THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 38, Issue of September 20, pp. 23169–23175, 1996
Printed in U.S.A.

An Engineered *Tetrahymena* tRNA^{Gln} for *in Vivo* Incorporation of Unnatural Amino Acids into Proteins by Nonsense Suppression*

(Received for publication, April 1, 1996, and in revised form, June 11, 1996)

Margaret E. Saks‡, Jeffrey R. Sampson‡, Mark W. Nowak, Patrick C. Kearney, Fangyong Du, John N. Abelson, Henry A. Lester, and Dennis A. Dougherty§¶

From the Division of Biology and §Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

A new tRNA, THG73, has been designed and evaluated as a vehicle for incorporating unnatural amino acids site-specifically into proteins expressed *in vivo* using the stop codon suppression technique. The construct is a modification of tRNA^{Gln}(CUA) from *Tetrahymena thermophila*, which naturally recognizes the stop codon UAG. Using electrophysiological studies of mutations at several sites of the nicotinic acetylcholine receptor, it is established that THG73 represents a major improvement over previous nonsense suppressors both in terms of efficiency and fidelity of unnatural amino acid incorporation. Compared with a previous tRNA used for *in vivo* suppression, THG73 is as much as 100-fold less likely to be acylated by endogenous synthetases of the *Xenopus* oocyte. This effectively eliminates a major concern of the *in vivo* suppression methodology, the undesirable incorporation of natural amino acids at the suppression site. In addition, THG73 is 4–10-fold more efficient at incorporating unnatural amino acids in the oocyte system. Taken together, these two advances should greatly expand the range of applicability of the *in vivo* nonsense suppression methodology.

Methods that facilitate the engineering of protein variants are critical to studies of molecular recognition and protein function. Initially, bacterial genetics provided the primary tool for making amino acid substitutions in proteins. Because some strains express a naturally occurring nonsense suppressor tRNA having an altered anticodon, an amino acid could be incorporated at specific sites in response to nonsense mutations that created stop codons in messenger RNA (1–3). The utility of the suppression approach was greatly increased once it became possible to engineer nonsense-suppressing anticodons into tRNAs, thereby allowing a broader range of amino acids to be incorporated at the site of interest (4, 5). Subsequently, it was shown that a nonsense suppressor tRNA could be aminoacylated using organic synthesis, allowing for the *in vitro* incorporation of unnatural amino acids into proteins in a site-directed manner (6). Compared with conventional site-directed mutagenesis, this methodology enabled a much broader range of side chain modifications, including the incorporation of biophysical probes and cross-linking agents, as well as modifications to the protein main chain (7).

More recently, the unnatural amino acid methodology has been extended to allow expression of modified proteins in intact cells by direct injection of the mutated mRNA and the chemically acylated suppressing tRNA into *Xenopus* oocyte cells (see Fig. 1) (8). A specific tRNA (MN3) was designed for use *in vivo*, and it was found (8) to be an improvement over the tRNA^{Phe}-derived suppressor (6, 7) that had been used in previous *in vitro* studies. Using MN3, unnatural amino acids related to Tyr were inserted into the nicotinic acetylcholine receptor (nAChR)[1] α subunit at several natural Tyr sites known to be important in agonist binding, and the results were evaluated using electrophysiological measurements.

New results described here, however, establish that MN3 does not constitute an optimal solution to the *in vivo* suppression problem. As in previous studies, we have used the nAChR (9–11) to evaluate nonsense suppression. This ion channel protein is highly compatible with the heterologous expression system of the *Xenopus* oocyte, has a number of well-studied mutation sites for evaluation, and gives readily characterizable electrophysiological responses to added agonist. By studying positions in the nAChR α subunit that are more tolerant with regard to substitution, we demonstrate that tRNA MN3 can lead to incorporation of *natural* amino acids at the mutation site along with the desired unnatural amino acid, probably because the chemically acylated tRNA MN3 is being reacylated (and/or edited) by synthetases that are endogenous to the *Xenopus* oocyte. In addition, previous *in vitro* studies (8) of MN3 indicated that the absolute efficiency of unnatural amino acid incorporation was low with this construct, and the *in vivo* studies appeared to support this. While this low efficiency could be compensated for in the initial studies mentioned above, subsequent work to be described here and elsewhere established that the apparent inefficiency of MN3 became a serious handicap at other mutation sites or in efforts to incorporate unnatural amino acids that differ in more substantial ways from the natural set.

The present work thus addresses two related challenges posed by the *in vivo* suppression method. 1) We sought to design suppressor tRNAs that are more efficient at incorporating an unnatural amino acid at the site of interest in the protein. 2) We sought to prevent or greatly reduce endogenous tRNA synthetases from editing and/or reacylating the suppressor tRNA, in order to prevent the inadvertent site-specific incorporation of a natural amino acid into the target protein.

We have now designed a new nonsense suppressor tRNA that can be used to deliver amino acids to more "promiscuous"

* This research was supported by National Institutes of Health Grants NS11756 (to H. A. L.), NS34407 (to D. A. D.), and GM48560 (to J. N. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Div. of Chemistry and Chemical Engineering–164-30, California Institute of Technology, Pasadena, CA 91125. Tel.: 818 395 6089; Fax: 818 564 9297; E-mail: dad@igor.caltech.edu.

[1] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; AChR, acetylcholine receptor; GlnRS, glutaminyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; NVOC, nitroveratryloxycarbonyl.

sites of nAChR and other proteins. The new design strategy begins with a eukaryotic tRNA that is already competent to translate UAG codons. In *Tetrahymena thermophila* and other ciliates, the UAG codon does not signal the termination of translation. Instead, it is translated as a glutamine by the $tRNA^{Gln}$(CUA) isoacceptor, producing a deviation from the "universal" genetic code (12). Evidently *T. thermophila* $tRNA^{Gln}$(CUA) has evolved the necessary properties to efficiently translate UAG codons in a variety of mRNA codon contexts. Indeed, previous *in vitro* translation experiments showed that when wheat germ (13) or rabbit reticulocyte (12) 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, (14–16) we have modified *T. thermophila* $tRNA^{Gln}$(CUA) to produce a new tRNA, THG73. The new tRNA satisfies both requirements 1 and 2 above and has greatly expanded the range of applicability of the nonsense suppression method (17, 18).

EXPERIMENTAL PROCEDURES

Materials—NVOC-protected dCA-amino acids were prepared according to literature procedures (19). *Fok*I restriction endonuclease and T4 RNA ligase were purchased from New England Biolabs. T7 RNA polymerase was purified using the method of Grodberg and Dunn (20) from the overproducing strain *Escherichia coli* BL21 harboring the plasmid pAR1219 (21).

Gene Construction and RNA Preparation—The genes for the *T. thermophila* $tRNA^{Gln}$(CUA) having either an A^{73} or G^{73} (THA73 and THG73, respectively) and flanked by an upstream T7 RNA polymerase promoter and a downstream *Fok*I restriction site were constructed from eight overlapping synthetic DNA oligonucleotides and cloned into pUC19 giving the plasmids pTHA73 and pTHG73, respectively (see Fig. 2*A*). Template DNA for transcription of tRNA lacking the 3′-terminal C^{75} and A^{76} was prepared by digesting the plasmid DNA with *Fok*I restriction endonuclease. Our previous tRNA gene, pMN3, was constructed in a similar manner (8). *In vitro* transcription of the linearized DNA template and purification of the truncated THA73 and THG73 RNA products were performed as described previously (22). MN3 transcript (lacking the 3′-terminal C^{75} and A^{76}) was prepared in the same manner as above from *Fok*I-linearized pMN3 (8). mRNA for the wild type and mutants (codon UAG) of the mouse muscle α, β, γ, and δ nAChR subunits were prepared by *in vitro* transcription of the appropriate linearized plasmid construct.

Ligation of dCA-Amino Acids to Suppressor tRNAs—The NVOC-protected dCA-amino acids were coupled to the THA73, THG73, and MN3 *Fok*I runoff transcripts using T4 RNA ligase (Fig. 1) (23). Ligation reaction mixtures contained 42 mM HEPES-KOH, pH 7.4, 10% Me_2SO (v/v), 4 mM dithiothreitol, 20 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 150 μM ATP, 10 μM tRNA transcript, 300 μM NVOC-protected dCA-amino acid, and 2,000 units/ml of T4 RNA ligase. After incubation at 37 °C for 2 h, the reaction mixtures were extracted once with an equal volume of phenol:$CHCl_3$:isoamyl alcohol (25:24:1, pH 4.5) and once with an equal volume of $CHCl_3$:isoamyl alcohol (24:1, pH 4.5), precipitated with 2.5 volumes of ethanol at −20 °C, dried, resuspended in 1 mM NaOAc (pH 4.5), and stored at −80 °C. The percentage of ligated product as judged by electrophoresis on high resolution 8% denaturing polyacrylamide gels was approximately 90% for the THA73 and MN3 transcripts and only 30% for the THG73 transcripts. The reported quantities of all ligated tRNAs have been corrected for ligation efficiency (30%).

Oocyte Microinjections and Electrophysiological Measurements—Prior to microinjection, the ligated NVOC-aminoacyl-tRNA was renatured by heating to 65 °C for 3 min in 1 mM NaOAc (pH 4.5). The NVOC protecting group was subsequently removed by irradiating the sample for 5 min with a 600-W xenon lamp equipped with WG-335 and UG-11 filters (Schott). The desired amounts of deprotected aminoacyl-tRNA and α, β, γ, and δ mRNAs were then mixed and immediately microinjected (50 nl) into *Xenopus* oocytes using published methods (24).

Electrophysiological measurements were carried out 18–30 h after injection using a two-electrode voltage clamp circuit. Oocytes were bathed in solutions containing 5 mM HEPES-NaOH (pH 7.4), 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 1 μM atropine. Macroscopic ACh-induced currents were recorded in response to bath application 200–800 μM ACh at a holding potential of −80 mV. All numerical and plotted data are from measurements obtained for four to eight individual oocytes and are reported as the mean ± S.E.

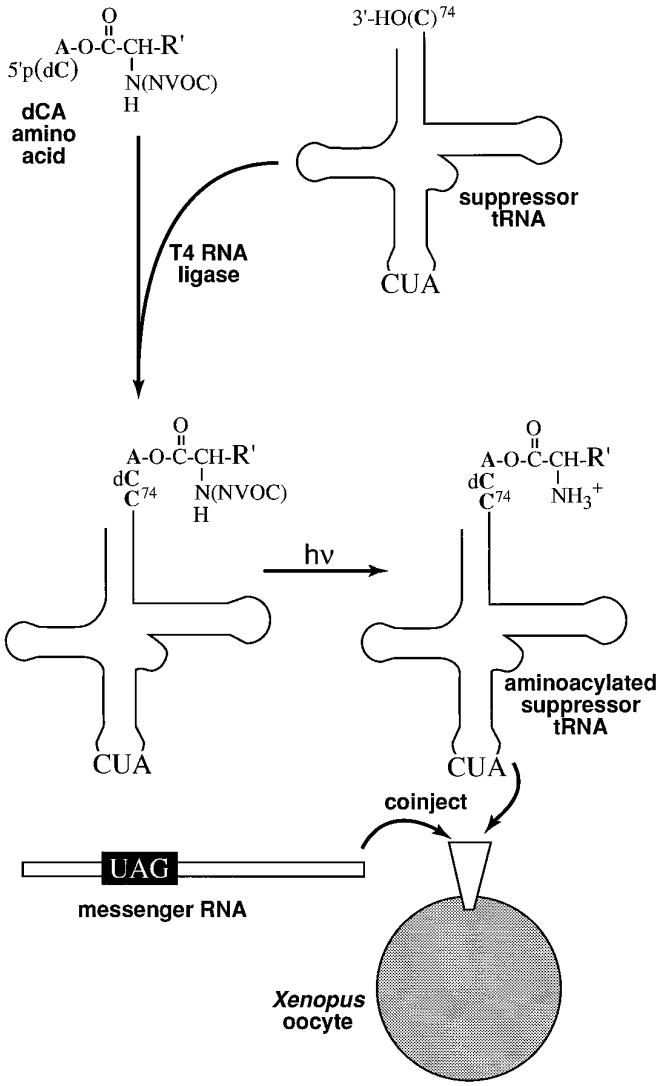

FIG. 1. **Scheme for incorporating unnatural amino acids into proteins expressed in *Xenopus* oocytes.**

RESULTS

Suppressor Design—Several factors must be considered in designing a new tRNA to meet the goals stated above. Translation efficiency depends not only on anticodon-codon base pairing, but also on other tRNA structural characteristics, including the sequence of the anticodon stem and loop (25) and the D-stem (26). In addition, post-transcriptional modification of nucleotides throughout the tRNA can affect translation efficiency (27).

Goal 1 requires a tRNA that can translate UAG codons with increased efficiency. Although the optimal anticodon stem/loop context for a CUA anticodon has been established for the *E. coli* translation system (25), the corresponding rules for eukaryotes have not been determined. We therefore chose to begin with *T. thermophila* $tRNA^{Gln}$(CUA), which naturally recognizes the UAG codon and efficiently suppresses UAG *in vitro* (12, 13).

Goal 2 requires a suppressor that cannot be recognized by the *Xenopus* aminoacyl-tRNA synthetases and therefore has a "null" *in vivo* amino acid identity. Biochemical studies (28, 29) and x-ray diffraction analyses (30, 31) indicate that *E. coli* glutaminyl-tRNA synthetase (GlnRS) recognizes the first three base pairs of the acceptor stem, the anticodon nucleotides

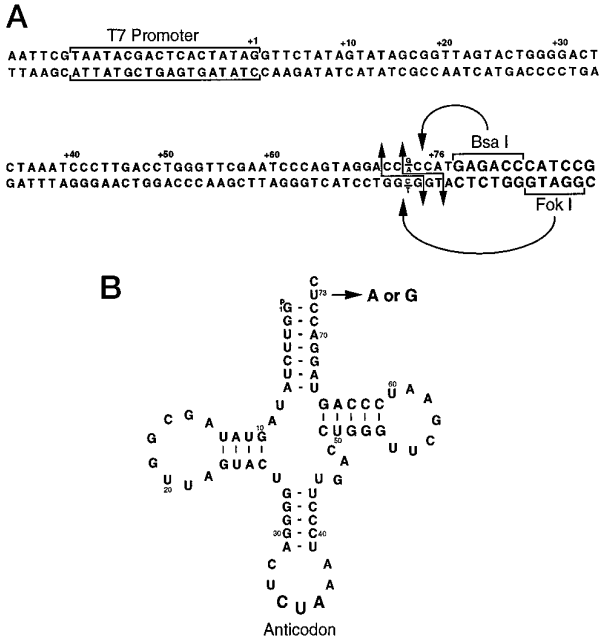

FIG. 2. **Design of the engineered *T. thermophila* tRNA^{Gln} CUA suppressor genes for *in vitro* transcription with T7 RNA polymerase.** *A*, the DNA sequence for the suppressor tRNA gene, the upstream T7 promoter, and downstream run-off restriction endonuclease sites are given. The tRNA gene sequence is based on the published *T. thermophila* tRNA^{Gln} RNA sequence (12). The recognition and corresponding cleavage site for the 3′-terminal *Fok*I restriction enzyme is indicated by *boldface letters* and associated *arrows*. *B*, nucleotide sequence for the THA73 and THG73 RNAs obtained from run-off transcription of *Fok*I-linearized DNA.

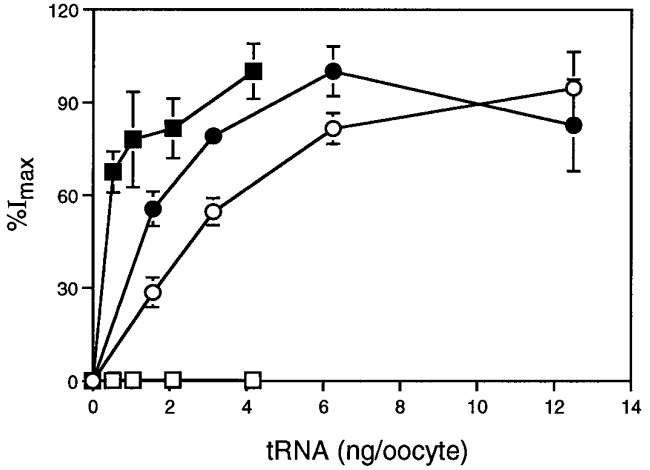

FIG. 3. **Concentration dependence of acylated (dCA-Tyr) and unacylated (dCA) forms of MN3 and THG73 suppressor tRNAs on the ACh-induced currents at position α180.** Oocytes were coinjected with a nAChR α180UAG:β:γ:δ mRNA concentration ratio of 100:1:1:1 (16 ng of total mRNA) and the indicated amount of acylated or unacylated tRNA. Currents were recorded at 200 μM ACh. The mean $\%I_{max}$ for each tRNA concentration was calculated by setting 100% at the highest mean current obtained for the acylated form of each suppressor tRNA (6.3 ng for MN3 and 4.2 ng THG73). The injected tRNA species are designated as follows: MN3-dCA (○), MN3-dCA-Tyr (●), THG73-dCA (□), THG73-dCA-Tyr (■).

(Py^{34}, U^{35}, and G^{36}), and a hairpin structure formed by RNA-RNA interactions between N^{73} and the phosphorus oxygen at position 72 of *E. coli* glutamine tRNAs. Eukaryotic GlnRS probably recognizes nucleotides in these regions as well. The G36A mutation in *T. thermophila* tRNA^{Gln}(CUA) that endows it with its distinctive codon coupling capacity is unlikely to affect aminoacylation by the cognate *T. thermophila* GlnRS but would be expected to reduce aminoacylation by other eukaryotic GlnRS. In support of these ideas, *in vitro* aminoacylation determinations showed that native *T. thermophila* tRNA^{Gln}(CUA) was efficiently aminoacylated by *T. thermophila* GlnRS but was not a substrate for wheat germ (13) or rabbit (12) GlnRS.

Thus, we investigated *T. thermophila* tRNA^{Gln}(CUA), anticipating that it might show both high translational efficiency and a potentially "null" amino acid identity in *Xenopus* oocytes. As a further precaution against recognition by *Xenopus* GlnRS, we constructed two mutants, U73A and U73G (Fig. 2*B*), which have been termed THA73 and THG73, respectively. These changes alter the wild type U^{73} that is likely to be recognized by eukaryotic GlnRS. These changes also facilitated the ligation of the 3′-terminal dCA to the T7 transcripts of tRNA^{Gln}(CUA) (see "Experimental Procedures"), because a pyrimidine was replaced by a more favorable purine at the position adjacent to the ligation junction (32).

Suppressor Evaluation—In these studies, an amino acid was chemically (rather than enzymatically) esterified to the 3′ OH of the dinucleotide dCA to form, for instance, dCA-Tyr (Fig. 1). Either dCA alone or aminoacylated dCA was then enzymatically coupled to the 3′ C^{74} of the tRNA to form either a full-length but unacylated tRNA (such as THG73-dCA), or a full-length chemically charged aminoacyl-tRNA (such as THG73-dCA-Tyr) (Fig. 1). Either of these full-length tRNAs was injected into oocytes along with nAChR mRNA. The response for oocytes injected with full-length, chemically acylated tRNA can be due to the insertion of the chemically acylated amino acid (the desired outcome) but also to the undesired insertion of amino acids that were coupled to the tRNA by the endogenous synthetases through either an editing mechanism or reacylation. The signal from oocytes injected with unacylated (dCA) suppressors can be used to estimate the extent of this endogenous acylation reaction, leading to the ratio $R_{uaa} = I_{tRNA\text{-}dCA\text{-}Tyr}/I_{tRNA\text{-}dCA}$ as an index of unnatural amino acid incorporation. R_{uaa} is a measure of the operational suppression efficiency.

Additional data of relevance to the efficiency question are provided by currents measured after injection of mRNA only. This experiment tests for read-through by the endogenous *Xenopus* translation system and generally produces small currents that vary among oocyte batches (see below).

Throughout this work we will use our suppressor tRNAs to deliver amino acids that are, in fact, natural, either Tyr or Leu. This is the most appropriate way to evaluate the tRNAs, removing any complications that might be introduced by the unnatural amino acid. However, we have previously shown that MN3 can deliver unnatural amino acids (8), and we have also found that THG73 is fully competent to deliver unnatural amino acids in a variety of contexts (17, 18).

Operational Suppression Efficiency—For studies of the comparative efficiencies of suppressor tRNAs, we focused on the α180 site of the nAChR. Fig. 3 compares currents obtained when oocytes were coinjected with α180UAG mRNA and with varying amounts of either the MN3 or the THG73 suppressor tRNAs. Observed currents reached 50% of I_{max} when less than 0.5 ng of THG73-dCA-Tyr was injected per oocyte. We find that 2–3-fold more MN3-dCA-Tyr was required to obtain the same relative signal, a small but likely significant effect.

From a practical viewpoint, the issue is not solely the amount of nAChR observed but rather the extent to which the suppression methodology gives current levels above those seen from controls involving tRNA-dCA (evaluating reacylation and editing) and injection of mRNA only (evaluating endogenous read-through). When this operational suppressor efficiency, expressed as R_{uaa}, is high, appropriate quantities of injected tRNA and mRNA will produce currents that can be confidently

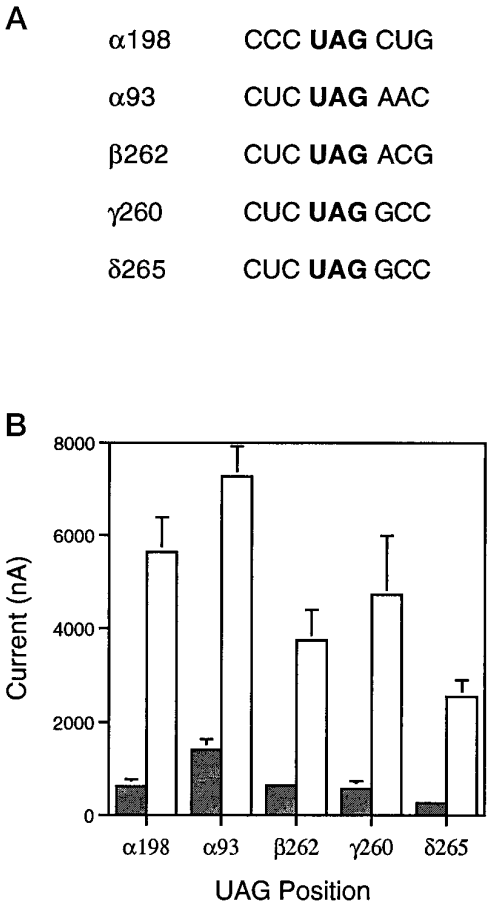

FIG. 4. **ACh-induced currents at five nAChR positions using acylated MN3 and acylated THG73 suppressor tRNAs.** *A*, codon contexts; *B*, observed currents. Oocytes were injected with 1.25 ng of tRNA dCA-Tyr for determinations at positions α198 and α93 and 1.25 ng tRNA dCA-Leu at positions β262, γ260, and δ265. Total injected amounts and concentration ratios (α:β:γ:δ) of nAChR subunit mRNAs for each test were as follows: α93UAG and α198UAG, 1.1 ng, 4:1:1:1; β262UAG, γ260UAG, and δ265UAG mRNA, 0.25 ng, 2:1:1:1. Currents were recorded at 400 μM ACh. *Shaded* and *open boxes* are for acylated MN3 and THG73, respectively.

TABLE I

ACh-induced current ratios (R_{uaa}) for the MN3, THA73, and THG73 suppressor tRNAs determined at two nAChR α subunit positions, 180 and 198, containing a UAG codon

Oocytes were coinjected with 12.5 ng (MN3 and THA73) or 4.2 ng (THG73) of acylated or unacylated tRNA and nAChR α180UAG or α198UG mRNA plus β, γ, and δ mRNA at a concentration ratio of 100:1:1:1 (16.1 ng of total mRNA). Current was recorded at 800 μM ACh. Oocytes injected with nAChR α180UAG mRNA only or nAChR α198UAG mRNA only (no tRNA) gave mean currents of 9 ± 3 nA or no detectable current, respectively.

UAG position	Suppressor tRNA	Mean current ± S.E. (nA)		R_{uaa} (dCA-Tyr/dCA)
		dCA	dCA-Tyr	
α198	MN3	3 ± 1	2950 ± 550	980
	THA73	13 ± 5	3640 ± 680	280
	THG73	4 ± 0.4	2720 ± 220	680
α180	MN3	3390 ± 1050	5460 ± 770	1.6
	THA73	4210 ± 380	7680 ± 950	1.8
	THG73	47 ± 23	4800 ± 870	100

attributed to receptor that has incorporated the desired unnatural amino acid. As shown in Fig. 3, significant currents were seen on injection of MN3-dCA, and current levels increased as the amount of injected MN3-dCA increased. In striking contrast, little or no current was measured for all tested concentrations of THG73-dCA (Fig. 3), indicating that MN3-dCA but not THG73-dCA was being acylated by an endogenous synthetase. Consequently, the operational efficiency (R_{uaa}) was as much as 100-fold higher for THG73 than for MN3 in this experiment (Fig. 3).

The muscle nAChR is composed of five subunits with the stoichiometry $\alpha_2\beta\gamma\delta$ (9–11). In our original experiments (8), the limiting factor for detectable signals was suppression of the UAG codon in α subunit mRNA by MN3, which was estimated to occur with only 10% efficiency *in vitro*. We therefore microinjected oocytes with a large excess of mRNA for α subunit variants to give a final subunit mRNA ratio of 100:1:1:1 (α:β:γ:δ). To assess whether the improved efficiency of THG73 *in vivo* could eliminate the need for this unusual subunit mRNA ratio, we measured the current that was obtained using a comparatively small amount of coinjected tRNA and varying amounts of the α180UAG mRNA subunit.

When the mRNA subunit ratio was 100:1:1:1 (16 ng of total mRNA), MN3-dCA-Tyr and THG73-dCA-Tyr gave comparable signals: 5000 ± 760 and 5200 ± 760 nA of current, respectively. However, when we dropped the subunit ratio to 4:1:1:1 (1.1 ng of total mRNA), MN3-dCA-Tyr and THG73-dCA-Tyr gave 530 ± 130 and 2200 ± 250 nA of current, respectively, establishing that only THG73 is able to produce large quantities of protein at the more conventional, 4:1:1:1 subunit ratio. These data again indicate that THG73 is a more efficient suppressor than MN3.

MN3 and THG73 were compared further at five positions in the nAChR subunits that have somewhat different codon contexts (Fig. 4*A*). Fig. 4*B* shows that the current due to UAG suppression at the five test positions ranged between 3 and 8 μA when THG73 was injected and that, in all cases, the current was at least 5-fold greater than that obtained for MN3. This result again indicates that THG73 is operationally a more efficient suppressor than is MN3 *in vivo*.

Acylation by the Endogenous Synthetases—To test for acylation of suppressor tRNA by an endogenous *Xenopus* synthetase, we studied oocytes co-injected with nAChR mRNA having a UAG mutation at several positions. Studies of the highly conserved position α198 (wild type = Tyr) indicate that aromatic amino acids are strongly preferred at this position (33). On the other hand, position α180 is not highly conserved when one considers both muscle and neuronal nAChR α subunits. That is, α180 is less restricted by amino acid type than α198. This suggests that α180 is the better site to seek evidence for reacylation of the tRNA, since the likelihood is greater that incorporation of a natural amino acid will still produce a functional (and hence detectable) receptor.

The operational suppression efficiency ratio R_{uaa} at position α198 was larger than 250 for all three engineered suppressor tRNAs (Table I). When evaluated at the more promiscuous position α180, however, R_{uaa} was 100 for THG73 but was less than 2 for both MN3 and THA73. This high ratio for THG73 is primarily due to the fact that oocytes injected with THG73-dCA had low currents, suggesting that THG73 is a much poorer substrate for endogenous synthetases. Thus, although THA73 and MN3 are adequate tRNAs for inserting unnatural amino acids at a highly restricted position like α198, THG73 is more versatile, since it functions properly at a more permissive site.

The highly conserved position termed Leu$^{9'}$ in the M2 region of all nAChR subunits presented a particularly sensitive test for acylation by endogenous synthetases or read-through by endogenous tRNAs, because many mutations at this position actually increase ACh sensitivity (34–36). Fig. 5*A* shows that when MN3 is the vehicle for incorporation of leucine at the 9′-position of the β subunit (β262) the dose-response relation

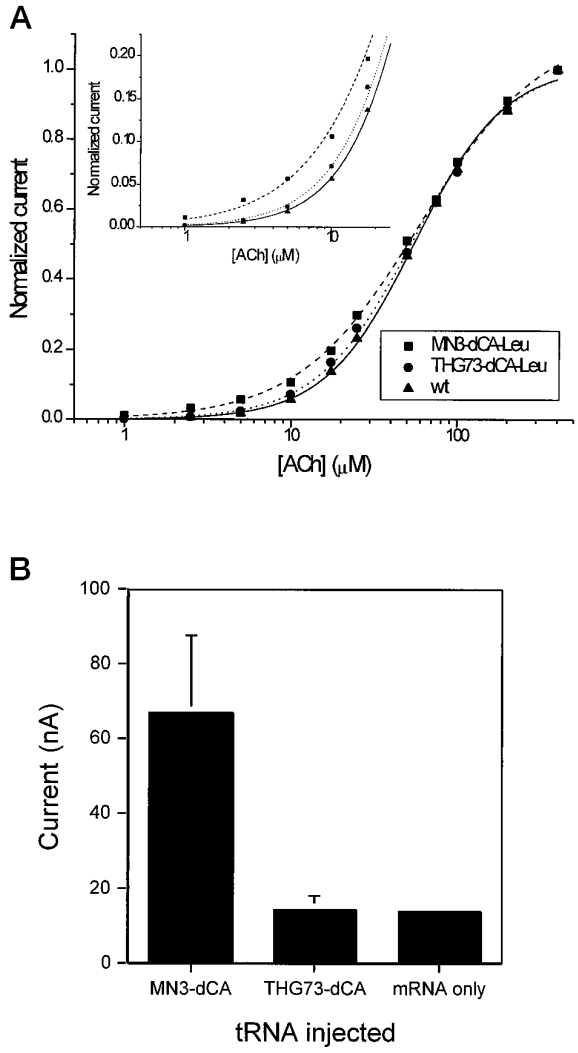

FIG. 5. **Suppression of nAChR β262UAG mRNA in *Xenopus* oocytes.** *A*, normalized dose-response relations for oocytes expressing wild-type nAChRs. Responses are shown for receptors expressed from wild type message (▲) or by suppression of the β262UAG message with either MN3-dCA-Leu (■) or THG73-dCA-Leu (●). Currents have been normalized to the 400 μM ACh response of each oocyte. Wild type currents were obtained by injecting a total of 0.1 ng of mRNA with a 2:1:1:1 concentration ratio (α:β:γ:δ). Suppression of the β262UAG message was performed by coinjecting 6 ng of tRNA with 0.63 ng of mRNA with a 2:1:1:1 concentration ratio (α:β:γ:δ). *B*, acetylcholine induced background currents at position β262UAG as a function of suppressor tRNA. Oocytes were coinjected with 6 ng of tRNA-dCA and 0.63 ng of mRNA (2:1:1:1 concentration ratio, α:β:γ:δ). Currents were recorded at 400 μM ACh.

deviates substantially from wild type at low concentrations of ACh. This indicates the presence of a small number of receptors having an EC_{50} less than wild type, revealing the incorporation of an unintended residue at this position. Further experiments (not shown) establish that the low EC_{50} component can become quite pronounced under different conditions with MN3. In clear contrast, when THG73 is used, the resulting dose-response curve superimposes on that for wild type, with no detectable low EC_{50} component (Fig. 5*A*). This is a very sensitive indication that suppression has proceeded with minimal reacylation when THG73 is used.

The sensitive measurements based on position 9′ do reveal a detectable signal with injection of the β262UAG mRNA but no tRNA of any type. This reflects a slight amount of read-through of UAG codons by tRNAs inherent to the *Xenopus* translation system. Importantly, this signal is not increased by coinjection of THG73-dCA (Fig. 5*B*). Thus, the lower end of the detection limit of suppression using THG73 is determined not by reacylation mechanisms, but rather by the read-through efficiency of the *Xenopus* system. Signals such as those in Fig. 5*B* are too small for systematic dose-response studies that might determine the tRNA responsible for the read-through. We emphasize, however, that this level of read-through represents less than 1% of our typical signal in a successful suppression experiment and therefore does not constitute a limiting factor at present.

DISCUSSION

The development of methods for controlled incorporation of unnatural amino acids greatly expands the range of structure/function studies available for complex proteins. To apply these methods to integral membrane proteins such as receptors, channels, and transporters, we previously adapted the nonsense suppressor/chemical acylation approach to the heterologous expression system of the intact *Xenopus* oocyte (8). This allows for site-specific, atomic scale modifications of proteins that can be studied in intact cells. A major challenge in developing this approach was to design a suppressor tRNA that efficiently translates UAG codons yet is not a substrate for any of the endogenous *Xenopus* synthetases. The present measurements unambiguously show that THG73 represents a significant improvement over our original suppressor MN3 with regard to both operational suppression efficiency and resistance to reacylation, greatly expanding the potential range of applicability of this methodology. In addition, the present work represents the first use of the nonstandard genetic code of ciliates for protein engineering in intact cells.

Efficiency of Suppression—The present study assesses the suppression efficiency of our engineered tRNAs by comparing the signals obtained from nAChR using the chemically acylated *versus* nonacylated versions of each tRNA. According to this criterion, THG73 is a more efficient suppressor than is MN3. However, it is difficult to precisely identify the major factors responsible for this improved efficiency. The nAChR possesses an $\alpha_2\beta\gamma\delta$ quaternary structure and is an integral membrane protein, so the production of functional receptors depends on many other factors besides suppression efficiency, such as post-translational receptor assembly and membrane localization steps. Also, both the sequences of MN3 and THG73 as well as the presence or absence of an amino acid are likely to affect interactions with the large multienzyme complexes that mediate eukaryotic protein synthesis (37, 38) and entry into the tRNA degradation pathway (39). Our experiments do not directly address these issues.

We contend, however, that chemically acylated THG73 is fairly efficient. Because the level of functional receptors increases with the injected tRNA concentration and reaches saturation, the injected THG73 must be effectively competing with the endogenous tRNAs. Moreover, the lowest concentration of injected THG73 roughly equals that of any individual endogenous tRNA isoaccepting species (40). It seems unlikely that the improved efficiency of THG73 relative to MN3 is due to differential stabilities of the tRNA·EF-1α·GTP ternary complexes, since EF1-α is present in relatively high concentrations in the oocyte (41) and should not be limiting. Rather, it is more likely that THG73 either interacts more productively with the UAG codon on the ribosome or competes more effectively with release factor than does MN3.

THG73 is derived from an *in vitro* transcription reaction and therefore possesses no modified nucleotides when injected. Sequence comparisons for a wide variety of eukaryotic tRNAs indicate that tRNAs having an A^{36} are expected to have an N^6-isopentenyl modification at A^{37} (i6A), adjacent to the anti-

codon (27). Of particular relevance here, this modification is known to be present in the native $tRNA^{Gln}$ (CUA) from *T. thermophila* and has been shown to increase suppression efficiency in yeast (41). We do not know whether THG73 is modified after injection by oocyte enzymes; but if not, it is likely that *in vitro*, post-transcriptional modification (42, 43) of A^{37} to i6A would further increase the suppression efficiency of THG73.

We emphasize that, viewing efficiency from a practical viewpoint, the present protocols routinely result in microampere currents from nAChR. This indicates that the quantity of protein produced is comparable with that produced in conventional *Xenopus* heterologous expression studies (although substantially more mRNA is being injected). Thus, we expect that unnatural amino acid incorporation can now be applied to the full range of systems that have been studied with the *Xenopus* heterologous expression system.

We anticipate that THG73 will also lead to improved efficiency and fidelity in *in vitro* studies of unnatural amino acid incorporation. The *in vitro* methodology has been used extensively in many systems, and considerable variability in efficiency of incorporation has been noted, including some unnatural residues that lead to no detectable incorporation with the existing methodology (7). Experiments should be attempted with THG73 acylated with the more problematical residues.

Lack of Reacylation—A major benefit of THG73 is the essentially complete elimination of receptors that have incorporated a natural amino acid resulting from acylation of the suppressor tRNA by an endogenous synthetase. Part of this improvement arises simply because the increased operational suppression efficiency allows the injection of severalfold less THG73, so that the absolute concentration of tRNA available for reacylation is lower than with MN3. The major improvement, however, derives directly from the diminished interactions with endogenous synthetases.

The tRNA elements recognized by synthetases are typically located in only a few regions of the tRNA (anticodon, acceptor stem, and N^{73}), (14–16). As such, nucleotide substitutions designed to decrease productive interactions with the cognate synthetase might lead to undesired, productive interactions with one of the other nineteen noncognate synthetases. Therefore, it was critical to assess whether THG73 was being acylated by endogenous synthetases. Our results thus far with the nAChR have revealed no significant complications due to endogenous reacylation of THG73. However, because this methodology will be applied to other proteins and to other heterologous expression systems, it is worthwhile to explicitly consider the likelihood of endogenous reacylation of THG73 by each of the aminoacyl-tRNA synthetases.

Table II summarizes such an analysis. If a residue is known to produce viable nAChR when inserted at a particular location, its cognate synthetase can be rejected on the grounds that no functional receptors are produced on injection of THG73-dCA. Mutagenesis studies at α198 and β262 allow a number of amino acids to be rejected. At α180 the wild type residue is Asp or Glu in muscle receptors, but in neuronal nAChR, a much broader range of residues is found. We assume that any residues that function at this site in the neuronal receptors will also function in the muscle receptor. This reasoning removes several other aminoacyl-tRNA synthetases from consideration (Table II).

A second criterion for eliminating synthetases as potential candidates for acylation of THG73 is a consideration of rules that govern tRNA recognition and identity (14–16). With the exception of the Ala, Ser, and Leu tRNAs, the important recognition elements for synthetases are typically located in the anticodon loop (positions 34–36) and/or position 73. Thus, the synthetases with the greatest likelihood of acylating THG73 would be those that recognize a C^{34}, U^{35}, A^{36}, and/or G^{73} in the cognate tRNA. Table II lists the nucleotides found at these positions in eukaryotic tRNAs for all 20 isoaccepting groups and the number of overlaps with the corresponding nucleotides of THG73. As shown in Table II, tRNAs for 12 isoaccepting groups have either zero or only one nucleotide in common with THG73 at these positions, indicating that their corresponding synthetases are unlikely to acylate THG73. Transfer RNAs for seven isoaccepting groups share two nucleotides in common with THG73. However, six of the seven synthetases (all but Gln) can be ruled out based on the AChR viability data.

TABLE II
Analysis of the potential for recognition of THG73 by all aminoacyl-tRNA synthetases

For the nAChR columns, × indicates an amino acid known to function at the appropriate site in muscle nAChR; n indicates a residue that is not found in muscle receptors but is found in neuronal nAChR. The synthetase columns list the nucleotide(s) found at the identified position in the appropriate isoaccepting group and the number of positions in which the residue is compatible with that found in THG73 (No. of overlaps). The residues found in THG73 are listed at the top of the columns.

Rejection based on viability in the nAChR				Rejection based on tRNA/synthetase recognition rules				
Amino acid	α180	α198	β262	G^{73}	C^{34}	U^{35}	A^{36}	No. of overlaps
Ala				A	N	G	C	0[a]
Cys			×	U	G	C	A	1
Asp	×			G	G	U	C	2
Glu	×			A	Y	U	C	2
Phe		×	×	A	G	A	A	1
Gly	n			A	N	C	C	0
His			×	U-A	G	U	G	1
Ile			×	A	(G)	A	U	0
Lys	n			G	Y	U	U	3
Leu			×	A	N	A	G	
					Y	A	A	(1)[a]
Met				A	C	A	U	1
Asn	n		×	G	G	U	U	2
Pro				C	N	G	G	0
Gln				U	Y	U	G	2
Arg	n			G	N	C	G	
					Y	C	U	1
Ser	n		×	G	N	G	A	
					G	C	U	(2)[a]
Thr	n		×	U	N	G	U	0
Val			×	A	N	A	C	0
Trp		×		A	C	C	A	2
Tyr		×		A	G	U	A	2

[a] For several isoaccepting groups, anticodon recognition is not crucial, but other key recognition elements are lacking in THG73 (14–16).

Based on the synthetase recognition rules and AChR viability data, we consider that only two synthetases deserve serious consideration as potential candidates for acylation of THG73. First, THG73 possesses C^{34} and U^{35}, which are known strong recognition elements for GlnRS, (28, 30), and we have no nAChR viability data for this amino acid. However, THG73 lacks the G^{36} and U^{73} that are likely to be strong recognition elements for the *Xenopus* GlnRS (28, 30). Moreover, *in vitro* acylation studies show that the native *T. thermophila* $tRNA^{Gln}$ is not a substrate for GlnRS from either plants (wheat germ) (13) or mammals (rabbit) (12). We therefore conclude that the *Xenopus* GlnRS is unlikely to acylate THG73 under the injection conditions presently employed.

This leaves LysRS as the only remaining candidate. Like GlnRS, LysRS can misacylate noncognate tRNAs that possess CUA anticodons (5). Lys is found in some neuronal nAChR α subunits at position 180, and we assume that functional receptor would have resulted had Lys been incorporated at 180 in the muscle-type nAChR studied here. In short, it remains a formal although unlikely possibility that reacylation by endog-

enous LysRS could become a complicating factor in other studies using TH73G.[2]

It should be noted that our test for recognition by an endogenous synthetase (the observation of functional receptor after injection of THG73-dCA) is quite stringent. In an actual experiment involving incorporation of an unnatural amino acid, a THG73-dCA-amino acid is injected; therefore, much less unacylated THG73-dCA becomes available for interaction with endogenous synthetases. Consistent with this argument, MN3-dCA alone produces currents that are ~60% of those obtained by injection of MN3-dCA-Tyr at α180 (Table I). However, treating ACh responses of Fig. 5*A* as the sum of two components (one produced by the wild type Leu and the second due to receptors with another, unknown residue) reveals that <10% of the total current from injection of MN3-dCA-Leu at β262 is due to presumed editing or reacylation of MN3-dCA formed from MN3-dCA-Leu. This suggests that reacylation may be much less of a problem when a charged tRNA is injected into the oocyte. Nonetheless, THG73 eliminates even this reacylation, an important consideration for quantitative experiments.

To summarize, in all cases studied here conditions can be found under which reacylation/editing by endogenous synthetases is not a complicating factor when THG73 is used. In other words, in the present system THG73 is *functionally* "null" with respect to its amino acid identity in the *Xenopus* oocyte. Although it has not been observed here, we acknowledge the possibility that, in a still more tolerant reporter system, endogenous reacylation might become a complicating factor. At present, however, the lower limit for detection of functional receptors is at the threshold of the read-through level of UAG codons inherent in the oocyte's translation apparatus. Although higher eukaryotic organisms do not possess conventional UAG-suppressing tRNAs having a CUA anticodon, a low level of read-through at UAG termination codons has been observed in a number of these systems (44). This read-through is presumably produced by "wobble-pairings" involving UAG codons and (*a*) the U/CUG anticodons of wild type glutamine tRNAs and/or (*b*) the GUA anticodon of tyrosine tRNAs that lack the queuine modification of the G^{34} in the anticodon (44).

General Applicability—Our engineered THG73 nonsense-suppressing tRNA is likely to have broad applicability. Works in progress in these labs establish that other proteins in addition to the nAChR can be studied. Also, nonsense suppression has been observed in mammalian cells (45–47), and since *Xenopus* and mammalian tRNAs strongly resemble each other, it seems probable that THG73 will possess a "null" amino acid identity in somatic cells lines as well. Thus, by combining new methodologies for introducing macromolecules into somatic cell lines with the use of our new nonsense-suppressing tRNA (THG73), it should now be possible to extend the benefits of unnatural amino acid incorporation to other eukaryotic systems.

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[2] Recent results with position α76 of the nAChR, which is Lys in the wild type receptor, give a large value for R_{uaa}, indicating no complications from reacylation by endogeneous LysRS (J. P. Gallivan, unpublished results from these labs).