

Identification of amino acids inserted during suppression of UAA and UGA termination codons at the *gag-pol* junction of Moloney murine leukemia virus

(translational readthrough/genetic code/retroviruses/tRNA/codon context)

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ABSTRACT Expression of the murine leukemia virus *pol* gene occurs by translational readthrough of an in-frame UAG codon between the *gag* and *pol* coding regions. In a previous study, we mutated the UAG codon to UAA or UGA and demonstrated that both of these termination codons could be suppressed in reticulocyte lysates and in infected cells with the same efficiency as UAG. We now report the identity of the amino acids inserted *in vitro* in response to UAA and UGA in fusion products containing the *gag-pol* junction region. The results show that UAA, like UAG, directs the incorporation of glutamine, whereas UGA directs the incorporation of three amino acids, arginine, cysteine, and tryptophan. To our knowledge, this is the first report indicating misreading of UAA as glutamine and UGA as arginine and cysteine in higher eukaryotes. Interestingly, although our protein synthesis system presumably contains other known UAG and UGA suppressors, these tRNAs did not suppress the termination codons in our experiments. Thus, it seems possible that the sequence surrounding the *gag-pol* junction not only promotes suppression but also helps determine which tRNAs function in suppression.

Readthrough suppression of termination codons is a mechanism of translational regulation that is used by a number of viruses infecting higher eukaryotes. The analysis of this phenomenon thus provides new information on the functional capabilities of the translational apparatus of these organisms. We have been investigating suppression in the murine leukemia virus (MuLV) system (1, 2). MuLV, like other mammalian type C retroviruses, regulates *pol* gene expression by translational readthrough of an in-frame UAG codon between the *gag* and *pol* coding regions (3–5). Suppression of this termination codon occurs with a frequency of about 5% (3–5) and is mediated by insertion of a glutamine residue at the position corresponding to the UAG (6). The readthrough product is a 200-kDa *gag-pol* fusion protein, which is cleaved at a late stage in virus assembly to give rise to protease, reverse transcriptase, and integrase (3).

To determine whether the signals that govern readthrough are unique for UAG, we used oligonucleotide-directed mutagenesis to change the UAG at the Moloney (Mo)-MuLV *gag-pol* junction to UAA or UGA (2). Interestingly, we found that these termination codons could be efficiently suppressed both in reticulocyte lysates and in infected cells (2). These results demonstrated that the suppression signals are not specific for UAG and that mammalian cells and cell extracts contain tRNAs capable of suppressing UGA and UAA codons which appear in a retroviral context. Similar findings have been reported by other investigators working

with tobacco mosaic virus (TMV) (7), Sindbis virus (8), and MuLV (9).

In the present study we have identified the amino acids inserted in response to UAA and UGA by using an *in vitro* MuLV suppression system (1). We find that UAA, like UAG, directs the incorporation of glutamine into protein, whereas UGA directs the incorporation of three amino acids, arginine, cysteine, and tryptophan. To our knowledge, misreading of UAA as glutamine and UGA as arginine and cysteine has not previously been reported in higher eukaryotes.

MATERIALS AND METHODS

Radioactive Amino Acids. [³H]Arginine (35 Ci/mmol; 1 Ci = 37 GBq), [³H]glutamine (50 Ci/mmol), and [³H]leucine (143 Ci/mmol) were purchased from Amersham. [³H]Tryptophan (30 Ci/mmol) and [³⁵S]cysteine (962 Ci/mmol) were obtained from New England Nuclear.

Construction of Plasmid Clones. The plasmids pEHS.UAG, pEHS.UGA, pEHS.UAA, and pEHS.CAG were constructed as follows. A double-stranded oligonucleotide consisting of a 5' *Eco*RI site, a short leader sequence, sequences from the Mo-MuLV *gag-pol* region (10), and a 3' *Hind*III site was inserted between the SP6 promoter of pGEM-4Z (Promega) and a portion of the *Staphylococcus aureus* protein A gene (11) (Fig. 1). Thus, the oligonucleotide was ligated at its 5' end to the *Eco*RI site of an *Eco*RI-*Sal* I fragment of the SP6 vector, and at its 3' end to the *Hind*III site of a *Hind*III-*Sal* I fragment of the plasmid pRIT2T (Pharmacia), containing the coding sequence for the protein A binding domain (nt 742–2001; ref. 11). In the course of the three-fragment ligation reaction, the SP6 segment was joined to the pRIT2T segment at the *Sal* I sites. The plasmids were each sequenced from the SP6 promoter region through the protein A binding domain by using a Sequenase kit (United States Biochemical) (data not shown).

Protein Synthesis and Analysis. The plasmids were digested with *Pst* I (which cleaves in the polylinker region of pRIT2T upstream of the *Sal* I site) and were transcribed with SP6 RNA polymerase (1). The resulting mRNAs were translated in rabbit reticulocyte lysates supplemented with calf liver tRNA (Promega) at 50 µg/ml and with the indicated radiolabeled amino acids (³H, 0.5 mCi/ml; ³⁵S, 0.85 mCi/ml), as described (1). Incubation was at 30°C for 30 min unless indicated otherwise. The protein products were isolated by binding to rabbit IgG-Sepharose (Pharmacia) (13) and analyzed in SDS/12.5% polyacrylamide gels.

Amino Acid Sequence Analysis. Large-scale protein synthesis reactions (0.25–2.0 ml) were performed. After purifi-

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Abbreviations: MuLV, murine leukemia virus; Mo-MuLV, Moloney MuLV; TMV, tobacco mosaic virus; nt, nucleotide(s).

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SP6	Leader	<i>gag</i>	TERM OR SENSE	<i>pol</i>	Protein A
(nt) 31	15	6	3	57	360

FIG. 1. Insert structure of plasmid clones. The schematic diagram illustrates the structure of the insert sequences that are transcribed by the SP6 promoter. The leader sequence encodes five amino acids from the chicken pre-lysozyme leader peptide (Met-Arg-Ser-Leu-Gly) (12). The termination or sense codons are TAG, TGA, TAA, or CAG. The diagram is not drawn to scale. nt, Nucleotides.

cation on IgG-Sepharose and gel electrophoresis, the resulting protein band was excised from the gel, eluted with water, and processed for amino acid sequence analysis by Edman degradation in a Beckman 890C automated spinning-cup sequenator (14).

RESULTS

Translation of SP6-Promoted Transcripts Containing Termination or Sense Codons. To determine the identity of amino acids inserted in response to termination or sense codons, we used an approach similar to the one described by Jacks *et al.* (15) in their analysis of ribosomal frameshifting in human immunodeficiency virus. We designed mini-construct plasmids with an SP6 promoter followed by a 15-nt leader sequence (with an AUG codon) linked to 6 nt from the 3' end of *gag*, a termination or sense codon (UAG, UAA, UGA, or the glutamine codeword CAG), 57 nt from the 5' end of *pol*, and the sequence encoding the binding domain of protein A (Fig. 1). SP6 transcripts of each plasmid were generated and translated in a rabbit reticulocyte lysate (1). Protein A fusion products ("suppressed products") were purified by binding to IgG-Sepharose and analyzed in an SDS/polyacrylamide gel (Fig. 2). Products in which suppression does not occur ("terminated products") are only seven residues long and were not detected; thus, the level of suppression is not directly measured in these experiments.

As illustrated in Fig. 2, a single band with the expected molecular mass of 18 kDa was obtained for each construct; no bands were observed in the absence of mRNA (lane 1). In the case of UAA, the protein product was not detected (lane 4) unless the reticulocyte lysate was supplemented with additional tRNA (lane 5); in this experiment the tRNA was from Mo-MuLV-infected NIH 3T3 mouse cells. This result is in accord with our previous finding (2) that translation of a 5-kb Mo-MuLV *gag-pol* mRNA containing the UAA codon (pMXH-UAA) is dependent on the addition of mouse cell tRNA (from infected or uninfected cells) or rabbit liver tRNA to the reticulocyte lysate. The amount of product generated with the CAG construct (lane 6) was about 4- to 5-fold greater than that observed with the UAG, UGA, or UAA constructs (lanes 2, 3, and 5, respectively), indicating that translation of the sense codon CAG is more efficient than suppression of any of the termination codons.

Amino Acid Sequence Analysis of the Protein A Fusion Products. It was of interest to determine the identity of the amino acids inserted *in vitro* in response to the termination and sense codons. The sequence of the first 20 amino acids present in the 18-kDa protein A fusion product is shown in Fig. 3; the position of the unknown residue (position 8) is

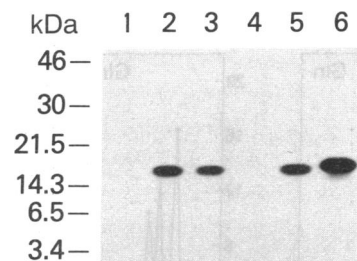


FIG. 2. Electrophoretic gel analysis of protein A fusion products synthesized in rabbit reticulocyte lysates. Procedures for transcription, translation, and isolation, and gel analysis of the fusion proteins are described in *Materials and Methods*. [³H]Leucine was used in the protein synthesis reactions. Addition of mRNA (designated by the codon at the *gag-pol* junction) was as follows. Lanes: 1, minus mRNA; 2, UAG; 3, UGA; 4, UAA; 5, UAA plus additional tRNA (40 μg/ml) from Mo-MuLV-infected NIH 3T3 cells (1); 6, CAG. The positions of molecular mass standards (kDa) are shown at left.

indicated by a boxed X. To obtain sufficient material for analysis, large-scale reactions containing 1 labeled amino acid and 19 unlabeled amino acids in excess were performed. The protein products were purified and sequenced as described in *Materials and Methods*.

Transcripts containing UAG, UAA, or CAG codons at the *gag-pol* junction were translated in reactions with [³H]-glutamine (Fig. 4A). The protein A fusion product should have glutamine at positions 11 and 13 (Fig. 3), and inspection of Fig. 4A shows that in each case, glutamine was detected in the 11th and 13th cycles of the Edman degradation. Since CAG is a glutamine codeword, it is not surprising that glutamine was inserted at position 8 in the CAG reaction. Glutamine was also incorporated at position 8 in response to the UAG-containing mRNA; this result indicates that the *in vitro* translation reaction mimics suppression *in vivo*, where it is known that the UAG codon at the MuLV *gag-pol* junction is read as glutamine (6). In addition, the data of Fig. 4A show that glutamine was inserted at position 8 in response to UAA, demonstrating that a glutamine isoacceptor can serve as a UAA suppressor in mammalian cells. Comparison of the UAG and UAA results with the data obtained for the CAG product (which can be viewed as a "standard" for glutamine incorporation) shows that the relative amounts of glutamine inserted at positions 8, 11, and 13 were the same in all three fusion proteins. This finding provides strong evidence that UAG and UAA, like CAG, are translated predominantly, if not exclusively, as glutamine at position 8. The data of Fig. 4B represent control experiments which show that [³H]leucine is found only at position 4 in the UAG, UAA, and CAG products, as predicted from the amino acid sequence (Fig. 3).

To determine which amino acid is inserted in response to UGA, we tested all amino acids whose codons differ from UGA by a single nucleotide and/or which have G at the second position: arginine, cysteine, glycine, leucine, serine, and tryptophan. Surprisingly, not one, but three amino acids—arginine, cysteine, and tryptophan—were found at position 8 (Fig. 5). Arginine, as anticipated (Fig. 3), was also found at positions 2 and 20. The frequency of insertion of arginine into position 8 was quite low, presumably reflecting competition between the labeled arginine and unlabeled tryptophan and cysteine, which were also present in the

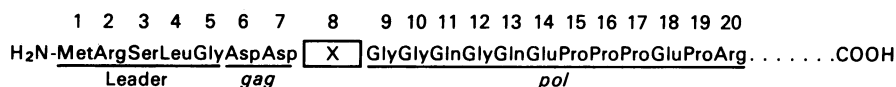


FIG. 3. Amino acid sequence at the Mo-MuLV readthrough site. The predicted amino acid sequence of the first 20 amino acids in the protein A fusion product is shown. The unknown residue at position 8 is indicated by a boxed X.

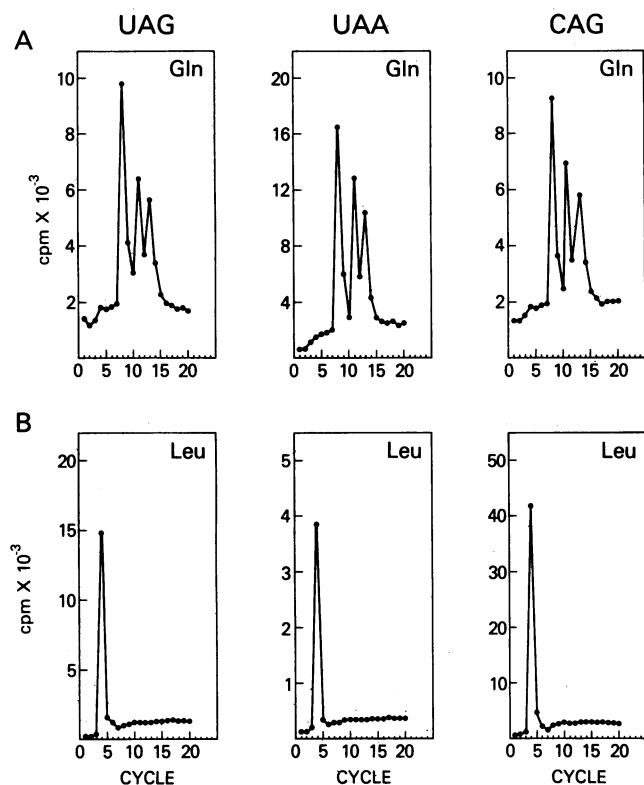


FIG. 4. Amino acid sequence analysis of protein A fusion products synthesized by mRNAs containing UAG, UAA, or CAG codons at the readthrough site. Protein synthesis reactions were carried out with [^3H]glutamine (A) or [^3H]leucine (B). The final volumes of the reaction mixtures in A were 1 ml for UAG and UAA and 0.3 ml for CAG; incubation of the UAG and UAA reactions was at 37°C for 20 min. The final volumes of the reaction mixtures in B were each 0.25 ml. Amino acid sequence analysis was performed by Edman degradation as described in *Materials and Methods*.

protein synthesis reaction. The tryptophan and cysteine peaks at position 8 were very sharp. The relatively high level of background radioactivity in the tryptophan profile was seen in repeated experiments and may reflect impurities or degradation products in the tryptophan preparation. Since the [^{35}S]cysteine had a specific activity ≈ 30 -fold greater than that of [^3H]tryptophan and [^3H]arginine, comparison of the radioactivity levels detected at position 8 suggests that the molar incorporation of cysteine was slightly lower than that of the other two amino acids. Experiments with serine, glycine, and leucine showed that serine was inserted at

position 3, glycine at positions 5, 9, 10, and 12, and leucine at position 4, as predicted from the sequence (Fig. 3); however, none of these amino acids was detected at position 8 (data not shown).

DISCUSSION

In the present study, we have used mini-constructs to identify the amino acids inserted *in vitro* in response to UAA and UGA termination codons placed at the MuLV *gag-pol* junction. We find that UAA, like UAG, directs the incorporation of glutamine, whereas three amino acids—arginine, cysteine, and tryptophan—are incorporated in response to UGA.

The observation that glutamine is inserted into the protein A fusion protein in response to UAG (Fig. 4A) emphasizes the parallel between suppression *in vitro* and *in vivo* (1, 2, 6) and supports the use of the *in vitro* system in this study. It is of particular interest that leucine is not incorporated at the UAG position (Fig. 4B), since calf liver tRNA, the major source of tRNA in the supplemented reticulocyte lysate (1), is known to contain two leucine amber suppressor tRNAs (16). This lack of leucine incorporation highlights the specificity of the MuLV suppression system.

While Kuchino *et al.* (17) reported that infection with Mo-MuLV alters the cellular profile of glutamine tRNAs, we found no difference between tRNAs of infected and uninfected cells (1). In contrast to the proposal of Kuchino *et al.* (17), our results (1, 2), as well as those of Panganiban (18), strongly suggested that suppression is mediated by a cellular tRNA that is neither altered nor induced as a result of virus infection.

The identity of the mouse cell glutamine isoacceptor that suppresses UAG at the MuLV *gag-pol* junction is not known. Kuchino *et al.* (17) isolated a minor glutamine tRNA that was reported to suppress the UAG codon of TMV RNA in a rabbit reticulocyte lysate. It is not clear that this tRNA can also suppress the MuLV UAG codon as suggested (17), however, since the MuLV and TMV systems are not entirely equivalent. Thus, in contrast to the situation with MuLV (Fig. 4), the tRNA specificity for *in vitro* suppression of the TMV UAG codon is less stringent and several tRNAs exhibit suppressor activity, including glutamine (17) and leucine (16), as well as the hypomodified tyrosine tRNA that is the TMV suppressor *in vivo* (19).

To date a mammalian UAA suppressor has not been isolated and indeed, UAA was once considered to be an absolute termination codon in mammalian cells (20, 21). Recently, it has been possible to demonstrate readthrough suppression of UAA in viral systems where the natural UAG codon [MuLV (2, 9) and TMV (7)] or UGA codon [Sindbis

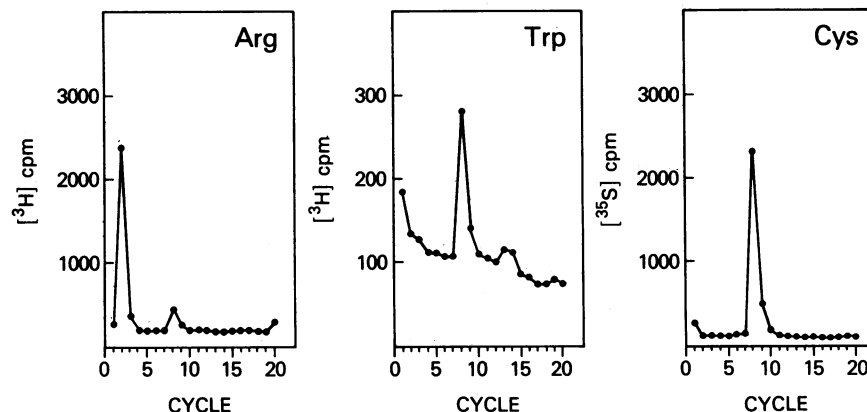


FIG. 5. Amino acid sequence analysis of protein A fusion products synthesized by mRNA containing UGA at the readthrough site. Protein synthesis reaction mixtures (final volume, 2 ml) contained [^3H]arginine, [^3H]tryptophan, and [^{35}S]cysteine, respectively, and were incubated at 37°C for 20 min.

virus (8)] was mutated to UAA. Presumably, in these cases, an appropriate nucleotide context (22, 23) was present that allowed suppression to occur (see below). The present results show that a glutamine isoacceptor can function as a UAA suppressor in mammalian cell extracts (Fig. 4A) and, to our knowledge, represent the first identification of an amino acid inserted in response to UAA in a higher eukaryote.

Suppression of UAA and UAG termination codons by glutamine tRNAs has a precedent in yeast. The yeast glutamine tRNA that can suppress UAA normally decodes CAA (24), while a different isoacceptor, which normally decodes CAG, suppresses UAG (25, 26). Whether UAA and UAG are suppressed by two distinct glutamine tRNAs in mammalian systems, as they are in yeast, is not clear. In this connection, it may be relevant that additional tRNA must be added to mammalian extracts for efficient suppression of UAA, but not of UAG (Fig. 2 and ref. 2).

The finding that UGA is decoded as three different amino acids (Fig. 5) is unique. Moreover, to our knowledge, misreading of UGA as cysteine and arginine has never been described in mammalian cells. Interestingly, the present results are in accord with early codon-recognition studies from Nirenberg's laboratory which showed that fractionated (27) and unfractionated (28) guinea pig liver tRNA acylated with cysteine binds to ribosomes in response to UGA. Subsequently, Hatfield (29) found that fractionated arginine, cysteine, and tryptophan tRNAs from calf liver show a weak response to UGA in the ribosomal binding assay.

It is of interest to consider the tRNAs that may mediate UGA suppression in the MuLV system. Tryptophan tRNA involvement in UGA suppression has already been noted in normal mammalian cells and bacteria. Thus, based on evidence that reticulocyte tRNA enriched for tryptophan acceptor activity promotes *in vitro* suppression of a UGA termination codon in β -hemoglobin mRNA, Geller and Rich (20) proposed that mammalian tryptophan tRNA can function as a UGA suppressor. In bacteria, wild-type tryptophan tRNA and a mutant tryptophan suppressor tRNA with a G \rightarrow A change at position 24 (30) decode the UGG tryptophan codon and UGA *in vitro* (31) and *in vivo* (32). The suppressor tRNA also decodes the UGU cysteine codon with low efficiency *in vitro* (33). Since mammalian tryptophan tRNA, like its bacterial counterpart (30), is expected to have a CCA anticodon, interaction with UGA may require C-A mispairing at the third position of the codon. Similarly, insertion of cysteine (UGU and UGC codons) in response to UGA would be expected to involve mispairing at the third position of the codon. Although arginine has six codons and several isoacceptors (29), the most likely candidate for suppressor activity would appear to be a CGA-decoding tRNA, which could suppress UGA by G-U mispairing at the first position of the codon, in analogy to the interactions of glutamine tRNAs with UAA and UAG in yeast (24-26), and possibly in MuLV. It is also possible that as yet unidentified specialized UGA suppressor tRNA(s) are involved in the readthrough suppression that is observed at the *gag-pol* junction.

One fundamental aspect of the suppression phenomenon that has not yet been systematically analyzed in eukaryotes is the nature of the signal or context required for suppression and the role of this signal in suppression. The fact that our "mini-constructs," which contain only two codons from *gag* and 19 codons from *pol*, exhibit suppression in our experiments suggests that this limited region of the viral mRNA may contain all the sequences needed for suppression. Taken together, our results show that mammalian cells contain either four or five distinct tRNA species (depending on whether the same glutamine tRNA is used in UAG and UAA suppression) that will suppress termination codons in the context of the MuLV *gag-pol* junction. [That so many tRNAs can function in this way would seem very difficult to

reconcile with the idea of viral induction of suppressor tRNAs (17).] It is also striking that while still other mammalian tRNAs capable of suppression have been described—i.e., leucine UAG suppressors (16) and a UGA suppressor that is acylated with serine (29, 34) and ultimately converted *in vivo* to selenocysteinyl-tRNA (35)—these tRNAs clearly do not suppress in our assay system (Fig. 4B; data not shown). This observation raises the intriguing possibility that the "context" not only affects the efficiency of suppression but also determines which tRNAs will be used for suppression.

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