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Two regions of the *Escherichia coli* 16S ribosomal RNA are important for decoding stop signals in polypeptide chain termination

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ABSTRACT

Two regions of the 16S rRNA, helix 34, and the aminoacyl site component of the decoding site at the base of helix 44, have been implicated in decoding of translational stop signals during the termination of protein synthesis. Antibiotics specific for these regions have been tested to see how they discriminate the decoding of UAA, UAG, and UGA by the two polypeptide chain release factors (RF-1 and RF-2). Spectinomycin, which interacts with helix 34, stimulated RF-1 dependent binding to the ribosome and termination. It also stimulated UGA dependent RF-2 termination at micromolar concentrations but inhibited UGA dependent RF-2 binding at higher concentrations. Alterations at position C1192 of helix 34, known to confer spectinomycin resistance, reduced the binding of f[^{3}H]Met-tRNA to the peptidyl-tRNA site. They also impaired termination *in vitro*, with both factors and all three stop codons, although the effect was greater with RF-2 mediated reactions. These alterations had previously been shown to inhibit EF-G mediated translocation. As perturbations in helix 34 effect both termination and elongation reactions, these results indicate that helix 34 is close to the decoding site on the bacterial ribosome. Several antibiotics, hygromycin, neomycin and tetracycline, specific for the aminoacyl site, were shown to inhibit the binding and function of both RFs in termination with all three stop codons *in vitro*. These studies indicate that decoding of all stop signals is likely to occur at a similar site on the ribosome to the decoding of sense codons, the aminoacyl site, and are consistent with a location for helix 34 near this site.

INTRODUCTION

During protein synthesis most of the information encoded in the mRNA is decoded into amino acid sequence by mRNA-tRNA basepairing. However, it is believed that proteins, the polypeptide chain release factors (RFs), decode the stop signals (1, 2). In *Escherichia coli* one of two factors, RF-1 or RF-2, bind to ribosomes when specific stop signals in the mRNA, UAG and UAA (for RF-1) or UGA and UAA (for RF-2), appear in the aminoacyl site (A site) on the small subunit. Following the initial decoding event which includes RF interaction with the ribosome the peptidyl transferase centre on the large subunit is converted into a hydrolase. This centre then cleaves the peptidyl-tRNA bond releasing the completed polypeptide.

The concept that the RF proteins recognised the stop signal in isolation has been challenged and work recently has focused on the role of the 16S rRNA in termination (3). It has been suggested that the RFs may not decode stop signals directly, but that they are initially decoded by base pairing with the 16S rRNA, then the factors bind to the ribosome and contribute to the hydrolysis event (2, 4). As yet no such highly specific role for the rRNA has been proven.

Several complementary regions of the 16S rRNA have been suggested. This possibility had been invoked originally by Shine and Dalgarno (5) when they noted complementarity between the extreme 3′ terminus of the *E.coli* 16S rRNA and stop codons. However, potential complementary sequences were found in few other organisms (6, 7). One potential location for stop signal decoding is in the A-site where the sense signals are decoded (8). This model is supported by *in vitro* experimental evidence using small designed mRNAs (9). These contained a reactive affinity residue (thio-U) as part of the a UAA stop signal placed in the ribosomal A-site. The residue crosslinked to both RF-2 and A1408 of the 16S rRNA. This nucleotide is located at the top of helix 44 (Brimacombe's numbering), and is weakly protected from chemical attack by an A site bound tRNA (10–12, Fig 1). A1408 forms part of a highly conserved single stranded region of sequence U^{5}mCA1408, complementary in three positions to UGA and two to UAA and UAG. This study suggested not only that direct recognition of the signal by the protein factor was occurring, but also that decoding of all the stop signals by this region of the rRNA was possible.

An alternate model has been suggested for the decoding of UGA specifically through base pairing with tandem triplets (1199–1204) in the central region of helix 34 (4, 13–16). In

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extension to this model it was suggested that UAA and UAG could be decoded by other complementary triplets between helices 34, 32, and 30, but these ideas have not been experimentally tested (Fig 1, 15, 17). The central region of helix 34 is located somewhat distant from the A site in current models of the 30S subunit (10, 11 Fig. 1). This termination model is based largely on the effects of specific *E.coli* mutants on *in vivo* suppression assays, which use stop codon readthrough by a tRNA as a negative test for termination. This model was initially based on the observation that a mutant with a deletion of a single base (C1054) in this helix had increased readthrough of some UGA codons, although not at the UAA or UAG codons tested (4). However context effects act on either termination or suppression, and the influence of the alterations in the rRNA on tRNA binding can also affect such results (18). The UGA-specific mutant can suppress all other termination codons under certain circumstances and may act by affecting the binding of tRNAs to the ribosome (19, 20).

To determine which regions of the 30S subunit are important for termination we investigated the *in vitro* effects of antibiotic probes whose target sites are now well characterised (21). Some of the antibiotics have been tested in part previously (22–26). It might be expected that if three codons are decoded by basepairing to different parts of the 16S rRNA, then differences between the two factors, or three codons might be detected by these antibiotic probes, whereas if all decoding occurs by basepairing at a single location no such discrimination would be expected.

METHODS

All procedures were essentially as described in Tate and Caskey (27). Ribosomes and release factors (RF-1 and RF-2) were isolated from *E.coli* MRE600. The C1192 wild type and altered ribosomes were isolated from HB101 containing pKK3535 derivatives (29). For preparation of ribosomal complexes containing f[^{3}H]Met-tRNA at the P site, 50 μl of a mixture containing 20 mM Tris–HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, 50 pmol 70S ribosomes, 10 nmol AUG, and 10–15 pmol of f[^{3}H]Met-tRNA (4000 c.p.m./pmol) were incubated for 20 min at 30°C. The effect of the antibiotics on the stability of the complexes was tested by incubating them with 100 μM antibiotic in buffer 1 (50 mM Tris–HCl, pH 7.2, 30 mM $Mg(OAc)_2$, 75 mM NH_4Cl) at 4°C for 10 min then at 20°C for 30 min and measuring the amount of f[^{3}H]Met-tRNA bound in the complex by collecting them on glass fibre filters. Most of the f[^{3}H]Met-tRNA remained bound (and therefore available for liberation by the RF) in each case; spectinomycin, 84% bound; hygromycin, 70%; neomycin, 82%; tetracycline, 94%. For *in vitro* termination, 5μl of this complex were added to 45μl of a solution to give final concentrations as buffer 1 with 5 pmol ribosomes and with 3 nmol of either UAA or UGA or UAG (codon directed reactions) or 10% v/v ethanol (peptidyl- tRNA hydrolysis reaction), and the antibiotic (0.01–1000μM). This mixture was preincubated for 10 minutes at 4°C before the addition of 1–5 μg RF-1 or RF-2 and then incubated for 30 minutes at 20°C. The f[^{3}H]Met released was extracted into ethyl acetate, and quantitated by liquid scintillation counting. To measure the binding of release factor to ribosomes, the reaction mixture contained 50 mM Tris-OAc, pH 7.2, 20 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 0.1 mg/ml bovine serum albumin, 5 or 10% v/v ethanol as indicated, 50 pmol of 70S ribosomes (1μM), 400 pmol UA[^{3}H]G or UG[^{3}H]A (100 c.p.m./pmol) and antibiotic (0.1–1000μM). This mixture was preincubated for 10 minutes at 4°C before the addition of 10–20 μg RF-1 or RF-2. Then the reaction was incubated for 20 minutes at 4°C; the complexes formed were collected on glass fibre filters.

RESULTS

Antibiotic probes to identify regions of the 30S subunit involved in termination

Four antibiotics, hygromycin, neomycin, spectinomycin, and tetracycline, whose binding sites on the 30S subunit have been well characterised (reviewed in 21, 28, 31) were used to investigate the regions of the 30S subunit important for release factor functions in the termination of protein synthesis. The regions perturbed by the binding of three of these antibiotics on current secondary and tertiary structural models of the 30S subunit are indicated in Fig 1. These target sites have been determined by combinations of crosslinking studies, translation studies *in vivo* and *in vitro*, rRNA protection studies and characterisation of resistant mutants (21, 28, 31).

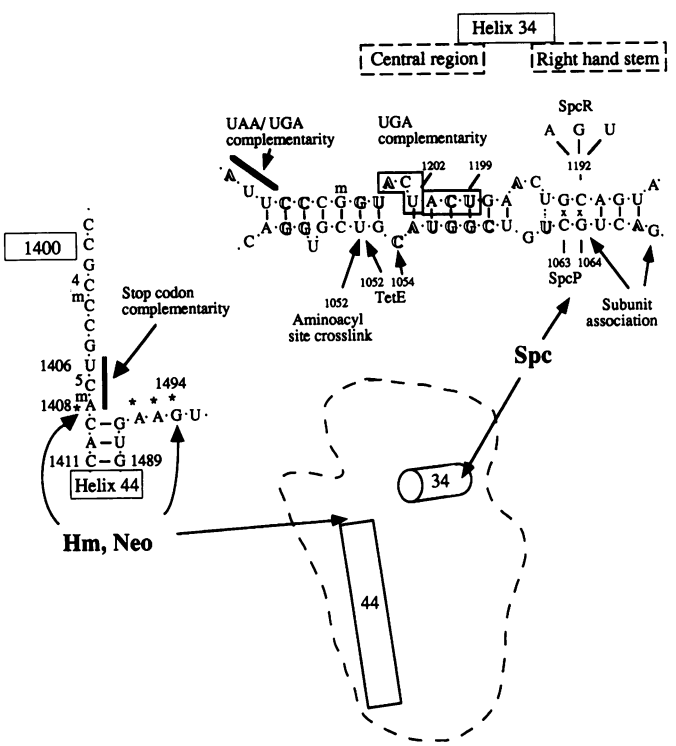

Figure 1. Regions of the 16S rRNA that may be involved in termination. Secondary structural models of the 1400 region, the base of helix 44 and helix 34 are shown with the approximate locations of these helices on the Brimacombe tertiary structural model for the small subunit (from 11, 38, 48, 49). Helix 44 is located in a similar position on the model of Noller and coworkers, but helix 34 higher in the head of the subunit (10, 49). The location of the regions affected by three of the antibiotics used in this study Spc (spectinomycin), Hm (hygromycin) and Neo (neomycin) are indicated (from 21, 11, 49). The nearby regions of potential complementarity to stop codons are also indicated, the two putative UGA complementary triplets are boxed (2, 4, 13, 15, 17). Asterisks, weak or strong protections by the A site tRNA (12); bold outlining, bases highly or moderately conserved in both (17) and (54); SpcR, spectinomycin resistant mutations; SpcP, Spectinomycin protection sites; TetE, bases whose reactivities are enhanced by tetracycline.

Spectinomycin. We tested spectinomycin as it specifically perturbs helix 34 of the 16S rRNA, near the potential site of UGA decoding. On binding it has been shown to protect nucleotides C1063, and G1064 from chemical attack (21). Furthermore single mutations in this region confer spectinomycin resistance (29). Spectinomycin has been thought to act primarily by interfering with the EF-G ribosome interaction, inhibiting translocation (28, 30).

Spectinomycin was first tested for its effect on the overall termination reaction *in vitro*. Ribosomal complexes f[^{3}H]Met-tRNA.AUG.70S were preincubated with the antibiotic before use for RF mediated, codon dependent termination.

The antibiotic had different effects on the RF-2 mediated termination reaction depending on the codon and factor used. Spectinomycin modestly *stimulated* RF-1 mediated UAG or UAA termination (Table). When present in micromolar amounts it stimulated almost two fold specifically UGA-dependent termination mediated by RF-2 (Table) but surprisingly had no effect on UAA-dependent termination. The data suggested that the interaction of spectinomycin with the small subunit perturbs the site of RF stop codon interaction, affecting each factor and codon combination in rather subtle ways.

Hygromycin, neomycin, tetracycline. The second group of antibiotics tested were aminoacyl site specific. Hygromycin is believed to bind to the region around A1408, the base crosslinked to thio-U in the stop signal (9). This antibiotic has been shown to protect nucleotide G1494 from chemical attack and increase the reactivity of A1408 (Fig 1). Furthermore methylation of A1408 or mutations in the basepair at the base of helix 44 (C1409 and G1491) confer hygromycin resistance (21, 31). The antibiotic inhibits translation by sequestering the tRNA in the A site and by causing A site miscoding (21). The region at the base of helix 44 is also implicated for neomycin binding as it protects A1408 and G1494 (21). The neomycin class of antibiotics also cause miscoding but in a way that can be distinguished from that of hygromycin. When we tested hygromycin and neomycin for their effects on termination, both strongly inhibited *in vitro* termination with both factors and all termination codons (Table). Each antibiotic was also tested over a range of concentrations (0.01μM $-$1000μM), neomycin gave 50% inhibition of termination reactions at 1μM, whereas 10μM hygromycin was needed to give a similar inhibition.

Tetracycline classically inhibits binding of tRNAs to the A site, but does not cause miscoding (12, 32). It may bind to A892 near the decoding site, as it protects this residue from chemical attack, but crosslinking studies indicate a major binding site may be on protein S7, also located near the decoding site (33, 34). It also has an unexplained effect on helix 34, enhancing the reactivity of U1052 and C1054. When tested tetracycline also inhibited *in vitro* termination with both factors and all three termination codons.

The effects of the antibiotics on the partial reactions of termination: codon dependent factor binding and codon independent hydrolysis

To identify at which stage of the termination reaction the antibiotics had their action, these antibiotics were also tested for their effect on the partial reactions of termination; first, the formation of an RF.UA[^{3}H]G/UG[^{3}H]A.ribosome complex, an indicator of codon dependent factor binding, and second the codon independent hydrolysis.

Initially we observed no effect of spectinomycin on stop codon binding of RF-1 to the ribosome. However, when the assay was made more sensitive, by the addition of bovine serum albumin, spectinomycin caused strong stimulation of RF-1 binding particularly at moderate concentrations (100μM, Table). In contrast spectinomycin showed only a slight stimulation of UG-A dependent RF-2 binding to the ribosome at low concentrations (1μm), but a higher concentration of the antibiotic (100μM) gave partial (62%) inhibition of UGA dependent RF-2 binding (Table). A similar degree of inhibition was also seen between 10μM and 1000μM antibiotic (data not shown). Inhibition of elongation was previously reported *in vitro* at 100μM (28, 30). Spectinomycin

Table 1. Effect of antibiotics on partial reactions of *in vitro* termination

		Codon dependent termination[a,b]		Formation of Complex RF.UGA/UAG.Ribosome[c]		Codon independent peptidyl tRNA hydrolysis[b]
		Percentage of activity without antibiotic[d]				
RF-1						
codon		UAA	UAG	UAA	UAG	None
Spectinomycin	1μM	131 ± 35	121 ± 27	120 ± 13	172 ± 36	102 ± 10
	100μM	114 ± 31	138 ± 30	189 ± 53	194 ± 47	106 ± 47
Hygromycin	100μM	18 ± 0	2 ± 1	0 ± 0	35 ± 23	21 ± 4
Neomycin	100μM	3 ± 6	12 ± 3	0 ± 0	34 ± 10	20 ± 14
Tetracycline	100μM	4 ± 0	24 ± 2	21 ± 20	58 ± 9	30 ± 4
RF-2						
codon		UAA	UGA	UAA	UGA	None
Spectinomycin	1μM	106 ± 17	177 ± 54	104 ± 1	115 ± 18	111 ± 4
	100μM	107 ± 20	120 ± 11	109 ± 10	62 ± 4	88 ± 18
Hygromycin	100μM	25 ± 10	17 ± 6	9 ± 6	22 ± 15	31 ± 4
Neomycin	100μM	0 ± 0	4 ± 2	0 ± 0	4 ± 2	16 ± 8
Tetracycline	100μM	0 ± 0	19 ± 10	0 ± 0	51 ± 40	44 ± 24

a. The reactions were carried out as described in Methods
b. Activities without antibiotic (100%) ranged from 0.25- 0.5 pmol (1000- 2000 c.p.m.) and backgrounds of 0.05 $-$ 0.1 pmol (200 $-$ 400 c.p.m.) were subtracted.
c. Activities without antibiotic ranged from 3 $-$ 7 pmol (300 $-$ 700 c.p.m.) and backgrounds of 1 $-$ 2 pmol (100 $-$ 200 c.p.m.) were subtracted.
d. Each number is the mean value ± the standard deviation from 3- 8 determinations

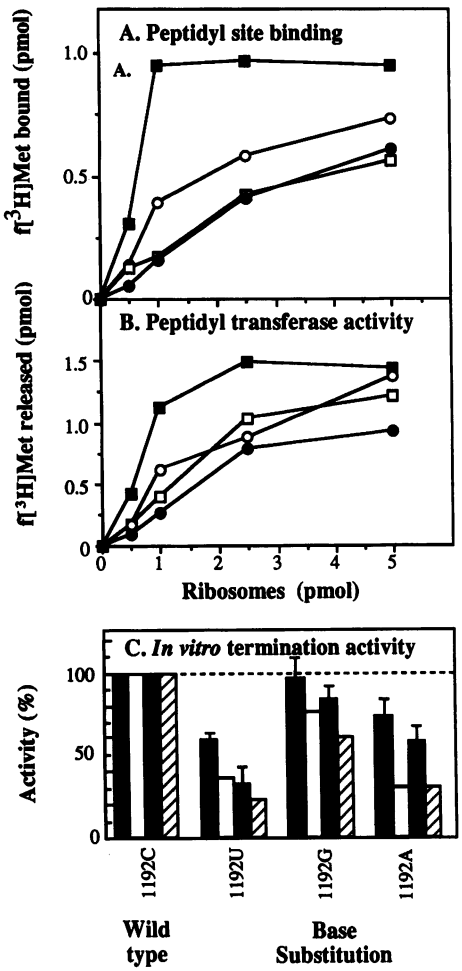

Figure 2. The effect of the alterations at position C1192 of the 16S rRNA. **A.** Binding of f[^{3}H]Met to the ribosomal Peptidyl (P) site. Wild type (C1192) filled squares; G1192 open circles; A1192, closed circles; U1192 open squares. **B.** Puromycin release of f[^{3}H]Met from complexes, labels as in 2A. **C.** *In vitro* termination activities. For overall *in vitro* termination, ribosomal substrate complexes containing 5pmol ribosomes (top points in A and B) were incubated with RF (0.5, 1.0, 1.5, 2 μg) and 0.1- 0.5 pmol (400−2000 c.p.m.) of f[^{3}H]Met were released, with backgrounds of (0.05- 0.1 pmol (200−400 c.p.m.) subtracted. Each experimental point was normalised to the overall peptidyl transferase activity for this alteration, to eliminate the effect due to the P site impairment, then divided by the normalised termination activities of the wild type ribosomes. The values obtained with the four amounts of RF were then averaged and presented as a percentage, with standard deviations as error bars. Each bar represents the average of four assays over a range of RF concentrations. Assays for the formation of complexes RF.UGA/UAG.ribosome (*in vitro* binding) were done in duplicate and not normalised as they should not be influenced by perturbations in the P site, the bars show the average of the duplicates. Binding activities ranged from 3−7 pmol (300−700 c.p.m.) and backgrounds of 1−2 pmol (100−200 c.p.m.) were subtracted. RF-1 *in vitro* termination (black bars), RF-1 *in vitro* binding (white bars); RF-2 *in vitro* termination (grey bars); RF-2 *in vitro* binding (striped bars).

had little effect on the other partial reaction, codon independent hydrolysis, whatever combination of factor and codon was used (Table).

The other antibiotics, as had been found with the overall termination assay, significantly inhibited the binding of both release factors to the ribosome, independent of the stop codon used. In the case of hygromycin 5% rather than 10% ethanol was used as the higher concentration reduced the inhibitory effect markedly (data not shown). The codon-independent hydrolysis mediated by the bound factor was also inhibited (Table).

These results have indicated two regions of the 30S subunit to be important for termination. One site important for all three codons and both factors is at or near the decoding site (the A site component of the decoding site) as defined by those antibiotics that bind in this region, particularly hygromycin. The other site is on helix 34 for RF-2 binding in response to UGA and for RF-1 binding by in response to both UAG and UAA as defined by spectinomycin. This region appears to have a much more subtle involvement, depending on the factor, codon and antibiotic concentration used, but has recently been implicated in a rRNA-mRNA basepairing model for termination. We further investigated the importance of this second region on termination by testing the effects of ribosomes altered in this helix.

The effects of spectinomycin resistant ribosomes on termination

Makosky and Dahlberg constructed 3 mutants containing different single base substitutions at C1192 in helix 34 (29). The mutations confer spectinomycin resistance and are located in or very near the major binding site of spectinomycin (21, 28).

Characterisation of the altered ribosomes. The altered ribosomes were isolated from *E.coli* HB101 grown in the presence of spectinomycin (50μg/ml). The proportion of altered ribosomes was determined by measuring the effect of spectinomycin (100μM) on the ability of a coupled cell free transcription/translation system (35) prepared from cells grown under the same conditions to synthesise protein. Spectinomycin insensitive ribosomes made up 74−95% of such preparations.

Before using these ribosomes in termination assays they were first tested for their ability to form normal substrate complexes. The ability of the ribosomes to form substrate complexes by the binding of f[^{3}H]Met-tRNA to the P site of ribosome, and the subsequent reaction of puromycin to release puromycin-f[^{3}H]Met from the complexes were measured.

All three variant ribosomes formed less stable substrate complexes with AUG and f[^{3}H]Met-tRNA than the wild type ribosomes, indicating significant perturbation of the P site. The 1192G variant formed somewhat more stable complexes than the other two variants (Fig. 2A). Other aliquots of the complexes were incubated with puromycin for 30 min at 20°C. In this assay the 50S peptidyl transferase catalyses the formation and release of f[^{3}H]Met-puromycin. With the altered ribosomes a reduction in the f[^{3}H]Met-puromycin released was also observed (Fig. 2B). This was likely to be a consequence of the reduced stable binding of f[^{3}H]Met-tRNA by these variants.

Substrate complexes containing 5 pmol of ribosomes were used for subsequent assays as this amount was needed to give comparable termination activities to those with unaltered ribosomes in the catalytic assay (top points Fig. 2B).

The effects of the alterations at C1192 on in vitro termination. The variant ribosomes were next tested for their effects on the overall termination *in vitro*. The ribosomal complexes were incubated with varying amounts of the two release factors as indicated. The results are normalised to the amount of puromycin dependent release, to eliminate the effect of the P site impairment. The variants A1192 and U1192 showed poor termination activity with both factors, and this activity was lower with RF-2

(Fig. 2C). In contrast the G1192 variant showed termination activity only slightly less than that of the wild type.

Codon dependent binding of both factors to the ribosome was also markedly reduced for RF-2 for the A1192 and U1192 variants, but UAG dependent RF-1 binding was less affected (Fig. 2C). The G1192 alteration also gave a slight reduction. This suggests that it is factor binding to the altered ribosomes that is primarily effected and that this reduces the overall termination reaction.

DISCUSSION

Using antibiotic probes we have identified as important both of the regions of the 30S subunit that have previously been suggested to be involved in termination. How can our data be reconciled with the ideas of how decoding of stop signals occurs (4, 9, 15, 17).

The decoding site. Antibiotics that bind the A site inhibit both codon dependent RF binding and the overall termination event, with all three codons and both release factors. Several studies have indicated that hygromycin and neomycin perturb and have their primary effects, in the decoding site at the base of helix 44 (nucleotides 1408, 1494, Fig. 1). The inhibition of termination by these miscoding antibiotics could be explained by a mechanism whereby on antibiotic binding perturbation of the decoding site causes ambiguity (miscoding) and the RF does not bind. This implies that the RF recognises the stop signal in the same location as the tRNA recognises the sense signal. Alternatively the antibiotic may provide direct steric hindrance of this region of the A site preventing RF contact with the stop signal, also indicating that part of the RF occupies a site very similar to that of the anticodon.

Tetracycline also gave a similar pattern of inhibition of termination to the miscoding antibiotics, indicating that the binding site of both RFs also includes the region of the aminoacyl tRNA binding site perturbed by tetracycline. This site has not yet been identified precisely, as discussed below.

Several other lines of evidence also point to the involvement of the A site in termination, notably competition *in vivo* between suppressor tRNAs and RF for decoding of stop codons at this site, and competition between EF-Tu and RFs *in vitro* (8). Further evidence of proximity between the RF-2 and base of helix 44 comes from a recent study in which a thio-U nucleotide in a termination signal could be crosslinked to both RF-2 and A1408 (9), one of the nucleotides affected by hygromycin or neomycin binding (21) and weakly protected by A site bound tRNA (12). Taken together this evidence strongly suggests that both factors directly contact the stop signal in the decoding site. However, some earlier studies also indicate that the release factor has some flexibility in this signal recognition site, as specific recognition *in vitro* still occurs after insertion of a few bases before the stop codon (36, 37).

Helix 34. Spectinomycin had different affects on RF-1 and RF-2 mediated termination reactions. It stimulated RF-1 mediated binding and termination, but stimulated only the UGA dependent *in vitro* termination reaction with RF-2. At higher concentrations spectinomycin partially inhibited RF-2 binding. Analysis of the spectinomycin-resistant ribosomes altered at position 1192 showed that they were impaired in the binding of tRNA to the P site. They were also impaired in both RF-2 and RF-1 dependent binding, but in this case RF-2 dependent activities were more affected than those for RF-1. This suggests that the structure of this region of helix 34 is generally important for termination, but in contrast to the base of helix 44, forms part of a domain showing some discrimination for codon and factor, and dependent upon the concentration of the antibiotic. The apparently contradictory effects of the antibiotic and resistant mutations are consistent with a model in which spectinomycin binding changes the conformation in this region, favouring factor binding. Alterations at 1192 however, may prevent both the antibiotic binding and the beneficial structural change.

Interestingly, we had initially observed only effects on RF-2 with UGA (8) which implied that this region was specific for this factor and codon, as initially suggested by Murgola et al. (4). However, as we increased the sensitivity of the assays more general effects were observed. This was also found in suppression studies with mutants in the central region of helix 34, they initially seemed specific for UGA (4, 16) but were shown subsequently to affect suppression of all three termination codons (19, 20).

What is the role of this region of helix 34? On binding, spectinomycin strongly protects two nucleotides (C1063, G1064) of the right hand stem of helix 34, and resistance can be conferred by mutations in G1064, or its basepairing partner C1192 (Right hand stem, Fig 1).

Several lines of evidence implicate this region in subunit association and translocation. Structural alterations occur in this region following subunit association, and the two G-C base pairs indicated by crosses may not be present in the 70S ribosome, but an adjacent non-canonical U-U may be (dotted line)(38, 39). Indeed, the region is essential for subunit association (40) and may be located at the subunit interface (11, 39) as kethoxal modification of 1064 and 1067 prevents subunit association (40, 17). Brimacombe's model also places this end of helix 34 on the interface side of the subunit (Fig 1, 11). Such a location would correlate with the major effect of spectinomycin being on translocation, specifically the EF-G cycle (30). The EF-G cycle is also inhibited by these alterations at C1192 (28). This finding supports our observation of impaired P site binding with these 1192 base substitutions, and indicates that the key point of this inhibition is likely to be the acceptance of the peptidyl-tRNA into the P site.

The central region of helix 34 adjacent to the right hand stem (Fig 1) had been previously shown to affect termination, particularly that mediated by RF-2 at UGA codons. This region of helix 34 is highly conserved, many nucleotides are conserved in small subunit RNAs (shown in bold outlining in Fig 1), but not however the two triplets proposed to base pair with UGA (neither are found in most Archea, 6, 7).

This central region of helix 34 is affected by ribosomal proteins S2, S3, and S5, and antibodies to all three of these proteins inhibit termination reactions (42, 8). S2 and S3 have been crosslinked to it (41) and S2 and S3 binding may induce a conformational change that brings S5 into contact with it (43). Interestingly, antibodies against S3 inhibit RF-2 functions more than those of RF-1 (42). Mutations in S5 may confer spectinomycin resistance (44), and antibodies against S5 are very inhibitory to termination reactions with both factors (42).

Sequence changes to the central region of the helix also effect termination reactions *in vivo*. Alterations in the first position of the sequences complementary to UGA, from U1202 or U1199 to C, were shown to cause an apparent UGA specific increase in readthrough by a natural tRNA at one very leaky UGA context,

~4.5% wildtype readthrough, although not at much tighter UAG and UAA contexts, with ~0.55% and ~0.015% readthrough respectively (45, 46). More recently however, the level of suppression in the three contexts was shown to be either increased or decreased (dependent on the mutation and codon) in the presence of suppressor tRNAs that normally increase the background level of suppression (20). A similar series of studies on bacteria containing a deletion or mutations in the nearby C1054 indicated at first UGA specific suppression in some contexts (4), but later increased suppression was found at all three stop codons with these ribosomes compared to wild type ribosomes in the presence of a suppressor tRNA (19). In a sucrose-gradient sedimentation assay, only the C1054 deletion showed impaired UGA directed RF-2 binding to 70S ribosomes (46).

Several recent studies have indicated that helix 34 may be much nearer to the aminoacyl site, and to helix 44. Firstly, a crosslink was observed between the 3' base of the A site codon and U1052 (47, 48, discussed in 49). As one turn of an A helix is 11 basepairs this base may be toward the same face of the helix as the spectinomycin interaction site (C1063, G1064). (However, there is some irregularity in this helix due to the bulge, and a conformational change during subunit association as discussed above.) Second, a functional interaction between the basepairs at the base of helix 44 and 1192 has been observed, a U1192 alteration restores the activity of ribosomes with four alterations at the base of helix 44 which retain the stem structure but with a different nucleotide composition (changing the 1409–1491 basepair from C-G to A-T and 1411–1389 from C-G to G-C, 50). Third, tetracycline which classically inhibits A site tRNA binding, and RF binding, also has an effect on helix 34. On binding it enhances the reactivity of both U1052 (the A site codon crosslink site) and C1054 (which when mutated caused suppression) (21, Fig 1). If the aminoacyl site, helix 34, helix 44, and ribosomal protein S7 were in close proximity this would help to clarify other studies on the tetracycline binding site on the ribosome (12, 32–34, 49, 55).

Given the apparent proximity of helix 34 to the A site component of the decoding site the simplest explanation of our data and those of others concerning this helix would be a model in which the RFs recognise the stop signals in the decoding site. Slight sequence and factor specific variations in the location of decoding events are likely (8, 51–53). In the case of the RF-2 recognising UGA this could involve nearby helix 34 and account for the apparent UGA specificity of some perturbations in parts of this helix. We are focussing now on whether a base paired structure between rRNA and the stop signal in the mRNA is a part of the substrate recognised by the release factor, a critical feature of the elucidation of mechanisms of codon recognition.

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