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Is the in-frame termination signal of the *Escherichia coli* release factor-2 frameshift site weakened by a particularly poor context?

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ABSTRACT

The synthesis of release factor-2 (RF-2) in bacteria is regulated by a high efficiency +1 frameshifting event at an in-frame UGA stop codon. The stop codon does not specify the termination of synthesis efficiently because of several upstream stimulators for frameshifting. This study focusses on whether the particular context of the stop codon within the frameshift site of the *Escherichia coli* RF-2 mRNA contributes to the poor efficiency of termination. The context of UGA in this recoding site is rare at natural termination sites in *E.coli* genes. We have evaluated how the three nucleotides downstream from the stop codon (+4, +5 and +6 positions) in the native UGACUA sequence affect the competitiveness of the termination codon against the frameshifting event. Changing the C in the +4 position and, separately, the A in the +6 position significantly increased the termination signal strength at the frameshift site, whereas the nucleotide in the +5 position had little influence. The efficiency of particular termination signals as a function of the +4 or +6 nucleotides correlates with how often they occur at natural termination sites in *E.coli;* strong signals occur more frequently and weak signals are less common.

INTRODUCTION

The mechanism used to decode translational stop signals during termination of the synthesis of a polypeptide is different from that used to decode sense codons during chain elongation. While the latter involves RNA:RNA interactions between three bases of the tRNA and mRNA at the decoding site of the small ribosomal subunit, the termination of protein synthesis involves interactions between the mRNA and a protein decoding molecule, the release factor, in place of the tRNA (1). Termination may involve further essential interactions between the release factor and the ribosome (2 and Pel,H.J., Rosenfeld,S. and Bolotin-Fukuhara,M., unpublished), and even between the release factor and the peptidyl-tRNA at the adjoining site (3). Despite wide acceptance of the different mechanisms mediating the decoding of sense and stop codons, protein synthesis termination signals have generally still been viewed as the originally proposed triplets: UAA, UGA and UAG (4,5). However, nucleotides both upstream and downstream of the codon may contribute to the termination signal. Restrictions in the upstream sequence could influence the nature of potential interactions of RF with the last amino acids and tRNA, and restrictions in the downstream sequence could reflect direct interaction of the RF with the mRNA itself (6).

Early experiments on non-cognate or cognate competitive suppression of these stop codons hinted that a region larger than the triplet codon might be important for the termination signal, since the efficiency of suppression was influenced by sequence context (7–11). Statistical analysis of the nucleotides surrounding natural stop codons (stop signals found at the end of genes) in genes from a wide range of organisms showed a strong bias in the nucleotides occurring in positions surrounding the codon, and particularly in the +4 position (the stop codon being +1 to +3) (12,13). In *Escherichia coli* we have shown that the termination efficiency of stop codons *in vivo* is indeed determined by the nucleotide immediately following the termination codon (+4) (14). The pattern is maintained in human cells where translation of an expressed type I 5′-deiodinase mRNA, in which an internal UGA encodes selenocysteine (Sec), has shown that the +4 nucleotide dramatically affects the competition between termination and Sec incorporation at this recoding site (15). Thus in genetic systems from these two kingdoms the +4 nucleotide significantly affects the efficiency by which a termination codon is decoded as a stop signal. While the effect of the identity of the +4 nucleotide is different between these prokaryotic and eukaryotic examples, in each case there is agreement between stop signal strength and frequency of occurrence at natural stop sites.

In addition, highly expressed genes use predominantly the strongest four-base stop signals (stop codon together with the following +4 nucleotide) (14,15), whereas stop signals at translational recoding sites (16) are mostly weak and are rarely used at natural termination sites (14,15). An example is UGAC which was determined to be the poorest termination signal in *E.coli* (14). This weak tetranucleotide stop signal occurs at the sites of two recoding events in *E.coli*, selenocysteine incorporation

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in the formate dehydrogenase mRNA (17,18) and at the +1 frameshift site in the translation of RF-2 (19).

The fact that the native signal at the RF-2 frameshift site, UGAC, was found to be significantly weaker than the other 11 four-base signals (14) raised the question of whether the effect was amplified in this particular context by the surrounding nucleotides. The sequence following the UGA termination triplet in the RF-2 frameshift site is CUA. Although suppression studies have not been carried out for UGAC**UA**, it has been demonstrated that UGAC**UG** and UGAC**UC** are strongly suppressed compared with some other UGAC contexts (for example, UGAC**AU**) (20). The occurrence of multiple CUA codons near the 5′ end of a coding sequence perturbs translation of the mRNA, possibly via a destabilising effect on the translational complex (21). This is likely to be because CUA is decoded by a rare tRNA (22); of 29 sense codons tested, CUA had the slowest rate of decoding (23).

In this study we have tested the proposal that CUA following a UGA termination codon might be an especially bad context for termination. All possible nucleotides were used in positions +4, +5 and +6 (3′ to the UGA) of the RF-2 frameshift site to create new contexts and the relative termination strengths of the resulting signals were determined.

MATERIALS AND METHODS

Materials

pMAL™-c2 plasmid and maltose binding protein (MBP) antibody, restriction enzymes and buffers, and T4 DNA ligase were purchased from New England Biolabs. [γ-^{32}P]dATP and Hybond transfer membranes were obtained from Amersham. Nitrocellulose transfer membranes were purchased from Schleicher and Schuell. Other chemical reagents were purchased from Sigma.

Deoxyoligonucleotides were either synthesised on site using an Applied Biosystems 380 B DNA synthesiser, or purchased from Macromolecular Resources, Colorado State University. Plasmid DNA was isolated using a Wizard™ Miniprep DNA purification kit (Promega). Cloned DNA was sequenced using a 373A AB1 Sequencer. Plasmids were electroporated into bacterial cells using an Electro Cell Manipulator© 600 (BTX).

Gel electrophoresis and protein transfer were performed using BioRad Mini-PROTEAN II electrophoresis cells and BioRad Mini Trans-Blot electrophoretic transfer cell. A GS-670 imaging densitometer (BioRad) was used for laser densitometry.

Computer sequence analysis

Statistical analyses of nucleotide sequences were performed on the set of *E.coli* data obtained from the 1995 release of the TransTerm Database (24). This database contains the sequence contexts around 3492 *E.coli* stop signals for 100 nucleotides (nt) before and after the stop codon. Only 3433 of these sequences had stop signals identified as far as the +6 nucleotide. The programme, ‘count_signal’, written by Mark Dalphin, was used to count the frequencies of various nucleotide patterns, such as UGAC, in both the termination position and in the 100 nt 3′ to the stop codon. If the 100 nt included another open reading frame, the counting stopped at the start of the open reading frame. The TransTerm database (24) was used to document how many stop signals of a defined length and sequence were present in any reading frame of the 100 nt of non-coding region immediately downstream of the natural termination sites of genes recorded in the database. For example, there are 4506 UGAN sequences in the non-coding regions of the recorded *E.coli* genes and 1494 (or 33%) are UGAU. Since there are 1081 UGAN sequences at natural termination sites we would expect 358 to be UGAU (33% of the 1081 UGAN stop signals). We looked for deviations from the ‘expected’ value using a normalisation scheme which we called ‘deviation’:

Deviation = (Observed – Expected)/Expected

Media and bacterial strains

Bacteria were grown in Luria Broth, and ampicillin (amp) resistance (final concentration 100 μg/ml) was used to select bacteria containing plasmids. Protein expression was induced from the P_{tac} promotor with 1 mM media concentration of IPTG (25). *Escherichia coli* strain TG1 was used for primary cloning. Plasmids were subsequently electroporated into *E.coli* strain FJU112 [Δ(lac pro) gyrA ara recA56/*10*, F′lacI^{Q1}] (26) for analysis of fusion proteins. Strain FJU112 has wild-type ribosomes and no suppressor tRNAs which could compete with termination or frameshifting events in our translational termination/frameshift assay.

Plasmid construction and analysis of expressed fusion proteins

Complementary deoxyoligonucleotides spanning the RF-2 frameshift window and containing UGACNA and UGACUN stop signal context series were annealed and directionally cloned into the pMal™ polylinker at *Eco*RI and *Sal*I sites using standard recombination techniques (25). The plasmid with the natural stop context UGACUA had been constructed previously (14). The plasmids were introduced into *E.coli* strain TG1 by electroporation (2.5 kV, 5–6 ms). Cells were selected for amp resistance. Recombinant clones were identified by colony hybridisation, using one of the oligonucleotides labelled with [γ-^{32}P]dATP as a probe. Positive transformants were screened for the presence of the RF-2 frameshift window by inducing expression of the fusion proteins. MBP fusion proteins were identified on a Western blot following electrophoresis. Plasmid DNA was isolated and the sequence was confirmed. The plasmids were electroporated into *E.coli* strain FJU112, fusion protein expression induced and the products analysed immunologically following Western blotting using MBP as described (14). Proportions of frameshift and termination products were determined by laser densitometry.

RESULTS

The context of the termination signal at the RF-2 frameshift site

The RF-2 frameshift site has the sequence CUA in positions +4, +5 and +6, following the stop codon UGA, as shown in Figure 1. This sequence is very rare at natural termination sites in *E.coli*; from 3492 genes currently available for analysis in the TransTerm database (24) there is only one possible natural termination site with this sequence, a putative unidentified open reading frame which terminates at a site overlapping with the initiation site of deoxyribopyrimidine photolyase. We have assumed this is a real termination site and have included it in our statistical analysis. Even so, the occurrence of UGACUA is much lower than could be anticipated from UGACUA frequencies in non-coding regions of the *E.coli* genome.

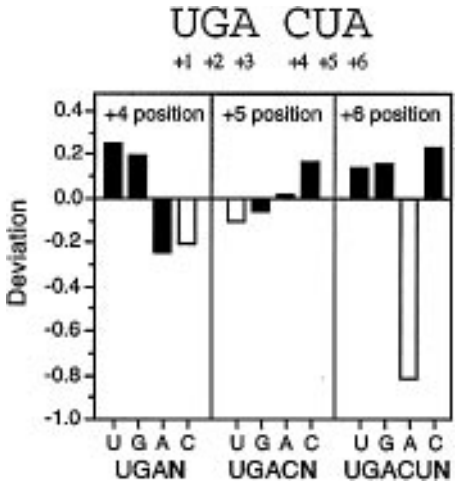

Figure 1. Statistical analysis of UGA contexts relevant to the RF-2 frameshift sequence. The stop signal context of the RF-2 frameshift site is shown at the top of the figure. Deviations between the observed and expected occurrences of each of the four nucleotides: UGAN, in the +4 position following natural UGA stop codons; UGACN, in the +5 position following natural UGAC signals; and UGACUN, in the +6 position following natural UGACU signals are indicated by bars above or below the 0.0 line. Bars above the line represent signals that occur more frequently than expected and bars below the line represent signals that occur less frequently than expected. The expected frequency of occurrence was calculated from the occurrences of the 4, 5 and 6 base sequences in non-coding regions spanning 100 bases downstream from termination codons. The deviation was then calculated as detailed in Materials and Methods. The nucleotide at position N is indicated for each bar. The open bars show the sequence found at the RF-2 frameshift site.

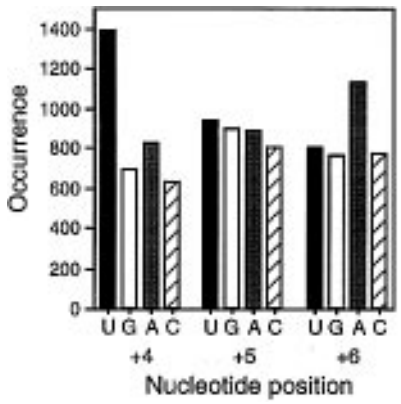

Figure 2. Frequency of occurrence of each of the four nucleotides in the +4, +5 and +6 positions following all three stop codons (UAA, UAG and UGA) in *E.coli* genes (24).

The frequency of occurrence of UGAN, UGACN and UGACUN at natural termination sites was compared with the expected frequency of these sequences calculated from the non-coding regions (with reference to the subgroup UGA for the UGAN series, the subgroup of UGAC sequences for the UGACN series and the subgroup of UGACU sequences for the UGACUN series). In this way the deviation between observed and expected frequency at a particular position was independent of that at previous positions. The expected tetra-, penta- and hexa-nucleotide frequencies were calculated by analysing the regions of DNA spanning 100 bases 3′ of the stop codons. This gave the expected frequencies of UGAN, UGACN and UGACUN compared with the observed occurrences of the sequences at natural termination sites.

The deviations between the observed and expected occurrences of each of the four nucleotides in the +4 position following natural UGA stop codons (UGAN), the +5 position following a UGAC sequence at natural signals (UGACN) and the +6 position following a UGACU sequence (UGACUN) show an interesting pattern as displayed in Figure 1. The nucleotide immediately 3′ of the UGA in the RF-2 frameshift site, C (+4 position) is found to be under-represented as a 4th base at UGAN natural termination sites, as is A, whereas U and G are over-represented. These differences between expected and observed occurrences reflect our experimentally determined stop signal strength, in that the under-represented UGAC and UGAA are weaker stop signals than the over-represented UGAU and UGAG (discussed below and in ref. 14).

The +5 nucleotide in the RF-2 frameshift site, U (+5 position) is found at below the expected frequency for this position at natural UGACN termination sites. At this 5th position the expected and observed values agree fairly closely for G and A, but there is some deviation between expected and observed for C. This suggests that a U present in the 5th position of a termination site, whether a natural or a recoding site, might be selected for or against by specific termination requirements.

A is very rare in the +6 position following UGACU natural termination sites, while the other nucleotides are over-represented in this position.

The nucleotides in positions +4 and +6 of the RF-2 frameshift site are rarely used following UGA and UGACU stop signals respectively. Are these nucleotides also under-represented in the downstream positions at other stop signals (UAA-containing, UAG-containing and other UGA-containing signals)? This is clearly the case for the 4th position where the low incidence of C is indeed found when all stop signals are considered and contributes, along with a high incidence of U, to the strong nucleotide bias at this position (Fig. 2). However, in position +6 a higher than expected incidence of A contributes significantly to the bias at this position when all stop signals are considered, and this contrasts with the low incidence of A as the 6th base in the UGACUN context (Fig. 1). The higher frequency of A is also apparent in non-coding regions and therefore must be independent of termination.

Experimental strategy to determine whether nucleotides following the termination signal affect signal strength

The statistical analysis (Figs 1 and 2) suggested that UGACUA is an unusual termination context. Experimentally, earlier suppression studies had shown that while UGAC contexts might be good for termination, there were exceptions to this general conclusion in cases where the sequence was followed by UG or UU (20). Is the competition between frameshifting and termination at the RF-2 frameshift site regulated by a particularly poor UGA termination context?

We previously examined the strength of each of the three termination codons with all contexts in the 4th position using a pMAL™ reporter construct and expression system with the RF-2 frameshift window cloned in-frame with the *malE* gene (14). An oligonucleotide spanning the RF-2 frameshift window, which contains a Shine–Dalgarno sequence (overlined), a ‘slippery’ run

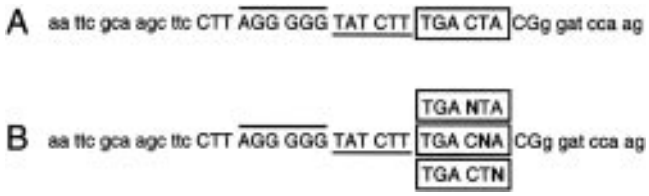

Figure 3. (**A**) The RF-2 frameshift window. The endogenous sequence of RF-2 is in capitals and restriction endonuclease sequences surrounding the frameshift window are in lower case. The six-base termination signal under investigation is enclosed in the box. Other motifs important for frameshifting are indicated; the Shine–Dalgarno sequence is overlined and the 'slippery' run of Ts is underscored. (**B**) The RF-2 frameshift window. The boxed sequences indicate the +4, +5 and +6 position redundancies (bold N) that were introduced to generate each series of clones.

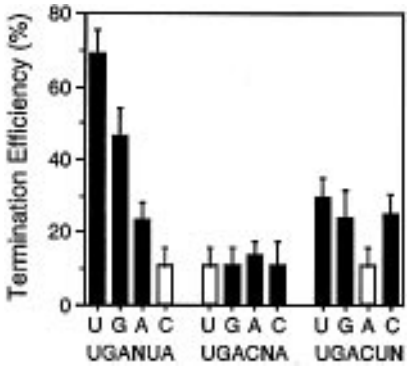

Figure 4. Influence on termination efficiency of substituting nucleotides at the 4th, 5th or 6th position of the RF-2 frameshift site UGACUA. The nucleotide in position N is indicated for each bar. The standard deviation is shown and was calculated from data derived from at least three experiments and in most cases from multiple isolates of the clone. For the UGACUA clone, the data from nine experiments was utilised. This clone was used in all three phases of the work and is represented by the open bars.

of Ts and leucine codon (underlined) and the TGA stop codon, is illustrated in Figure 3A. Oligonucleotides containing redundancies in the +4, +5 and +6 positions following the TGA were synthesised. These oligonucleotides were used to generate a series of constructs that contained the stop codon contexts; TGANTA, TGACNA and TGACTN (Figure 3B). *In vivo* transcription and translation of plasmids containing the RF-2 frameshift window produces two fusion proteins; a 44 kDa product when synthesis stops at the termination signal in the frameshift site and a 53 kDa frameshift product when a +1 frameshift event occurs and translation is halted further downstream. The constructs were expressed in FJU112, a wild-type strain of *E.coli* which has normal ribosomes and no suppressor tRNAs. Independent isolates of the variant clones were analysed for termination signal strength in three separate experiments. The wild-type UGACUA clone is common to all three series, and therefore the results from nine experiments have been combined for this clone.

A stronger termination signal results in more termination product compared with the amount of frameshift product. The converse is true if the termination signal is weaker. Therefore, the effects on termination signal strength of each of the nucleotides in positions +4, +5 and +6 following the stop signal can be measured from the proportions of the two products.

Figure 4 shows the termination efficiencies of the three series of clones, UGA**N**UA, UGAC**N**A and UGACU**N**. Confirming the results found previously, there is a hierarchy of termination signal strengths dependent upon the 4th nucleotide with a 7-fold range in their competitiveness with frameshifting in the order, UGA**U**UA > UGA**G**UA > UGA**A**UA > UGA**C**UA. Altering the nucleotide at position +5 had no significant effect upon the poor termination signal strength resulting from the 4th position C. In contrast, the nucleotide present at the 6th position increased termination strength by 2–3-fold. The natural sequence has A in this position (open bar) and this sequence was the weakest termination signal of the series, that is for the UGACUN contexts, termination efficiency varied with N = A < G ≈ C ≈ U. With C, G or U in position +6 the termination signal strength was raised to a level similar to that observed for UGA**A**UA.

Correlation of termination efficiency and codon usage

The occurrences of nucleotides at the 4th and 6th positions, following UGA and UGACU respectively, were non-random (Fig. 1). While the particular stop signal context of the RF-2 frameshift site (UGACUA) is rare at natural termination signals, signals with a nucleotide other than C in the +4 position are more common. All 5th base contexts of the type UGACNA and 6th base contexts of the type UGACUN are relatively infrequent at natural termination sites (Table 1), presumably as a consequence of the rarity of UGAC termination signals (14).

Table 1. Occurrence of UGA**N**UA, UGAC**N**A and UGACU**N** stop signals at natural termination sites

Sequence	Expected	Observed	% Total
UGA**U**UA	34	41	1.19
UGA**G**UA	16	19	0.55
UGA**A**UA	35	23	0.67
UGA**C**UA	10	1[a]	0.03
UGAC**U**A	10	1[a]	0.03
UGAC**G**A	21	10	0.29
UGAC**A**A	30	17	0.50
UGAC**C**A	13	9	0.26
UGACU**U**	15	9	0.26
UGACU**G**	19	12	0.35
UGACU**A**	10	1[a]	0.03
UGACU**C**	12	8	0.23

From the 3433 *E.coli* gene sequences examined, there were 10 461 six-base 'stops' found in all three reading frames in the non-coding regions (100 bases) adjacent to the natural stops of *E.coli* genes. The 'Expected' column is calculated from the proportion of the 10 461 non-coding stops that each six-base signal represents. For example, UGAUUA occurs 105 times in the non-coding region, hence we expect to find it $105/10\,461 \times 3433 = 34.46$ times in the natural stop position (rounded to 34). The 'Observed' column is the actual occurrence of each six-base signal in the 3433 natural stops. The '% Total' column is the observed termination signals expressed as a percentage of the 3433 natural site signals.

[a]Putative site of an unidentified reading frame.

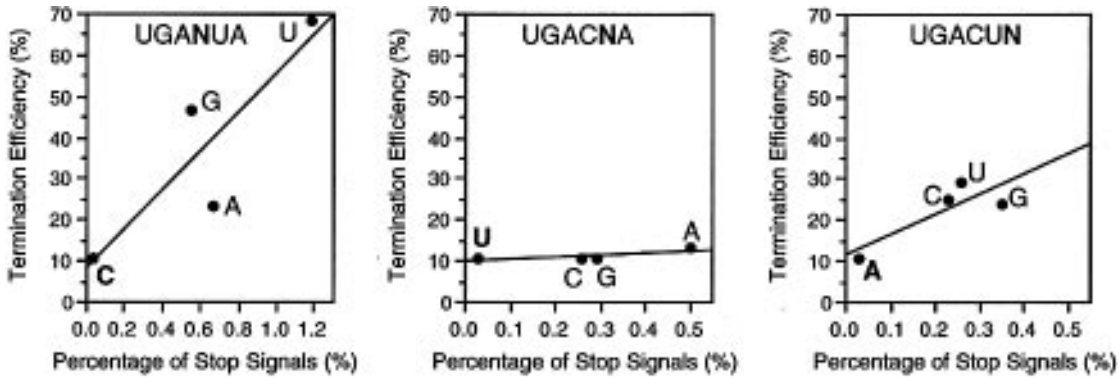

Figure 5. Linear regression analysis was used to display the relationship between termination efficiencies at UGA**N**UA, UGAC**N**A and UGACU**N** stop signals and the percentage occurrence of each signal in *E.coli*. The correlation coefficients (r) are: 0.88 for UGANUA, 0.75 for UGACNA and 0.84 for UGACUN signals.

We used linear regression analysis to display how the relative termination efficiencies in the UGANUA, UGACNA and UGACUN contexts correlated with their use (Fig. 5). The occurrence of each six-base signal with these contexts was calculated as a percentage of all stop codons as in Table 1, from the 3433 *E.coli* genes listed in the TransTerm database for which all six nucleotide positions were identified (24).

At positions +4 and +6 of termination signals the usage bias relates to termination efficiency, with correlation coefficients of $r \approx 0.88$ for UGANUA and $r \approx 0.84$ for UGACUN contexts. The usage bias at position +5 correlates less well with the termination efficiency, $r \approx 0.75$. This relationship at positions +4 and +6, suggests that the role of positions +4 and +6 in UGA-containing stop signals for RF-2-mediated termination of protein synthesis might be important. Any variations in the use of nucleotides in the +5 position may have been influenced by factors independent of the termination mechanism.

DISCUSSION

Recoding sites often contain in-frame stop codons, and therefore 'recoding' represents a failure of that stop codon to specify efficient termination of protein synthesis at the site. Although termination is usually by far the predominant event, the RF-2 frameshift site is an exception since frameshift and termination events occur with comparable frequency. The stimulators of frameshifting, in this case a Shine–Dalgarno-like sequence followed by a high density of Us immediately before the frameshift point (27), either could override a normal stop codon, or the stop signal may be partly responsible for its own failure. A ribosomal pause at the stop codon within the frameshift site is thought to be critical for the high efficiency of frameshifting (28).

The duration of the ribosomal pause at the stop codon might be a key contributor to efficient frameshifting. There is considerable bias in the position following stop codons at natural termination sites (12,13), and a critical determinant of the pause might be how the nucleotide in this position affects the rate of decoding of the stop signal. There was a 7-fold difference in the +4 nucleotide's ability to influence the competitiveness of the UGA as a stop codon against the +1 frameshifting event (Fig. 4). In the original study we calculated relative rates of RF-2 selection (29) at the various stop codon contexts. UGAC, the stop signal found at the RF-2 frameshift site, was the slowest of all the possible UGAN, UAAN or UAGN signals to be decoded, selecting RF at a rate some 50-fold less than UAAU, the most rapidly decoded signal, and ~30-fold less than the UGA-containing signal, UGAU (14).

Other site-specific context features of the RF-2 frameshift site may contribute to the length of the pause at the stop codon and influence conclusions of stop signal strength. UGAC might not be such a poor termination signal generally, and indeed suppression studies had indirectly suggested that UGAC contexts might be good termination sites (20). While it was not possible to study the influence of the nucleotides upstream of the stop codon at the RF-2 frameshift site because of the effect on the other stimulators of the event (Shine–Dalgarno, spacing to frameshift site, homopolymeric run or the frameshift codon itself), further analysis of the downstream region was possible.

The current study has focussed on the +5 and +6 downstream nucleotides at the RF-2 frameshift site. The statistical analysis of the +5 position suggested that where UGAC is found at natural sites there is no selection against a particular nucleotide in the next position, whereas there is significant selection against A in the +6 position following UGACU (Fig. 1). There is only one UGACUA sequence out of 30 UGACUN sequences at natural sites in the pool of 3433 genes examined, whereas 10 UGACUA sequences would be expected statistically (Table 1). It is interesting that A is usually the favoured nucleotide in the +6 position of a termination signal (Fig. 2).

The experimental study reinforced predictions from these theoretical analyses; the UGAC signal was equally weak whatever the nucleotide in the +5 position, but any of the three nucleotides U, G or C in the +6 position significantly strengthened the competitiveness of the termination signal (Fig. 4). There was a correlation between the frequency of occurrence of UGANUA and UGACUN at natural sites and their experimentally-determined strengths (Fig. 5). In contrast, while U was underrepresented in the +5 position for UGACNA signals, there was no correlation between this occurrence and experimentally-determined signal strength.

How might the +4 and +6 nucleotides have such an effect on the rate of selection of release factor at the stop codon? We have shown by site-directed crosslinking that the RF is in close physical contact with the codon during recognition (30). The release factor protein in this position can presumably make contact with nucleotides outside the primary recognition determinant, the codon itself. The statistical bias, particularly in the +4

position, to a lesser extent in the +6 to +10 positions and in addition the –2 position (6), may be an indicator that there are favoured contexts for effective RF recognition of stop signals extending on both sides of the codon. It is likely that the determinants for RF recognition of a particular sequence could vary from one context to another. For example, the +6 nucleotide may be important for UGACUN contexts, as shown in the current study, but have less effect in other contexts.

The RF-2 frameshift site has been conserved among prokaryotic organisms in five of the six sequences reported so far, and the stop codon UGA and +4 nucleotide C have been maintained in each case [the first example of an RF-2 gene which lacked the frameshift site was found in *Streptomyces coelicolor* (31)]. The conserved +4 C is at the third base position of the first codon, GAC, after frameshifting and might have been expected to vary at least to a U without penalty if protein sequence alone was the determining factor. There is less conservation in the +5 and the +6 positions (two sequences have A and three have U in the +6 position). It is interesting that the +8 and +9 positions are also conserved in all sequences although some of this conservation might reflect a requirement to maintain protein sequence of the gene product (positions 1 and 2 of an amino acid codon).

The termination signal within the RF-2 frameshift site is an integral part of the regulatory event aimed at controlling the completion of the synthesis of RF-2 molecules, and it is particularly weak because of the +4 C and, to a lesser extent, the +6 A which keep the rate of RF selection at the signal slow enough for high efficiency frameshifting.

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