RNA (2001), *7*:969–978. Cambridge University Press. Printed in the USA.

Altered discrimination of start codons and initiator tRNAs by mutant initiation factor 3

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ABSTRACT

IF3 is essential for ensuring the fidelity of the initiation step of translation in bacterial cells. Mutations at residues R99 and R131 in the C-terminal domain of the factor have previously been shown to increase initiation from the non-canonical GUA codon. Here we show that these mutant forms of IF3 fail to discriminate against initiation from many different non-AUG codons. They also enhance the activity of mutant tRNAs carrying changes in the three consecutive G-C pairs that are conserved in the anticodon stem of initiator tRNAs. In addition, the IF3 mutants stimulate initiations from leaderless mRNAs and from internal initiation codons, in the absence of any SD–anti-SD interaction. These results indicate that IF3 ensures the accuracy of initiation by inspecting both the codon–anticodon pairing and unique features of the initiator tRNA as well as suppressing initiation from other potential start sites within the mRNA.

Keywords: IF3; initiation; translation; tRNA

INTRODUCTION

The selection of the correct initiation codon on the ribosome is a critical step in translation as it establishes the translational reading frame of an mRNA and also serves as a major control point in gene expression. Selection of the initiation codon on bacterial mRNAs involves the coordinated activities of the ribosome, initiator tRNA, and at least three initiation factors, IF1, IF2, and IF3. Much of the fidelity of this step in protein synthesis is due to the activities of IF3, which discriminates against nonstandard initiation codons and against noninitiator tRNAs. In addition, IF3 stimulates the activities of the other two initiation factors and aids in dissociating 70S ribosomes, thereby ensuring the availability of free subunits for initiation (Gualerzi & Pon, 1990).

Several conserved features of initiator tRNAs distinguish them from elongator tRNAs; these include the absence of base pairing between bases 1 and 72 in the acceptor arm and the presence of three consecutive G-C base pairs in the anticodon stem (Fig. 1). An unpaired C1-A72 as well as other conserved sequence elements in the acceptor stem are important for interaction of the initiator tRNA with both IF2 and the transformylase enzyme (Mangroo et al., 1995). The presence of three consecutive G-C pairs in the anticodon stem is a conserved feature of initiator tRNAs from eubacteria and archaebacteria and of eukaryotic cytoplasmic initiator tRNAs (Sprinzl et al., 1989). Mutagenesis experiments showed that these G-C pairs were critical for initiator tRNA activity, specifically at the step of binding to the P site on the ribosome (Seong & RajBhandary, 1987). Moreover, toeprinting experiments indicated that an initiator tRNA fragment containing just the anticodon stem loop could be selected effectively by IF3 in initiation complexes and that the three consecutive anticodon stem G-C pairs were essential for IF3 sensing of initiator tRNA identity (Hartz et al., 1990).

The *infC* gene encoding IF3 is essential (Olsson et al., 1996); however, *infC* mutants have been obtained in several laboratories and many of these mutant forms of IF3 display decreased discrimination against nonstandard AUG initiation codons (Haggerty & Lovett, 1993, 1997; Sacerdot et al., 1996; Sussman et al., 1996). Although most (83%) *Escherichia coli* mRNAs initiate with AUG, 14% initiate with GUG and 3% utilize UUG as initiation codons (Blattner et al., 1997). Among the genes that utilize the GUG initiation codon is *recJ*, encoding a single-strand specific DNA exonuclease in-

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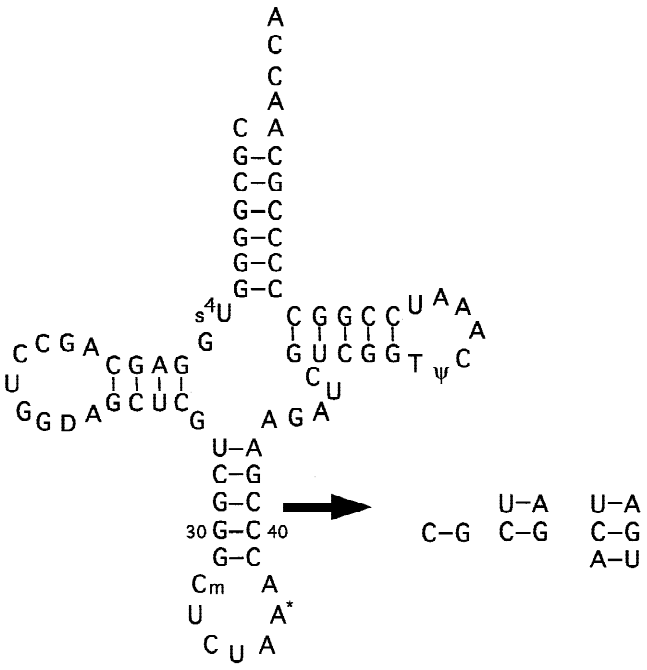

FIGURE 1. Structure of the UAG-decoding derivative of the *metY*-encoded $tRNA_i^{Met}$. A* indicates the ms^2i^6A modification found in the U35 A36 mutant of *metY*-derived tRNA. The anticodon stem mutants analyzed here (C30:G40, U29:A41 C30:G40, and U29:A41 C30:G40 A31:U39) are indicated on the right.

volved in genetic recombination and UV repair. The *recJ154* mutation alters this initiation codon to GUA, rendering the cell UV sensitive and defective in recombination functions. Several *infC* mutations were isolated as allele specific, extragenic suppressors of the *recJ154* mutation and these were subsequently found to effect suppression by stimulating initiation from the noncanonical GUA codon (Haggerty & Lovett, 1993, 1997). We have now carried out an extensive characterization of these IF3 mutants in vivo. Our analysis indicates that all the discriminatory properties of IF3 are altered in these mutants. As observed with other IF3 mutants (Sussman et al., 1996; Sacerdot et al., 1999), the R99L and R131P mutants enhance initiation from CUG and AUN codons, and increase expression of leaderless mRNAs. In addition, our data indicate that the mutant factors fail to discriminate against initiator tRNA derivatives carrying alterations in the three conserved G-C base pairs in the anticodon stem. These data are consistent with a model in which ribosome bound IF3 inspects (directly or indirectly) the unique features of initiator tRNA bound to an initiation codon in the ribosomal P site and actively discriminates against codon–anticodon mismatches and noninitiator tRNAs. Finally, we have also shown that the mutant factors stimulate initiations from internal sites in the mRNA, downstream of the start codon. This suggests that another, under-appreciated function of the wild-type IF3 may be to suppress initiation events from such internal, ectopic start codons within the mRNA.

RESULTS

IF3 mutants stimulate initiation from non-AUG codons

The proposal that IF3 mutations stimulated initiation from non-AUG codons came from a genetic analysis of *infC* mutants that acted as extragenic, allele-specific suppressors of a *recJ* mutation containing a GUG → GUA alteration in its initiation codon (Haggerty & Lovett, 1993). Similar effects on initiation have also been observed with other IF3 variants (Sacerdot et al., 1996; Haggerty & Lovett, 1997). We have previously used a series of *lacZ* constructs containing altered initiation codons to show that nucleotide changes in the decoding center of 16S rRNA lead to a loss of fidelity at both A and P sites during elongation and at the P site during initiation and showed that initiation occurred at the altered initiation codon (O'Connor et al., 1997). Using the same series of constructs, we have asked if the IF3 mutants isolated as suppressors of the *recJ153* mutation affected discrimination of non-AUG codons. The data presented in Table 1 show that the R99L and R131P IF3 variants stimulated initiation five- to sixfold from all AUN and CUG codons tested. When the initiation codon was changed to UAG in the pSG500 construct, very little activity was detected in wild-type cells. Although this level of activity was increased in the R99L and R131P IF3 mutants, these levels of β-galactosidase (124 and 150 units, respectively) were minuscule compared to those observed in the AUN and CUG

TABLE 1. Effect of mutant IF3 on initiation from non-AUG codons.

	IF3 mutants		
lacZ construct	wt	R99L	R131P
Non-AUG initiation codon			
pSG25-AUG	25,858 ± 597	22,201 ± 1,567	21,559 ± 2,032
pSG414-UUG	7,016 ± 759	11,953 ± 439	10,119 ± 1,089
pSG431-GUG	11,607 ± 498	12,288 ± 820	14,207 ± 600
pSG413-CUG	585 ± 61	3,633 ± 211	2,921 ± 282
pSG415-AUC	461 ± 71	2,657 ± 145	2,491 ± 265
pSG416-AUA	501 ± 42	2,781 ± 619	2,550 ± 54
pSG417-AUU	535 ± 73	3,287 ± 446	2,828 ± 159
pSG500-UAG	5 ± 1	124 ± 13	152 ± 9
Internal nitiation			
p304-UUG	1,714 ± 234	6,859 ± 614	6,665 ± 561
p302-UUA	11 ± 1	87 ± 7	63 ± 5
p203-GUG	387 ± 52	1,214 ± 138	968 ± 123
p212-CUC	8 ± 2	77 ± 14	73 ± 14
p211-AUC	30 ± 2	207 ± 5	147 ± 10
p220-CUA	9 ± 1	112 ± 9	136 ± 31
p222-CUG	30 ± 2	401 ± 63	280 ± 15

Units of β-galactosidase measured in wild-type or mutant IF3 strains carrying the indicated *lacZ* plasmids. Cells were grown in minimal medium with required supplements and antibiotics. Each number is the mean (± *SD*) of assays from at least three independent cultures.

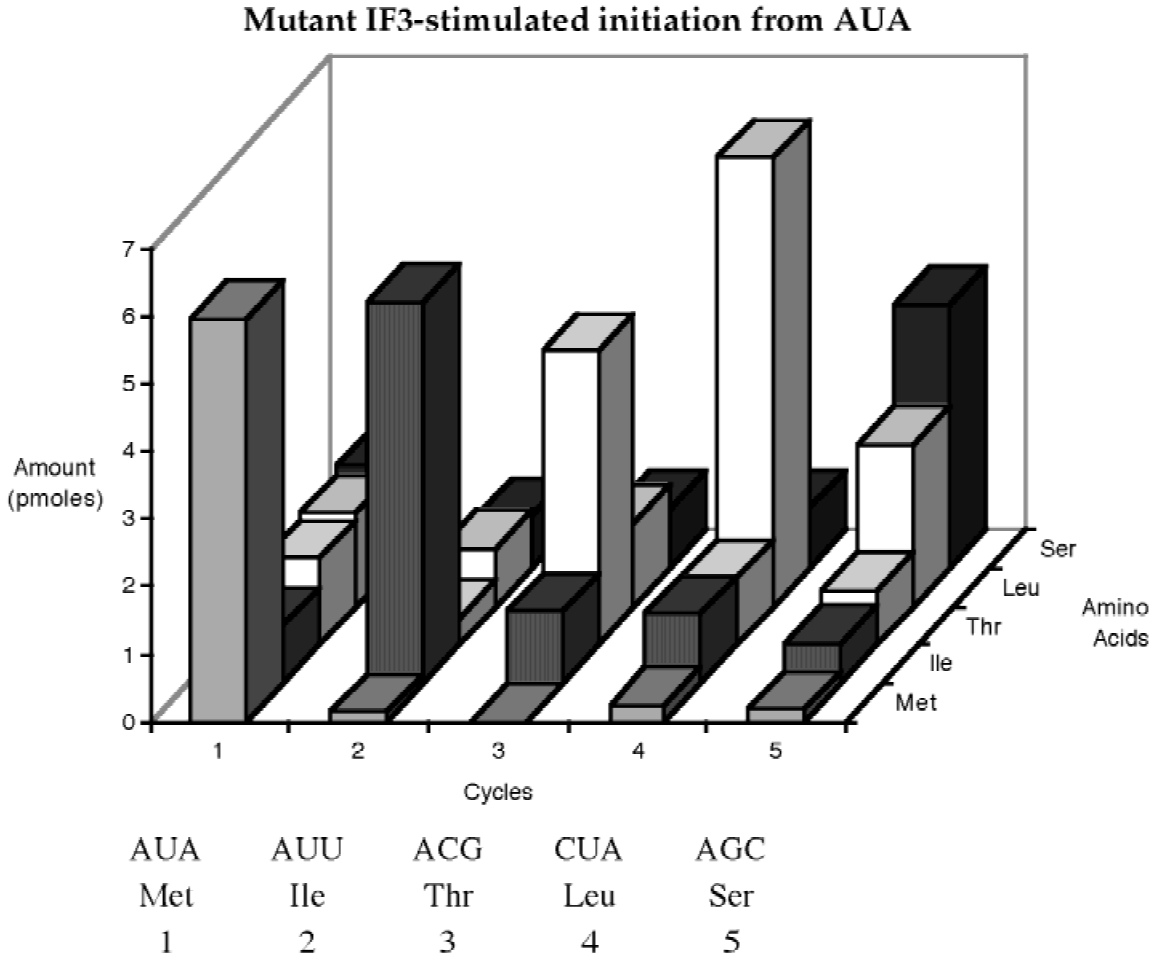

FIGURE 2. N-terminal sequencing to determine the influence of altered IF3 on the site of initiation used in the pSG416 *lacZ* construct. β-Galactosidase was purified from the R131P IF3 mutant containing the pSG416 plasmid. The yield in picomoles for selected PTH amino acids analyzed for the first five cycles is shown.

constructs (2,491–3,633 units). Because all of these constructs are related to each other by one or two nucleotide changes, this indicates that the high levels of activity seen with the AUN and CUG initiation constructs in the mutant IF3 background were dependent on the identity of the altered initiation codon and not on other potential initiation codons downstream in the *lacZ* mRNA. N-terminal sequencing of β-galactosidase isolated from the R131P IF3 mutant containing plasmid pSG416 (where the AUG initiation codon of *lacZ* had been changed to AUA) confirmed that initiation occurred faithfully at the AUA codon (Fig. 2). Significantly, initiation from the AUG and GUG codons that are normally used to initiate with high efficiency was unaffected by either of the mutations in IF3 and initiation from UUG was only marginally increased in the mutant strains. These data indicate that although mutations in IF3 did not affect initiations from canonical, cognate AUG, UUG, and GUG codons, discrimination of near cognate, non-AUG initiation codons was compromised.

Altered discrimination of initiating tRNAs by IF3 mutants

IF3 discriminates against elongator tRNAs and selects for initiator tRNAs in 30S initiation complexes (Risuleo et al., 1976; Hartz et al., 1990). The sequence and structure of the tRNA anticodon loop together with the three consecutive G-C pairs in the anticodon stem are critical for IF3 discrimination (Hartz et al., 1990). Genetic analysis of initiator tRNA has been facilitated by the construction of a specialized initiator tRNA whose anticodon has been altered so that it can now initiate from UAG codons. The availability of such tRNAs together with a CAT reporter gene construct that contains a UAG initiation codon means that the activity of specific tRNA mutants can be analyzed in isolation (Varshney & RajBhandary, 1990). Using this system, it has been shown that mutagenesis of the three consecutive G-C anticodon stem pairs led to successive decreases in initiation frequency from the UAG codon of

TABLE 2. Effect of mutant IF3 on activities of initiator tRNAs.

	IF3 mutants		
metY tRNA mutants	Wild-type	R99L	R131P
Wild-type	8 ± 2	202 ± 6	110 ± 5
U35 A36	7,491 ± 1,276	9,485 ± 1,622	ND
C30:G40/U35 A36	1,141 ± 141	6,277 ± 481	ND
U29:A41 C30:G40/U35 A36	732 ± 141	3,907 ± 262	3,639 ± 602
U29:A41 C30:G40 A31:U39/U35 A36	503 ± 67	2,564 ± 353	2,878 ± 689

Units of β-galactosidase obtained in wild-type or IF3 mutant strains carrying a *lacZ* plasmid with a UAG initiation codon (pSG500) and each of the indicated tRNA mutants on pRSVCATam1.2.5-derived-plasmids (Varshney & RajBhandary, 1990). Cells were grown in minimal medium with required supplements, tetracycline (12.5 mg/L) and ampicillin (200 mg/L). Each β-galactosidase activity measurement represents the mean (± *SD*) of assays from at least three independent cultures.

the CAT reporter gene (Mandal et al., 1996). Based on the results of the toeprinting studies cited above, we considered it likely that the low activity of these altered initiator tRNAs was due, in part at least, to IF3 discrimination, which senses these mutant tRNAs effectively as noninitiator species. We asked, therefore, if this discrimination was ameliorated in IF3 mutants. The wild-type strain and both of the IF3 mutants were transformed with a *lacZ* plasmid, pSG500, in which the initiation codon was altered to UAG. These strains were then transformed with plasmids carrying the wild type or each of the altered initiator tRNAs (Fig. 1). Assays of β-galactosidase from each of the IF3/initiator tRNA combinations showed that the R99L and R131P IF3 mutants stimulated the activity of each of the mutant initiator tRNAs approximately fivefold (Table 2). As a result of the changes to the anticodon loop, the initiator tRNAs used here are charged with glutamine (Schulman & Pelka, 1985; Varshney & RajBhandary, 1990). N-terminal sequencing of β-galactosidase isolated from the R99L IF3 mutant carrying plasmid pSG500 confirmed that glutamine was inserted at the UAG initiation codon (Fig. 3), with no evidence of initiation from internal sites or initiation with methionine. These data indicate that IF3 mutants that fail to discriminate against non-AUG codons also fail to discriminate against noninitiator tRNAs. Because of unanticipated problems with plasmid instability, we were unable to measure the activities of the wild-type UAG-decoding tRNA (i.e., the U35 A36 mutant with no changes in the anticodon stem) or the C30:G40/U35 A36 mutant in the slow-growing *infC135* strain carrying multiple plasmids and expressing high levels of β-galactosidase. However, the activity of the wild-type, U35 A36, UAG-decoding initiator tRNA was unaffected by the R99L IF3 mutant, indicating that, as expected, IF3 does not discriminate against wild-type initiator tRNA decoding of a cognate initiation codon. These data indicate that the defect in initiator tRNAs carrying alterations in the three anticodon stem G-C pairs was due, at least in part, to discrimination by wild-type IF3.

Altered expression of leaderless mRNAs in IF3 mutants

In *E. coli*, all chromosomally encoded mRNAs contain untranslated leader regions preceding the initiation codon. However, several phage and transposon-encoded mRNAs are leaderless when expressed in *E. coli* (Tedin et al., 1999). Previous work had shown that both ribosomal protein S1 together with initiation factors IF2 and IF3 (Moll et al., 1998; Tedin et al., 1999; Grill et al., 2000) were important for initiation complex formation on leaderless mRNAs and that IF3 in particular discriminated against the authentic AUG initiation codon in the leaderless c*I* mRNA (Tedin et al., 1999). Using either wild-type (pdb wt) or mutant (pdb*) c*I-lacZ* fusions (Shean & Gottesman, 1992), we have asked if the IF3 mutants analyzed here affect expression of this leaderless mRNA. The data presented in Table 3 show that both the R99L and R131P IF3 mutants enhanced leaderless cI expression modestly whereas expression of two leadered mRNAs encoding *RpoH-lacZ* (Nagai et al., 1991) and *LysU-lacZ* (Ito et al., 1993) fusion proteins was unaffected. Consistent with these results, previous experiments by Tedin et al. (1999) showed that the same *infC135* mutant used here increased the expression of a *tetR-lacZ* fusion substantially (sevenfold). This suggests that IF3 recognizes the authentic initiation codon on leaderless mRNAs as a nonstandard start codon and, in the absence of any stabilization afforded by the SD–anti-SD interaction found in most leadered mRNAs, IF3 destabilizes initiation complex formation on leaderless mRNAs. Consequently, the activity of IF3 is an important element in determining the expression of leaderless mRNAs.

IF3 modulates initiation from internal initiation sites

In the course of our earlier work on frameshifting (Falahee et al., 1988; O'Connor et al., 1989), we observed that several of our *lacZ* reporter gene constructs had

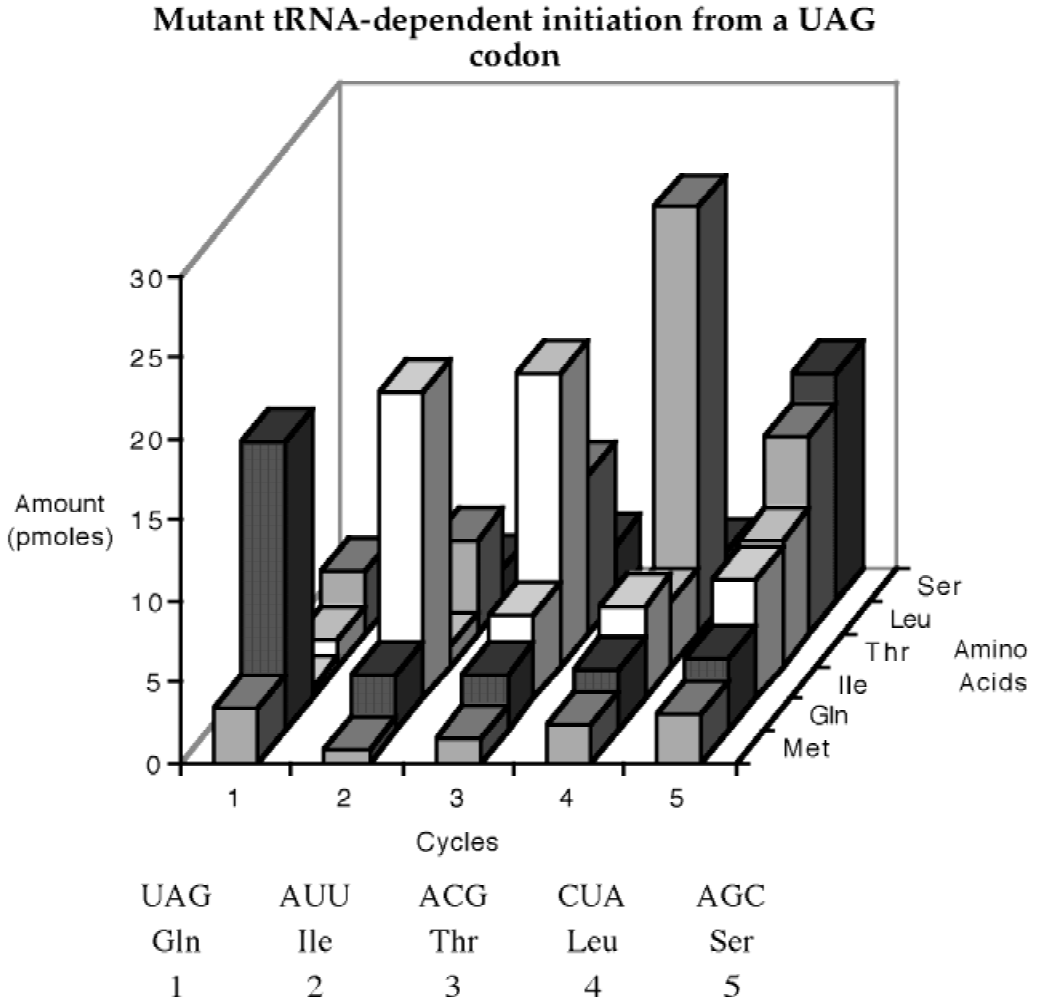

FIGURE 3. N-terminal sequencing to determine the influence of altered IF3 on initiation by mutant UAG-decoding initiator tRNA. β-Galactosidase was purified from the R99L IF3 mutant containing the pSG500 plasmid and expressing the U29:A41 C30:G40 A31:U39/U35 A36 mutant initiator tRNA. The yield in picomoles for selected PTH amino acids obtained for the first five cycles is shown. The lower panel depicts the mRNA sequence and the experimentally determined amino acid sequence.

unanticipated high levels of activity. Subsequent mutagenesis and peptide sequencing of the expressed β-galactosidase showed that in many instances, such high basal levels of expression originated from initiation at internal sites within the mRNA, often at non-AUG codons. Given the effects of the IF3 mutants on several atypical initiation events described above, we asked if mutations in IF3 affected such initiations from internal initiation codons. Each *lacZ* frameshift construct contains an authentic AUG initiation codon and a downstream frameshifting window. To synthesize β-galactosidase, (1) ribosomes must initiate from the authentic AUG codon and frameshifting must occur within this window delimited by stop codons or (2) initiation in a different reading frame must occur within the frameshifting window or downstream from it. A variation on the latter possibility is that rather than *de novo* binding of 30S subunits to the internal start site, reinitiation at internal positions may occur by backscanning following termination at the downstream stop codon (Adhin & van Duin, 1990). In wild-type IF3 cells, the p304 +1 frameshift construct displays a high basal ac-

TABLE 3. Effect of mutant IF3 on initiation from leadered and leaderless mRNAs.

	IF3 mutants		
mRNA/plasmid	Wild-type	R99L	R131P
λcI/pdb wt	811 ± 43	1,224 ± 112	2,145 ± 199
λcI/pdb*	120 ± 17	276 ± 26	513 ± 39
rpoH/pFRP103-15A	885 ± 17	993 ± 74	1,114 ± 93
lysU/pRP92	376 ± 35	461 ± 26	432 ± 46

Units of β-galactosidase measured in wild-type or IF3 mutant strains carrying the indicated *lacZ* fusion plasmid. Cells were grown in minimal medium with the required supplements and antibiotics. Each number is the mean (± *SD*) of assays from at least three independent cultures.

TABLE 4. *lacZ* Plasmid sequences.

Plasmid	Relevant feature	Sequence
Non-AUG initiation codon constructs		
pSG25	Wild-type lacZ	**AUG** AUU ACG CUA AGC UUG GCA CUG
pSG413	CUG initiation	**CUG** AUU ACG CUA AGC UUG GCA CUG
pSG414	UUG initiation	**UUG** AUU ACG CUA AGC UUG GCA CUG
pSG431	GUG initiation	**GUG** AUU ACG CUA AGC UUG GCA CUG
pSG415	AUC initiation	**AUC** AUU ACG CUA AGC UUG GCA CUG
pSG416	AUA initiation	**AUA** AUU ACG CUA AGC UUG GCA CUG
pSG417	AUU initiation	**AUU** AUU ACG CUA AGC UUG GCA CUG
pSG500	UAG initiation	**UAG** AUU ACG CUA AGC UUG GCA CUG
Internal initiation constructs		
p304	+1 frameshift	**AUG** AAA AGC U*UA A*AC GUUG CAA CC*U AA*C GGC CCU AAU UCA CUG
p302	+1 frameshift	**AUG** AAA AGC U*UA A*AC GUUA CAA CC*U AA*C GGC CCU AAU UCA CUG
p203	−1 frameshift	**AUG** AAA AGC UU*U AA*C GUGUG C*UA G*GC CCU AAU UCA CUG
p212	−1 frameshift	**AUG** AAA AGC UU*U AA*C GUCUC C*UA G*GC CCU AAU UCA CUG
p211	−1 frameshift	**AUG** AAA AGC UU*U AA*C GUAUC C*UA G*GC CCU AAU UCA CUG
p220	−1 frameshift	**AUG** AAA AGC UU*U AA*C GUCUA C*UA G*GC CCU AAU UCA CUG
p222	−1 frameshift	**AUG** AAA AGC UU*U AA*C GUCUG C*UA G*GC CCU AAU UCA CUG

The codon corresponding to the position of the wild-type *lacZ* initiation codon is indicated in bold. Alternative, internal initiation codons are underlined and stop codons in the relevant reading frames are italicized in the internal initiation constructs. The final CUG leucine codon in the sequences corresponds to codon 7 of the wild-type *lacZ* gene.

tivity (approximately 6% of wild-type *lacZ*) and the presence of either IF3 mutation increases this activity fourfold (Table 1). Inspection of the p304 sequence (Table 4) shows the presence of a UUG codon in the +1 reading frame 14 nt downstream of the AUG codon. Alteration of this +1 frame UUG codon in p304 to UUA (in plasmid p302) abolishes virtually all activity in wild-type and mutant IF3 strains, indicating that initiation from UUG is indeed responsible for the high expression seen in p304. N-terminal sequencing of β-galactosidase isolated from the R99L IF3 mutant carrying p304 showed that initiation did indeed occur exclusively at the internal UUG codon in the IF3 mutant strain (Fig. 4). Similarly, the p203 construct contains an internal GUG codon (Table 4) and has a high expression level in wild-type cells (387 units of β-galactosidase, Table 1). When this GUG codon in p203 was altered to CUG, CUA, AUC, or CUC, (constructs p222, p220, p211, and p212, respectively) expression diminished severely, indicating that initiation in p203 most likely derives from internal initiation at GUG. When the activities of these various constructs were measured in the R99L and R131P IF3 mutants, substantial increases in activities were observed with all constructs (Table 1). Although increased substantially over the level found in the wild-type strain, expression of p302, p212, p211, p220, and p222, which are all predicted to have poor internal initiation sites, was still very low in the IF3 mutants. However, expression from the p304 and p203 constructs containing UUG and GUG internal initiation codons, respectively, was substantial in these strains. Comparison of these expression levels with those obtained with the pSG414 and pSG431 constructs indicates that although the mutant IF3 had no effect on initiation from UUG and GUG when these codons were at the authentic initiation site, the altered factor stimulated initiation from these codons when they were at internal sites in the mRNA. This suggests that wild-type IF3 suppresses initiations even from cognate codons when they are at internal positions in the message and acts to direct initiation from the authentic start codon exclusively.

DISCUSSION

In this article, we have demonstrated that mutant forms of IF3 lose the ability to discriminate against codon–anticodon mismatches in the P site of initiating ribosomes and fail to eject tRNAs lacking elements normally essential for initiator tRNA activity. Moreover, the same IF3 mutants fail to discriminate against initiation from internal start codons downstream of the authentic AUG or from the AUG at the 5′ ends of leaderless mRNAs that are usually recognized as nonstandard initiation codons by wild-type IF3. These phenotypes are most easily interpreted as loss-of-function mutations in IF3. Together, these data indicate that IF3 is involved in ensuring the fidelity of both codon and tRNA selection and modulates the interaction between these two ligands on the initiating ribosome.

Although AUG is overwhelmingly the most common codon used to initiate protein synthesis in all organisms, GUG and UUG are also used efficiently and with appreciable frequency. Because wild-type IF3 does not discriminate against GUG and UUG codons, clearly some latitude is tolerated at the first codon position; of all NUG codons, only CUG is discriminated against. Consistent with this interpretation, previous work with IF3 mutants (Sussman et al., 1996; Sacerdot et al.,

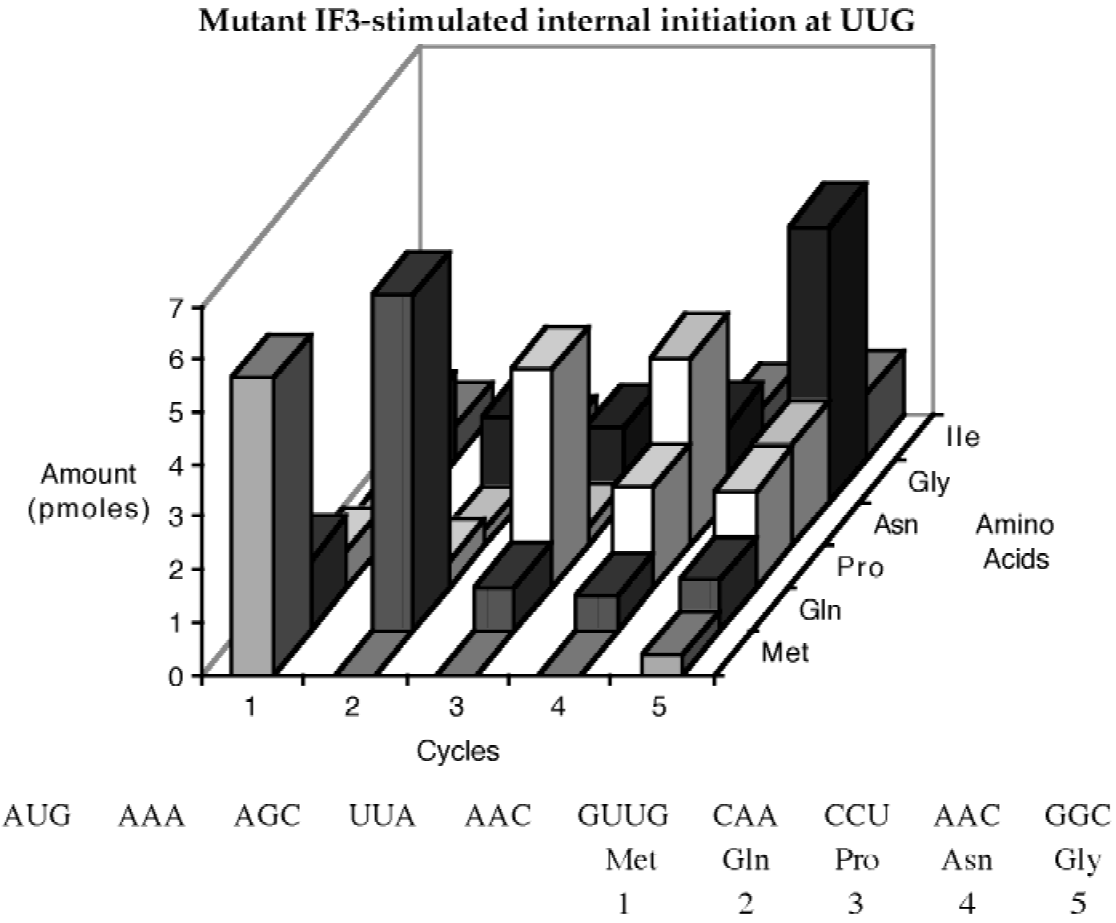

FIGURE 4. N-terminal sequencing to determine the influence of altered IF3 on initiation from internal sites in the p304 *lacZ* construct. *β*-Galactosidase was purified from the R99L IF3 mutant containing the p304 *lacZ* plasmid. The yield in picomoles for selected PTH amino acids analyzed for the first five cycles is shown on the upper panel and the lower panel depicts the mRNA sequence aligned with the experimentally determined amino acid sequence.

1999) as well as the data presented here (Table 1) showed that initiation from AUG and GUG was completely unaffected by mutant IF3 and only minor increases in initiation from UUG were observed. However, initiation from CUG, AUU, AUA, and AUC constructs was strongly stimulated by mutant IF3, indicating that wild-type IF3 discriminates strongly against these codons. In agreement with this, in vitro experiments have shown that initiation from AUU was strongly repressed by excess IF3 and was more dependent on IF2, when compared to initiation from AUG (La Teana et al., 1993). Translation of the *infC* message occurs from the atypical AUU codon and repression of initiation from this start codon underlies autoregulation of *infC* expression by intracellular IF3 levels (Butler et al., 1987). Discrimination against non-AUG initiations appears to be due to sensing of codon–anticodon mismatches by IF3, as such discrimination was eliminated in the presence of an engineered initiator tRNA with perfect complementarity to any of several noncanonical initiation codons (Meinnel et al., 1999).

Toeprinting experiments have shown that most of the elements necessary for IF3 sensing of initiator tRNA identity are in the anticodon stem loop and the conserved G-C pairs in the anticodon stem are essential for IF3 recognition (Hartz et al., 1990). Mutagenesis of these residues (either singly or in combination) resulted in a loss of initiator tRNA activity both in vitro and in vivo (Seong & RajBhandary, 1987) and subsequent work showed that alterations to the G30:C40 pair had the most severe effect (Mandal et al., 1996). Using an AUG-dependent, IF-stimulated ribosome binding assay, these initiator tRNA mutants were shown to be specifically defective in binding to the ribosomal P site (Seong & RajBhandary, 1987). Formally, such defective tRNA binding could be due either to a distorted anticodon conformation that cannot be accommodated in the ribosomal P site, or to ejection of what are effectively noninitiator tRNAs by IF3. Mutagenesis of the critical G30:C40 pair in the UAG-decoding initiator tRNA reduced its initiator activity to 15% of the wild-type tRNA (Table 2). However, when the initiator activity of the same tRNA was assayed in the R99L IF3 mutant, its activity was 66% of wild type (Table 2). Similar increases in activity were seen with the double and triple mutant tRNAs in the mutant IF3 strains. These data

thus suggest that the impaired P site occupancy seen with these tRNAs is due, in part at least, to their rejection by IF3, which senses them as noninitiator tRNAs.

Discrimination against non-AUG codons in 30S initiation complexes appears to be due to IF3-mediated destabilization, rather than to any inherent instability of the mRNA–tRNA–30S complex (La Teana et al., 1993). Both SD–anti-SD base pairing as well tRNA–mRNA interactions contribute to the stability of initiation complexes formed on mRNAs containing 5′ untranslated leader regions. Because no mRNA–rRNA base pairing occurs on leaderless mRNAs, such initiation complexes may not be able to withstand IF3-mediated destabilization. A similar interpretation may explain the effect of IF3 on initiation from internal start codons: The mRNAs supporting internal initiation from non-AUG codons that we have examined lack recognizable SD sequences upstream of the (internal) start codon (Table 4). Initiation complexes formed on these mRNAs may be particularly susceptible to IF3-mediated destabilization because codon–anticodon mismatches are not offset by any compensating SD–anti-SD interaction.

IF3 is a two-domain protein and the structures of the separate domains have been solved by X-ray crystallography and NMR spectroscopy (Biou et al., 1995; Garcia et al., 1995a, 1995b). The C domain appears to contain most of the elements necessary for binding to 30S subunits and dissociation of 70S ribosomes (Garcia et al., 1995b). Mutations affecting the fidelity of initiation have been isolated in both domains. The R99 and R131 variants studied here, as well as the D106, E134, and P176 mutants (Sacerdot et al., 1996, 1999; Sussman et al., 1996) are all in the C-terminal domain, whereas the Y75 (Sussman et al., 1996) mutant lies in the N-terminal domain and the G71 (Sacerdot et al., 1999) mutant is at the beginning of the interdomain linker region (De Cock et al., 1999). The mutant R99 and R131 *E. coli* factors have not been assayed for their ability to bind 30S subunits. However, mutagenesis of R99, R133, and a number of neighboring residues in *Bacillus stearothermophilus* IF3 decreased factor binding to 30S subunits (Sette et al., 1999). The *E. coli* P176 IF3 mutant had a decreased affinity for 30S subunits and the D103 mutant displayed a lack of binding specificity and bound to both ribosomal subunits (Sacerdot et al., 1999). In contrast, the Y75 and G71 mutants in the N-terminal domain only affected the discriminatory properties of the factor and did not affect its affinity for 30S subunits (Sussman et al., 1996; Sacerdot et al., 1999). A model to explain these differential interactions of IF3 mutants with the ribosome, all of which compromise the fidelity of initiation, has been proposed by Sacerdot et al. (1999). According to this model, the analyzed N-domain mutants affected the proofreading function directly, whereas the C-terminal mutants decreased the concentration of factor bound to 30S subunits, allowing initiation to occur in the absence of proofreading by IF3.

IF3 has been localized on the 30S subunit by cryoelectron microscopy (McCutcheon et al., 1999). When the crystal structure of IF3 was modeled into the electron densities obtained from electron microscopic reconstructions of the 30S–IF3 complexes, the C-terminal domain appeared to be located close to the anticodon loop of the initiator tRNA and the linker region appeared to approach the initiation codon and, according to this model, IF3 inspects the tRNA–codon complex directly. A fundamentally different orientation of IF3 on the 30S subunit has been proposed by Sette et al. (1999) in which the N and C domains of the factor bridge distant elements of the 30 S subunit and IF3 senses the tRNA–codon complex only indirectly. The question thus remains unresolved whether IF3 inspects tRNA–mRNA initiation complexes directly or instead effects a conformational change in the ribosome, perhaps akin to that adopted by the A site during the proofreading stage of tRNA selection (Pape et al., 2000) favoring dissociation of noncognate initiation codons and noninitiator tRNAs.

MATERIALS AND METHODS

Bacterial strains and plasmids

The 2 *infC* (*srjA*) mutants used here were obtained from Tim Haggerty and Susan Lovett, Brandeis University (Haggerty & Lovett, 1993, 1997). The ability of these mutants to stimulate nonstandard initiation events was explored in a pilot experiment using a Δ*(lac-pro)* derivative of the *infC135* strain. These experiments showed that the *infC* mutation increased initiation from internal GUG and UUG codons in *lacZ* (see Results). To facilitate their subsequent analysis, both *infC* mutations were moved into the Δ*(lac-pro)* strain, MC41. An *aroD::Tn10* derivative of MC41 was first constructed and was then transduced to Aro^+ with phage P1 prepared on each of the *infC* mutants. These strains were designated MC171 (*infC134*) and MC172 (*infC135*). The presence of the *infC* mutation was assessed by the ability of selected transductants to stimulate initiations from internal GUG and UUG sites in a *lacZ* construct and by DNA sequencing of an *infC*-containing PCR fragment amplified from these strains. In addition, *infC135* strains had a distinct growth defect and both mutants formed wrinkled colonies on LB agar plates containing calcium and high concentrations (2%) of glucose.

The pACYC184-derived plasmids containing *lacZ* genes with altered initiation codons have been described previously (O'Connor et al., 1997). Additional constructs were made by ligating pairs of complementary oligonucleotides (containing the desired initiation codon) with *Eco*RI and *Hin*dIII overhangs into *Hin*dIII/*Eco*RI-digested pSG25 (O'Connor & Dahlberg, 1993). All plasmid constructions were verified by plasmid sequencing. Plasmids containing internal initiation codons were constructed earlier for analysis of frameshifting by mutant tRNAs (O'Connor et al., 1989). The relevant sequences of all *lacZ* plasmids used in this work are shown in Table 4.

Leaderless *lacZ-cI* fusion plasmids (Shean & Gottesman, 1992) were obtained from Max Gottesman, and *rpoH-lacZ* (Nagai et al., 1991) and *lysU-lacZ* (Ito et al., 1993) fusion plasmids were obtained from T. Yura and Y. Nakamura, respectively.

Plasmids expressing initiator tRNAs were derived from pRS-VCATam1.2.5 (Varshney & RajBhandary, 1990) and also contained either a wild-type *metY* gene encoding the initiator tRNA or the indicated mutant tRNAs.

β-Galactosidase assays and purification

Cells to be assayed for β-galactosidase activity were grown in minimal E medium (Vogel & Bonner, 1956) containing glucose (0.2%) thiamine, proline, and casamino acids (0.2%) together with any necessary antibiotics. β-Galactosidase was assayed as described previously (O'Connor & Dahlberg, 1993; O'Connor, 1998). For protein purification, cells were grown in rich medium and disrupted with a French press. β-Galactosidase was purified from the postribosomal supernatants of these lysates by immunoaffinity chromatography (5 Prime → 3 Prime, Boulder, Colorado) and sequenced as described previously (O'Connor et al., 1989).

ACKNOWLEDGMENTS

We are grateful to Drs. Susan Lovett and Tim Haggerty for supplying the *infC* mutants, to Dr. George Q. Pennabble for his continued forbearance and to David G. Warter for inspiration and enthusiasm. We are indebted to R. Schackmann, Raymond Gesteland, and John Atkins for peptide sequencing. This work was supported by Grants #GM19756 to A.E.D. and #R37 GM17151 to U.L.R. from the U.S. National Institutes of Health.

Received February 1, 2001; returned for revision March 15, 2001; revised manuscript received April 9, 2001

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