 1/10 volume 250 m*M* EDTA, pH 8.0, and 1/10 volume 250 m*M* NaOAc, pH 5.0. The mixtures are extracted once with an equal volume of phenol (saturated with 300 m*M* NaOAc, pH 5.0):$CHCl_3$:isoamyl alcohol (25:24:1). THG73 is precipitated with 2 volumes of ethanol at −20°, dried under vacuum, and resuspended in 500 μl TE buffer. The product is purified on a preparative gel [8% acrylamide:bisacrylamide (19:1) in 7 *M* urea, 1× TBE; 4 mm thick, 40 cm long]. Before loading the sample, the gel is run at 500 V for 30 min. Samples are prepared for loading by adding 500 μl of BPB/XC dye solution to the resuspended THG73 followed by heating at 65° for 3 min. No more than 500–600 μg crude THG73 is loaded onto the gel. Gels are run at 500 V until the xylene cyanol band migrates 25–30 cm. The lower half of the gel is wrapped in plastic wrap and placed on a thin-layer chromatography plate with fluorescent indicator. The band corresponding to THG73 is visualized by short-wavelength UV light and outlined with a marker. The THG73 band is cut out, transferred to a 10-ml tube, and crushed with a glass rod (the tube and rod are previously baked overnight at 180°). To elute THG73, 5 ml of elution buffer is added, and the mixture is gently agitated overnight at 4°. After centrifugation at 6000 rpm in a clinical centrifuge for 15–20 min, the supernatant is transferred to a disposable empty column (Bio-Rad Laboratories Inc., Hercules, CA) to remove any remaining acrylamide and stored at 4°. The gel is eluted again with 3 ml of elution buffer for 5 hr at 4°, and the supernatant is isolated as above. The combined supernatants are transferred to 30-ml glass centrifuge tubes. Ethanol (25 ml) is added to precipitate THG73, and the mixture is placed at −80° for 24 hr and then at −20° for 24 hr. THG73 is pelleted by centrifugation at 7000 rpm in a HB4 rotor at −10° for 1 hr. The pellet is dried under vacuum, resuspended in 400 μl TE buffer, and transferred to a 1.7-ml Eppendorf tube. The product is precipitated a second time with 100 μl of 8 *M* NH_4OAc and 1 ml ethanol at −20° overnight. THG73 is pelleted a final time by centrifugation at 15,000 rpm at 4° for 30 min, washed with cold 70% ethanol, dried under vacuum, and resuspended in 40 μl water. The RNA concentration is determined from UV absorption (A_{260}), assuming that an OD of 1 at

260 nm corresponds to 40 μg/ml of RNA. The purity is assessed by analytical PAGE as described earlier.

Purification of tRNA-THG73 Using Modification of Qiagen Total RNA Kit (Method 2)

Buffers and Solutions

Wash Buffer (Prepared in DEPC-Water)

Volume	Reagent	Final concentration
2.5 ml	1 *M* MOPS, pH 7.0	50 m*M* (21 m*M* Na^+)
6.61 ml	4 *M* NaCl	529 m*M* Na^+
7.5 ml	Ethanol	15% ethanol
33.39 ml	DEPC–water	
50 ml		550 m*M* Na^+

800 mM Na^+ Elution Buffer (Prepared in DEPC–Water)

Volume	Reagent	Final concentration
2.5 ml	1 *M* MOPS, pH 7.0	50 m*M* (21 m*M* Na^+)
9.74 ml	4 *M* NaCl	779 m*M* Na^+
7.5 ml	Ethanol	15% ethanol
30.26 ml	DEPC–water	
50 ml		800 m*M* Na^+

1000 mM Na^+ Elution Buffer (Prepared in DEPC–Water)

Volume	Reagent	Final concentration
2.5 ml	1 *M* MOPS, pH 7.0	50 m*M* (21 m*M* Na^+)
12.24 ml	4 *M* NaCl	979 m*M* Na^+
7.5 ml	Ethanol	15% ethanol
27.76 ml	DEPC–water	
50 ml		1000 m*M* Na^+

For the Qiagen kit purification procedure (method 2), the tRNA transcription reaction is mixed with 2.5 ml Qiagen buffer QRL1 containing 25 μl 2-mercaptoethanol. The solution is mixed with 22.5 ml Qiagen buffer QRV2 and applied to a Qiagen tip-500 column preequilibrated with 10 ml Qiagen buffer QRE. Wash buffer is eluted until the A_{260} of the eluent is less than 0.05 (typically 40–50 ml). THG73 is eluted with 2 $\times$ 7.5 ml of 800 m*M* NaCl elution buffer then 4 $\times$ 7.5 ml of 1000 m*M* NaCl elution

buffer into six 30-ml glass centrifuge tubes (previously rinsed with ethanol and baked overnight at 180°). UV spectra are taken of a 300-μl aliquot of each 7.5-ml fraction; the fractions with high A_{260} contain the THG73 product. THG73 is precipitated by the addition of 1 volume (7.5 ml) 2-propanol to each fraction followed by centrifugation at 13,000 rpm in a JA-20 rotor (Beckman Instruments, Fullerton, CA) at 4° for 30 min. Pellets are each reconstituted in 200 μl DEPC–water, transferred to 1.7-ml Eppendorf tubes, and reprecipitated by the addition of 20 μl 3 *M* NaOAc, pH 5.0, and 600 μl cold ethanol. After storage overnight at −20°, the tRNA is repelleted by centrifugation at 14,000 rpm at 4° for 15 min. The pellets are rinsed with 100 μl cold 70% ethanol, dried under vacuum, redissolved in 100 μl DEPC–H_2O and stored at −80°. An aliquot (1 μl) is removed from each fraction and run on a small (0.75-mm-thick, <8-cm-long) analytical polyacrylamide gel (conditions as given earlier) to assess yield and purity. Typically the middle four fractions contain the bulk of the THG73 product; these are combined and the final concentration of tRNA is quantified by UV absorption (A_{260}), assuming that an OD of 1 at 260 nm unit corresponds to 40 μg/ml of RNA.

Organic Chemistry

Preparation of dCA Amino Acids

For the most part the procedures of Schultz and co-workers are followed, and the appropriate references should be consulted.[14,15]

Synthesis of dCA

This multistep sequence is carried out using the published procedure[14,15] with small modifications as described elsewhere.[3] Note that the *in vivo* nonsense suppression methodology generally operates on a much smaller scale than the *in vitro* protocol. Therefore, smaller quantities of reagents such as dCA are consumed. The total synthesis sequence is carried out on a scale to produce ~2 g of final dCA product, which is enough for a very large number of experiments. After HPLC purification, dCA can be stored indefinitely at −80° under an inert atmosphere. Alternatively, dCA can be synthesized on an automated DNA synthesizer.[16]

[14] S. A. Robertson, C. J. Noren, S. J. Anthony-Cahill, M. C. Griffin, and P. G. Schultz, *Nucleic Acids Res.* **17,** 9649 (1989).

[15] J. Ellman, D. Mendel, S. Anthonycahill, C. J. Noren, and P. G. Schultz, *Methods Enzymol.* **202,** 301 (1991).

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

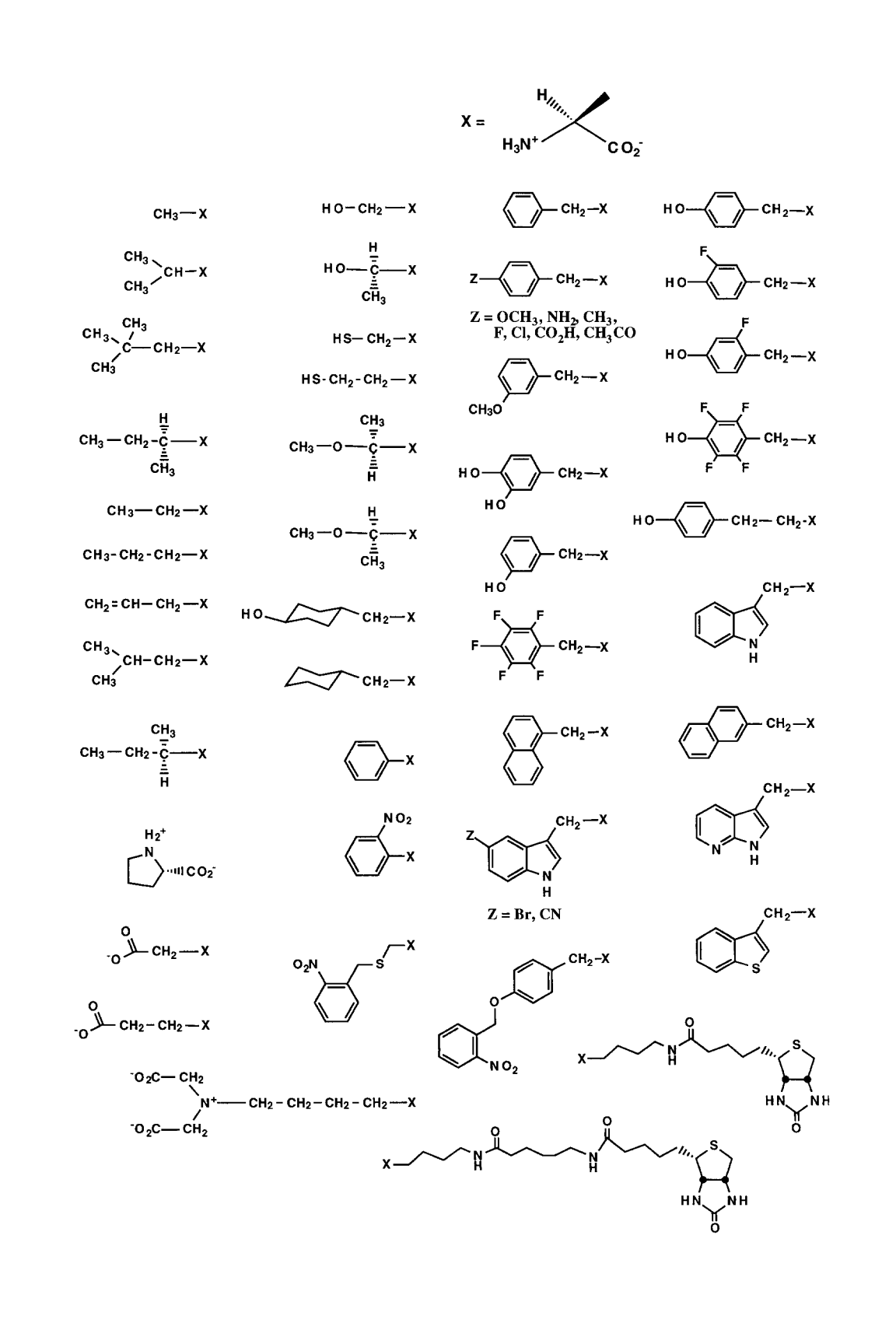
Synthesis of Unnatural Amino Acids

This aspect of the protocol is highly variable, depending on the desired structure. In many cases, the unnatural amino acid is prepared by modification of a natural amino acid, while many others are commercially available. The NRC Biotechnology Research Institute Peptide/Protein Chemistry Group maintains an excellent listing of commercially available amino acids at http://aminoacid.bri.nrc.ca:1125. A representative list of amino acids (natural and unnatural) that have been successfully incorporated by the *in vivo* nonsense suppression methodology is given in Fig. 3. Note also that racemic amino acids can be used, because only L-amino acids, and not D-amino acids, are incorporated.[17]

Typically, the amino group is protected as the *o*-nitroveratryloxycarbonyl (NVOC) group, which is subsequently removed photochemically according to the previous protocols. However, for amino acids that have a photoreactive side chain an alternative must be used. We have used the 4-pentenoyl (4PO) group, a protecting group first described by Madsen *et al.*[18] Lodder *et al.*[19] have also shown that the 4PO group is compatible with the tRNA systems. We present here a representative procedure based on the unnatural amino acid (2-nitrophenyl)glycine (Npg).[20]

N-4PO-DL-(2-Nitrophenyl)glycine. The unnatural amino acid DL-(2-nitrophenylglycine) hydrochloride is prepared according to published procedures.[21,22] The amine is protected as the 4PO derivative as follows.[18,19] To a room temperature solution of (2-nitrophenyl)glycine hydrochloride (82 mg, 0.35 mmol) in H_2O:dioxane (0.75 ml:0.5 ml) is added Na_2CO_3 (111 mg, 1.05 mmol) followed by a solution of 4-pentenoic anhydride (70.8 mg, 0.39 mmol) in dioxane (0.25 ml). After 3 hr the mixture is poured into saturated $NaHSO_4$ and extracted with CH_2Cl_2. The organic phase is dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residual oil is purified by flash silica gel column chromatography to yield the title compound (73.2 mg, 75.2%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.06 (dd, J=1.2, 8.1 Hz, 1H), 7.70 (ddd, J=1.2, 7.5, 7.5 Hz, 1H), 7.62-7.53 (m, 2H), 6.21 (s, 1H), 5.80 (m, 1H), 5.04–4.97 (m, 2H), 2.42–2.28 (m, 4H). HRMS calculated for $C_{13}H_{14}N_2O_5$ 279.0981, found 279.0992.

[17] V. W. Cornish, D. Mendel, and P. G. Schultz, *Angew. Chem. Int. Ed. Engl.* **34,** 621 (1995).
[18] R. Madsen, C. Roberts, and B. Fraser-Reid, *J. Org. Chem.* **60,** 7920 (1995).
[19] M. Lodder, S. Golvine, and S. M. Hecht, *J. Org. Chem.* **62,** 778 (1997).
[20] P. M. England, H. A. Lester, N. Davidson, and D. A. Dougherty, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11025 (1997).
[21] A. L. Davis, D. R. Smith, and T. J. McCord, *J. Med. Chem.* **16,** 1043 (1973).
[22] S. Muralidharan and J. M. Nerbonne, *J. Photochem. Photobiol. B: Biol.* **27,** 123 (1995).


X =
Z = OCH3, NH2, CH3, F, Cl, CO2H, CH3CO
Z = Br, CN

N-4PO-DL-(2-Nitrophenyl)glycinate Cyanomethyl Ester. The acid is activated as the cyanomethyl ester using standard conditions.[14,15] To a room temperature solution of the acid (63.2 mg, 0.23 mmol) in anhydrous dimethylformamide (DMF, 1 ml) is added $N(C_2H_5)_3$ (95 μl, 0.68 mmol) followed by $ClCH_2CN$ (1 ml). After 16 hr the mixture is diluted with $(C_2H_5)_2O$ and extracted against H_2O. The organic phase is washed with saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residual oil is purified by flash silica gel column chromatography to yield the title compound (62.6 mg, 85.8%) as a yellow solid. ^1H NMR (300 MHz, $CDCl_3$) δ 8.18 (dd, $J=1.2$, 8.1 Hz, 1H), 7.74–7.65 (m, 2H), 7.58 (ddd, $J=1.8$, 7.2, 8.4 Hz, 1H), 6.84 (d, $J=7.8$ Hz, 1H), 6.17 (d, $J=6.2$ Hz, 1H), 5.76 (m, 1H), 5.00 (dd, $J=1.5$, 15.6 Hz, 1H), 4.96 (dd, $J=1.5$, 9.9 Hz, 1H), 4.79 (d, $J=15.6$ Hz, 1H), 4.72 (d, $J=15.6$ Hz, 1H), 2.45–2.25 (m, 4H). HRMS calculated for $C_{16}H_{17}N_3O_5$ 317.1012, found 317.1004.

N-4PO-(2-Nitrophenyl)glycine-dCA. The dinucleotide dCA is prepared as reported by Ellman *et al.*[15] with the modifications described by Kearney *et al.*[3] The cyanomethyl ester is then coupled to dCA as follows. To a room temperature solution of dCA (tetrabutylammonium salt, 20 mg, 16.6 μmol) in anhydrous DMF (400 μl) under argon is added N-4PO-DL-(2-nitrophenyl)glycinate cyanomethyl ester (16.3 mg, 51.4 μmol). The solution is stirred for 1 hr and then quenched with 25 m*M* ammonium acetate, pH 4.5 (20 μl). The crude product is purified by reversed-phase semipreparative HPLC (Whatman, Clifton, NJ Partisil 10 ODS-3 column, 9.4 mm × 50 cm), using a gradient from 25 m*M* NH_4OAc, pH 4.5 to CH_3CN. The appropriate fractions are combined and lyophilized. The resulting solid is redissolved in 10 m*M* acetic acid/CH_3CN and lyophilized to afford 4PO-Npg-dCA (3.9 mg, 8.8%) as a pale yellow solid. ESI-MS M^- 896 (31), [M-H] 895 (100), calculated for $C_{32}H_{36}N_{10}O_{17}P_2$ 896. The material is quantified by UV absorption ($\varepsilon_{260} \approx 37{,}000\ M^{-1}\ cm^{-1}$).

Chemical Acylation of tRNAs and Removal of Protecting Groups

The α-NH_2-protected dCA-amino acids or dCA are enzymatically coupled to the THG73 *Fok*I runoff transcripts using T4 RNA ligase to form

FIG. 3. Representative amino acids, both natural and unnatural, that have been successfully incorporated into proteins expressed in *Xenopus* oocytes by the *in vivo* nonsense suppression method. Absence from this list does not imply that a particular unnatural amino acid would not be compatible with the system.

a full-length chemically charged α-NH_2-protected aminoacyl-THG73 or a full-length but unacylated THG73-dCA.[23]

2.5× Reaction Mix

Volume	Reagent	Concentration in 2.5× mix	Final concentration in reaction
25 μl	400 m*M* HEPES, pH 7.5	100 m*M*	40 m*M*
10 μl	100 m*M* DTT	10 m*M*	4 m*M*
25 μl	200 m*M* $MgCl_2$	50 m*M*	20 m*M*
5 μl	7.5 m*M* ATP	375 μ*M*	150 μ*M*
10 μl	5 mg/ml acetylated BSA	0.5 mg/ml	0.2 mg/ml
1 μl	RNase inhibitor (40 U/μl)	0.4 U/μl	0.16 U/μl
24 μl	DEPC-H_2O		
100 μl			

Prior to ligation, 10 μl of THG73 (1 μg/μl in water) is mixed with 5 μl of 10 m*M* HEPES, pH 7.5. This tRNA/HEPES premix is heated at 95° for 3 min and allowed to cool slowly to 37°. Ligation reactions (40 μl) are set up as follows:

Ligation Reaction

Volume	Reagent	Final concentration
15 μl	tRNA-THG73/HEPES premix (see text)	0.25 μg/μl
16 μl	2.5× reaction buffer	1×
4 μl	α-NH_2-protected dCA-amino acid (3 m*M* in DMSO)*	300 μ*M*
2.4 μl	T4 RNA ligase (20 U/μl)	1.2 U/μl
2.6 μl	DEPC-H_2O	
40 μl		

* The final DMSO concentration in the ligation reaction must be 10%.

After incubation at 37° for 2 hr, DEPC–H_2O (52 μl) and 3 *M* sodium acetate, pH 5.0 (8 μl), are added and the reaction mixture is extracted once with an equal volume of phenol (saturated with 300 m*M* sodium acetate, pH 5.0) : $CHCl_3$: isoamyl alcohol (25 : 24 : 1) and once with an equal volume of $CHCl_3$: isoamyl alcohol (24 : 1) then precipitated with 2.5 volumes of cold ethanol at −20°. The mixture is centrifuged at 14,000 rpm at 4° for 15 min, and the pellet is washed with cold 70% (v/v) ethanol, dried under vacuum, and resuspended in 7 μl 1 m*M* sodium acetate, pH 5.0. The amount of α-NH_2-protected aminoacyl-THG73 is quantified by measuring A_{260},

[23] T. E. England, A. G. Bruce, and O. C. Uhlenbeck, *Methods Enzymol.* **65,** 65 (1980).

and the concentration is adjusted to 1 μg/μl with 1 m*M* sodium acetate, pH 5.0.

The ligation efficiency may be determined from analytical PAGE. The α-NH_2-protected aminoacyl-tRNA partially hydrolyzes under typical gel conditions, leading to multiple bands, so the ligated tRNA is deprotected (as described later; see section on Oocyte Injection) prior to loading. Such deprotected tRNAs immediately hydrolyze on loading. Typically, 1 μg of ligated tRNA in 10 μl BPB/XC buffer (described earlier) is loaded onto the gel, and 1 μg of unligated tRNA is run as a size standard. The ligation efficiency may be determined from the relative intensities of the bands corresponding to ligated tRNA (76 bases) and unligated tRNA (74 bases). We typically find a 30% ligation efficiency for THG73; the side products are presumably due to self-ligation.

Generation of mRNA

High-Expression Plasmid Constructs

For the nonsense codon suppression method, it is desirable to have the gene of interest in a high-expression plasmid, so that functional responses in oocytes may be observed 1–2 days after injection. Among other considerations, this minimizes the likelihood of reacylation of the suppressor tRNA.

A high-expression plasmid (designed by C. Labarca) was generated by modifying the multiple cloning region of pBluescript SK+. At the 5′ end, an alfalfa mosaic virus (AMV) sequence was inserted, and at the 3′ end a poly(A) tail was added, providing the plasmid pAMV-PA (Fig. 4). mRNA transcripts containing the AMV region bind the ribosomal complex with high affinity, leading to 30-fold increase in protein synthesis.[24] Including a 3′ poly(A) tail has been shown to increase mRNA half-life, therefore increasing the amount of protein synthesized.[25] The gene of interest was subcloned into pAMV-PA such that the AMV region is immediately 5′ of the ATG start codon of the gene (i.e., the 5′ untranslated region of the gene was completely removed). The plasmid pAMV-PA is available from C. Labarca at Caltech.

Site-Directed Mutagenesis

Any suitable site-directed mutagenesis method may be used to change the codon of interest to TAG. We have successfully used the Transformer

[24] S. A. Jobling and L. Gehrke, *Nature* **325,** 622 (1987).
[25] P. Berstein and J. Ross, *Trends. Biochem. Sci.* **14,** 373 (1989).

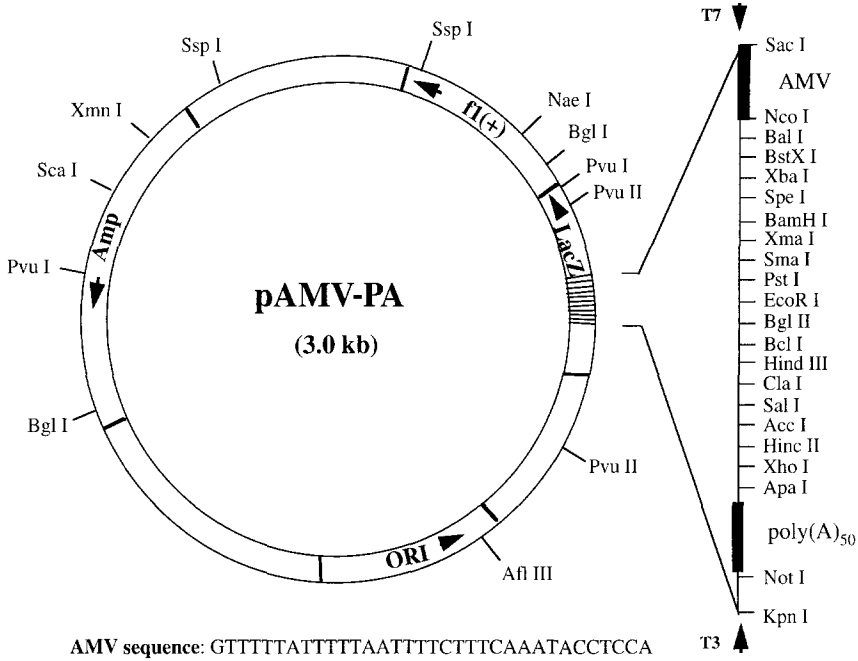


FIG. 4. pAMV-PA high-expression plasmid.

kit (Clontech, Palo Alto, CA), the Altered Sites kit (Stratagene, La Jolla, CA), and standard polymerase chain reaction (PCR) cassette mutagenesis procedures.[26] With the first two methods, a small region of the mutant plasmid (400–600 base pairs) is subcloned into the original plasmid. With all methods, the inserted DNA regions are checked by automated sequencing over the ligation sites. If the plasmid of interest naturally has TAG as its stop codon, this must be mutated to either TAA or TGA, to prevent the suppressor tRNA from inserting amino acids at the termination position.

In vitro mRNA Synthesis

The pAMV-PA plasmid constructs are linearized with *Not*I, and mRNA transcripts are generated using the mMessage mMachine T7 RNA polymerase kit (Ambion, Austin, TX). We have found that mRNA generated with this kit consistently expresses well when injected into *Xenopus* oocytes.

[26] S. N. Ho, H. D. Horton, J. K. Pullen, and L. R. Pease, *Gene* **77,** 51 (1989).

Oocytes

Oocyte Injection

Before using the α-NH_2-protected aminoacyl-tRNA for oocyte experiments, the α-NH_2 protecting group must be removed. We primarily use one of two protecting groups, either NVOC or 4PO.[18] The NVOC group is removed by irradiating 1 μl of tRNA solution for 5 min with a 1000-W Hg(Xe) arc lamp equipped with WG-335 and UG-11 filters (Schott, Duryea, PA). The filters are placed in a water-filled chamber to cool them and to remove infrared wavelengths. The 4PO group is removed by treating 0.5–1 μl of tRNA solution with an equal volume of saturated aqueous I_2 for 10 min.

Following deprotection, the tRNA is mixed with mRNA and microinjected into *Xenopus* oocytes using published methods[27] (50 nl/oocyte). The amounts of tRNA and mRNA must be optimized for each aminoacyl-tRNA and for each position within a given protein. We typically inject 5–25 ng tRNA per oocyte. Note that the endogenous level of total tRNA in an oocyte is approximately 50 ng, although any individual tRNA is present in much smaller amounts. We typically base the amount of injected UAG-mutant mRNA on the amount of analogous wild-type mRNA needed to provided a reasonable signal level. Since suppression may be only 10–15% efficient in some cases (see *In Vitro* Protein Translation section), it is often necessary to inject 10–50 times the amount of UAG mRNA relative to the wild-type mRNA to achieve comparable responses.

For heteromultimeric proteins, one must also consider the stoichiometry of the functional protein. For example, the muscle nAChR consists of four subunits, α, β, γ, and δ, in a 2 : 1 : 1 : 1 ratio. When carrying out suppression experiments in the α subunit, we typically inject the α UAG mRNA along with the wild-type β, γ, and δ mRNAs in a (2-20) : 1 : 1 : 1 ratio.

Electrophysiologic Measurements and Expression Levels

Whole-cell electrophysiologic measurements using a two-electrode voltage-clamp circuit are typically performed 18–48 hr after injection. With the nonsense codon suppression method, we routinely observe whole-cell currents on the order of microamperes from a variety of ion channels, including the muscle nAChR, the G-protein-coupled inward-rectifier K^+ (GIRK) channel, and the *Shaker* K^+ channel. In addition, we have measured single-channel currents for the nAChR using the patch-clamp technique.[4]

[27] M. W. Quick and H. A. Lester, *in* "Ion Channels of Excitable Cells" (T. Narahashi, ed.) pp. 261–279. Academic Press, San Diego, 1994.

Assessing and Optimizing Efficiency and Fidelity of Nonsense Codon Suppression in *Xenopus* Oocytes

Assessing mRNA-Dependent Background Currents

It is important to determine whether injection of the UAG mRNA without a suppressor tRNA leads to functional protein. This could occur if (1) an endogenous tRNA misreads the UAG codon or (2) an endogenous *Xenopus* subunit substitutes for the UAG mutant subunit. In some instances with the nAChR, we have observed currents when only UAG mRNA is injected, although these currents are generally small (<20 nA) and can be minimized by reducing the amount of injected mRNA.

Assessing Reacylation of THG73

The desired outcome from coinjecting oocytes with an aminoacylated suppressor tRNA and UAG mRNA is to synthesize protein incorporating the desired unnatural amino acid at the UAG position. However, if endogenous synthetases replace the unnatural amino acid on the tRNA with a natural amino acid (editing and/or reacylation), this can lead to an uncontrolled mixture of amino acids at the UAG position.

To assess the extent of reacylation, if any, oocytes are coinjected with uncharged tRNA (tRNA-dCA) and UAG mRNA. When examining a new position in a protein one *must* perform this experiment to ensure that (1) reacylation is not occurring or (2) reacylation is not a contaminating factor. For initial studies with THG73,[7] we tested for reacylation by coinjecting oocytes with tRNA-dCA and nAChR α180UAG, β, γ, and δ mRNAs. We chose position 180 since it is not highly conserved among known α subunits, and a wide range of natural amino acids are observed at this position. For these experiments, THG73 reacylation currents in response to 50–200 μM ACh were routinely <10 nA. Based on these findings, we initially thought that reacylation of the suppressor tRNA-THG73 was not occurring. However, more recent experiments at other positions within the α subunit have demonstrated reacylation currents ranging from 50 nA to as high as 1 μA. In almost all cases, this current could be reduced or eliminated by lowering the amounts of tRNA and mRNA injected.

Assessing Fidelity of Amino Acid Incorporation

When examining a new mutant, one would like to verify the fidelity of amino acid incorporation, which may be done in several ways. First, the wild-type protein should be reconstituted by incorporating the wild-type residue using the nonsense suppression method. Second, a mutant prepared

by conventional site-directed mutagenesis may be compared to the same mutant obtained using nonsense suppression. We initially verified the fidelity of nonsense codon suppression in oocytes by inserting the wild-type tyrosine or mutant phenylalanine residues at positions α93, α190, and α198 of the mouse muscle nAChR.[2] Receptors obtained from nonsense codon suppression displayed indistinguishable characteristics (EC_{50} values and Hill coefficients) when compared to receptors obtained from wild-type or conventional mutant mRNAs, thus validating the method.

Additional Issues with Nonsense Suppression Method

Potential Concerns for Heteromultimeric Protein Expression

A potential complication in the oocyte expression of heteromultimeric proteins is promiscuity in a subunit–subunit assembly. For example, when one expresses the muscle nAChR in oocytes, it is possible to obtain receptors composed of only α, β, and γ subunits ("δ-less" receptors) or α, β, and δ subunits ("γ-less" receptors) simply by omitting either the δ- or γ-subunit mRNA, respectively.[28,29] Expression of δ-less or γ-less receptors could occur in suppression experiments with δ- or γ-subunit UAG mRNAs, respectively, if the suppression efficiency is poor. The δ-less or γ-less receptors could then be mistaken for receptors containing the desired unnatural amino acid. To avoid this problem, properties of the δ-less or γ-less receptors should be compared to those of receptors observed in the suppression experiment.

For many proteins like the muscle nAChR, one does not normally worry about stoichiometry variations in conventional expression experiments. But for some proteins, like those comprising the neuronal nAChRs, varying subunit compositions are a normal concern. For example, injecting oocytes with neuronal nAChR α_3 and β_4 mRNAs in mole ratios of 0.91 : 0.09, 0.5 : 0.5, and 0.09 : 0.91 leads to receptors with ACh EC_{50} values of 170, 40, and 25 μM (M. W. Nowak, unpublished results, 1997). Presumably these receptors differ in their α_3/β_4 stoichiometry. In such cases, suppression experiments with either subunit may give results difficult to distinguish from the normal stoichiometry variation.

Additional Potential Complications of the Nonsense Suppression Method

If the nonsense UAG codon is near the 3′ end of the mRNA coding region, poor suppression efficiency may yield mostly truncated protein,

[28] P. Charnet, C. Labarca, and H. A. Lester, *Biophys. J.* **59,** 527a (1991).
[29] C. Czajkowski, C. Kaufmann, and A. Karlin, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6285 (1993).

which is nevertheless functional. In this case, one could mistake the truncated protein for the protein incorporating the desired unnatural amino acid. To check for this, one may inject the UAG mRNA without suppressor tRNA to determine whether a functional protein is produced.

Related to this, truncated proteins may retain enough assembly domains to function as dominant negative mutants. In such cases, the amounts of mRNA must be optimized to control the dominant negative effect and to maximize expression. However, we note that in many cases, such as at the nAChR 9′ position in the second transmembrane domain, suppression experiments work well although dominant negative effects are observed when no suppressor tRNA is injected.[4]

What if a Functional Response is not Observed?

In some cases, a suppression experiment does not result in a functional response. This could occur because (1) the unnatural amino acid is not incorporated into the protein or (2) the unnatural amino acid is incorporated but the mutant protein is nonfunctional. To address the first possibility, an *in vitro* translational assay can be performed as described next. In the second case, an alternative protein detection method that does not require a functional response should be used. For the muscle nAChR, we have employed an α-bungarotoxin binding assay to detect expression of nonfunctional receptors. If a toxin binding assay is unavailable, a convenient epitope such as c-*myc*[30] may be inserted into an extracellular region of the protein and an antibody binding assay performed.

In vitro Translation as a Test for Suppression

Buffers and Solutions

*4× Separating Gel Buffer**

Amount	Reagent	Final concentration
18.17 g	Tris-Cl	1.5 *M*
4 ml	10% sodium dodecyl sulfate (SDS)	0.4%
	H_2O (bring final volume to 100 ml)	

* Adjust pH to 8.8.

[30] P. A. Kolodziej and R. A. Young, *Methods Enzymol.* **194,** 508 (1991).

*4× Stacking Gel Buffer**

Amount	Reagent	Final concentration
6.66 g	Tris-Cl	0.55 *M*
4 ml	10% SDS	0.4%
	H_2O (bring final volume to 100 ml)	

* Adjust pH to 8.8.

*10× Running Buffer**

Amount	Reagent	Final concentration
3.03 g	Tris-Cl	0.25 *M*
10 ml	10% SDS	1%
14.4 g	Glycine	1.92 *M*
	H_2O (bring final volume to 100 ml)	

* Adjust pH to 6.8.

Sample Buffer

Amount	Reagent	Final concentration
2.5 mg	Bromphenol blue	0.025%
2.5 ml	4× Stacking buffer	1×
2 ml	Glycerol	20%
2 ml	10% SDS	2%
0.5 ml	2-Mercaptoethanol (add just before using)	5%
3 ml	H_2O	
10 ml		

40% Acrylamide Solution (19:1)

Amount	Reagent	Final concentration
38 g	Acrylamide	38%
2 g	Bisacrylamide	2%
	H_2O (bring final volume to 100 ml)	

5% Stacking Gel

Amount	Reagent	Final concentration
6.25 ml	40% Acrylamide (19:1) solution	5%
12.5 ml	4× Stacking gel buffer	1×
10 μl	TEMED*	
100 μl	10% Ammonium persulfate*	
	H_2O (bring final volume to 50 ml)	
50 ml		

* The TEMED and ammonium persulfate are added just prior to pouring the gel.

10% Separating Gel

Amount	Reagent	Final concentration
12.5 ml	40% Acrylamide (19:1) solution	10%
12.5 ml	4× Separating gel buffer	1×
10 μl	TEMED*	
100 μl	10% Ammonium persulfate*	
	H_2O (bring final volume to 50 ml)	
50 ml		

* The TEMED and ammonium persulfate are added just prior to pouring the gel.

An *in vitro* translation system may be employed to test the efficiency of suppression for a specific UAG mRNA and aminoacyl-tRNA independent of protein function. This method can assess the mRNA quality, chemical ligation of THG73, and the ability of the particular unnatural amino acid to pass through the protein translational machinery. We have employed a rabbit reticulocyte lysate *in vitro* translational system (Promega, Madison, WI). Reactions are set up as follows:

In vitro Translational Reaction Premix for 10 Reactions

Volume	Reagent
70 μl	Rabbit reticulocyte lysate
2 μl	1 m*M* Amino acids (without methionine)
8 μl	[^{35}S]Methionine (1000–1200 Ci/mmol)
2 μl	RNase inhibitor (40 U/μl)

In vitro Translational Reaction

Volume	Reagent
8.2 μl	*In vitro* translational reaction premix
1 μl	1 μg/μl mRNA
1 μl	1 μg/μl Deprotected aminoacyl-tRNA

For control reactions, UAG mRNA and deprotected aminoacyl-tRNA (or tRNA-dCA) are replaced with 1 μl DEPC–water and 1 μl 1 m*M* sodium acetate, pH 5.0, respectively. After incubation at 30° for 2 hr the reactions are terminated by the addition of 10 μl of sample buffer and run on a 5% separating/10% stacking SDS–polyacrylamide gel. ^{14}C-labeled markers are run as standards. Gels are fixed for 30 min with 45:10:45 methanol:acetic acid:water, 30 min with Entensify A (DuPont, Wilmington, DE), and 30 min with Entensify B (DuPont, Wilmington, DE), dried under vacuum, and exposed to X-ray film overnight. Using this method, we estimate that

the *in vitro* nonsense codon suppression efficiency of THG73 is 10–15%, although the *in vivo* efficiency may be higher or lower in any particular case.

Summary

A general method for the incorporation of unnatural amino acids into ion channels and membrane receptors using a *Xenopus* oocyte expression system has been described. A large number of unnatural amino acids have been incorporated into the nAChR, GIRK, and *Shaker* K^+ channels. Continuing efforts focus on incorporating unnatural amino acids that differ substantially from the natural amino acids, for example, residues that include fluorophores. In addition, we are addressing the feasibility of incorporating unnatural amino acids into ion channels and membrane receptors in mammalian cells.

Acknowledgments

We thank the NIH (NS-11756 and NS-34407) for support of this work.

[29] High-Level Expression and Detection of Ion Channels in *Xenopus* Oocytes

By THEODORE M. SHIH, RAYMOND D. SMITH, LIGIA TORO, and ALAN L. GOLDIN

Introduction

Since the initial demonstration by Miledi and co-workers that ion channels and neural receptors can be functionally expressed in *Xenopus* oocytes,[1–3] this system has become a standard for demonstrating that a specific cloned cDNA encodes a functional channel or receptor. Many different ion channels and receptors have been expressed in oocytes for functional analysis (reviewed in Refs. 4 and 5), and oocytes have been used for

[1] E. A. Barnard, R. Miledi, and K. Sumikawa, *Proc. R. Soc. Lond.* **215,** 241 (1982).
[2] R. Miledi, I. Parker, and K. Sumikawa, *EMBO J.* **1,** 1307 (1982).
[3] C. B. Gundersen, R. Miledi, and I. Parker, *Nature* **308,** 421 (1984).
[4] N. Dascal, *CRC Crit. Rev. Biochem.* **22,** 317 (1987).
[5] T. P. Snutch, *Trends Neurosci.* **11,** 250 (1988).

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