

The Signal for Translational Readthrough of a UGA Codon in Sindbis Virus RNA Involves a Single Cytidine Residue Immediately Downstream of the Termination Codon

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The nucleotide sequences surrounding termination codons influence the efficiency of translational readthrough. In this report, we examined the sequence requirement for efficient readthrough of the UGA codon in the Sindbis virus genomic RNA which regulates production of the putative viral RNA polymerase, nsP4. The UGA codon and its neighboring nucleotide sequences were subcloned into a heterologous coding context, and readthrough efficiency was measured by cell-free translation of RNA transcripts in rabbit reticulocyte lysates. The CUA codon immediately downstream of the UGA codon was found to be sufficient for efficient translational readthrough. Further mutagenesis of residues in the CUA triplet demonstrated that mutations at the second or third residues following the UGA codon (U and A, respectively) had little effect on readthrough efficiency. In contrast, replacement of the cytidine residue immediately downstream of the UGA codon with any of the other three nucleotides (U, A, or G) dramatically reduced the readthrough efficiency from approximately 10% to less than 1%. These results show that a simple sequence context can allow efficient readthrough of UGA codons in a mammalian translation system. Interestingly, compilation studies of nucleotide sequences surrounding eukaryotic termination codons indicate a strong bias against cytidine residues immediately 3' to UGA termination codons. Taken together with our results, this bias may reflect a selective pressure for efficient translation termination for most eukaryotic gene products.

Protein translation usually stops at one of the three termination codons: UAG (amber), UGA (opal), and UAA (ochre). However, termination can sometimes be leaky, and translation continues until a second termination codon is reached. As a result, an extended polypeptide can be produced. This phenomenon is called translational readthrough. Translational readthrough occurs in both prokaryotic and eukaryotic systems (reviewed in reference 37). Some viruses utilize this mechanism to produce small amounts of essential viral proteins. For example, bacteriophage Q β produces a minor coat protein by readthrough of a UGA codon (38). Tobacco mosaic virus (TMV, a plant RNA virus) synthesizes its RNA replicase via readthrough of a UAG codon (29). Moloney murine leukemia virus (MuLV, a type C retrovirus) uses translational readthrough of a UAG codon to produce viral reverse transcriptase (30, 41). Sindbis virus (SIN) and several other alphaviruses require translational readthrough of a UGA codon for the synthesis of the putative viral RNA-dependent RNA polymerase (36). In addition to viral systems, translational readthrough has also been observed in cellular mRNAs. For example, the mRNA encoding β -globin contains a leaky UGA codon (13). Some cellular enzymes contain selenocysteine residues at their active sites, which are decoded by UGA codons (35).

In general, translational readthrough is mediated by two types of tRNAs. Suppressor tRNAs, which can specifically recognize termination codons, are present in small amounts in both prokaryotes and eukaryotes (37). Naturally occurring suppressor tRNAs have been implicated in performing some specialized cellular functions (15), such as the insertion of a

selenocysteine residue at the active site of certain selenocysteine-containing enzymes (19, 20). Some normal tRNAs also have the ability to read termination codons, although at lower efficiency. For example, the leaky UGA codon in β -globin mRNA is misread by the cellular tRNA^{Trp} (13). Translational readthrough of UAG and UAA codons in TMV RNA is mediated by a cytoplasmic tRNA^{Tyr} with a G ψ A anticodon (43). In the context of MuLV RNA, a Gln residue is inserted in response to a UAG or UAA codon while three amino acid residues (Trp, Arg, and Cys) can be inserted in response to a UGA codon (10).

An important determinant of translational readthrough efficiency can be the nucleotide sequence 3' to the termination codon. This phenomenon has been well documented in prokaryotic systems in which the nucleotide immediately downstream of a termination codon is most influential (7, 9, 12, 32, 40). In bacteria, a purine residue at this position usually facilitates translational readthrough (1, 2, 8, 27). Recently, it has been reported that 3' context effects on translational readthrough also occur in eukaryotic systems (11, 33, 39). Readthrough of the UAG codon in TMV RNA is facilitated by a six-nucleotide sequence immediately downstream of the termination codon (33). For MuLV, the nucleotide context required for translational readthrough of the UAG codon appears to be more complex and includes the sequence immediately following the termination codon as well as a pseudoknot structure further downstream (11, 39). In this report, we have examined the nucleotide context which determines the efficient readthrough of the UGA codon in SIN RNA. In contrast to the more complex signals regulating translational readthrough of the UAG codons in TMV and MuLV RNAs, we found that efficient readthrough of the UGA codon in SIN RNA requires only a single cytidine residue immediately 3' to the termination codon.

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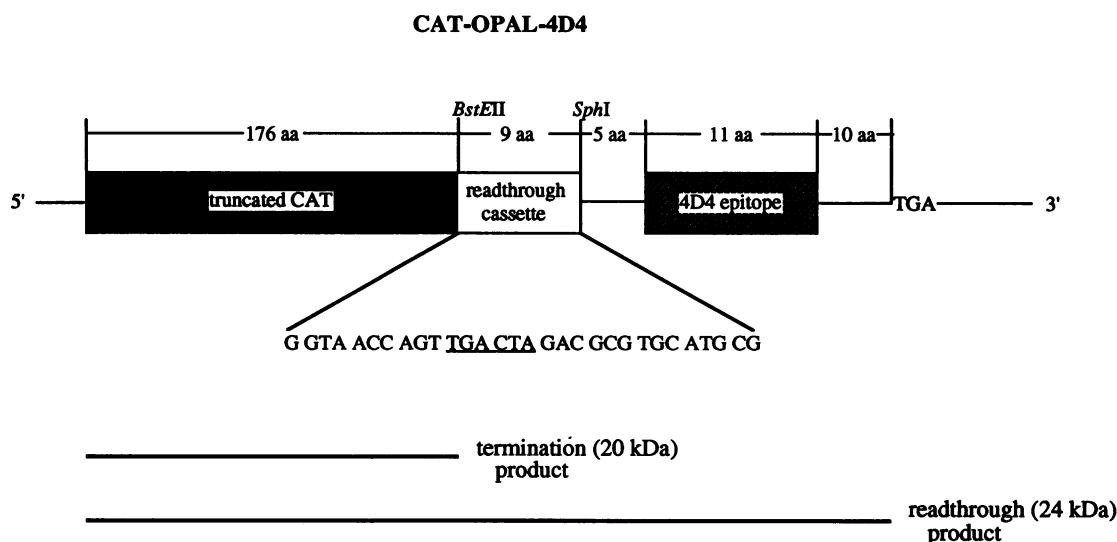


FIG. 1. Schematic structure of the translational readthrough module (CAT-OPAL-4D4). In the readthrough cassette (open box), only the UGA termination codon (underlined) and the 3' downstream codon CUA (underlined) are from SIN RNA. The flanking sequences are chosen not to match SIN sequences at the corresponding positions. The 5' and 3' sequences of the readthrough cassette contain engineered *BstEII* and *SphI* restriction sites (indicated). Through the *BstEII* and *SphI* sites, the readthrough cassette is fused in frame between a truncated CAT gene (5', closed box) and the 4D4 epitope of Rift Valley fever virus (3', hatched box). An SP6 promoter is present 5' to the CAT gene to allow production of CAT-OPAL-4D4 transcript RNA *in vitro* by using SP6 RNA polymerase. Upon translation in rabbit reticulocyte lysates, translational readthrough of the UGA codon in this heterologous context can be examined. The correct termination and readthrough products are expected to be approximately 20 and 24 kDa, respectively, in molecular mass and immunoprecipitable by anti-CAT antibody (both products) and anti-4D4 antibody (only the readthrough product). The transcription plasmid containing CAT-OPAL-4D4 was constructed in the original plasmid Prot25CATa⁺ (a gift from Henry Huang, Department of Molecular Microbiology, Washington University School of Medicine). The readthrough cassette and the 4D4 epitope were inserted at the *NcoI* and *SspI* restriction sites, respectively, in the CAT gene of Prot25CATa⁺. The readthrough cassette was constructed by annealing two chemically synthesized complementary oligonucleotides. The sequence of the plus-sense oligonucleotide is shown in the figure. By using T4 DNA ligase, the readthrough cassette was ligated to Prot25CATa⁺, which had been linearized by *NcoI* digestion and treated with mung bean nuclease to produce blunt ends. The resulting plasmid, whose structure was verified by DNA sequence analysis, was termed pCAT-OPAL. By the same approach, the 4D4 epitope was created by annealing two 45-mer complementary oligonucleotides containing a 5' *XbaI* site, the 33-nucleotide sequence encoding the 4D4 epitope, and a 3' *SspI* site (25). The 4D4 epitope was inserted at the *SspI* site of pCAT-OPAL, which had been linearized by a partial digestion with *SspI* (there are two *SspI* sites at nucleotides 601 and 964, respectively, in the plasmid Prot25CATa⁺). The correct insertion at the *SspI* site at position 601 was confirmed by DNA sequence analysis. The final construct, called pCAT-OPAL-4D4, contains the CAT-OPAL-4D4 module shown in the figure (not drawn in scale). aa, amino acids.

In SIN genomic RNA, a leaky UGA codon interrupts the nonstructural polyprotein-coding region between nsP3 and nsP4 (36). Since nsP4 is believed to be the viral RNA polymerase, translational readthrough of the UGA codon, which occurs at about 10% efficiency in reticulocyte lysates (see below), is necessary for viral RNA replication. To determine the sequences necessary for efficient translational readthrough of this UGA codon, we first subcloned a SIN cDNA fragment containing the UGA codon together with 485 upstream and 124 downstream bp into an SP6 transcription vector (31) containing the SIN capsid protein gene. The predicted translation product was a chimeric polypeptide with this portion of nsP3-nsP4 fused in frame between amino acid residues 227 and 228 of the SIN capsid protein. Cell-free translation of the resulting RNA transcripts in lysates of rabbit reticulocytes yielded the expected termination and readthrough products (~43 and 57 kDa, respectively) with readthrough efficiency of about 10% (data not shown), the same as that observed for the UGA codon in its original SIN RNA context (see below). These results suggested that the signal necessary for efficient readthrough of the UGA codon was contained within this 612-base region. A second chimeric construct was made in which the inserted sequence was shortened to 9 bp: the UGA codon and the two

downstream codons (CUAACC). About 10% readthrough of the UGA codon was also observed in this context (data not shown), indicating that the sequence(s) determining efficient readthrough resides within the CUAACC sequence immediately 3' the UGA codon.

To further characterize the readthrough signal(s), we designed an SP6 transcription vector (called pCAT-OPAL-4D4) to facilitate mutagenesis of the UGA codon and 3' flanking sequences (see Fig. 1 for details). The readthrough cassette contained the SIN UGA codon and the 3' CUA codon flanked by nucleotides chosen to be different from the SIN sequence at the corresponding positions. This cassette, which readily allowed engineering of substitutions by using synthetic oligonucleotides, was fused in frame between a truncated chloramphenicol acetyltransferase (CAT) gene (5') and the sequence encoding the 4D4 epitope of Rift Valley fever virus (3') (17). The structure of the predicted translation products allowed identification of the termination and readthrough products by size and by immunoprecipitation by using CAT- and 4D4-specific antibodies.

To determine the readthrough efficiency of the UGA codon in this context, *in vitro* transcripts were produced from pCAT-OPAL-4D4 templates by using SP6 RNA polymerase. The transcripts were translated in rabbit reticulo-

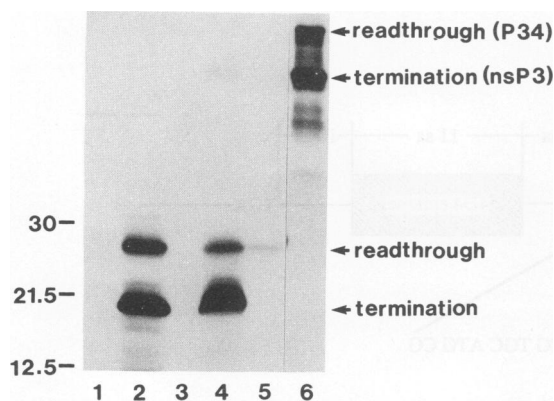


FIG. 2. The CUA triplet immediately downstream of the UGA codon provides enough information for efficient readthrough. Shown is a fluorogram of an SDS-13% polyacrylamide gel containing ^{35}S -labeled protein products from translation in rabbit reticulocyte lysates (Promega Biotec). Translation reactions (12.5 μl of reaction mixture) were carried out according to the manufacturer's protocols in the absence or the presence of exogenous template RNAs (~50 ng). The template RNAs were produced by *in vitro* transcription from *Xho*I-linearized pCAT-OPAL-4D4 by using SP6 RNA polymerase (26). Molecular mass standards (in kilodaltons) are indicated on the left. Termination and readthrough products are indicated on the right. Lane 1, no exogenous RNA added. Lane 2, the translation was directed by the *in vitro* transcript of pCAT-OPAL-4D4. Lane 3, same as lane 2, except that the translation products were immunoprecipitated with pre-immune rabbit serum before SDS-PAGE. Lane 4, same as lane 2, except that the translation products were immunoprecipitated with anti-CAT antibody (5' prime-3' prime, Inc.) before SDS-PAGE. Lane 5, same as lane 2, except that the translation products were immunoprecipitated with anti-4D4 antibody (provided by J. Smith, U.S. Army Medical Research Institute of Infectious Diseases) before SDS-PAGE. The 4D4-specific antibody reacted poorly but specifically with the readthrough product, and the fluorogram was overexposed in order to visualize this product. Lane 6, the translation was directed using an *in vitro* transcript encoding the entire nsP3 and nsP4 of SIN derived from pTM3/SINnsP34 (22). In this case, the termination product was the nsP3 protein (76 kDa) whereas the readthrough product was the P34 polyprotein (145 kDa). For quantitation, ^{35}S -labeled termination and readthrough products were excised from the gels, eluted from the gel slices, and measured by liquid scintillation counting (6). On the basis of the incorporation of [^{35}S]methionine and the methionine content in the termination and readthrough products, the efficiency of translational readthrough was determined to be $10\% \pm 1\%$ (both lanes 2 and 6). Standard deviations were calculated by using the data from three independent experiments.

cyte lysates, and the protein products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two translation products were observed (Fig. 2, lane 2), whose apparent molecular weights corresponded to those of the predicted termination and readthrough products (20 and 24 kDa, respectively). The identification of the major polypeptide (20 kDa) as the termination product and the minor polypeptide (24 kDa) as the readthrough product was further confirmed by immunoprecipitation assays. The 20-kDa product was immunoprecipitated by anti-CAT antibody (Fig. 2, lane 4), and the 24-kDa product was immunoprecipitated by both anti-CAT and anti-4D4 antibodies (Fig. 2, lanes 4 and 5). None of the polypeptides was precipitated by pre-immune rabbit serum (Fig. 2, lane 3). On the basis of the incorporation of [^{35}S]methionine into the termination and

readthrough products, the readthrough efficiency was calculated to be approximately 10% (Fig. 2), which was the same as that observed for the UGA codon in the original SIN RNA context, as measured by translation of a control transcript encompassing the entire nsP3-nsP4-coding region (Fig. 2, lane 6). In the latter construct, the termination product was the 76-kDa nsP3 protein and the readthrough product was the 145-kDa P34 polyprotein (Fig. 2, lane 6). We also examined translational readthrough of the UGACUA module placed in two other heterologous contexts: the gene encoding the SIN capsid protein (see above) and the M gene of mouse hepatitis virus. In the latter case, the UGACUA module was fused in frame between codons 123 and 124 of the M gene, which had previously been subcloned into a transcription vector (3). As before, the readthrough efficiency in reticulocyte lysates for either of these contexts was ~10% (24). These results suggest that efficient readthrough of the UGA codon in SIN RNA requires no more than the three downstream nucleotides (CUA).

To further define the readthrough signals residing in the CUA triplet, substitutions were introduced at each of the three nucleotide positions. Transcripts containing these mutations were produced *in vitro* by using SP6 RNA polymerase and translated in rabbit reticulocyte lysates, and the protein products were analyzed by SDS-PAGE. As shown in Fig. 3A, replacement of the C residue with any of the other three nucleotides (U, A, or G) (Fig. 3A, lanes 3 to 5) dramatically decreased the readthrough efficiency from 10% to less than 1% (which was the background level and therefore the limit of detection for these assays). Changes in the two downstream nucleotides (U and A) (Fig. 3A, lanes 6 to 11) had little effect on the readthrough efficiency of the UGA codon. When the UGA codon was replaced with either of the other two termination codons (UAG or UAA), the readthrough efficiency dramatically decreased to background levels (Fig. 3A, lanes 12 and 13). When the UGA codon was replaced by a sense codon (UGG, decoded as Trp), as expected, only one translated product, which comigrated with the readthrough product, was observed (Fig. 3A, lane 14).

It can be seen that the mutations at the second nucleotide (U) downstream of the UGA codon resulted in a slight mobility shift of the readthrough products (Fig. 3, lanes 6 to 8). At the protein level, the Leu residue (encoded by CUA) was replaced by Arg (CGA), Pro (CCA), or Gln (CAA), respectively, in these readthrough products. It seems likely that certain properties of the readthrough products (e.g., conformation, charge, or SDS-binding) were affected by these amino acid substitutions, leading to mobility shifts detectable by SDS-PAGE. To test this possibility, the UGA codon was replaced with the codons encoding Trp (UGG), Arg (CGA), and Pro (CCA), respectively. The resulting transcripts were translated in rabbit reticulocyte lysates, and the translation products were analyzed by SDS-PAGE. Because the termination codon (UGA) was replaced with sense codons, only one translation product was observed, corresponding to the readthrough product (Fig. 3B). Interestingly, it was found that these sense replacements sometimes resulted in translation products with distinguishable electrophoretic mobilities (Fig. 3B). With the Trp replacement (Fig. 3A, lane 14; Fig. 3B, lane 1), the translation product comigrated with the readthrough product of the wild-type transcript (Fig. 3A, lane 2) whereas the translation products with Arg or Pro sense replacements (Fig. 3B, lanes 2 and 3) migrated more slowly and correlated with the readthrough products of the mutant transcripts (Fig. 3A,

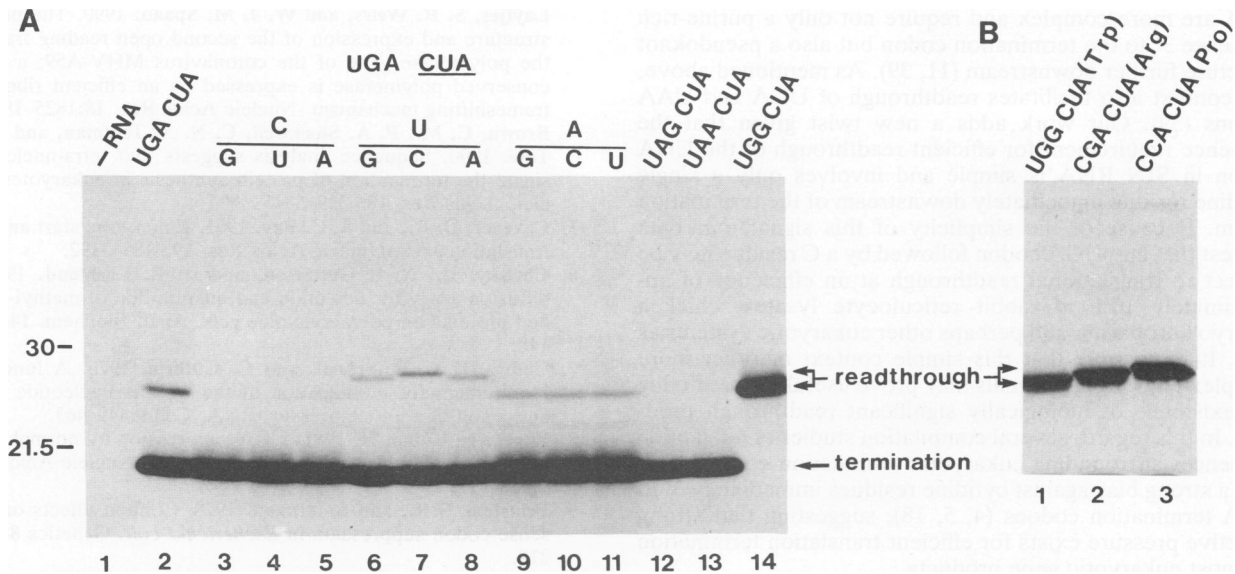


FIG. 3. The C residue immediately downstream of the UGA codon is the major determinant for efficient translational readthrough. Shown are fluorograms of SDS-13% polyacrylamide gels containing ³⁵S-labeled cell-free translation products programmed by in vitro transcripts of pCAT-OPAL-4D4 and its mutant derivatives. The wild-type sequence UGACUA and the specific mutations are shown on top of the figure. Molecular mass standards (in kilodaltons) are indicated on the left. Readthrough and termination products are indicated. (A) Lanes: 1, no exogenous RNA added; 2, the translation was directed by the wild-type transcript from pCAT-OPAL-4D4; 3 to 5, the translations were directed by the transcripts containing mutations at the first position downstream of the UGA codon; 6 to 8, the translations were directed by the transcripts containing mutations at the second position downstream of the UGA codon; 9 to 11, the translations were directed by the transcripts containing mutations at the third position downstream of the UGA codon; 12 and 13, the translations were directed by the transcripts which had the UGA codon replaced by the other two termination codons (UAG and UAA), respectively; 14, the translation was directed by the transcript which had the UGA codon replaced by a sense codon (UGG, decoded as Trp). (B) The translations were directed by the transcripts which had the UGA codon replaced by Trp codon (UGG, lane 1), Arg codon (CGA, lane 2), and Pro codon (CCA, lane 3). Readthrough efficiency was determined as described in the legend to Fig. 2. The readthrough efficiencies for the wild-type transcript and its mutant derivatives were $10\% \pm 1\%$ (lane 2), $<1\%$ (lanes 3 to 5), $8\% \pm 1\%$ (lanes 6 to 11), and $<1\%$ (lanes 12 and 13). All mutant constructs were created by replacing the readthrough cassette in pCAT-OPAL-4D4 (see Fig. 1) with synthetic oligonucleotides containing the indicated mutations. Oligonucleotides were ligated directly into pCAT-OPAL-4D4, which had been digested with *Bsr*EII and *Sph*I. The mutations were identified and verified by DNA sequence analysis.

lanes 6 and 7). These data indicate that the observed mobility shift was due to a single amino acid substitution in the readthrough product and shed light on the identity of the amino acid residue inserted at the UGA codon of SIN RNA. Recently, it was reported that three amino acid residues (Trp, Arg, or Cys) could be inserted in response to translational readthrough of a UGA codon in an MuLV RNA context in rabbit reticulocyte lysates (10). It was likely that the UGA codon in SIN RNA was recognized by one or more of these three misreading tRNAs. The data presented in Fig. 3, however, eliminate the possibility that the UGA codon in SIN RNA is primarily decoded as Arg, since an Arg residue at this position (Fig. 3B, lane 2) or a codon downstream (Fig. 3A, lane 6) resulted in a product with slower mobility, in comparison to the authentic readthrough product (Fig. 3A, lane 2). On the other hand, Trp replacement of the UGA codon led to a translation product (Fig. 3A, lane 14), which comigrated with the authentic readthrough product. The data are consistent with the contention that translational readthrough of the UGA codon in SIN RNA may result from misreading by the cellular tRNA^{Trp}, as has been documented in both prokaryotic and eukaryotic systems (8, 13, 16, 42).

Besides the cell-free translation studies reported here, the importance of the 3' downstream C residue for efficient translational readthrough of the UGA codon in vivo has also been demonstrated in the context of SIN-infected chicken embryo fibroblast cells (21). Although a detailed character-

ization of the readthrough signal could not be performed by using this approach, a silent C-to-U change at the position 3' to the UGA codon was shown to greatly reduce translational readthrough, as evidenced by diminished accumulation of products containing the nsP4 region (21). In spite of this reduction in the level of an essential replicase component, this SIN mutant was still capable of efficient replication in these cells. In addition, when the UGA codon between SIN nsP3 and nsP4 was replaced by a UAA or a UAG codon, both mutants could replicate efficiently, implying that readthrough had occurred even though these products were not readily detected (23). Although it is not known whether the SIN RNA context contains signals which might enhance the readthrough of these other termination codons, these observations raise the possibility that termination codons may never signal an absolute stop for translation.

Until recently, little was known about context effects on translational readthrough in eukaryotic systems, and the mechanism(s) involved has yet to be elucidated. Accumulating data suggest that readthrough efficiency can be modulated by diverse RNA signals and depends, as expected, on the particular translation apparatus being studied. Experiments by Skuzeski et al. (33) have revealed that translational readthrough of the UAG codon in TMV RNA requires a six-nucleotide sequence (CAR YYA) immediately downstream from the termination codon. In contrast, the signals for translational readthrough of the UAG codon in MuLV

RNA are more complex and require not only a purine-rich sequence 3' to the termination codon but also a pseudoknot structure further downstream (11, 39). As mentioned above, this context also facilitates readthrough of UGA and UAA codons (39). Our work adds a new twist given that the sequence requirement for efficient readthrough of the UGA codon in SIN RNA is simple and involves only a single cytidine residue immediately downstream of the termination codon. Because of the simplicity of this signal, our data suggest that any UGA codon followed by a C residue may be subject to translational readthrough at an efficiency of approximately 10% in rabbit reticulocyte lysates, chicken embryo fibroblasts, and perhaps other eukaryotic systems as well. It is possible that this simple context or other more complex readthrough signals may prove useful for predicting the existence of biologically significant readthrough products. In this regard, several compilation studies of nucleotide sequences surrounding eukaryotic termination codons indicate a strong bias against cytidine residues immediately 3' to UGA termination codons (4, 5, 18), suggesting that strong selective pressure exists for efficient translation termination for most eukaryotic gene products.

The readthrough signals found in TMV RNA (33), MuLV RNA (11, 39), and SIN RNA (this report) may involve different mechanisms. In prokaryotic mRNAs, the readthrough signals are similar to that identified in SIN RNA. Translational readthrough of UAG and UGA codons in bacteria is usually facilitated by a single purine residue (A or G) 3' to the termination codon (1, 2, 8, 27). In addition, a CUC or CUG triplet 3' to the UGA codon also enhances translational readthrough (8). Three mechanisms have been proposed to explain this effect of the 3' nucleotide on translational readthrough: (i) the recognition of termination codons by the polypeptide release factors could be influenced by the nucleotide 3' to the termination codons (32); (ii) the recognition of termination codons by misreading tRNAs might be affected by the adjacent 3' nucleotide via stacking energy (14); and (iii) the misreading tRNA and the tRNA recognizing the following codon may interact within the ribosome (34). The fitness (or stability) of this tRNA-tRNA interaction, which could be affected by the nucleotide immediately downstream of the termination codon, might then influence the efficiency of the elongation step and translational readthrough (2). Several lines of evidence argue against the first possibility. It has been reported that the context effect is dependent on specific suppressor tRNAs. In other words, the same context may have opposite effects if the translational readthrough is mediated by different suppressor tRNAs (2). Furthermore, context effects have also been observed for suppression of missense mutations (28). So far, the experimental data appear to support but not distinguish between the last two possibilities.

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