A single mutation in 16S rRNA that affects mRNA binding and translation-termination

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ABSTRACT

A single base change in 16S rRNA (C726 to G) has previously been shown to have a dramatic effect on protein synthesis in *E. coli* (1). This paper more specifically details the effects of the mutation on mRNA binding and translation-termination. The *in vitro* technique of toeprinting (2) was used to demonstrate that 30S subunits containing the mutation 726G had an altered binding affinity for mRNA by comparison to the wild type. In addition, expression of the mutant ribosomes *in vivo* resulted in exclusive suppression of the UGA nonsense codon. This effect was supported by *in vitro* studies that showed the mutant ribosomes to have an altered binding affinity for Release Factor-2.

INTRODUCTION

The functional involvement of rRNA sequences during catalytic stages of the translation cycle is by now a well documented phenomenon (3). Here we present further evidence for the participation of a region of 16S rRNA in both mRNA binding and translation-termination. It has been previously reported that a C to G transversion at base 726 in 16S rRNA, dramatically affects protein synthesis (1). Nucleotide C726 forms part of a two base paired stem (G725, C726 / G731, C732) immediately downstream of a single stranded region (4). In close proximity to this region are nucleotides G693, protected by P site bound tRNA (5), and U723, one of a limited number of bases outside of the 3′ decoding region that is alternately accessible to chemical probes during the active-inactive transition (6). Ribosomal protein S21 has been cross linked to this vicinity as well as to the 3′ end of 16S rRNA (7). Ribosomal proteins S11 and S15 map to this region which has been placed in the platform adjacent to the cleft of the 30S subunit (8,9). This location is consistent with a number of studies which implicate S11 in the codon:anticodon interaction and S21 at initiation, exposing the 3′ end of 16S rRNA so that it is accessible for base pairing and translation of messenger RNAs (10,11).

The presence of the 726G mutation *in vivo* caused three distinct categories of changes on protein synthesis: the induction of some of the heat shock proteins by the wild type ribosomes in response to aberrant protein synthesis, alternate levels in the expression of wild type proteins and, the synthesis of novel peptides of varying sizes. This paper presents evidence that can account for all these observed changes. In addition, these data can be interpreted as there being a link between termination and initiation events involving a dynamic intra-molecular RNA/RNA interaction, thereby providing a clue as to the functional significance of the 720's region.

MATERIALS AND METHODS

Strains and vectors

Plasmid pRS170 contains the sequence from −92 to +170 of bacteriophage T4 gene 32 (12) and was a gift from L. Gold. Plasmid pKK3535 contains the complete *E. coli rrnB* operon including its natural promoters P_1 and P_2 (13) and the derivative pKK726G contains the C to G transversion at base 726 in the 16S rDNA, as previously described (1). Plasmid pNO2680 contains the *E. coli rrnB* operon under control of the lambda leftward promoter (14) and the derivative pL726G contains the 726G mutation. Ribosomes encoded by pKK726G constituted <15% of the total 70S population, whereas ribosomes encoded by pL726G represented approximately 30%, following two hours induction at 42°C.

Preparation of ribosomes and subunits

Cells were harvested during mid-logarithmic growth and washed once in buffer A (10mM Tris-HCl pH7·6, 10mM $MgCl_2$, 150mM KCl, 1mM DTT). All subsequent steps were performed at 0−4°C. The cells were broken by grinding in the presence of aluminia and buffer A and the cell debris removed by successive centrifugation steps (12000 rpm, 10 min. then 16000 rpm, 60 min., Sorval SS34 rotor). The ribosomes were pelleted from the supernatant by centrifugation (50000 rpm, 3h, Beckman Ti60 rotor) and resuspended in buffer B (10mM Tris-HCl pH7·6, 15mM $MgCl_2$, 250mM NH_4Cl, 1mM DTT). Insoluble material was removed by centrifugation (16000 rpm, 1h., Sorval SS34 rotor) and the ribosomes fractionated by sedimentation through 10−30% (w/v) sucrose gradients (20000 rpm, 16h., Beckman SW28 rotor). The 70S subunits were pooled and dissociated in the presence of 10mM Tris-HCl pH7·6, 1·5mM $MgCl_2$, 60mM KCl, 1mM DTT. The resulting 30S subunits were purified by sedimentation through 10−30% (w/v) sucrose gradients, pelleted

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as above and resuspended in buffer A. The subunits were heat activated at 37°C for 30 min. prior to the toeprint inhibition assay.

Toeprinting assay

The conditions were as described by Hartz, McPheeters and Gold (15). The mRNA template was synthesized *in vitro* using the purified 350 base pair PvuII fragment from pRS170. The components for *in vitro* transcription were supplied by Pharmacia. The 10μl toeprinting reactions were prepared in standard buffer (10mM Tris-acetate, pH7·4, 60mM NH_4Cl, 6mM β-mercaptoethanol, 10mM Mg-acetate) and contained 5pmol of 30S subunits, 50pmol of uncharged tRNA (as specified) and 0·2pmol of the *in vitro* transcript. The reactions were preincubated on ice for 10 min. prior to the addition of reverse transcriptase and shifting to 37°C. Toeprinting reactions performed in the presence of drugs were achieved by preincubation of the 30S subunits with a 10 fold molar excess of the drug (relative to the 30S subunit concentration) for 5 min. at 0°C, followed by the addition of $tRNA_f^{Met}$ and a further 5 min. incubation at 0°C.

Measurement of β-Galactosidase activity

β-galactosidase measurements were performed according to Miller (16). Whole-cell assays were carried out in a final volume of 1·0 ml Z-buffer at 28°C and begun following the addition of 200μl o-nitro-phenyl-β-D galactopyranoside (4mg ml^{-1}). The reaction was terminated following the addition of 500μl 1M Na_2CO_3. The lysates were clarified by centrifugation prior to reading the A_{420}. The activity was measured as β-gal units = $[10^3 (A_{420})] / [OD_{600} \times \text{time (min)} \times \text{vol. (ml)}]$.

In vivo labelling of RF-2 termination factor

Radioactively labelled RF-2 protein was isolated from the overproducing strain SU1675 pPY1025 pKB4 (synRF-2) kindly provided by R. Weiss and L. Yang. Cells were grown in supplemented M9 minimal medium (16) lacking methionine and cysteine and containing 100μg/ml ampicillin and 50μg/ml spectinomycin for plasmid selection. After growth to 0·5 OD, episomal transcription was started by adding IPTG to a final concentration of 1·5mM. Following 2 hours of induction, [^{35}S]-methionine was added (500μCi/100ml cell culture) and cells were incubated at 37°C for a further 10−12 hours. The termination factor was purified from freeze-cracked cell lysates (17) following the method of Caskey *et al.* (18). The purity of the preparation was routinely checked by one- and two-dimensional polyacrylamide gel electrophoresis. Estimates of the specific activity of the [^{35}S]-RF-2 protein preparations varied from 0·05−1·0μCi/nmole.

Binding of RF-2 to 70S ribosomes

The interaction between 70S ribosomes and RF-2 termination factor was standardly determined in 200μl TMK-buffer (20mM Tris-HCl pH 7·5, 20mM $MgCl_2$, 100mM KCl) containing 20−50 pmoles heat activated (40°C, 30 min.) 70S ribosomes, 2·5nmoles termination codon (UGA or UAA) and varying amounts of [^{35}S]-RF-2 protein (2·5−500pmoles) in the presence of 6% (v/v) ethanol (19). Equilibrated termination complexes (0−4°C, 30 min.) were analyzed by isokinetic centrifugation on linear sucrose gradients (5−30% (w/v), Beckman SW 40 rotor) in TMK-buffer containing 6% (v/v) ethanol at 36000 rpm and 4°C for 4·5 hours (20). After centrifugation the gradients were fractionated (400μl fractions) and both the A_{260} and radioactivity profiles were determined. The recovery of [^{35}S] counts was in the range of 90−95%. Nonspecific binding of labelled RF-2 was determined using either no termination codon or the RF-1 specific UAG triplet codon. The binding data were finally analyzed by applying a double reciprocal plot analysis.

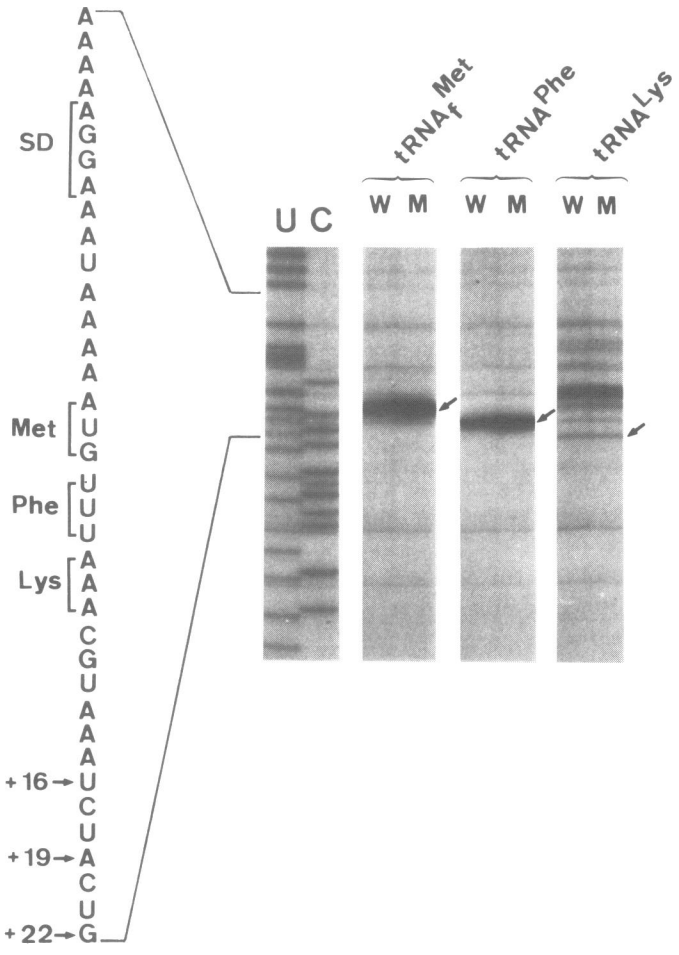

Figure 1. In vitro analysis of 30S·mRNA·tRNA complex formation by wild type and mutant 30S subunits. Stable initiation-like complex formation on phage T4 gene 32 mRNA in the presence of either wild type (W) or mutant (M) 30S subunits and uncharged tRNA molecules, inhibit further migration of reverse transcriptase thus causing the premature stop/toeprint signals. The toeprints are generated 16 nucleotides 3′ to the first nucleotide of the sense codon. Sequence information of the mRNA template along with U and C sequencing tracks are to the left.

RESULTS

Effect of ribosomes containing 726G on mRNA binding

The technique of toeprinting (2) was employed to determine the ability of the mutant ribosomes to bind to mRNA *in vitro*. The formation of a stable initiation-like complex consisting of 30S ribosomal subunits and a cognate tRNA bound to mRNA inhibits further migration of reverse transcriptase from a point downstream of the ribosome binding site, thus resulting in a premature stop or 'toeprint'. A toeprint is generated 16 nucleotides 3′ to the first nucleotide of a sense codon, dependent on the appropriate cognate tRNA used during the assay.

Purified 30S subunits were obtained from translationally active 70S ribosomes, from transformants of either pNO2680 (wild type) or pL726G (approximately 30% of which contained the mutation 726G in the 16S rRNA (1)). The 30S subunits were

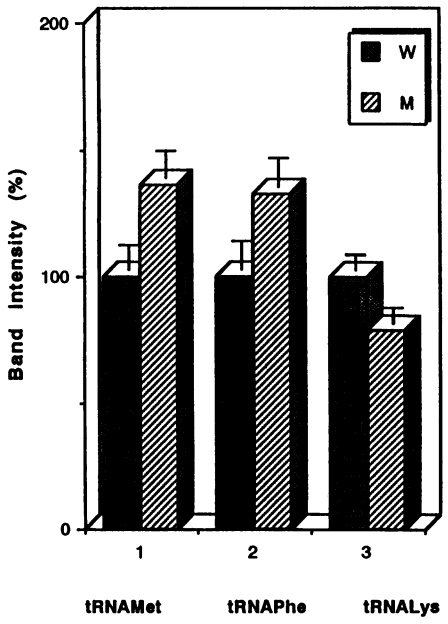

Figure 2. Densitometric analysis of toeprint signals generated by 30S·mRNA·tRNA complexes on phage T4 gene 32 mRNA. Band intensities of the toeprint signals generated in the presence of either wild type (W) or mutant (M) 30S subunits and uncharged tRNA molecules (as indicated), were obtained by densitometric scans (at 525nm) of non-saturated autoradiograms. Error bars indicate standard deviations for ≥3 independent experiments. Wild type signals were set at 100%.

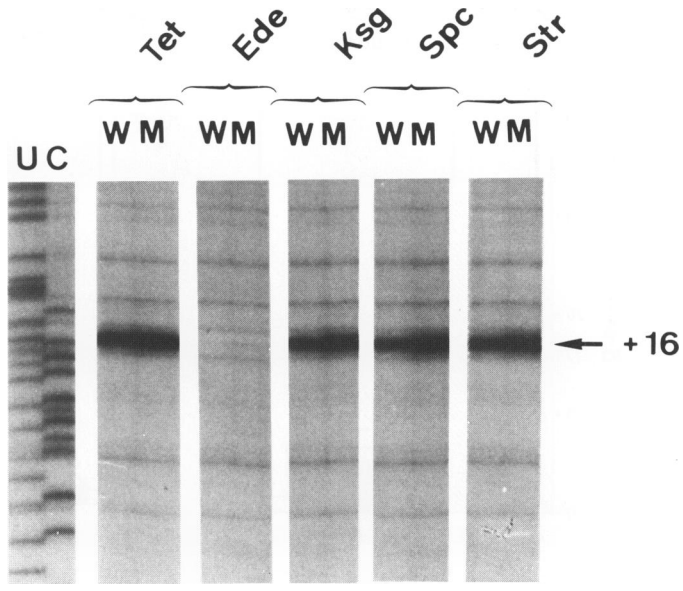

Figure 3. In vitro analysis of 30S·mRNA·tRNA complex formation in the presence of antibiotics. Toeprint inhibition experiments (Figure 1) were used to determine the affect of antibiotics on stable initiation-like complex formation in the presence of either wild type (W) or mutant (M) 30S subunits, phage T4 gene 32 mRNA and $tRNA_f^{Met}$. (Tet-tetracycline; Ede-edeine; Ksg-kasugamycin; Spc-spectinomycin; Str-streptomycin).

used for the generation of stable complexes with T4 gene 32 mRNA. This mRNA is characterized by a weak Shine-Dalgarno motif upstream of the AUG initiation codon (designated codon position +1), followed immediately by codons for phenylalanine (+2) and lysine (+3). A strong toeprint signal was obtained with both wild type and mutant populations of 30S subunits in the presence of either $tRNA_f^{Met}$ or $tRNA^{Phe}$ (Figure 1). These complexes generated toeprints 16 nucleotides, 3′ to the first nucleotide of the appropriate codon. In the presence of $tRNA^{Lys}$, multiple toeprints were observed each corresponding to an appropriate distance from one of the multiple lysine codons on the mRNA, present both upstream and downstream of the AUG initiation codon.

The strength of the toeprint reflects the stability of the initiation-like complex (22). It was evident that the population of 30S subunits that contained approximately 30% mutant ribosomes, generated a stronger stop signal in the presence of either $tRNA_f^{Met}$ or $tRNA^{Phe}$ than with wild type 30S subunits alone. The density of the toeprint signals were measured and are presented in Figure 2. In the presence of the mutant 30S subunits and $tRNA_f^{Met}$ the toeprint signals were increased by $37 \pm 10\%$ by comparison to those generated by wild type 30S subunits. Similarly, in the presence of $tRNA^{Phe}$ the toeprint was $33 \pm 11\%$ more intense in the mutant track by comparison to that of the wild type. These figures could be most simply explained in terms of the mutant 30S subunits having an increased binding affinity for the mRNA. The enhanced stop signals were not observed in the presence of $tRNA^{Lys}$. The intensity of the toeprints generated by the 30S subunit populations binding at codon position +3 in the presence of $tRNA^{Lys}$, showed a $21 \pm 6\%$ decrease in the presence of the mutant ribosomes.

The effect of various antibiotics on initiation-like complex formation were also assayed using the toeprinting technique. The reason for this was based on the following rationale. Mutations in ribosomal protein S2 confer resistance to kasugamycin (23). The 726G mutation affects ribosomal protein S2 binding (1), and therefore may conceivably alter the interaction of this drug with the mutant ribosomes. In addition, S2 is footprinted to helix 34, a region associated with resistance to spectinomycin (a C to U base change at 1192 (24)). Finally edeine is associated with base U693, which is in close proximity to C726 (5); again the presence of the 726G mutation could have some affect on the interaction by edeine with the 30S subunits. Therefore, the 30S subunits were treated with various antibiotics prior to the addition of the uncharged tRNAs and reverse transcriptase. It was evident that the presence of the antibiotics did not affect initiation-like complex formation for either the mutant or wild type ribosomes, with the exception of edeine (Figure 3). The inhibitory effect by edeine on ternary complex formation (30S·$tRNA_f^{Met}$·mRNA) reflects the effect of this antibiotic on the binding of mRNA to the small subunit (25).

Expression of 726G caused read through of UGA nonsense codons

In vivo expression of the mutant ribosomes resulted in the synthesis of novel peptides (1). Since these included high molecular weight proteins it was proposed that these may be products generated by the read through of nonsense codons on polycistronic mRNAs. To test this hypothesis, transformants of pKK726G were screened for their ability to read through each of the three stop codons. This was achieved *in vivo* utilizing a system in which ribosomes must translate through a stop codon in order to synthesize functional β-galactosidase (26). Three test strains were available: DEV1-*lac* Z UAG, DEV14-*lac* Z UAA and DEV15-*lac* Z UGA. The levels of enzyme activity reflected the level of readthrough activity. Each of the DEV strains were

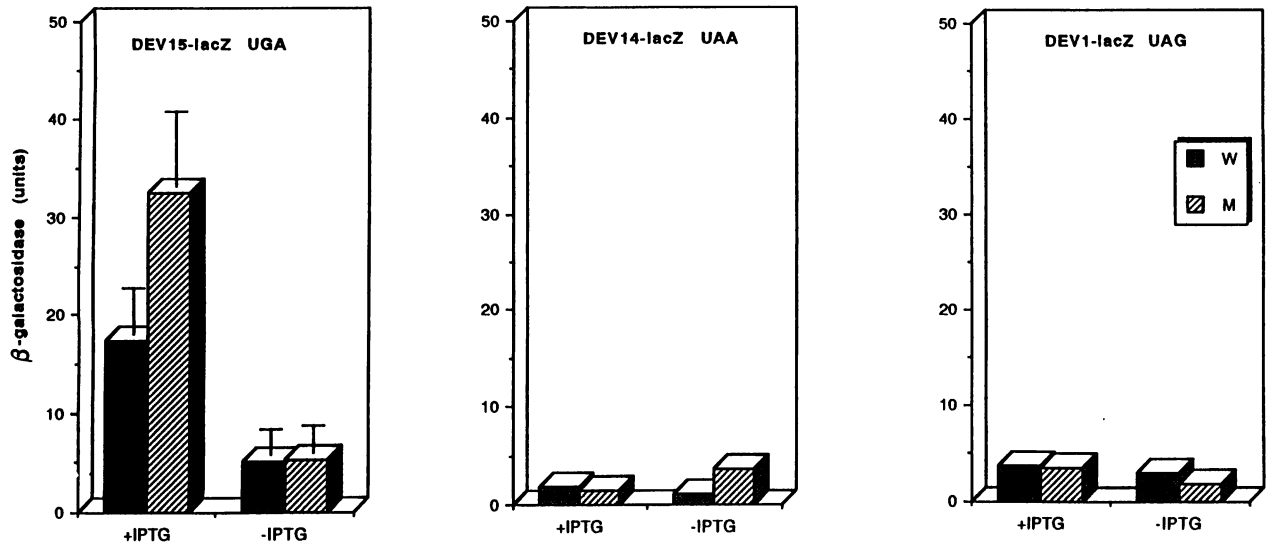

Figure 4. *In vivo* suppression data in DEV-*lacZ* transformants. Synthesis of functional β-galactosidase in the DEV-*lacZ* strains transformed by either pKK3535 (W) or pKK726G (M), is dependent on suppression of the specified nonsense codon and the levels of enzyme activity (β-galactosidase) reflect the level of suppression activity. Readthrough levels were recorded in the presence (+) and absence (−) of IPTG. The standard deviations are indicated by error bars.

transformed by pKK726G and the levels of β-galactosidase activity measured. Transformants of the wild type plasmid pKK3535 were used as a control to estimate the background levels of termination suppression. A summary of the data for nonsense suppression in transformants of DEV-*lac* Z strains is presented in Figure 4. Recognition of UAA and UAG stop codons was not impaired indicating that the interaction by RF-1 with the ribosomes was not functionally altered. By contrast, DEV15-*lac* Z UGA transformed by pKK726G showed a significant increase in the level of UGA suppression. Taking into account the background levels of β-galactosidase activity (that is, both in the absence of IPTG and following induction in transformants of pKK3535) there was a statistically significant increase greater than 2 fold in the level of readthrough of the UGA stop codon by transformants of pKK726G. Synthesis of functional β-galactosidase additionally implied that the mutated ribosomes neither affected the accuracy of translation initiation nor elongation.

Ribosomes containing 726G affect RF-2 binding

Termination at UGA nonsense codons is dependent on the function of RF-2. Therefore, the interaction of RF-2 with ribosomes containing the mutation 726G was analyzed *in vitro*, that is the ability to form a termination complex dependent on the presence of triplet stop codons and [^{35}S]-methionine labelled RF-2. Ribosomes obtained from transformants of pKK726G showed over a two fold increase in the binding affinity for the termination factor, by comparison to the wild type control, in the presence of either UAA or UGA triplet stop codons (Figure 5).

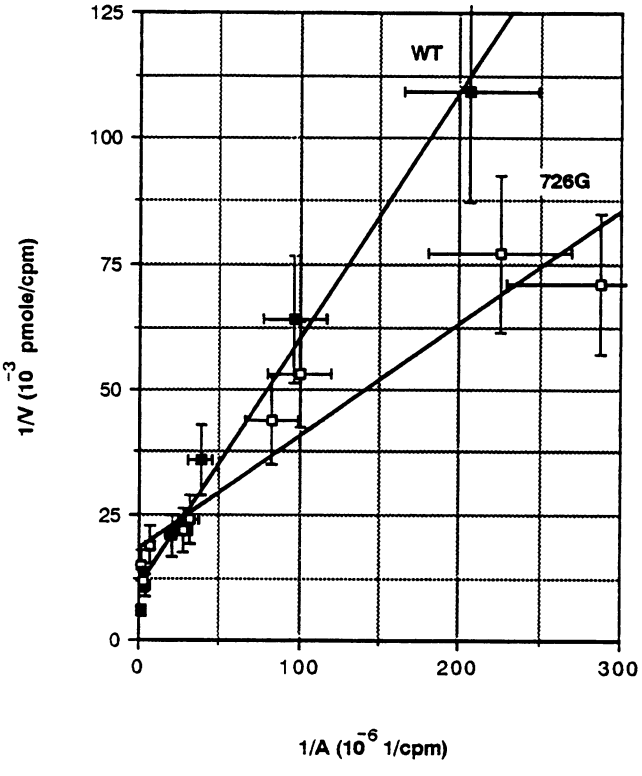

Figure 5. Double reciprocal plot analysis for the binding of RF-2 to 70S ribosomes. The reported data points are the average of ≥3 independent determinations with relative errors of ±20% as indicated by the error bars. V = RF-2 protein bound (cpm)/70S ribosomes (pmole); A = unbound RF-2 protein (cpm). Relative apparent association constants (rel. $K_{app.}$) were determined by a linear least square fit of the data points with the slope of the regression line representing $1/nK_{app.}$. Assuming a 1:1 molar stoichiometry of the two molecules in the complex the relative apparent association constant for wild type ribosomes was set to 1.0. Accordingly, for ribosomes from 726G mutant cells a relative value of 2.5 was calculated. Binding data were identical for both UGA and UAA stop codons. WT denotes wild type.

DISCUSSION

This paper describes the effects caused by a single base change in 16S rRNA on both mRNA binding and translation-termination. In the presence of tRNAs cognate for the +1 and +2 codons on the T4 gene 32 mRNA, the mutant ribosome population had

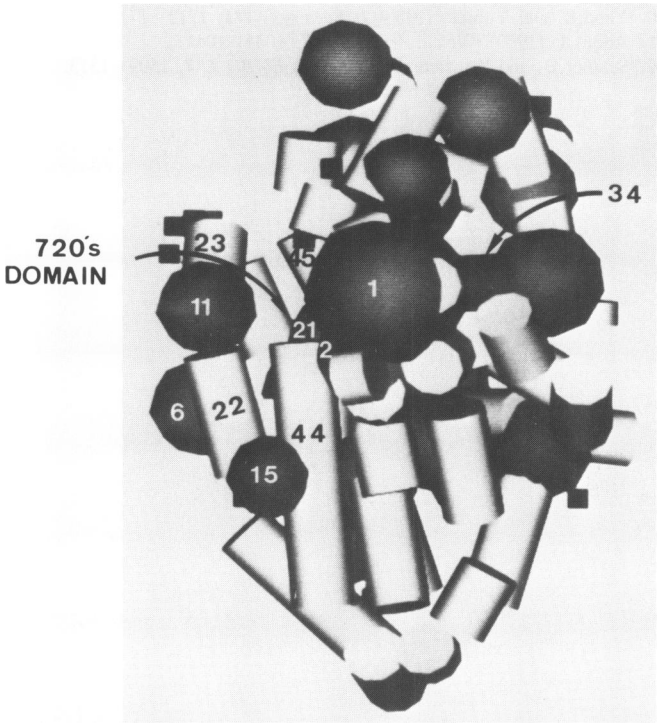

Figure 6. Tertiary structure model of the *E. coli* 30S subunit denoting the location of proteins associated with the initiation and termination domains (Redrawn with kind permission from R. Brimacombe). Annotation of RNA helices (cylindrical elements, black numbering) and proteins (spheres, white numbering) was according to Schüler and Brimacombe (32).

a higher affinity for the mRNA than the wild type (Figures 1 and 2). The opposite was observed in the presence of $tRNA^{Lys}$ (Figures 1 and 2) which has multiple codons encoding lysine located upstream and downstream (+3) of the initiation codon. Ribosomes containing the 726G mutation essentially behaved as wild type during initiation-like complex formation in the presence of various antibiotics (Figure 3). Transformants of pKK726G also caused the readthrough of the UGA nonsense codon (Figure 4), whilst termination at UAA and UAG stop codons was unaffected. *In vitro* termination complexes dependent on either UAA or UGA triplet codons and RF-2 showed that the mutant ribosomes had an increased binding affinity for the termination factor (Figure 5).

The presence of the 726G mutation has a dramatic effect on the levels of protein synthesized *in vivo* (1). This phenomenon can in part be explained by the altered mRNA binding event characteristic of the mutant ribosome populations (Figure 1). It has been reported that the potential of 30S subunits to bind MS2 messenger is dependent on the presence of ribosomal protein S21, and is accompanied by exposure of the 3′ terminus of 16S rRNA (10,11). Whilst the mutant 70S ribosomes contained S21, the free 30S subunit pool had a decreased affinity for S21 (1). Therefore, the functional binding of S21 to the mutant ribosomes may be altered in such a manner as to cause the altered mRNA binding event (Figures 1 and 2). The 3′ terminus of 16S rRNA is proposed to be involved in an intra-molecular interaction (10,27). Immediately upstream of base 726 is a sequence motif (721−725: GGUGG) complementary to the 3′ end of 16S rRNA (1535−1539:CCUCC). The cross linking of S21 to these two regions (7) and that S21 has helix destabilising properties (11), are consistent with the proposal that S21 functions to destabilise this intra-molecular base pairing interaction, in order to expose the 3′ terminus of 16S rRNA , thereby permiting initiation complex formation. In the case of the mutated ribosomes, the addition of a G residue at base 726 creates an extensive stretch of consecutive G nucleotides (721−727:GGUGGGG) thus likely creating a conformational change (by base stacking) that could interfere with the base pairing to the 3′ end of the 16S rRNA. Therefore, in the mutant 30S subunits the 16S 3′ end is freely available to base pair with the message, in line with the observed increased affinity by these ribosomes for the mRNA (Figures 1 and 2). The data show that this holds true for complex formation attributable to either of the first two codons on the mRNA. In the case of $tRNA^{Lys}$, complex formation is clearly a different interaction since a decrease in mRNA binding was observed (Figures 1 and 2). Whether this 'position dependent' phenomenon reflects conformational changes in either the 30S subunit and/or the mRNA, remains unknown. It can not however, be explained in terms of attenuation since the messenger lacks the required secondary structural elements (22). That the ribosomes containing the 726G mutation essentially behaved as wild type in the presence of the antibiotics tested (Figure 3), was indicative of the specificity of the mutation for defined steps within the translation cycle.

Ribosomes containing the 726G mutation suppress termination at UGA nonsense codons *in vivo* (Figure 4). Taking into account the percentage of mutant ribosomes encoded by pKK726G, this mutation caused the same degree of UGA suppression as mutants in helix 34 of 16S rRNA (such as 1199 and 1202), that are believed to directly base pair with the UGA termination codon on the mRNA (Göringer, unpublished data). For the 726G mutation, the suppression activity can most simply be attributed to the mutant ribosomes having some effect on RF-2 binding. The *in vitro* binding data are in support of this since in the presence of both RF-2 specific termination triplet codons, an increased association constant was measured (Figure 5). The result of this can be interpreted as the termination complex no longer having the necessary degree of flexibility required for its function, leading to the UGA suppression seen *in vivo* (Figure 4). The increased binding constant can be attributed to one of two reasons: 726G directly alters an rRNA contact point for RF-2 (that is, making it more accessible) or, the mutation leads to a secondary effect by altering the binding of a ribosomal protein, for example S2 and/or S21 (1). Interestingly, ribosomes lacking S15, mapped to the region surrounding C726 (29), suppress UGA nonsense codons (30). Note, the binding of S15 to 16S rRNA containing 726G was not altered (1).

Suppression of UGA nonsense codons on polycistronic mRNAs has two effects that would account for the *in vivo* pattern of protein synthesis: the synthesis of novel peptides and a decrease in the level of translation of 3′ proximal encoding sequences.

Although the mutation 726G had an affect on both an initiation-like event and translation-termination, the functional domains for these two events have been established to involve apparently distal regions, the 3′ end of 16S rRNA and helix 34 respectively (Figure 6). Could the 720's region represent a functional domain that links these two events? Atkins proposed that to achieve ongoing translation of polycistronic mRNAs the ribosome may have evolved some mechanism that provides an intimate association between termination and initiation (31). Sequences surrounding

the initiation codons have a high preponderance of either UAA or UGA within the first three to seven codons upstream of the translation start site (31). The presence of these stop codons suggest that RF-2 may interact with this region of the mRNA. This, along with the data presented here is consistent with the following proposal: termination factor RF-2 serves an additional role to prevent the 720's domain from sequestering the 3′ end of 16S rRNA thus ensuring internal reinitiation on polycistronic mRNAs.

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This manuscript is dedicated to the memory of Professor H.G. Wittmann.

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