doi:10.1006/jmbi.2000.3672 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2000) 299, 1–15

JMB

Interaction of Translation Initiation Factor IF1 with the *E. coli* Ribosomal A Site

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Initiation Factor 1 (IF1) is required for the initiation of translation in *Escherichia coli*. However, the precise function of IF1 remains unknown. Current evidence suggests that IF1 is an RNA-binding protein that sits in the A site of the decoding region of 16 S rRNA. IF1 binding to 30 S subunits changes the reactivity of nucleotides in the A site to chemical probes. The N1 position of A1408 is enhanced, while the N1 positions of A1492 and A1493 are protected from reactivity with dimethyl sulfate (DMS). The N1-N2 positions of G530 are also protected from reactivity with kethoxal. Quantitative footprinting experiments show that the dissociation constant for IF1 binding to the 30 S subunit is 0.9 μM and that IF1 also alters the reactivity of a subset of Class III sites that are protected by tRNA, 50 S subunits, or aminoglycoside antibiotics. IF1 enhances the reactivity of the N1 position of A1413, A908, and A909 to DMS and the N1-N2 positions of G1487 to kethoxal. To characterize this RNA-protein interaction, several ribosomal mutants in the decoding region RNA were created, and IF1 binding to wild-type and mutant 30 S subunits was monitored by chemical modification and primer extension with allele-specific primers. The mutations C1407U, A1408G, A1492G, or A1493G disrupt IF1 binding to 30 S subunits, whereas the mutations G530A, U1406A, U1406G, G1491U, U1495A, U1495C, or U1495G had little effect on IF1 binding. Disruption of IF1 binding correlates with the deleterious phenotypic effects of certain mutations. IF1 binding to the A site of the 30 S subunit may modulate subunit association and the fidelity of tRNA selection in the P site through conformational changes in the 16 S rRNA.

Keywords: decoding region; IF1; RNA-protein interactions; translation initiation; conformational change

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Introduction

Initiation of translation establishes the correct reading frame on the mRNA, and thus, is one determinant of translational accuracy. Moreover, translational regulation occurs at the initiation step. Proper initiation requires three factors in *Escherichia coli*, IF1, IF2·GTP, and IF3, as well as a specialized initiator tRNA. Many details of the mechanism of initiation have not yet been ascertained. In prokaryotes, initiation begins on the 30 S subunit (for reviews, see Draper, 1996; Gualerzi & Pon, 1990; Hershey, 1987). The 30 S subunit binds to the translation initiation region on the mRNA, which contains a Shine-Dalgarno sequence complementary to the 3′ end of 16 S rRNA spaced four to seven nucleotides from the start codon. The initiation step in translation is different from elongation in that the initiator tRNA is recruited directly to the peptidyl-tRNA site (P site) instead of the aminoacyl-tRNA site (A site). When the fMet·tRNA^{fMet} is bound in the P site to the correct AUG initiation codon, the 50 S subunit is recruited, GTP is hydrolyzed, and the factors leave the 70 S initiation complex. At the end of the initiation process, the P site is filled with the initiator tRNA, and the A site is vacant awaiting the first aminoacyl-tRNA to be delivered as a ternary complex with the elongation factor EF-Tu.

The functions of the protein factors in initiation are slowly being discerned. IF3 is a subunit dissociation factor, and IF2 is a ribosome-dependent GTPase that hydrolyzes GTP once the 50 S subunit joins the 30 S initiation complex (Gualerzi & Pon, 1990). Together, IF2 and IF3 act on the 30 S

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0022-2836/00/010001–15 $35.00/0

initiation complex to select for charged and formylated initiator tRNA correctly contacting the AUG start codon in the P site (Hartz *et al*., 1989; Meinnel *et al*., 1999). IF1 has had no specific function attributed to it except to stimulate the binding and activities of the other two factors and to assist them in selecting the initiator tRNA once a 70 S initiation complex has formed (Hartz *et al*., 1989). Nonetheless, IF1 is essential for cell viability and cells deficient in IF1 exhibit few polysomes (Cummings & Hershey, 1994).

IF1 is small (71 amino acid residues), basic, and a member of the oligonucleotide/oligosaccharide binding fold family of proteins, which includes RNA-binding proteins such as aspartyl-tRNA synthetase, ribosomal protein S1, and the cold shock protein CspA (Bycroft *et al*., 1997; Sette *et al*., 1997), and eukaryotic initiation factors eIF2α and eIF1A (Gribskov, 1992; Kyrpides & Woese, 1998). Cleavage of 16 S rRNA between A1493 and G1494 with the bacteriocin cloacin DF13 specifically disrupts the function of IF1 (Figure 1(a); Baan *et al*., 1976). Moazed and co-workers (1995) have shown that IF1 footprints the decoding region of 16 S rRNA: IF1 binding to the 30 S subunit protects A1492 and A1493 from modification by dimethyl sulfate (DMS) and causes an enhancement of reactivity at A1408. In addition, IF1 protects G530 from attack by kethoxal. This set of nucleotides is also protected by A-site bound tRNA (Figure 1(b); Moazed & Noller, 1986, 1990) and is adjacent to the cloacin DF13 cut that causes loss of IF1 activity. These data taken together strongly suggest that IF1 is an RNA-binding protein that sits in the A site of the 30 S subunit.

The binding of IF1 to the ribosomal A site leads to two questions. What is the function of IF1 in the A site, and what is the structure of the interaction? Initiation is composed of two related activities, fidelity of tRNA selection in the P site and subunit association, that are modulated by the initiation factors. IF3 functions to inspect the codon-anticodon interaction in the P site (Hartz *et al*., 1989; Meinnel *et al*., 1999). Chemical modification experiments have shown that IF3 causes changes in reactivity of nucleotides directly adjacent to those footprinted by P-site bound tRNA (Moazed *et al*., 1995; Muralikrishna & Wickstrom, 1989), localizing IF3 to the P site. The affinities of the A and P sites for tRNA may be reciprocally linked (Green & Noller, 1997). Binding of IF1 to the A site may influence the binding of tRNA to the P site. In addition to the tRNA binding sites, both IF1 and IF3 alter the reactivity of nucleotides called ''Class III'' sites that are protected by tRNA, 50 S subunits, or aminoglycoside antibiotics (Figure 1 (b); Moazed & Noller, 1986, 1987; Moazed *et al*., 1995; Noller, 1991). These sites reflect the interdependence of subunit association and tRNA binding, and changes in their reactivities may be the result of a conformational change in 16 S rRNA.

Recently, the low resolution crystal structures of both the 30 S subunit alone and the 70 S ribosome complexed with tRNA have been reported (Cate *et al*., 1999; Clemons *et al*., 1999). In both structures the penultimate stem of 16 S rRNA, which contains the decoding region A site, is found at the subunit interface. Each of the class III regions implicated in initiation factor binding, the 790 loop, the 900 region, and the A1413·G1487 base-pair, are also found near each other at the subunit interface, although details differ between the two structures. The 530 loop has not yet been located in either electron density.

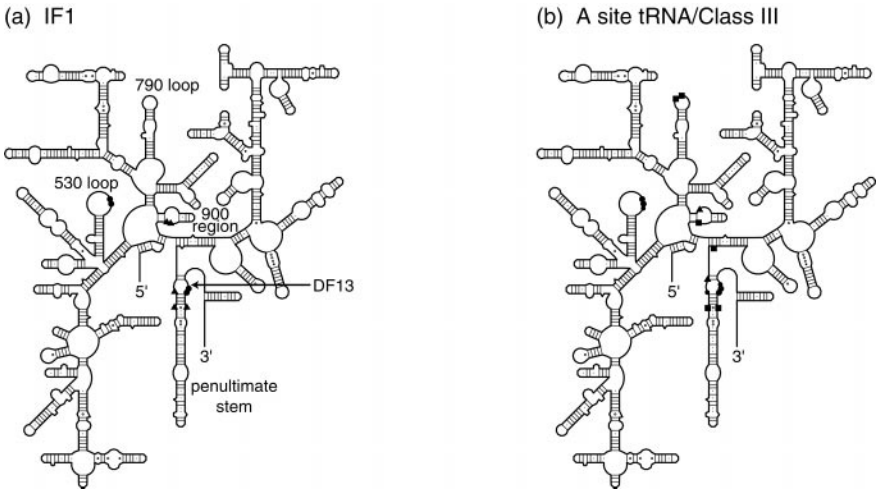

Figure 1. Secondary structure of *E. coli* 16 S rRNA. (a) Nucleotides affected by the binding of IF1 to 30 S subunits (Moazed *et al*., 1995; this study). (●) Nucleotides protected by the binding of IF1. (▲) Nucleotides enhanced in reactivity by the binding of IF1. An arrow indicates the location of the cloacin DF13 cleavage that inactivates IF1 function (Baan *et al*., 1976). (b) Bases affected by A-site bound tRNA or class III sites affected by tRNA, 50 S subunits, or aminoglycoside antibiotics (Moazed & Noller, 1986, 1987). (●) Nucleotides protected by A-site bound tRNA. (▲) Nucleotides whose reactivity is enhanced by A-site bound tRNA. (■) Nucleotides protected by class III ligands.

In the absence of a high resolution structure of the IF1-30 S subunit complex, chemical modification experiments have two advantages. First, quantitative data can be derived from titration experiments. Second, structural changes in the RNA upon ligand binding can be detected. IF1 binding to 30 S subunits primarily affects the reactivities of nucleotides in the 530 loop and the 1400-1500 decoding region. No ribosomal proteins are known to bind to this latter region of 16 S rRNA (Powers & Noller, 1995), and thus are unlikely to contribute to the IF1 binding site. In addition, these regions of RNA are among the most highly conserved ribosomal RNA sequences (Gutell, 1994), and thus may be functionally important to initiation. The purpose of this study is to characterize the RNA requirements for IF1 binding to the 30 S subunit by the use of quantitative footprinting and site-directed mutagenesis. We show that in addition to the changes in reactivity in the A site caused by IF1 previously reported, IF1 enhances the reactivity of a subset of the class III nucleotides of 16 S rRNA. Furthermore, IF1 binding has been tested by footprinting experiments on 30 S subunits that have been mutated in the decoding region and 530 loop. Particular mutations perturb the local RNA structure and disrupt IF1 binding. Disrupted IF1 binding correlates with the deleterious phenotypes of several mutants. The results suggest a model in which IF1 modulates a specific conformational change on the ribosome during initiation.

Results

Binding of IF1 to wild-type 30 S subunits

Upon binding of IF1 to wild-type 30 S subunits, several nucleotides in 16 S rRNA change their reactivity to chemical modifying agents as monitored by primer extension. It was previously reported that IF1 binding protects the N1 position of A1492 and A1493 from DMS attack and causes an enhancement of reactivity at the N1 position of A1408. Also, G530 is protected from attack by kethoxal at the N1-N2 positions (Moazed *et al*., 1995). We have extended these results in two ways. First, the intensity of the bands in the modification experiments was quantitated using a PhosphorImager in order to measure binding affinities. Wild-type 30 S subunits were titrated with increasing amounts of IF1 (Figure 2(a)). A binding curve drawn based on the modification level at A1492 gives an apparent dissociation constant of 0.9 μM for IF1 (Figure 2(b)). This value agrees with binding constants calculated from protection levels at A1493 and G530, and those determined by other methods (Celano *et al*., 1988; Zucker & Hershey, 1986). Second, chemical modification experiments have revealed changes in reactivity of other nucleotides. In addition to the signals described above, A908, A909, and A1413 are enhanced in reactivity to DMS at the N1 position when IF1 binds, while G1487 is enhanced in reactivity at the N1-N2 positions to kethoxal (Figure 2(a)). Finally G529 is also protected from attack by kethoxal, although the reverse transcriptase stop at this position may be due to the enzyme ''stuttering'' because of the very strong modification at G530 (Figure 2(a); for discussion see Moazed & Noller, 1990).

IF1 binding to wild-type 30 S subunits affects the reactivities of nucleotides in the 1400-1500 decoding region, the 530 loop, and a subset of class III sites (Figure 1(a)). A-site-bound tRNA is believed to directly contact nucleotides in the 1400-1500 region (Yoshizawa *et al*., 1999; Cate *et al*., 1999), whereas it is thought to affect class III and 530 loop nucleotides *via* conformational changes in the 16 S rRNA (Moazed & Noller, 1986, 1990; Powers & Noller, 1993b). Since the modification pattern of IF1 bound to the 30 S subunit overlaps that of A-site-bound tRNA, we reasoned that the primary binding site for IF1 would be in the A site decoding region. Therefore, we constructed mutations in the decoding site and 530 loop of 16 S rRNA and tested for IF1 binding by chemical modification and primer extension to determine the RNA requirements for IF1 binding to the 30 S subunit.

Analysis of 30 S subunits containing mutant 16 S rRNA

Since *E. coli* contains seven genomic copies of the ribosomal RNA (*rrn*) operon, and because mutations in highly conserved residues are often deleterious or lethal, plasmid copies of the gene were mutagenized. Therefore, ribosomes purified from these strains contain a mixture of both wild-type and mutant 30 S subunits. To monitor exclusively the mutant subunits in the mixture *in vitro*, silent mutations in the 16 S gene were made in addition to the mutation of interest. These silent mutations formed two separate allele-specific primer binding sites for reverse transcriptase so that the only modification signals detected would be from the mutant subunits (Powers & Noller, 1993a). With two sets of priming site constructs, the IF1 footprint at G530 can be monitored in the presence of mutations in the decoding region and the modifications in the 1400-1500 region can be monitored in the presence of a mutation in the 530 loop.

The reactivity of nucleotides in the 1400-1500 region of 16 S rRNA were monitored by priming site V (G1514C, G1515C, C1520G, C1521G; Figure 3; Powers & Noller, 1993a). Priming site I (G592U, U593G, G646C, C647A; Figure 3) was newly engineered to monitor nucleotides in the 530 region. Previous attempts at creating a priming site for this region failed because all the mutations tried were lethal when expressed at high levels (Powers & Noller, 1993a). Subsequent chemical probing data suggest that the mutated region of 16 S rRNA interacts with ribosomal protein S16 (Powers & Noller, 1995). To ensure that priming

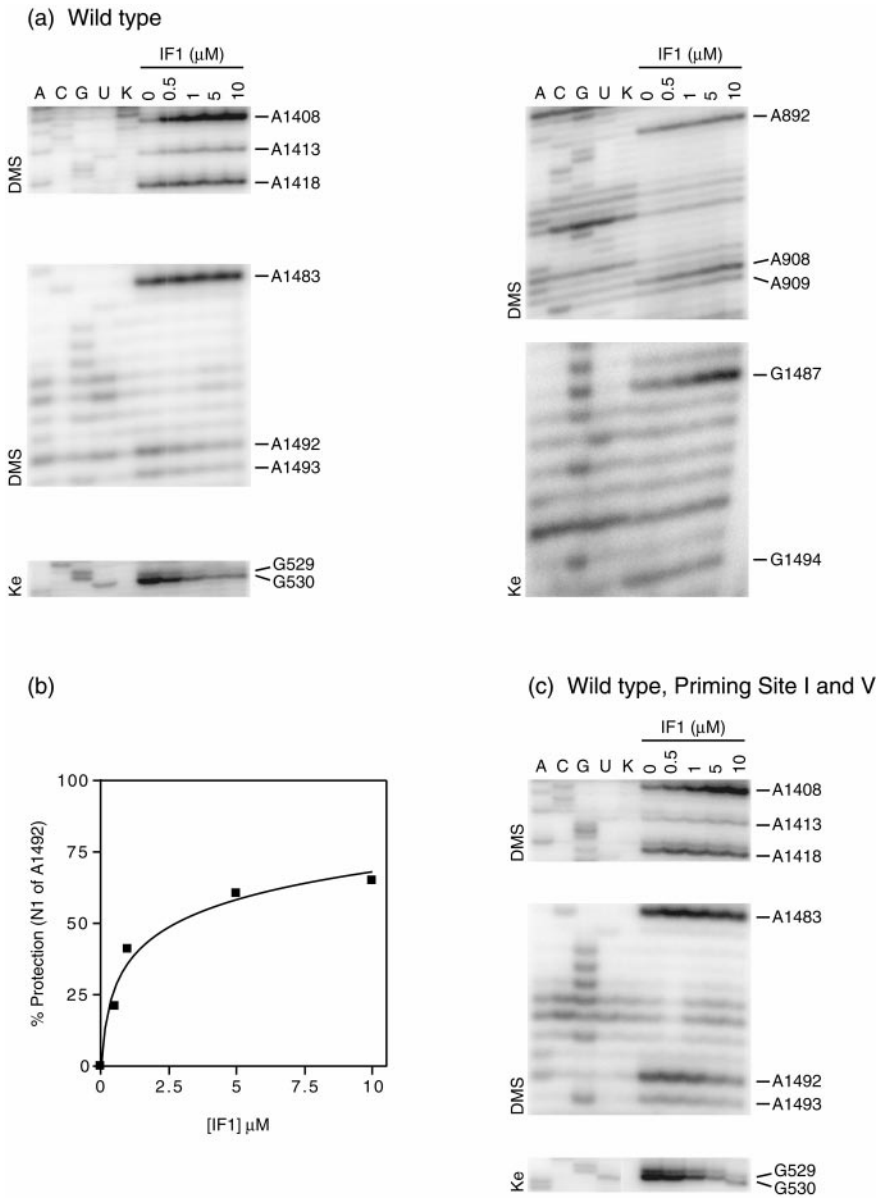

Figure 2. Autoradiographs showing the changes in reactivity of bases in 16 S rRNA in the presence of IF1. DMS and kethoxal modification gels are marked DMS and Ke, respectively. (a) Wild-type. 12.5 pmol of 30 S subunits in a 50 μl volume were probed with DMS or kethoxal and primer extension was performed as described in Materials and Methods. IF1 was present at the concentration indicated. A, C, G, U are dideoxy sequencing lanes. K is an extension of unmodified 16 S rRNA. (b) Plot of the percent protection of the N1 of A1492 as a function of IF1 concentration. An average was calculated from the phosphorimaged data from five independent modification experiments. (c) 30 S subunits containing a wild-type sequence except for the presence of priming site I or V in a wild-type background (plasmid pKK3535 in strain DH1; Brosius *et al*., 1981). Experiment performed as in (a).

site I would be a set of silent mutations, a region of the 590-650 helix between the areas shown to be important for S8 and S15 binding was chosen for mutagenesis (Batey & Williamson, 1996; Mougel *et al*., 1993). Potentially viable base-pair substitutions were selected from the 16 S rRNA phylogeny. The 592U·647A base-pair exists in the Gram-positive bacteria *Arthrobacter globiformis* 16 S sequence; the 593G·646C base-pair exists in the Gram-positive *Mycoplasma gallisepticum* 16 S sequence (Gutell, 1994).

Priming site I was initially expressed in *E. coli* strain DH1 from the plasmid pSTL102, which contains the entire *rrnB* operon under the control of the natural P_1P_2 promoters and carries the spectinomycin resistance mutation C1192U (Triman *et al*., 1989). These cells grew like wild-type control cells containing pSTL102 without priming site I at 30, 37, and 42 °C in the presence of 100 μg/ml ampicillin and 50 μg/ml spectinomycin (data not shown). This test demonstrates that the cells could survive when their only source of translating ribosomes

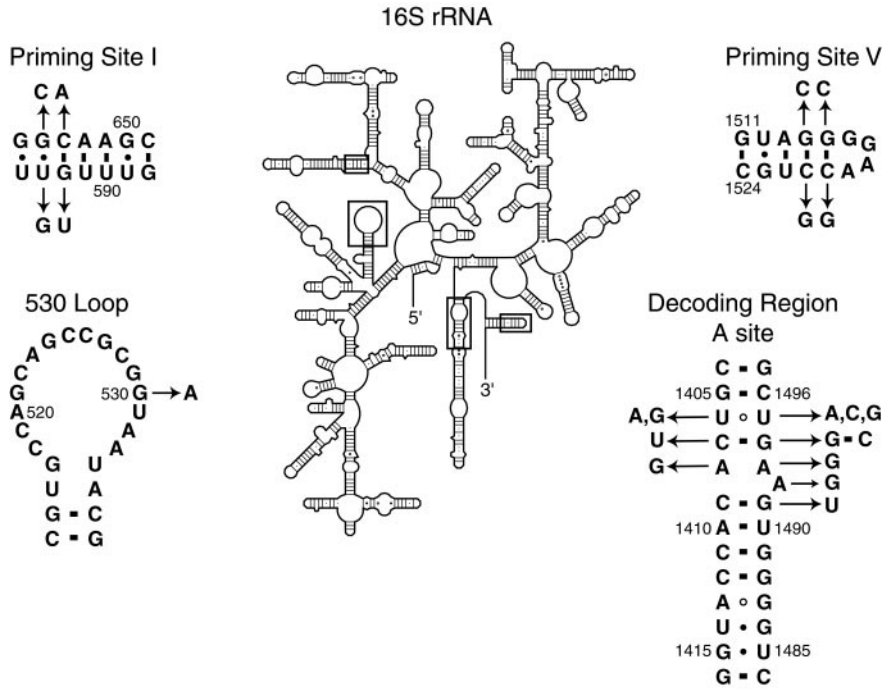

Figure 3. Secondary structure of 16 S rRNA with the locations of priming site I, priming site V, the decoding region A site, and the 530 loop boxed. The mutations that comprise priming site I and V are shown (this study; Powers & Noller, 1993a), as well as the mutations made in the decoding region and 530 loop.

contained priming site I, indicating that the mutations have no major deleterious effect. Finally, Figure 2(c) demonstrates that subunits carrying the priming site I or V mutations in an otherwise wild-type background give a wild-type IF1 footprint in the regions they monitor.

We have characterized IF1 binding to a set of ribosomes carrying mutations that confer growth phenotypes that are viable, mildly deleterious, and dominant lethal when coexpressed with wild-type 16 S rRNA (Figure 3; Table 1). Cells containing the U1406A, A1408G, and G1491U mutations are viable at the high levels of mutant rRNA expressed from the natural P_1P_2 promoters in pKK3535 (DeStasio & Dahlberg, 1990; Recht *et al*., 1999a). Additionally, A1408G cells are resistant to the aminoglycoside antibiotics, neomycin and kanamycin (among others), and G1491U cells are resistant to paromomycin in strain HB101 (DeStasio *et al*., 1989; Recht *et al*., 1999b). Cells tolerate the mutations U1406G, C1407G + G1494C, U1495A, U1495C, and U1495G at the lower levels of rRNA expressed from the vector pLK35 where the natural promoters have been replaced by the phage λ P_L promoter (Recht *et al*., 1999a). G530A, C1407U, A1492G, and A1493G are dominant lethal and their expression from the P_L promoter must be repressed by the conditional cI repressor for cell viability (Powers & Noller, 1990; Thomas *et al*., 1988; Yoshizawa *et al*., 1999). Ribosomes containing the G530A mutation are impaired in EF-Tu-mediated A-site tRNA binding, but not in non-enzymatic tRNA binding (Powers & Noller, 1993b). In contrast, 30 S subunits containing the A1492G or A1493G mutations show decreased non-enzymatic binding of A-site tRNA (Yoshizawa *et al*., 1999). Finally, the C1407U mutation causes frameshifting, stop-codon read-through, and translation initiation from non-AUG start codons *in vivo* (O'Connor *et al*., 1997).

Polysome profile analysis suggests which step in translation is affected by the ribosomal mutations in question. The incorporation of mutant 16 S rRNA into actively translating ribosomes was quantified from the fractions of a sucrose density gradient. Table 1 summarizes data from this study and others. High levels of rRNA (>50 %) containing the viable mutations U1406A, A1408G, and G1491U are found in all fractions. 16 S rRNA containing the C1407G + G1494C mutations is also found equally in all fractions, but at about half the level of RNA compared to a strain carrying the comparable wild-type plasmid with priming site V. The amount of 16 S rRNA containing the mutations, U1406G, U1495A, U1495C, or U1495G decreases in the polysome fractions, indicating that these mutations affect one of the early stages of translation. 16 S rRNA containing the C1407U or G530A mutations is under-represented in polysome fractions. 16 S rRNA containing the A1492G mutation is slightly decreased in the 70 S fraction, but is present at higher levels in the 2X and 3X polysome fractions. 16 S rRNA containing the A1493G mutation is represented equally in all fractions.

Table 1. Percent of plasmid-encoded 16 S rRNA present in the various fractions of the polysome profile

Mutation in 16 S rRNA	Plasmid	Viability	Lysate	30 S	70 S	2X	3X
Wild-type Control[a]	pKK3535	wild-type	65	65	66	59	62
Wild-type Control[a]	pLK35	wild-type	31	33	36	36	40
Wild-type Control[b]	pLK45cI857	wild-type	34	38	34	35	37
U1406A[a]	pKK3535	high expression	62	69	56	51	50
U1406G[a]	pLK35	low expression	22	20	5	9	12
C1407U[b]	pLK45cI857	dominant lethal	30	48	24	28	32
C1407G + G1494C[a]	pLK35	low expression	30	20	23	24	24
A1408G[a]	pKK3535	high expression	61	51	66	58	58
G1491U[c]	pKK3535	high expression	59	75	68	68	69
A1492G[b]	pLK45cI857	dominant lethal	33	40	29	34	38
A1493G[b]	pLK45cI857	dominant lethal	31	29	26	28	25
U1495A[a]	pLK35	low expression	45	56	31	20	17
U1495C[a]	pLK35	low expression	46	58	48	32	22
U1495G[a]	pLK35	low expression	40	71	31	30	16
G530A[d]	pLK45, pcI857	dominant lethal	nd	68	36	10	7.5

The amount of plasmid-encoded 16 S rRNA was determined using priming site V as a marker. Values reported are the average of at least two experiments. 2X and 3X designate fractions from the sucrose gradient containing two and three ribosomes per mRNA, respectively. pKK3535 contains the natural *rrnB* promoters; pLK35 and pLK45cI857 contain the P_L promoter from phage lambda. The presence of the cI857 allele represses expression from the P_L promoter.

nd, not determined.

[a] Recht *et al.* (1999).

[b] This study.

[c] M. I. Recht & J. D. P. (unpublished).

[d] Powers & Noller (1990).

Perturbations in 16 S rRNA structure caused by decoding region mutations

To determine the effects of the mutations on the local structure of 16 S rRNA, chemical probing was performed on the mutant ribosomes in the absence of IF1. Primer extension was performed on two to four independent modification experiments per mutant, and the bands were quantitated using a PhosphorImager. The basal level of reactivity at each modified nucleotide in the absence of IF1 was compared to the modification level at A1418 or A1483, whose reactivities are independent of IF1 concentration. The results are summarized qualitatively in Table 2 (0 μM IF1).

None of the mutations in the 1400-1500 region affected the basal reactivity of G529 or G530. The mutation G530A did not affect the basal reactivity of any of the nucleotides in the A site, but the basal reactivity of A1483, further down in the penultimate stem, was increased significantly in the absence of IF1 (Figure 4(a), 0 μM IF1 lane). When position 530 is an adenosine, it is only mildly reactive to DMS at the N1 position (Figure 4(a), 0 μM IF1 lane) and no kethoxal modification is observed at G529 (data not shown).

Nearly every mutation in the A site has an effect on baseline chemical reactivities in the region, indicating potential changes in local structure or disrupted tertiary interactions (Table 2, 0 μM IF1). For example, the local structure of the A site is very sensitive to different mutations at U1406 and U1495. Although the U1406G mutation does not affect the basal reactivities of any of the nucleotides in the A site (Figure 4(b), 0 μM IF1 lane), in 30 S subunits carrying the U1406A mutation, the reactivity of A1492 increases relative to the wild-type (Table 2, 0 μM IF1). When U1495 is changed to an adenosine, the reactivity at A1408 is increased (Figure 4(c), 0 μM IF1 lane). When U1495 is a cytosine, reactivity at A1408 is increased and reactivity at A1493 is decreased (Table 2, 0 μM IF1). However, when U1495 is a guanosine, reactivity at A1408 is decreased, while reactivity at A1492 is increased (Figure 4(d), 0 μM IF1 lane). In addition, A1410(N1) is slightly reactive to DMS and A1483(N1) is very reactive in U1495G mutant subunits.

The C1407G + G1494C mutation does not affect the basal reactivities of nucleotides in the A site (Table 2, 0 μM IF1). However, in subunits carrying the C1407U mutation, A1493 is much less reactive to DMS and the N1-N2 positions of G1494 are very reactive to kethoxal (Figure 5(a), 0 μM IF1 lane), suggesting that a 1407U·G1494 base-pair does not form in these mutant subunits. In 30 S subunits carrying the G1491U mutation, the basal reactivity of A1492 is increased and C1409 and A1410 become weakly reactive to DMS (Table 2, 0 μM IF1; data not shown).

Changing any of the reactive adenosines to guanosine in the A site also has consequences for local structure. In 30 S subunits containing the A1408G mutation, the reactivity of the N1 of A1492 is decreased relative to wild-type (Figure 5(b), 0 μM IF1 lane) and 1408G itself is not reactive to DMS at the N7 position or to kethoxal at the N1-N2 positions (data not shown). In 30 S subunits containing the A1492G mutation, A1408(N1) is much less reactive to DMS than in wild-type subunits (Figure 5(c), 0 μM IF1 lane). Spontaneous reverse transcriptase stops occur at G1494 and 1492G mak-

Table 2. Modification level of nucleotides in 16 S rRNA that change reactivity in the presence of IF1

	16 S rRNA mutations												
Modified nucleotide	None	U1406A	U1406G	C1407U	C1407G + G1494C	A1408G	G1491U	A1492G	A1493G	U1495A	U1495C	U1495G	G530A
A1408 (DMS, N1)													
0 μM	+	+	+	+	+	nd	+	±	+	++	++	±	+
1 μM	++	++	++	+	++		++	+	+	++	++	+	++
5 μM	+++	+++	++	++	+++		+++	++	++	+++	+++	++	+++
10 μM	++++	++++	+++	++	++++		++++	++	++	++++	++++	+++	++++
A1413 (DMS, N1)													
0 μM	±	±	±	±	±	±	±	±	±	±	±	±	±
1 μM	+	±	+	+	+	±	±	+	±	+	+	±	+
5 μM	++	+	++	++	++	+	+	++	+	++	++	+	++
10 μM	+++	++	++	+++	++	+	++	+++	+	++	+++	++	+++
A1492 (DMS, N1)													
0 μM	+++	++++	+++	+++	+++	++	++++	nd	++	+++	+++	++++	+++
1 μM	++	++	+++	+++	+++	++	++		++	++	++	+++	++
5 μM	+	+	++	+++	++	++	+		++	+	+	+++	+
10 μM	±	±	+	++	+	+	±		+	±	±	++	+
A1493 (DMS, N1)													
0 μM	++	++	++	+	++	++	++	++	nd	++	+	++	++
1 μM	+	+	++	+	++	++	+	++		+	+	++	+
5 μM	±	±	++	+	++	++	±	++		±	±	++	±
10 μM	±	±	+	±	+	++	±	++		±	±	++	±
G530 (Kethoxal, N1-N2)													
0 μM	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	nd
1 μM	++	++	++	++++	++	++++	+++	++++	++	+++	+++	++	
5 μM	+	+	+	+++	+	++++	+	++++	++	+	+	+	
10 μM	±	±	±	++	±	+++	±	+++	+	±	±	+	

++++, strongest modification; ±, marginal modification; nd, reactivity at this nucleotide could not be determined because it is absent in the mutated 16 S rRNA.

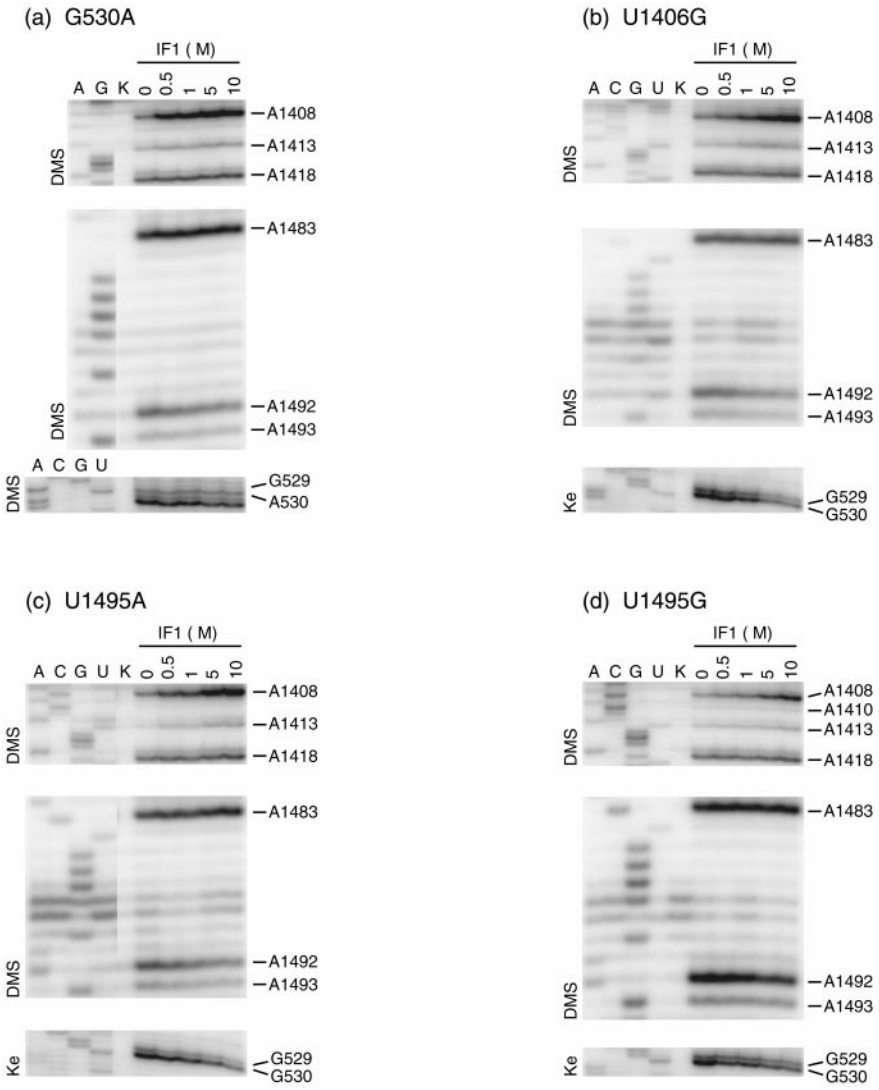

Figure 4. Autoradiographs showing chemical probing experiments with mutant 30 S subunits containing allele-specific priming sites I and V. 12.5 pmol of 30 S subunits were probed in a 50 μl volume with DMS or kethoxal and primer extension was performed as described in Materials and Methods. IF1 was present at the concentration indicated. A, C, G, U are dideoxy sequencing lanes. K is an extension of unmodified 16 S rRNA. DMS and kethoxal modification gels are marked DMS and Ke, respectively. (a) G530A. (b) U1406G. (c) U1495A. (d) U1495G.

ing it difficult to assess the reactivity of 1492G in this mutant. In the A1493G mutant variant, A1492 is less reactive to DMS and 1493G itself is reactive to kethoxal (Table 2; data not shown).

Minor discrepancies in the baseline reactivities of nucleotides in the A site in 30 S subunits containing mutations at U1406 and U1495 between the results reported here and those reported by Recht *et al*. (1999a) are explained by differences in methods. Recht *et al*. (1999a) performed aniline cleavage on the DMS modification experiments and the additional bands arising from the N7 of guanosine modifications alter the primer extension properties of the surrounding nucleotides. For example, G1494 is strongly modified at the N7 position affecting bands in the 1490 region. Also, it is more difficult for reverse transcriptase to read through to the 1400 region because of the number of extra stops due to the G(N7) modifications.

Binding of IF1 to mutant 30 S subunits

In order to assess the affinity of IF1 for 30 S subunits carrying mutations in their 16 S rRNA, chemical modification experiments were performed in the presence of 0, 0.5, 1, 5, and 10 μM IF1, followed by reverse transcription from allele-specific priming sites I and V (Figures 4 and 5). Primer extension was performed on two to four independent modification experiments per mutant, and the bands were quantitated using a PhosphorImager. Modification signals in the A site were normalized to a control band at A1483 whose reactivity is independent of IF1 concentration. Table 2 summarizes the changes in reactivities of A1408, A1413, A1492, A1493, and G530 for subunits carrying each mutation in the presence of different concentrations of IF1. Monitoring the modification level of G530 in the presence

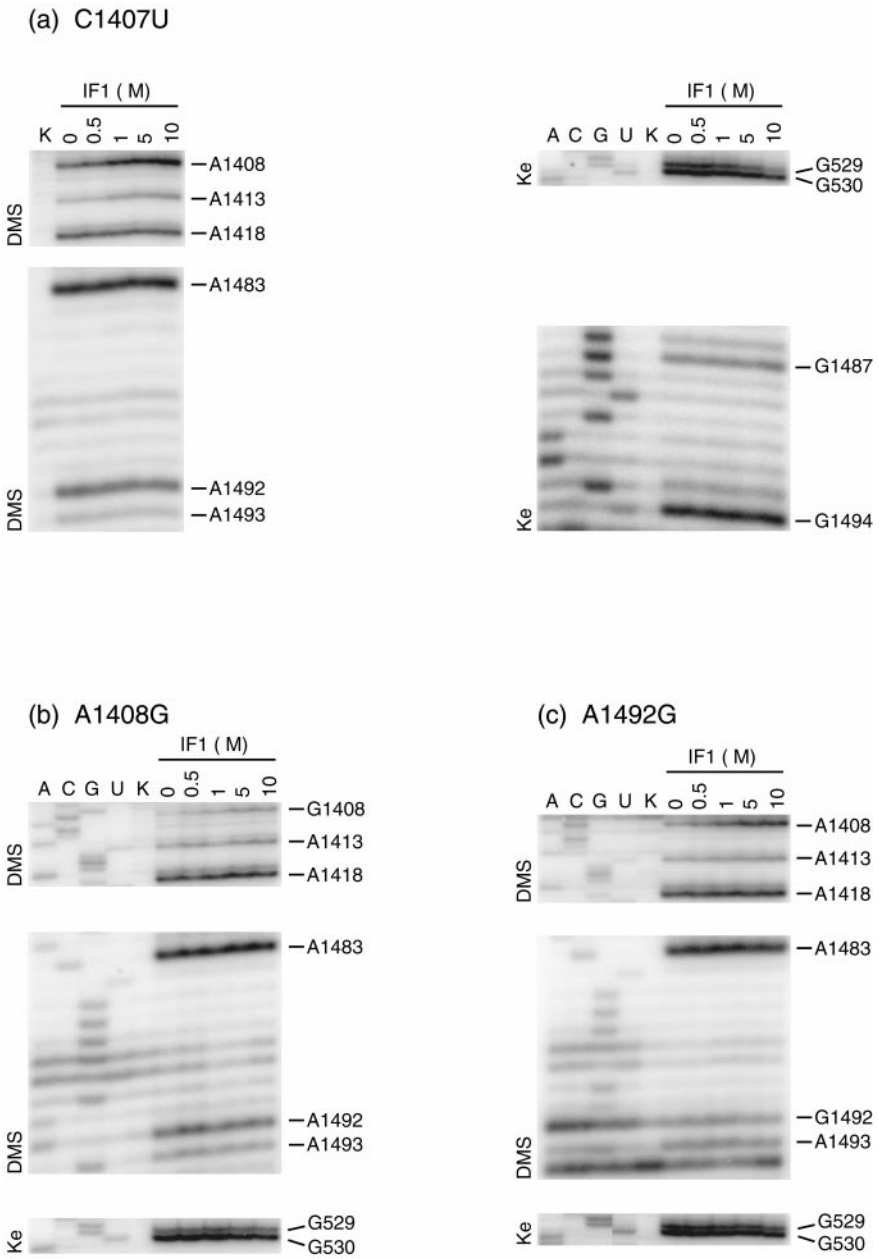

Figure 5. Autoradiographs showing chemical probing experiments with mutant 30 S subunits containing allele-specific priming sites I and V. 12.5 pmol of 30 S subunits were probed in a 50 μl volume with DMS or kethoxal and primer extension was performed as described in Materials and Methods. IF1 was present at the concentration indicated. A, C, G, U are dideoxy sequencing lanes. K is an extension of unmodified 16 S rRNA. DMS and kethoxal modification gels are marked DMS and Ke, respectively. (a) C1407U. (b) A1408G. (c) A1492G.

of mutations in the 1400-1500 region and *vice-versa* allows comparison between the different mutant subunits and wild-type when the mutated nucleotide eliminates a modification signal or affects the baseline reactivities of the neighboring nucleotides. Based on those criteria, the mutations fall into two classes, those that allow essentially wild-type IF1 binding and those that show a strong reduction in IF1 binding. Mutations that allow wild-type binding are: U1406A, U1406G, C1407G + G1494C, G1491U, U1495A, U1495C, U1495G, and G530A. Mutations that strongly disrupt IF1 binding are: C1407U, A1408G, A1492G, and A1493G.

IF1 bound to mutant ribosomes containing the G530A mutation at wild-type levels (Table 2; Figure 4(a)), consistent with the preservation of local structure in the A site in the absence of IF1. IF1 also binds to 30 S subunits containing the G1491U mutation like wild-type (Table 2), which correlates with the viability of cells expressing the mutation at high levels.

The U1406·U1495 base-pair is universally conserved (Gutell, 1994). IF1 can bind to 30 S subunits

containing mutations at either position at wild-type levels as judged by the protection at G530 (Figure 4(b)-(d); Table 2). However, a guanosine at either 1406 or 1495 disrupts the IF1 reactivity pattern in the 1400-1500 region. Mutation of U1406 to a G has a deleterious effect on the IF1 footprint at positions A1492 and A1493 in the A site, although the 530 region shows a wild-type footprint (Figure 4(b); Table 2). Subunits carrying the mutation U1495G show a greatly reduced enhancement at A1408 and protections at A1492 and A1493, although the footprint at G530 appears largely unaffected (Figure 4(d); Table 2).

The C1407·G1494 base-pair is also universally conserved (Gutell, 1994). The C1407U mutation has a drastic effect on IF1 binding. The enhancement at A1408 and protections at A1492, A1493, and G530 are far below wild-type levels (Figure 5(a); Table 2). Since the baseline reactivities of nucleotides in the decoding region in the absence of IF1 suggest that the 1407U·G1494 base-pair does not form, it seems likely that IF1 requires a base-pair in those positions for binding. Flipping the base-pair to 1407G·1494C retains Watson-Crick pairing, based on chemical reactivities in the absence of IF1, and shows essentially wild-type IF1 binding. In the presence of IF1, the enhancement at A1408 and protection at G530 are at the same level as wild-type. However, the protections at A1492 and A1493 are not as strong (Table 2).

Mutation of any of the three adenosines in the A site, 1408, 1492, or 1493 to a guanosine has an extremely detrimental effect on IF1 binding (Figure 5(b), (c); Table 2). In each case, the enhancement at A1408, and the protections at A1492, A1493, and G530 are greatly reduced. These mutations also affect the local structure in the A site in the absence of IF1. The weak background modification seen at 1408G is due to a low level of non-specific priming to the wild-type rRNA in the mixture (Figure 5(b)). The lack of protection by IF1 at G530, A1492, or A1493 is consistent with little IF1 binding to 30 S subunits carrying the 1408G mutation.

A1413 is the only class III site that was monitored for every mutation. Generally its enhancement in reactivity in each of the mutants relative to wild-type follows the trend set by A1408. However, in the case of subunits carrying the U1406A, C1407G + G1494C, and G1491U mutations, the enhancement at A1413 was lower than wild-type levels, even though the rest of the modification signals indicated wild-type IF1 binding. Conversely, in the case of subunits containing the C1407U and A1492G mutations, the enhancement at A1413 was near wild-type levels even though the rest of the modification signals indicated that IF1 was not binding to these mutant subunits. The modification level of G1487 was investigated for 30 S subunits carrying the mutations C1407U, A1408G, A1492G, and A1493G (Figure 5(a); data not shown). In each instance, the N1-N2 positions of G1487 were not enhanced in reactivity to kethoxal, correlating with the lack of IF1 binding indicated by the other modification signals (Figure 5(a); data not shown). The baseline level of reactivity at A1413 and G1487 is not very high and may be sensitive to changes in local structure due to mutations in the surrounding nucleotides. To determine more precisely how decoding region mutations affect the class III sites, it will be necessary to investigate their effects at A908 and A909.

Discussion

We have determined the complete set of nucleotides that are affected by IF1 binding to 30 S subunits, nucleotides in the decoding region A site and a subset of the class III sites. Through mutational analysis of the A site, we have discerned which of these nucleotides are critical for IF1 binding. The C1407·G1494 base-pair and the three adenosines, A1408, A1492, and A1493 are required for optimal IF1 binding. Since certain bases were better tolerated at different positions in the sequence than others, it appears that the overall conformation of the internal loop is more important to IF1 binding than the identity of the nucleotide at a certain position. Mutations lower in the penultimate stem were not tested.

The structure of an oligonucleotide model that mimics the A site has been solved by NMR (Figure 6; Fourmy *et al*., 1996, 1998; Yoshizawa *et al*., 1998). The upper stem formed by nucleotides 1404-1407 and 1494-1497 is closed by a non-canonical U·U base-pair and a Watson-Crick C·G pair. In the absence of ligand, A1408, A1492, and A1493 define an internal loop, in which A1408 and A1493 form a base-pair mediated by a single hydrogen bond between the A1408(N1) and the A1493(N6), while the unpaired A1492 stacks below A1493, serving to widen the major groove. The binding of IF1 must alter the conformation of this base-pair because the N1 of A1408 becomes highly reactive to DMS. IF1 may be mainly contacting the RNA backbone in this region. However, the wild-type probing data do not distinguish between major or minor groove binding of IF1 to the A site. The reactivities of the N7 positions of G1491 or G1494 to DMS or the N7 positions of A1408, A1492, A1493 to diethylpyrocarbonate in the major groove do not change upon binding of IF1, nor is any nucleotide in this region protected from hydroxyl radicals, a probe of the backbone in the minor groove, by the binding of IF1 (K. D. D. & J. D. P., unpublished results).

IF1 binding is sensitive to the conformation of the internal loop formed by A1408, A1492, and A1493 (Figure 6). Mutation of any of the three adenosines to a guanosine severely disrupts IF1 binding. Loss of IF1 affinity could result from the introduction of a bulky amino group in the minor groove or from an alteration of the overall conformation of the internal loop. In the NMR structure of a 1408G variant oligonucleotide, both the geo-

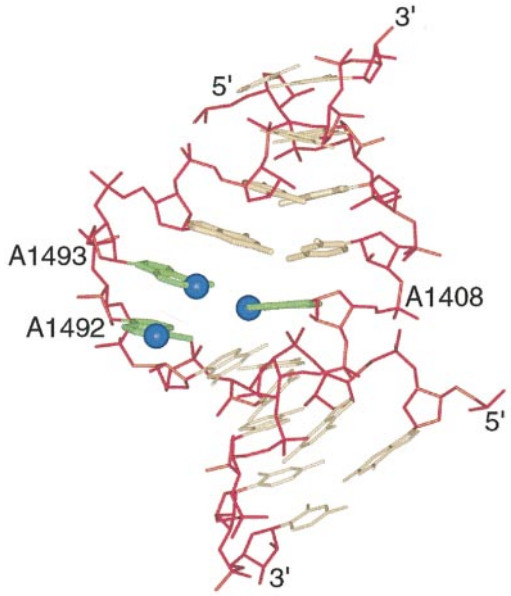

Figure 6. NMR structure of an oligonucleotide that mimics the A site of 16 S rRNA, nucleotides 1404-1412 and 1488-1497 (Fourmy *et al.*, 1998), viewed from the minor groove side of the internal loop. N1 positions (blue spheres) of A1408, A1492 and A1493 (green) are highlighted.

metry of the 1408·1493 purine·purine pair and the overall backbone conformation of the internal loop was altered (S. R. Lynch & J. D. P., unpublished results). Changing A1492 or A1493 to a G interferes with possible direct contacts between a tRNA-mRNA helix and the N1 positions of those adenosines in the minor groove (Yoshizawa *et al.*, 1999). It is possible that IF1 directly contacts A1492 and A1493, since in these experiments IF1 binding can cause a footprint at these residues in the absence of message, although this conclusion must be supported by further experiments.

Although the A1408G, A1492G, and A1493G mutations are all detrimental to IF1 binding, mutant rRNA was found in all polysome fractions (Table 1), suggesting that translation initiation was not abolished. This result is puzzling because IF1 is an essential gene (Cummings & Hershey, 1994) and is required for proper 70 S initiation complex formation (Hartz *et al.*, 1989). Either IF1 binding to the mutant subunits cannot be detected by chemical modification methods because the identity of the footprinting signal has changed, or the need for IF1 has been reduced in the mutant subunits. For A1408G, this would explain the wild-type growth phenotype. In the case of A1492G and A1493G, the fact that mutant rRNA that is impaired in A-site tRNA binding (Yoshizawa *et al.*, 1999) is found in the actively translating pool of ribosomes would contribute to the lethal phenotype.

Structurally, the C1407·G1494 base-pair provides the context for the A-site internal loop (Figure 6). The C1407G + G1494C mutant preserves Watson-Crick base-pairing, and presumably the geometry of the loop, and has less of an effect on IF1 binding, although it is not entirely like wild-type. Consistent with this result, the C1407G + G1494C mutation is found at low levels equally in all polysome fractions (Table 1). In contrast, IF1 has a much lower affinity for 30 S subunits carrying the C1407U mutation, which disrupts the overall conformation of the internal loop. G1494 is highly reactive to kethoxal at the N1-N2 positions (Figure 5(a)), suggesting that a U1407·G1494 base-pair does not form, which may in turn perturb the conformation of the three adenosines. In this case, structural perturbations that disrupt IF1 binding may explain the phenotypic defects associated with initiation. The polysome profile of cells carrying the C1407U mutation has larger subunit peaks (Thomas *et al.*, 1988). This defect in subunit association could explain why rRNA containing the C1407U mutation is excluded from the 70 S and polysome fractions (Table 1). This correlates with the dominant lethal phenotype of these mutant cells and their ability to initiate protein synthesis from non-AUG start codons (O'Connor *et al.*, 1997). Disruption of the structure of the A site prevents IF1 binding and may lead to inaccurate tRNA selection in the P site.

Mutations at the U1406·U1495 base-pair have different effects on local structure, although they allow wild-type levels of IF1 binding. Mutations of U1406 or U1495 to an adenosine create Watson-Crick pairing at these positions, while mutation of U1495 to a cytosine preserves a pyrimidine·pyrimidine pair. Mutation of U1406 or U1495 to G creates a G·U pair and disrupts reactivity changes in the 1400-1500 region upon IF1 binding, although there are wild-type footprints at G530. The disruption may be caused by the geometry of the G·U pairs formed or the changes in local structure that propagate down the stem in these mutants.

rRNA containing U1406A mutation can be expressed at high levels, and is found in all gradient fractions, which correlates with wild-type IF1 binding. rRNA containing the U1406G, U1495A, U1495C, or U1495G mutations can only be expressed at low levels and is enriched in the 30 S subunit fraction, although IF1 binds to 30 S subunits carrying these mutations at wild-type levels. More detailed functional assays are required to discern the translational defect of these mutations.

A mutation in the 530 loop does not influence IF1 binding in the A site. 30 S subunits containing the mutation G530A have a wild-type chemical reactivity pattern in the presence of IF1. It has been previously shown that G530A gives a lethal phenotype when coexpressed with wild-type and that 16 S rRNA containing the G530A mutation is not found in the polysome fractions (Powers & Noller, 1990). G530A ribosomes are impaired in ternary complex-mediated tRNA binding to the A site, but not in non-enzymatic binding of tRNA to the A site, suggesting that the 530 region is important for

interacting with EF-Tu, not with tRNA in the A site. Also the dominant lethal mutation G530U, when reconstituted into 30 S ribosomes could correctly form 30 S and 70 S initiation complexes, but was impaired in forming the first peptide bond (Santer *et al*., 1993). Our results suggest that IF1 is directly contacting the 1400-1500 region and affecting the 530 loop *via* a conformational change in the structure of 16 S rRNA.

The set of nucleotides affected by IF1 overlaps with those affected by A-site bound tRNA, although there are some differences. A-site bound tRNA protects A1408(N1) and G1494(N7) from DMS (Moazed & Noller, 1986, 1990), while IF1 enhances the reactivity of A1408 and does not affect G1494. The differences in the reactivity pattern suggest that the A site adopts a different conformation in response to IF1, than in response to tRNA. IF1 also enhances the reactivity of a subset of the class III sites, A1413, G1487, A908, and A909. The class III sites are protected by tRNA, 50 S subunits (Moazed & Noller, 1986), and certain antibiotics (Moazed & Noller, 1987). Moazed *et al*. (1995) also demonstrated that IF3 protects another class III site, G791, enhances G1487, and probably A1413 as well.

The crystal structure of the 30 S subunit places the 900 region close to the decoding region A site (Clemons *et al*., 1999). The 790 loop interacts with the 900 region, and these two regions form bridges of electron density that extend toward the 50 S subunit in the 70 S ribosome crystal structure (Cate *et al*., 1999). In both structures, the penultimate stem is seen as a long RNA helix on the surface of the 30 S subunit facing the subunit interface, and the 900 region docks somewhere in the vicinity of 1413-1418 and 1483-1487. The bend in the long penultimate stem is different in both structures, as well as the angle between the penultimate stem and the 900 region, suggesting a conformational difference between the 30 S subunit and a 70 S ribosome complexed with tRNAs. When IF3 was localized on the 30 S subunit using cryo-electron microscopy, IF3 appeared to induce a change in the relative positions of the head and platform of the 30 S subunit (McCutcheon *et al*., 1999). IF3 promotes subunit dissociation, whereas IF1 binding increases both the rates of subunit association and dissociation (Godefroy-Colburn *et al*., 1975). The footprinting and structural data suggest that IF1 and IF3 may act to control subunit association by influencing the conformation of class III sites.

In addition to manipulating the association state of the ribosomal subunits, IF1 may have an additional role during initiation in ensuring translational fidelity. Mutations that alter ribosomal accuracy of tRNA selection affect the reactivity of certain class III sites to chemical probes (Allen & Noller, 1989). A local change in conformation in the A site caused by the binding of IF1 may be transmitted to the accuracy center of the 30 S subunit, thus tying the fidelity apparatus used by the A site during elongation to the process of tRNA selection in the P site during translation initiation.

Materials and Methods

Preparation of IF1

The IF1 overproducing strain, *E. coli* host strain K12ΔH1Δtrp containing pXR201 with the *infA** gene under control of the heat-inducible lambda P_L promoter (Calogero *et al*., 1987), was a kind gift from Claudio Gualerzi. One liter cultures in LB media (10 g of bactotryptone, 5 g of yeast extract, 5 g of sodium chloride, 1 mM sodium hydroxide) containing 60 μg/ml ampicillin were grown to an A_{600} of 0.7-0.9 at 30 °C. In order to induce overexpression, cultures were rapidly brought to 42 °C by swirling in a 60 °C water bath. The cultures were further incubated in a 42 °C water bath for 20 minutes and at 37 °C for three hours. Cells were pelleted and stored at −20 °C.

Purification steps were carried out at 4 °C. Frozen cells were resuspended in 25 ml of 20 mM sodium phosphate (pH 7.0) and lysed by passage through a french press. The crude lysate was spun at 30,000 ***g*** for 15 minutes. The supernatant was passed through a 0.2 μm filter and loaded onto a 10 ml FPLC Resource S column (Pharmacia). IF1 was eluted from the column by a 0 to 1 M sodium chloride gradient in 20 mM sodium phosphate (pH 7.0). The fractions containing IF1 were extensively dialyzed against water and then lyophilized. IF1 was resuspended in 20 mM Tris-HCl (pH 7.0), 5 % (v/v) glycerol and stored in small aliquots at −80 °C.

Site-directed mutagenesis

Priming site V

The U1406A/V and A1408G/V mutations were introduced into pKK3535, the U1406G/V, C1407G +G1494G/V, U1495A/V, U1495C/V, U1495G/V mutations were introduced into pLK35, and the A1492G/V and A1493G/V mutations were introduced into pLK45cI857 as described previously (Recht *et al*., 1999a; Yoshizawa *et al*., 1999). pLK45 G530A/V (Powers & Noller, 1990) was a kind gift from the Noller laboratory. G1491U/V and C1407U/V, were introduced into the 16 S rRNA gene by site-directed mutagenesis, using the method described by Kunkel *et al*. (1987) with the modifications for use with BlueScript by Evnin & Craik (1988). Mutations were constructed in pBS-V (T. Powers and H.F. Noller, unpublished results), which contains the *Apa*I-*Xba*I fragment from pSTL102 (Triman *et al*., 1989) with allele-specific priming site V. Oligonucleotides used for mutagenesis were as follows: C1407U, 5′-CCG-CCC-GT**T**-ACA-CCA-TG-3′; G1491U, 5′-ACT-GGG-GT**T**-AAG-TCG-TA-3′. The fragment from pSTL102 contains the spectinomycin-resistance mutation (C1192U). All decoding site mutants were made both with and without this additional mutation. All probing experiments were performed on 30 S subunits containing the wild-type sequence (C1192), except for U1406A/V, C1407U/V, and G530A/V. No differences were seen between probing experiments with C1192/V or C1192U/V 30 S subunits (data not shown). Strain XL-1 Blue was used as the recipient of the mutagenesis reactions. Mutations were identified by dideoxy sequencing of the pBS-V derivatives. These mutations were introduced into either pKK3535 (G1491U) or pLK45cI857

(C1407U) for expression of 16 S rRNA using the unique *Apa*I and *Xba*I sites present in all three plasmids. Strain DH1 was used as the recipient of the final constructs. The presence of the mutations in the final constructs was verified by dideoxy sequencing analysis.

Priming site I

Priming site I was introduced into plasmid pSC-KA which contains the small *Kpn*I-*Apa*I fragment of pLK35 in BlueScript (T. Powers and H.F. Noller, unpublished results) *via* site-directed mutagenesis as described above. Oligonucleotides for mutagenesis were as follows: G646C + C647A, 5′-TGA-TAC-TG**C**-**A**AA-GCT-TGA-3′; G592U + U593G, 5′-GGC-GGT-TT**T**-**G**TA-AGT-CAG-3′; G530A, 5′-CAG-CCG-CG**A**-TAA-TAC-GG-3′. Priming site I was introduced into pSTL102, pKK3535, pLK35, and pLK45cI857 *via* an *Apa*I-*Hin*dIII partial digest. Priming site I was paired with each of the A-site mutations in a two step process. Priming site V was removed by cloning the *Apa*I-*BstE*II fragment of pBS-V mutant derivatives into pBS-16 S-AX which contains the wild-type *Apa*I-*Xba*I fragment of pKK3535. Then the mutations were put with priming site I *via* an *Apa*I-*Xba*I digest of the appropriate parent vector containing priming site I. G530A was paired with priming site I *via* site-directed mutagenesis of pSC-KA/I and put into pLK45cI857 *via* the *Kpn*I-*Apa*I sites unique to those vectors. Strain DH1 was used as the recipient of the final constructs. The presence of the mutations in the final constructs was verified by dideoxy sequencing analysis.

Preparation of 30 S subunits

Wild-type 30 S subunits from *E. coli* MRE600 and the mutant 30 S subunits described above were prepared exactly as described (Recht *et al*., 1999a). Subunits were stored in small aliquots at −80 °C in a storage buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM magnesium chloride, 100 mM ammonium chloride, and 6 mM 2-mercaptoethanol.

Chemical modification

A concentrated aliquot of wild-type or mutant 30 S subunits in storage buffer was thawed on ice and brought to 20 mM magnesium chloride by the addition of 1/10 volume of 100 mM magnesium chloride. The subunits were then activated at 40-42 °C for 30 minutes. The subunits were then diluted to a concentration of 250 nM in binding buffer, 80 mM potassium cacodylate (pH 7.5), 10 mM magnesium chloride, 55 mM ammonium chloride, 1 mM dithiothreitol, 0.01 % (w/v) octaethyleneglycol mono *n*-dodecyl ether (Fluka), and aliquotted into individual reaction tubes, 12.5 pmol of subunits per reaction. 5 μl of freshly diluted 10X stock of IF1 in water were added to the appropriate tubes for a total volume of 50 μl and incubated at 37 °C for five minutes and at room temperature for 30 minutes. Two microliters of DMS diluted 1:6 with ethanol (or water) was added to the appropriate tubes and incubated for five minutes at 37 °C. The reaction was stopped by adding 50 μl of a solution of 280 mM sodium acetate and 950 mM 2-mercaptoethanol followed by ethanol precipitation. Subunits were resuspended in an extraction buffer containing 280 mM sodium acetate, 0.5 % (w/v) sodium dodecyl sulfate, 12 mM EDTA, and extracted three times with phenol and two times with chloroform. Modified RNA was concentrated by ethanol precipitation and resuspended in 20-50 μl water.

Kethoxal modification was performed as described above except, after the binding step, 2.5 μl of kethoxal (37 mg/ml in 20 % (v/v) ethanol) was added. Subunits were modified for five minutes at 37 °C and an equal volume of a solution of 250 mM potassium borate and 150 mM sodium acetate was added followed by ethanol precipitation. Subsequent steps were the same except the extraction buffer also contained 25 mM potassium borate and the final resuspension was in 25 mM potassium borate.

Primer extension

Primer extension of wild-type 16 S rRNA was performed as described (Stern *et al*., 1988). Primer extension from priming site V was performed as described (Recht *et al*., 1999a) except that the extension step was performed at 42-45 °C. Primer extension from priming site I was performed following a modified version of Douthwaite *et al*. (1989). A 20 nucleotide primer complementary to nucleotides 625 to 646 of priming site I 16 S rRNA was annealed to the rRNA in hybridization buffer (225 mM K-Hepes (pH 7.0), 450 mM potassium chloride) by heating to 70 °C for three minutes and at 58 °C for 15 minutes. Following the annealing of the primer, extension was performed as in Stern *et al*. (1988).

The intensities of the bands were quantitated using a PhosphorImager. To account for small differences in the loading and extension of each lane, the modification intensities of nucleotides in the 1400-1500 region were normalized to the modification level at A1483, whose modification is independent of IF1 concentration. Specifically, normalization factors were calculated by dividing the intensity of the band at A1483 at each IF1 concentration by the intensity of the band at A1483 at 0 μM IF1. The intensity of the modification signals at A1408, A1413, A1492, and A1493 were each multiplied by the normalization factor for the appropriate concentration of IF1 to obtain the normalized value. The modification level at G530 was not normalized to another band because there are no other strong kethoxal modifications in that region with which to compare.

Preparation of polysomes and quantification of mutant 16 S rRNA

Freshly inoculated 50 ml cultures in LB medium (10 g of bactotryptone, 5 g of yeast extract, 10 g of sodium chloride, 1 mM NaOH) and 100 μg/ml ampicillin were grown at 30 °C until an A_{600} of ∼0.05. Cultures were switched to a 42 °C water bath and grown for another 2.5 hours. Polysomes were prepared and analyzed exactly as described in Recht *et al*. (1999a).

Acknowledgments

We thank Claudio Gualerzi for the IF1 overproducing strain, and Harry Noller, Kate Lieberman, and other members of the Noller laboratory for providing bacterial strains, plasmids, and discussions. We also thank Peter Sarnow, Karla Kirkegaard and members of their laboratories for use of the PhosphorImager. We thank Steve Douthwaite, Michael Recht, and Satoko Yoshizawa for help with ribosome preparation and discussions, Michael

Recht, Dominique Fourmy, Steve Lynch, Satoko Yoshizawa, and Harry Noller for critical reading of the manuscript, and Steve Lynch for Figure 6. This work was supported by NIH grant GM51266.

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Edited by D. E. Draper

(Received 23 December 1999; received in revised form 6 March 2000; accepted 7 March 2000)