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Tandem termination signals: myth or reality?

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Abstract In two *Escherichia coli* genomes, laboratory strain K-12 and pathological strain O157:H7, tandem termination codons as a group are slightly over-represented as termination signals. Individually however, they span the range of representations, over, as expected, or under, in one or both of the strains. In vivo, tandem termination codons do not make more efficient signals. The second codon can act as a backstop where readthrough of the first has occurred, but not at the expected efficiency. UGAUGA remains an enigma, highly over-represented, but with the second UGA a relatively inefficient back up stop codon.

Key words: Termination signal; Tandem codon; *Escherichia coli* translation

1. Introduction

A surprising finding when the first gene sequences emerged was the discovery of tandem termination codons in the coat genes of the *Escherichia coli* bacteriophages R17, f2 and MS2 [1–3] and in the A_1 gene of bacteriophage Qβ [4]. It was estimated that up to 13% of protein synthesis termination signals in *E. coli* might be tandem termination codons [5], and that tandem signals may have evolved to ensure efficient termination of protein synthesis [6,7]. The second termination codon was seen as 'a fail safe' mechanism [8]. A dogma was established that tandem termination signals were especially effective.

Detailed studies of the characteristics of termination signals have now revealed T as the preferred base following the termination codons of *E. coli*, and more generally this fourth base was a critical part of the termination signals [9,10]. For example, the most highly expressed genes of *E. coli* use TAAT or TGAT almost exclusively [9,11]. This means TAATNN or TGATNN signals might be expected to occur more frequently than signals with other bases in the +4 position, and among these will be the tandem termination codons, for example TAATAA or TAATAG or TAATGA. The critical question then is whether tandem termination codons are particularly selected for their advantages or whether they occur as a consequence of the selection of T as the +4 base.

Termination codons are decoded by protein release factors. Site-directed crosslinking has demonstrated that in *E. coli* the decoding release factor contacts not only the stop codon but also the following three nucleotides [12]. The three nucleotides immediately 3′ to the stop codon also significantly affect the strength of the termination signal [13]. This provides two possible mechanisms whereby tandem termination codons may be particularly effective, as six base termination signals, or with the second stop codon acting as a backstop stop codon if the first was miscoded by a near cognate tRNA.

The long standing belief that tandem termination codons are efficient termination signals in protein synthesis has to date escaped rigorous examination. In this study we have asked the following questions: (i) do tandem termination codons occur with unusual frequency in *E. coli*?; (ii) are tandem termination codons as sequence elements particularly good termination signals?; (iii) does the second termination codon act as a backstop where they occur?

2. Materials and methods

2.1. Database analysis

Statistical analyses of nucleotide sequences were performed on two complete *E. coli* genomes after entry into the Transterm database [14]. The genome sequences were from *E. coli* strains K-12 MG1655 ([15], GenBank accession number U00096) and O157:H7 EDL933 ([16], GenBank accession number AE005174). A subset of genes from each genome with high codon adaptive index values [17] calculated from published relative synonymous codon usage tables [18], were selected and analysed separately. The abundance of termination signals was counted using 'count_signalx'. This program counts signals both at the end of coding sequences (termination signals) and in the non-coding sequence spanning 99 nucleotides immediately 3′ of termination codons. Deviation from anticipated signal abundance (as determined by the frequency of the signal in the 99 nucleotides downstream of termination codons) was detected with a Poisson approximation of a binomial distribution [19].

2.2. Translation termination assays

Two in vivo assays assessing termination signal strength were used in this work. In the 3A′ translation reporter gene [20] termination of protein synthesis is in competition with readthrough by near cognate or suppressor tRNA, and the protein products from both translational events are purified by affinity chromatography, identified and quantitated after SDS–PAGE. The efficiency of termination was determined by the proportion of protein arising from termination at the first termination codon compared to total protein expressed from the reporter gene. In the pMAL in vivo translation termination assay [10] the competitive event is +1 frameshifting and the termination and frameshifting products are detected by immunoblotting after SDS–PAGE and Western transfer. The intensities of both bands were quantitated.

An array of termination contexts was cloned into each reporter gene using redundant oligonucleotide pairs as previously described [20,10]. The identities of individual plasmid constructs were then confirmed by sequencing.

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2.3. Media and bacterial strains

For both the pMAL and 3A′ expression systems ampicillin resistance was used to select bacteria containing plasmids. For the pMAL system, bacteria were grown in Luria broth and protein expression was induced from the P_{tac} promoter with 1 mM IPTG. For the work using the 3A′ translation readthrough assay, bacteria were grown in minimal media with all 19 L-amino acids and glycine added at recommended concentrations [21] and protein expression was induced from the P_{trc} promoter with 0.5 mM IPTG.

E. coli strains DH5α, TG1 or MC1061 [22] were used for the primary cloning. For the expression studies using the pMAL in vivo translation termination assay the *E. coli* strain FJU_{112} [D*(lac pro) gyrA ara recA56/10, F′lacI*^{Q1}] [23] was used. This strain has wild-type ribosomes and no suppressor tRNAs. The 3A′ readthrough assay work involved five *E. coli* K-12 strains: 'wild-type' XAc, UAG suppressor strains XA101 and XA102, UAA suppressor strain XA105, and UGA suppressor strain CDJ64 [24].

3. Results and discussion

3.1. Are tandem termination codons over-represented in E. coli?

The occurrence of tandem termination codons at the ends of genes in two published *E. coli* genomes, *E. coli* K-12 strain MG1655 [15] and *E. coli* O157:H7 strain EDL933 [16], was determined using the Transterm database [14] and a signal counting program (count_signalx).

Despite the two *E. coli* strains containing very different numbers of genes (O157:H7 has 1128 more genes than K-12) the proportion of genes having tandem termination codons was comparable at around 7% (316 tandem termination codons from 4288 genes in K-12, 395 from 5416 genes in O157:H7). This is slightly higher than what would be predicted if the sequence after the stop codon was completely random in an organism with around 50% GC content (*E. coli* K-12 has a GC content of 50.8%), where the probability of seeing a second stop codon is 4.7% (T: A or G: A or G but excluding TGG). However, this ignores the bias for T following stop codons which has previously been demonstrated [9]. If this bias is taken into account then 7.3% of termination signals are expected to be tandem termination codons, that is, strikingly similar to what is found.

Whether the nine individual tandem combinations of termination codons were significantly over-represented or not was tested statistically using two-tailed Poisson approximations of binomial distributions (Fig. 1). The expected use of the signals was based on dinucleotide signal use in the non-coding region immediately following all genes. Thus, the expected frequency of AA, AG or GA was used to predict how often tandem signals would form at TAAT, TGAT or TAGT termination signals (throughout this paper T is used where genomic sequences are considered whereas U is used in experimental results involving translation).

If tandem termination codons conferred selective advantage on the organism the expectation was that they would be over-represented. There were only three of the nine combinations of termination codons in tandem that were found at a significantly different frequency from that expected in the two *E. coli* strains analysed. TAATGA and TGATGA were both significantly over-represented, while TAATAG was significantly under-represented. Three other combinations were found only in one of the strains at a frequency significantly different from that expected. TAGTGA and TGATAG were significantly over-represented in *E. coli* strain O157:H7, but at the expected frequency in *E. coli* K-12. TAATAA was under-represented in *E. coli* strain K-12. A similar analysis was performed for a highly expressed subset of genes, as predicted by codon adaptive index values for the genes. Significantly in this important group of genes none of the tandem termination codons was over- or under-represented. However, the very few TAG terminating genes (only two in K-12, three in O157:H7) in the high CAI data all used tandem termination signals (TAGTAA and TAGTAG).

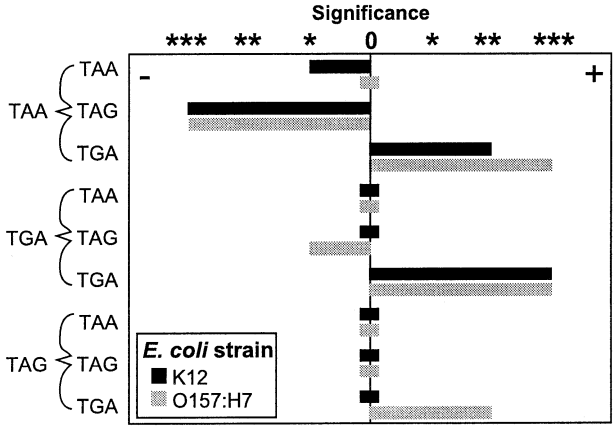

Fig. 1. Bias in the use of tandem termination signals in the *E. coli* K-12 and O157:H7 genomes. Expected use of tandem termination signals was predicted from dinucleotide frequencies in non-coding regions 100 nucleotides downstream from termination codons in all *E. coli* genes. Significance was determined from a two-tailed Poisson approximation of a binomial distribution, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$.

Of particular interest were the four pairs of stop codons that were significantly over-represented in either one or two *E. coli* strains.

3.2. Are six base termination signals arising from tandem termination codons more efficient?

That the sequence downstream of termination codons has an effect on termination efficiency in competition with suppression by tRNA is well established (reviews: [25–27]). We have demonstrated specifically that the three nucleotides immediately 3′ to a stop codon can significantly affect termination efficiency in competition with a +1 frameshifting event [13]. Interactions between mutant RF1 and P-site peptidyl-tRNA appear to be influenced by the identity of the two nucleotides following the stop codon UAG [28]. Crosslinking between +4 and +6 positions in the mRNA and decoding release factor suggests that there may be a direct effect of sequence 3′ to the stop codon upon RF decoding of the stop codon [12].

The efficiency of tandem termination codons as six base termination signals was measured using the pMAL in vivo translation termination assay where the termination of protein synthesis is in competition with +1 frameshifting at the RF2 frameshift site [10]. The RF2+1 frameshift occurs at an internal termination codon, UGA in the RF2 mRNA. Key determinants for frameshifting are a Shine–Dalgarno like element [29] with critical sequence and spacing from the frameshift site [30], a slippery run of four uracil nucleotides encompassing a critical CUU codon immediately before the UGA, and the stop codon itself [10,31]. All data collected to date suggest that altering the sequence downstream of the termination codon in the RF2 frameshift site affects frameshifting efficiency by affecting release factor decoding of the termination codon.

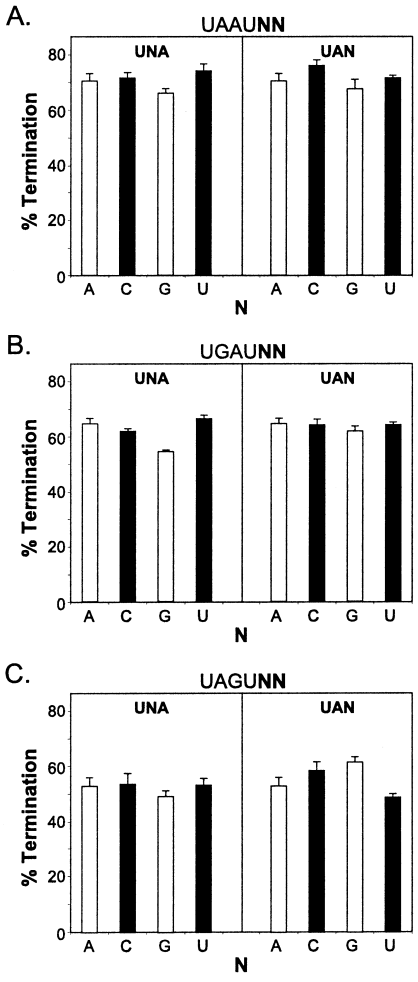

Fig. 2. The strength of tandem termination signals in the pMAL translation termination assay. Translation termination efficiencies of pMAL translation termination constructs with 3′ termination contexts UNA and UAN were measured for UAA (A), UGA (B) and UAG (C) termination signals. The bars representing tandem termination signals are white while non-tandem bars are black. Mean values from at least three experiments with at least two independent isolates of each construct are presented. Error bars are S.E.M.

The RF2 frameshift window containing different 3′ termination contexts was cloned into a multiple cloning site at the 3′ end of the maltose binding protein gene in the vector pMAL-c2. This enabled the immunological detection of termination (44 kDa) and frameshift (52 kDa) products after separation by SDS–PAGE and Western transfer.

Four series of constructs were made where each termination codon was followed by UNA, UNG, UAN or UGN (Fig. 2). This enabled a comparison of the termination efficiency of tandem stop codons with that of closely related six base termination signals. Termination efficiency was determined in *E. coli* strain FJU_{112}. The data in Fig. 2 are from at least three experiments utilising at least two independent isolates of each construct. A selection of the experimental data for the 3′ UNA and UAN series is shown. Studies with 3′ UNG or UGN constructs were also carried out (data not shown) to complement the data from the UNA and UAN series.

For the UAA tandem termination signals (Fig. 2A), TAATGA and TAATAG had been over-represented (and TAATAA under-represented) in at least one of the *E. coli* strains. These tandem termination signals, however, were no stronger or weaker respectively than their closely related signals. Indeed UAAUGA was slightly weaker than the other members of the UAAUNA series.

TGATGA had been significantly over-represented at the ends of *E. coli* genes (Fig. 1). However, the data for the UGAUNN tandem termination signals (Fig. 2B) do not support this tandem termination signal being a 'superior' six base termination signal. In the UGAUNA series of plasmids the over-represented UGAUGA signal was the poorest context of the four in the series. When the sixth base of the signal was altered (UGAUGN and UGAUAN) no effect on termination efficiency was seen. Indeed all of the UGA starting tandem termination signals were unremarkable or poorer termination contexts than related signals. For UGAUGN signals all four constructs supported between 54 and 56% termination (data not shown).

The tandem UAGUAG was a somewhat stronger signal than most related contexts, although not exclusively as UAGUAC was just as strong a termination context (Fig. 2C). The tandem signal UAGUAA was a weaker context than UAGUAG and UAGUAC, and UAGUGA was in the weakest group with four related contexts (Fig. 2C and data not shown).

These studies showed that there was no correlation between over- or under-represented signals and their signal strength. Over-represented or under-represented tandem termination signals were not especially strong or weak termination signals when considered as six base termination signals. For example, TAATGA (found 1.4-fold more frequently than predicted) was a weaker translation termination signal than the rest of the UAAUNA series. The real enigma was TGATGA, found over 2-fold more frequently than predicted from dinucleotide frequencies, but a weaker signal than the rest of the UGAUNA series of constructs, and equivalent to all other constructs of the UGAUGN series.

Is another property of the signal important for the bias in signal use? Could UGA signals be particularly in need of a backstop stop codon, and might this be the explanation for the extreme bias of TGATGA termination signals? Low level readthrough of a UGA codon is essential for the formation of viable bacteriophage Qβ particles [4]. UGA is also read through to produce the phage λ O′ protein [32]. The normal *E. coli* $tRNA^{Trp}$ can decode UGA [33], and readthrough of UGA with this tRNA is growth phase dependent [34], suggesting that it may be a regulatory mechanism used by *E. coli*.

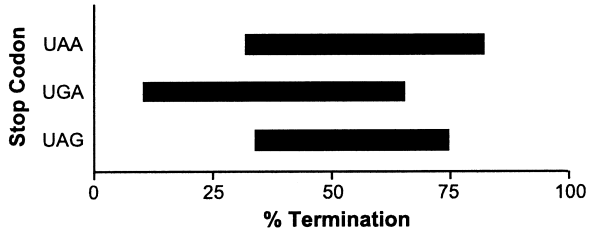

Fig. 3. Range of termination signal strengths observed for different termination codons. Termination efficiency was measured for UAANNN, UGANNN and UAGNNN termination signals in the pMAL translation termination assay. The range of signal strength was determined from 41 UAA constructs, 52 UGA constructs and 64 UAG constructs.

The incorporation of selenocysteine by specialised elongation factor and tRNA also occurs at UGA codons [35].

We have measured the termination efficiency of many six base termination signals and the range observed for each termination codon group is presented in Fig. 3. Efficiencies of all three stop codons were significantly affected by the nucleotides immediately 3′ to the stop codon as indicated by the broadness of the bars. As a group the UGA signals were the poorest, and this is consistent with the concept that the abundance of UGAUGA stop signals might reflect a backstop function. However, confounding this idea, only TGATGA tandem codons of the three TGANNN group are over-represented.

3.3. How effective are UGAUGA signals as backstops?

To measure the effect of tandem termination signals on readthrough of stop codons a well established in vivo readthrough versus termination assay was used [20]. In the 3A′ assay termination results in a two domain 15.6 kDa protein and readthrough a three domain 23.9 kDa protein. These were purified using affinity chromatography and separated by SDS–PAGE.

The readthrough of stop codons was measured using a series of plasmids containing the termination contexts UGAUNA and UGAUGN, as well as at the specific tandem termination codons, UAGUAG, UAGUAA and UAAUAA. Readthrough was measured in a wild-type *E. coli* strain, XAc, and *E. coli* strains containing suppressor tRNA (CDJ45, XA101, XA102 and XA105). Termination was measured in the suppressor strain containing tRNA cognate for the first stop codon of the tandem signals.

In the wild-type strain no significant difference in readthrough was found for either the UGAUNA or UGAUGN series of constructs (data not shown), with 1–2.5% readthrough observed. In the UGA suppressor strain CDJ64 (Fig. 4A) the tandem signal UGAUAA permitted no readthrough, and at UGAUGA significant readthrough still occurred (11%). This was less than observed for the non-tandem signals (15–20%). The improvement of signal strength by the presence of a second stop codon was less than expected for UGAUGA. It was anticipated that if each stop codon in tandem had similar efficiencies, then having two stop codons would proportionally decrease the readthrough efficiency observed at that signal (i.e. with 10% readthrough observed with one stop codon, expect 1% readthrough with two stop codons). In contrast to this expectation readthrough of UGAUGA was reduced by only 2-fold compared with UGAUCA rather than 10-fold. Since UGAUGC and UGAUGA were equivalent six base termination signals in the pMAL assay this suggested that termination at the second stop codon was not as efficient as the decoding at the first stop codon, or conversely that readthrough of the second stop codon was more efficient.

Data from three other tandem termination signals in wild-type *E. coli* or suppressor strains are presented in Fig. 4B to reflect the range of results obtained. UAGUAA allowed no readthrough under both conditions (the second codon UAA was not suppressed by the UAG suppressor). The second codon of UAAUAA permitted a low level of readthrough in both wild-type and suppressor strains, modestly increased in the UAA suppressor strain. In contrast even with two 'UAGs' in the UAGUAG signal there was 12% readthrough in the two UAG suppressor strains (data for strain XA102 not shown). Here the backstop was relatively ineffective. If tandem stop codons were an evolutionary mechanism to avoid readthrough by cognate or near cognate tRNAs then mixed tandems would provide more protection against readthrough

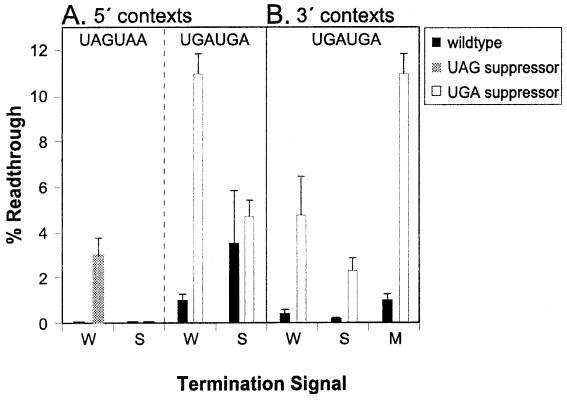

Fig. 5. Influence of 5′ and 3′ contexts on the effectiveness of tandem termination signals. A: Readthrough has been measured in wild-type (black bars) and the appropriate suppressor strains (UAG grey bar, UGA white bars) as indicated, using UAGUAA and UGAUGA constructs differing in their 5′ context. 'W' means a weak context, 'S' a strong context (unpublished data). The 5′ contexts used were: UAG 'S', GUCGCG; UAG 'W', GAUGGA; UGA 'S', AAUUUC; UGA 'W', GAAACU. B: Readthrough has been measured with UGAUGA constructs having strong (S), average (M) and weak (W) 3′ contexts (unpublished data) in wild-type (black bars) or the suppressor strain (white bars) as indicated. The 3′ contexts used were: 'S', UUU; 'W', CUA; 'M', ACU. Mean values from at least three experiments with two independent isolates of each construct are presented. Error bars are S.E.M.

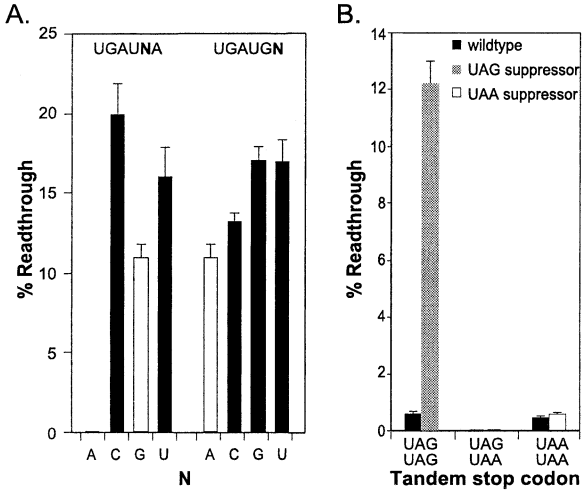

Fig. 4. Tandem termination signals in the 3A′ protein synthesis termination assay. A: Readthrough efficiencies of constructs with termination contexts UGAUNA and UGAUGN in the UGA suppressor strain CDJ64. Bars representing tandem termination signals are white while non-tandem bars are black. B: Readthrough at UAGUAG, UAGUAA and UAAUAA tandem signals in strain XAc (wild-type *E. coli*, black bars), and suppressor strains XA101 (UAG suppressor, grey bars) and XA105 (UAA suppressor, white bars). Mean values from at least three experiments with two independent isolates of each construct are presented. Error bars are S.E.M.

than repetitions of one stop codon. The enigma of TGATGA being the most over-represented signal remains.

As each stop codon of a tandem signal will have a different upstream and downstream context it is likely that they will have different decoding efficiencies. To determine whether the upstream and downstream context was affecting decoding at tandem signals several constructs tested whether either the 5′ or 3′ contexts of tandem termination signals had significant influence (Fig. 5).

The upstream and downstream contexts used for this study were the ones previously characterised in our laboratory as being strong (S) or weak (W) contexts for termination (unpublished data). They have been used together with the 3′ context used in the experiments in Fig. 4, which is indicated as 'M' (medium). The effect of the contexts is stop codon dependent (unpublished data), thus different sequences were used upstream and downstream of the different tandem signals.

The termination signal UAGUAA in the wild-type strain XAc did not allow readthrough regardless of 5′ context. Against prediction, under competition from the suppressor tRNA the stronger 5′ context allowed 3% readthrough. The over-represented UGAUGA signal was sensitive to 5′ context in both wild-type (XAc) and suppressor (CDJ64) strains. In wild-type strain (XAc) the 5′ 'S' context allowed more readthrough than the 5′ 'W' context, while in strain CDJ64 the expected hierarchy of signal strength was observed with the 'S' context permitting less readthrough than the 'W' context.

UGAUGA signals were also sensitive to the 3′ context in both wild-type and suppressor strains. Readthrough ranged from 0.5 to 2% (wild-type) and from 2 to 11% (suppressor strain). Hence context can influence the effectiveness of the tandem termination signal (although not always as predicted from the rules determined for single codons (unpublished data)). These data do not appear, however, to provide the missing link for why TGATGA is so common at the end of genes using TGA as a stop codon.

4. Conclusion

Tandem termination codons, discovered with the first gene sequences from RNA bacteriophages, have been seen as a fail safe mechanism against competition from near cognate translational events. The two *E. coli* genomes, the laboratory strain K-12, and the pathological strain O157:H7 that differ by over 1000 genes have a similar occurrence of tandem termination codons. As a group they are slightly elevated over that expected but this can be accounted for by the strong preference for T in the fourth base position as part of an efficient stop signal. The tandem termination codons do not make more efficient signals than those where these three bases do not form a second stop codon. While the second stop codon does indeed act as a backstop where readthrough of the first codon has occurred, the effectiveness of the backstop can be affected by wider 5′ and 3′ contexts. UGAUGA is an enigma, occurring at the highest frequency of tandem stop codons, despite the fact that the second UGA is a much poorer back up stop codon than expected. The idea that tandem termination codons have been exquisitely engineered as highly efficient termination signals for protein synthesis seems to be more myth than reality.

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