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Discrimination of 5′-terminal start codons by translation initiation factor 3 is mediated by ribosomal protein S1

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Abstract The interrelation between ribosomal protein S1 and IF3 in recognition/discrimination of 5′-terminal start codons by 30S ribosomes has been studied using in vitro toeprinting. The study has been performed with two naturally occurring leaderless mRNAs, λ *c*I and phage r1t *rro* mRNA, as well as with an artificial leaderless mRNA derived from the *E. coli ompA* gene. We show that in the absence of S1, IF3 does not discriminate against the authentic 5′-terminal start codon of both *c*I and *rro* mRNA. Since IF3 was able to exert its proofreading function for initiator $tRNA_f^{Met}$ on 30S ribosomes lacking S1, this observation cannot be attributed to a lack of binding to or action of IF3 on 30S(−S1) ribosomes. In contrast to leaderless mRNAs, ternary complex formation occurs in the presence of IF3 with 30S ribosomes when the start codon is preceded by a short 20-nucleotide 5′-untranslated region containing a canonical Shine and Dalgarno sequence. This suggests that 5′-terminal start codons are recognised by IF3 as non-standard because of the lack of 16S rRNA-mRNA contacts.

Key words: Leaderless mRNA; Ribosomal protein S1; Translation initiation factor 3

1. Introduction

Leaderless mRNAs starting directly with the 5′-terminal start codon are found in all genetic systems, from bacteriophages to human mitochondria [1]. Very little is known how these mRNAs are recognised by the translational apparatus and how these mRNAs can compete in bacteria for ribosomes with mRNAs harbouring a canonical ribosome binding site (rbs). Despite the lack of a Sine and Dalgarno sequence (SD-sequence) some of these leaderless mRNAs are translated with a high efficiency [1] suggesting that mechanisms or conditions exist which allow for an efficient recognition of these mRNAs by ribosomes.

It has been shown that the translational efficiency of a leaderless λ *c*I-*lacZ* transcript is enhanced in an *E. coli rpsB* mutant containing S2-deficient ribosomes [2]. The effect of the *rpsB* mutation was attributed to an increased accessibility of the anti-downstream box, which has been suggested to be located in the penultimate stem of 16S rRNA [3,4], for the downstream box positioned in the immediate vicinity of the start codon of *c*I mRNA [2]. However, the existence of this interaction has been questioned recently [5]. No *cis*-acting downstream elements required for the interaction of leaderless mRNAs with ribosomes have as yet been demonstrated by experiment [5]. In fact, the leaderless λ *c*I mRNA was found to be translated with almost the same efficiency in both a *B. stearothermophilus* and an *E. coli* in vitro translation system despite the fact that the respective putative anti-downstream boxes differ significantly [6]. Moreover, it has been recently shown that the removal of the 5′-untranslated region (UTR) of the *S. lividans* viomycin phosphotransferase gene [7] and the *S. acrimycini* chloramphenicol acetyltransferase gene [8], respectively, still allows translation of the resulting mRNAs in *E. coli*. It remains therefore questionable whether the ability of *E. coli* ribosomes to translate leaderless mRNAs can be attributed to interactive sequences.

Balakin et al. [9] have shown that 70S tight-coupled ribosomes have an intrinsic high affinity for the 5′-terminal start codon on λ *c*I mRNA. These authors suggested that translation initiation of leaderless mRNAs may be achieved by 70S ribosomes. So far, there is only circumstantial evidence that this may indeed occur. It has been reported that expression of a leaderless λ *c*I-*lacZ* mRNA is unaffected in the presence of kasugamycin [10], an antibiotic known to stimulate the release of $f-Met-tRNA_f^{Met}$ bound to 30S subunits but much less to 70S subunits [11]. Furthermore, when the authentic 5′-terminal start codon of different leaderless constructs was abutted with an upstream 4-base leader containing a 5′-terminal out of frame AUG, translation of the corresponding leaderless mRNAs was prevented [8,12]. This suggested that the 5′-terminal position of the initiation codon is an important parameter for translation initiation of a leaderless mRNA.

A commonly held perception is that mRNAs are recognised by a homogeneous population of ribosomes and that ribosome-mRNA interactions are mainly determined by intrinsic features of the individual mRNA. However, our recent studies have revealed that translation initiation factor 3 (IF3) discriminates against the 5′-terminal AUG codon on both the leaderless λ *c*I and phage P2 gene *V* mRNA in vitro and it has been shown that elevated levels of IF3 decreased the translational efficiency of a leaderless *tetR-lacZ* construct in vivo [13]. This finding suggested that a sub-population of 30S ribosomes devoid of IF3 may be recruited for translation initiation of leaderless mRNAs.

Ribosomal protein S1 was found to be dispensable for 30S initiation complex formation on leaderless mRNAs as well as on mRNAs which contained a short 5′-UTR [6]. Here, we have tested whether there is an interrelation between the function of IF3 and S1 in 30S initiation complex formation on leaderless mRNAs. In the presence of IFs and S1, the ternary complex with 30S subunits was shifted to internal start codons preceded by a putative or an authentic SD sequence with either leaderless mRNA used, whereas in the absence of S1,

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Abbreviations: rbs, ribosome binding site; SD-sequence, Shine and Dalgarno sequence; IF, translation initiation factor

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IF3 did not abolish ternary complex formation on 5′-terminal start codons.

2. Materials and methods

2.1. Plasmid and PCR templates for preparation of mRNAs

Plasmid pAXL2 [13] bearing the first 63 codons of the λ *c*I gene under transcriptional control of the T7 gene *10* promotor served as a template for in vitro transcription of *c*I mRNA. After linearisation of plasmid pAXL2 with *Sma*I, in vitro transcription with T7 RNA polymerase was performed, and the 190-nt long run-off transcript was used for the in vitro toeprinting studies.

Plasmid pUH100 [14] was used for generating PCR templates suitable for in vitro transcription of *ompA*Δ117 mRNA with T7 RNA polymerase. PCR was performed with primers V8 (5′-GGGCTCTAGAGTAATACGACTCACTATAGATGATAACGAGGCGCAAAAAATG-3′) containing a T7 promoter and primer *Ava*II [14]. T7-RNA polymerase directed transcription generated a 131 nt long mRNA.

Plasmid pIR12 [15] harbouring the entire *rro* gene of *L. lactis* phage r1t was used to obtain PCR templates for transcription of *rro* mRNA. PCR was performed with primers L9 (5′-CCGTCTAGACGTAATACGACTCACTATAGATGAAAAAAATACGACTACCTGAAATGATAG-3′) and M9 (5′-AAAGAATTCGAGTTGTGAACTGATTTT-3′). Primer L9 contained the T7 gene *10* promoter. In vitro RNA synthesis with T7 RNA polymerase yielded a 257-nt long transcript.

2.2. Preparation of E. coli 30S ribosomes and depletion of S1

Ribosomes used in this study were prepared from *E. coli* MRE600 as described [16]. 30S ribosomes were depleted for protein S1 by affinity chromatography using a poly(U)-Sepharose 4B column (Pharmacia) as described previously [17]. The depletion of protein S1 was verified by Western-blotting with anti-S1 antibodies according to standard protocols. *E. coli* initiation factors were kindly provided by C.O. Gualerzi, University of Camerino, Italy.

2.3. Primer extension inhibition analysis (toeprinting)

Reverse primers used for toeprinting on λ *c*I, *ompA*Δ117, and *rro* mRNAs were O-8 (5′-GGGGTTATAAGC-3′), *Ava*II [14], and Q9 (5′-ATCAAAATCTTCAACCATGGG-3′), respectively. The toeprinting assays were performed with 30S ribosomes using 5′-end-labeled primers as specified by Hartz et al. [18] with the exception that the M-MLV reverse transcriptase was added to the dNTP mix prior to addition of the annealing mix. The final ratio of 30S subunits, tRNA(s), mRNA, was 1 pmol, 5 pmol, and 0.1 pmol, respectively. IFs were added as indicated in the legends to the figures.

3. Results

3.1. Leaderless mRNAs used in this study

The mRNAs used in this study are depicted in Fig. 1. The secondary structures present in the 5′-coding region of λ *c*I mRNA have been mapped by enzymatic probing [13]. The 5′-

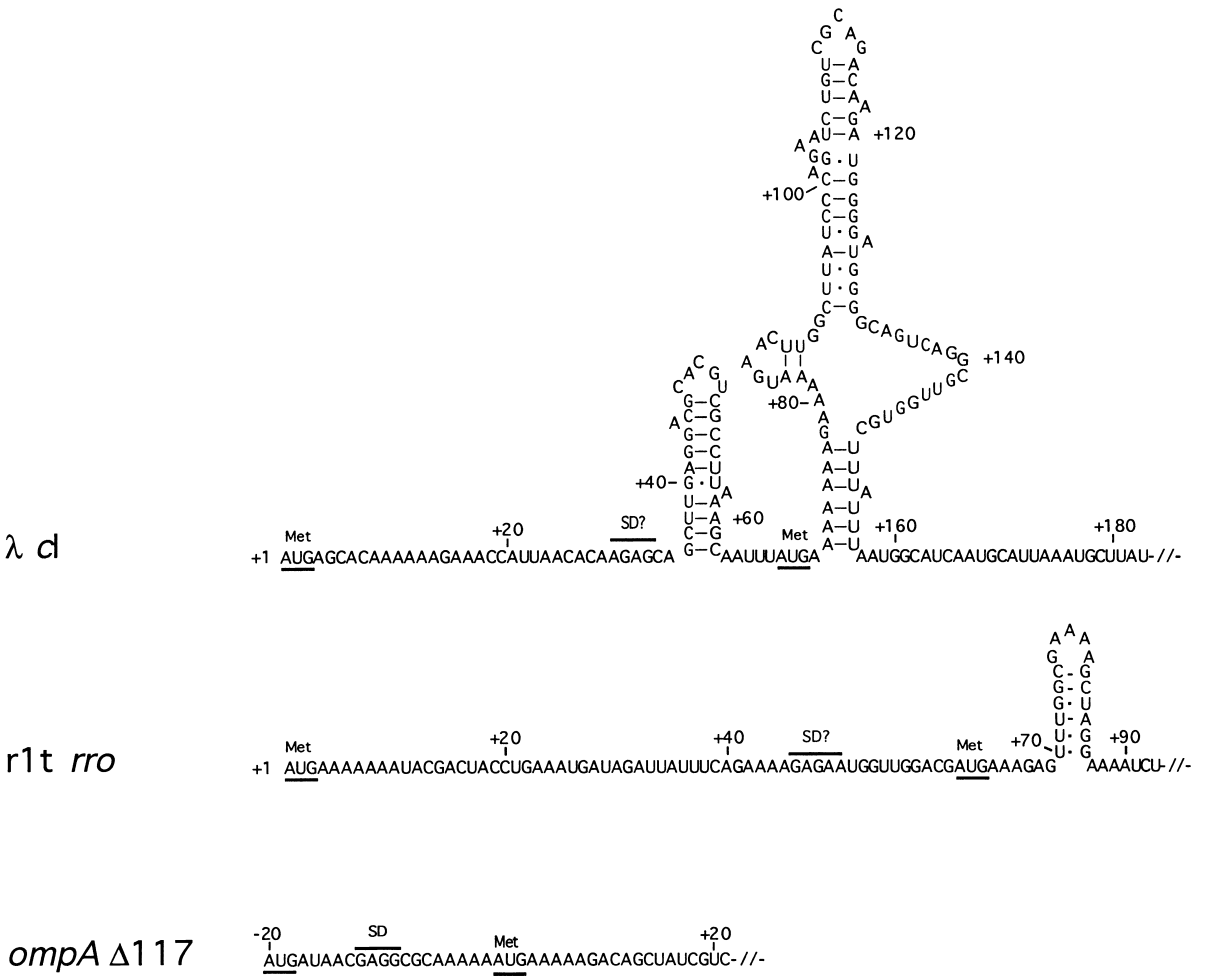

Fig. 1. Depiction of mRNAs used in this study. The 5′-terminal and internal start codons as well as the putative internal SD sequences in both λ *c*I and r1t *rro* mRNA are indicated by bars. The *ompA*Δ117 mRNA contains a 5′-terminal start codon upstream of the authentic rbs of *ompA*.

end of the *ompA*Δ117 mRNA has been mapped also and was found to be single stranded [6]. The secondary structure shown in the *Lactococcus lactis* phage r1t *rro* mRNA [15] was predicted using the program Hairpin included in PC/Gene. Note that in both *c*I and *rro* mRNAs the internal start codons are preceded by a putative SD sequence (Fig. 1). The *ompA*Δ117 mRNA contains the authentic *ompA* ribosome binding site downstream of the 5′-terminal AUG.

3.2. Discrimination by IF3 of AUG1 on λ cI mRNA and on phage r1t rro mRNA depends on ribosomal protein S1

We have recently shown that 30S ribosomes form a ternary complex on *c*I mRNA over the 5′-terminal start codon as well as over the internal codon AUG68 (see Fig. 1) which is preceded by a putative SD sequence [13]. However, as shown in Fig. 2, in the absence of ribosomal protein S1, 30S initiation complex formation did not occur at AUG68 (Fig. 2, lane 10). At first glance this result agreed with previous observations that ribosomal protein S1 was shown to be required for ternary complex formation on internal rbs of several *E. coli* mRNAs [6,9,19,20] as well as on heterologous mRNAs [21], which are faithfully translated in *E. coli* despite of the absence of an apparent SD sequence. Nevertheless, further experiments revealed that IF3 in fact discriminated against the 5′-terminal start codon with 30S ribosomes on *c*I mRNA [13]. The presence of IF3 directed the ternary complex exclusively to the internal AUG codon 68 in *c*I mRNA (Fig. 2, lane 3). Due to the apparent interrelation between S1 and IF3 dependent start codon partitioning on *c*I mRNA, we tested whether IF3 dependent discrimination against AUG1 on *c*I mRNA would occur with 30S ribosomes depleted for S1. As shown in Fig. 2, lanes 6–9, even a 4-fold excess of IF3 over 30S(−S1) subunits did not direct the ternary complex to the internal start codon AUG68, nor did it affect selection of the authentic start codon. Thus, in the absence of S1, IF3 apparently does not discriminate against AUG1 on *c*I mRNA. In contrast, S1 was required for recognition of the internal codon AUG68 (Fig. 2, lanes 2–5). Efficient ternary complex formation on AUG68 occurred up to an IF3/30S ratio of 1 (Fig. 2, lane 3) where the 30S subunits are exclusively directed to the internal start codon. When IF3 was added in excess over ribosomes, the toeprint signal corresponding to the internal AUG68 was greatly diminished (Fig. 2, lanes 4 and 5).

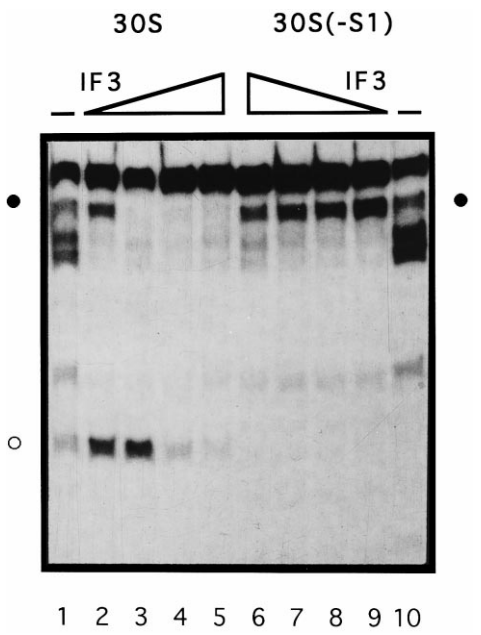

Fig. 2. S1 dependent start codon discrimination on *c*I mRNA by IF3. Lanes 1 and 10: Toeprinting with 30S and 30S(−S1) ribosomes in the absence of IFs. Lanes 2–9: The molar ratio of ribosomes/IF3 was 5:1 (lanes 2 and 9), 1:1 (lanes 3 and 8), 1:2 (lanes 4 and 7) and 1:4 (lanes 5 and 6), respectively. The toeprint signals obtained for AUG1 and AUG68 are marked by a filled and an open circle, respectively.

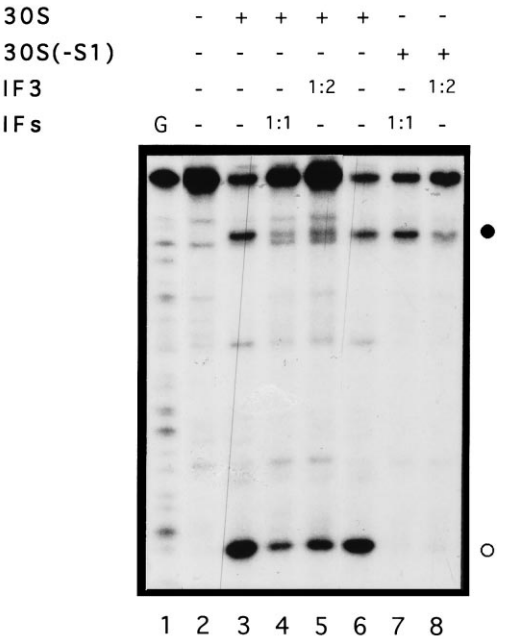

Fig. 3. S1 dependent start codon discrimination on *rro* mRNA by IFs. Lane 1: Sequencing reaction; lane 2: primer extension in the absence of ribosomes. Lanes 3 and 6: Toeprinting with 30S ribosomes in the absence of IFs. Lane 4: Toeprinting with 30S subunits in the presence of IFs (IF1–IF3), which were added in a 1:1 ratio with ribosomes. Lane 5: Toeprinting with 30S subunits in the presence of IF3, which was added in a molecular ratio of 2:1 over ribosomes. Lanes 7 and 8: Toeprinting with 30S(−S1) ribosomes in the presence of IFs (lane 7) and IF3 (lane 8), respectively. The toeprint signals obtained for AUG1 and AUG61 are marked by a filled and an open circle, respectively.

Next, we tested whether 5′-terminal start codon discrimination by IF3 with *E. coli* 30S ribosomes represents a general phenomenon independent of sequence requirements of the 5′-coding region or the source of the mRNA. We used phage r1t *rro* mRNA which is naturally translated in *L. lactis*. As shown in Fig. 3, 30S initiation complex formation at the authentic start codon (AUG1) was reduced in the presence of either all three initiation factors (lane 4) or in the presence of IF3 alone (lane 5). As well, S1 was required for ternary complex formation on the internal AUG61 codon (Fig. 3, lanes 3–6). In the absence of S1, and in the presence of IFs (Fig. 3, lane 7) or IF3 alone (Fig. 3, lane 8) a ternary complex was only observed for the 5′-terminal start codon.

3.3. IF3 dependent discrimination of 5′-terminal start codons cannot be attributed to a lack of action of IF3 on 30S(−S1) ribosomes

To further verify the effects of S1 and IF3 on 5′-terminal start codons as well as to show that IF3 functions in the

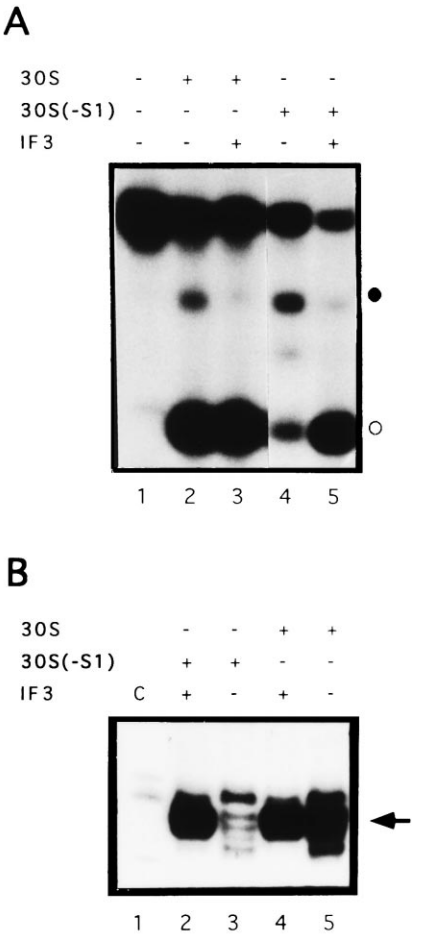

Fig. 4. Effects of ribosomal protein S1 and IF3 on ternary complex formation on *ompA*Δ117 mRNA and 'proofreading' for initiator tRNA by IF3 on 30S(−S1) ribosomes. A: Toeprint analysis performed with 30S and 30S(−S1) ribosomes in the absence or presence of IF3. Lane 1: Primer extension in the absence of ribosomes and initiator tRNA. The toeprinting signals resulting from 30S (lane 2) and 30S(−S1) (lane 4) initiation complex formation over the 5′-terminal start codon and the authentic start codon of *ompA* are indicated by a filled and an open circle, respectively. IF3 was added in a 1:1 ratio with 30S (lane 3) and 30S(−S1) (lane 5) ribosomes. B: IF3 selects for initiator tRNA on 30S(−S1) ribosomes. Lane 1: Sequencing reaction. Toeprinting on the authentic rbs of *ompA*Δ117 mRNA was performed with a tRNA mix ($tRNA_f^{Met}$; $tRNA^{Glu}$; $tRNA^{Lys}$) and 30S(−S1) (lanes 2 and 3) or 30S (lanes 4 and 5) ribosomes in the presence of IF3 (1:1 ratio with ribosomes) (lanes 2 and 4) or in the absence of IF3 (lanes 3 and 5). The arrow indicates the position of the toeprint signal obtained for the authentic start codon of *ompA*.

absence of S1, we made use of *ompA*Δ117 mRNA (Fig. 1), where the 5′-proximal *ompA* rbs is recognised by 30S(−S1) ribosomes [6], and which in addition contains a 5′-terminal AUG codon. First, we tested whether S1/IF3 affect start codon partitioning on *ompA*Δ117 mRNA in the same manner as shown above for both λ *c*I and r1t *rro* mRNA. As shown in Fig. 4A, lanes 2 and 4, in the absence of IFs a ternary complex was formed over both the 5′-terminal AUG and the authentic start codon of *ompA* with both 30S and 30S(−S1) ribosomes. 30S subunits showed a high preference for the authentic AUG start codon of *ompA*. However, in contrast to the situation observed with leaderless mRNAs, in the presence of IF3 a ternary complex was formed on the internal AUG preceded by the canonical SD of *ompA* regardless of whether 30S (Fig. 4A, lane 3) or whether 30S(−S1) ribosomes (Fig. 4A, lane 5) was used. This finding seemed to exclude the possibility that the lack of discrimination against 5′-terminal start codons by IF3 with 30S(−S1) ribosomes on leaderless mRNAs could result from either a reduced affinity or action of IF3.

To examine this further, we tested whether IF3 can select initiator $tRNA_f^{Met}$ over elongator tRNAs on 30S(−S1) translation initiation complexes at the *ompA* rbs in *ompA*Δ117 mRNA. In the absence of IF3 several toeprint signals were obtained which resulted from ternary complex formation with either 30S(−S1) (Fig. 4B, lane 3) or 30S (Fig. 4B, lane 5) ribosomes over several in and out of frame codons cognitive for the different tRNAs used. In the presence of IF3, selection for initiator tRNA occured with both 30S(−S1) (Fig. 4B, lane 2) and 30S (Fig. 4B, lane 4) ribosomes. Again, this demonstrated that ternary complex formation on 5′-terminal start codons with 30S(−S1) ribosomes in the presence of IF3 does not result from a lack of binding to or action of IF3 on 30S(−S1) subunits.

4. Discussion

We have previously shown that ribosomal protein S1 is not required for 30S initiation complex formation on leaderless mRNAs as well as on mRNAs bearing a 5′ proximal rbs when it is free of structure [6]. From these experiments we concluded that S1 is required only when the rbs contains secondary and/or tertiary structures interfering with the formation of the ternary complex. Here, we have shown that S1 mediates IF3 dependent discrimination against 5′-terminal start codons (see Figs. 2 and 3). Toeprinting [2,5,6,9] and hydroxyl radical footprinting (U. Bläsi, unpublished) studies with 30S ribosomes in the presence of initiator tRNA have confirmed that coverage of the 5′ initial coding region of leaderless mRNAs is indistinguishable from that of mRNAs containing a canonical rbs and a 5′-untranslated leader sequence. Given the suggested binding geometry of S1, the mRNA binding sites of which have been mapped upstream of the AUG [20], on leaderless mRNAs the '5′-edge' of the 30S ribosome including protein S1 would protrude into the solvent. Apparently, this creates a situation which cannot withstand the kinetic proofreading function [22,23] exerted by IF3. In contrast, in the absence of S1, IF3 does not abolish 30S initiation complex formation even when IF3 is added in excess (see Fig. 2). This effect was not observed with *ompA*Δ117 mRNA which has a short 5′-terminal extension (20 bases) upstream of the AUG initiating codon. However, even here the 30S subunit would protrude into the solvent since the mRNA region protected by 30S ribosomes from hydroxyl radical attack has been shown to extend up to −35 relative to the A of the initiation codon [24]. There are two possibilities to explain this observation. Either on *ompA*Δ117 the SD/anti-SD interaction is sufficient to withstand the proofreading function exerted by IF3 or S1 provides additional contacts with the short 5′-leader region. Since 30S binding to oligonucleotides with SD-like sequences occurred in the absence and presence of S1 and IF3 [25], respectively, we favour the first possibility.

In line with previous studies [6,20], S1 is required for ternary complex formation on internal AUG codons in both *c*I

and *rro* mRNA both of which are preceded by a putative SD sequence. However, with 30S ribosomes an excess of IF3 reduced ternary complex formation on both AUG68 of *c*I mRNA (Fig. 2, lanes 4 and 5) and AUG61 of *rro* mRNA (I. Moll, unpublished). It thus appears that on both mRNAs the internal rbs is recognised as non-standard by IF3. In the *c*I mRNA the putative SD sequence 5′-GAG-3′ (see Fig. 1) is separated by a stem loop structure from the AUG68 [13], while in *rro* mRNA the putative SD sequence 5′-GAG-3′ is separated from the AUG start codon by 12 nt, a spacing considered to be unfavourable [26]. These pecularities could account for the concentration dependent effect exerted by IF3 on formation of the ternary complex at the internal starts.

These studies with three different leaderless mRNAs indicated that the effects exerted on *E. coli* 30S initiation complex formation by S1 and/or IF3 appear to be independent of the primary sequence context of the immediate 5′-coding region of the leaderless mRNAs. The data favour a model in which productive 30S initiation complex formation on the 5′-terminal start codon of leaderless mRNAs in *E. coli* is accomplished by a sub-population of 30S ribosomes which either lack IF3 and/or S1. The determination of the stoichiometry of S1 on *E. coli* ribosomes in vivo and in vitro [27] suggested that 30S subunits devoid of S1 are present in the cell. It remains to be seen whether similar mechanisms exist in the low G/C group of Gram-positive bacteria which appear to lack a functional homolog of S1 [28].

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