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Translation initiation with 70S ribosomes: an alternative pathway for leaderless mRNAs

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ABSTRACT

It is generally accepted that translation in bacteria is initiated by 30S ribosomal subunits. In contrast, several lines of rather indirect *in vitro* evidence suggest that 70S monosomes are capable of initiating translation of leaderless mRNAs, starting with the A of the initiation codon. In this study, we demonstrate the proficiency of dedicated 70S ribosomes in *in vitro* translation of leaderless mRNAs. In support, we show that a natural leaderless mRNA can be translated with crosslinked 70S wild-type ribosomes. Moreover, we report that leaderless mRNA translation continues under conditions where the prevalence of 70S ribosomes is created *in vivo*, and where translation of bulk mRNA ceases. These studies provide *in vivo* as well as direct *in vitro* evidence for a 70S initiation pathway of a naturally occurring leaderless mRNA, and are discussed in light of their significance for bacterial growth under adverse conditions and their evolutionary implications for translation.

INTRODUCTION

According to the general textbook view, translation initiation in prokaryotes proceeds by binding of 30S ribosomes to mRNA through interactive RNA sequences, the Shine and Dalgarno (SD) sequence on mRNA and the anti-SD sequence located at the 3′end of 16S rRNA (1–3). The conversion of a binary 30S ribosome–mRNA complex into a 30S-mRNA–initiator–tRNA ternary complex, wherein the ribosome is tethered to the mRNA by both the SD–anti-SD interaction and the codon–anti-codon interaction, is further governed by three initiation factors (IF1–IF3) which control the fidelity of this first step in translation initiation, i.e. the selection of the correct start codon (4). In addition to this pathway, there is experimental evidence for a second *t*ranslation *i*nitiation *p*athway (*tip*), wherein a 30S-initiator–tRNA complex is recruited to canonical mRNAs (5,6). As recently reported by Mayer *et al.* (7), IF2 appears to act as a carrier for initiator–tRNA to the ribosome, and would thus augment the formation of the 30S-initiator–tRNA complex. Consistent with the latter *tip* is the lack of evidence that *cis* elements on leaderless mRNAs, starting directly with the 5′ terminal start codon, contribute to ribosomal recognition (8–12). Taken together with the stimulatory effect of IF2 on leaderless mRNA translation, it has been concluded that the start codon is the only constant and necessary ribosomal recognition element on leaderless mRNAs, and that start codon recognition of leaderless mRNAs requires a ribosome–initiator–tRNA complex, an intermediate equivalent to that obligatorily formed during translation initiation in eukaryotes (13,14).

Regardless of whether the 30S ribosomal subunit binds first either to mRNA or to initiator–tRNA, it is generally accepted that translation initiation in prokaryotes proceeds via an obligatory dissociation of ribosomes and only the 30S subunit is believed to associate with natural occurring mRNAs and initiator–tRNA. While there is ample evidence for transient 30S translation initiation complexes formed at the 5′ end of a mRNA (15), it is an open question whether translational re-initiation at start codons internal to a polycistronic mRNAs is brought about by non-dissociated 70S ribosomes. Typically, translation re-initiation events in bacteria occur when a downstream translational start site is in close vicinity to or overlaps with the termination codon of an upstream gene. Although a canonical SD sequence facilitates such re-initiation events, it appears not to be essential (16,17). It is unknown whether re-initiation events occur in the presence of initiation factors, especially IF3, which would restrict ribosomal P-site access to fMet-tRNA$_f^{Met}$ (3). The translational coupling of the coat and lysis gene of phage fr was shown to be dependent on the non-canonical UUG start codon of the lysis gene (18). This finding could indicate that re-initiation of the lysis gene is accomplished by 70S ribosomes on which IF3 may not exert its proofreading function for initiator-tRNA. In addition, based on studies of the *Escherichia coli lac* (19) and *str* (20) operons it has been suggested that 70S ribosomes cross the boundary between translationally coupled genes. More light on potential 70S re-initiation events has been shed through the use of *E.coli* temperature-sensitive mutants in the gene encoding the ribosome recycling factor (RRF) (21,22). RRF together with EFG is required for disassembly of the translational post-termination complex (23–25). Inactivation of the RRF protein resulted *in vitro* (26) as well as *in vivo* (21) in unscheduled translational re-initiation downstream from a termination codon. These experiments suggested that 70S ribosome are not dislodged from the mRNA upon termination, and that they are indeed capable of translation initiation at

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downstream translational starts. Moreover, inactivation of RRF *in vivo* does not affect translational coupling at the coat/lysis gene boundary of RNA phage GA, which is again indicative for 70S re-initiation events (22).

Using the primer extension inhibition assay (toeprinting; 27), Balakin *et al*. (28) have first shown, that 70S ribosomes, in contrast to 30S ribosomal subunits, show a high preference for the 5′ terminal AUG of leaderless phage λ *c*I repressor mRNA. Using the same assay, O'Donnell and Janssen (29) reported likewise for several leaderless mRNAs that 70S initiation complexes are intrinsically more stable than 30S initiation complexes. These findings are in agreement with filter binding assays performed in our laboratory (30) and by Udagawa *et al*. (31), which revealed a 5- to 10-fold higher retention of leaderless mRNAs in the presence of $tRNA_f^{Met}$ with 70S ribosomes when compared to 30S subunits. Udagawa *et al*. (31) have used the cell-free PURE translation system to scrutinize the 70S *tip* for leaderless mRNAs. Although these authors observed translation of leaderless *c*I mRNA with 70S ribosomes in the absence of initiation factors, an mRNA containing a canonical ribosome binding site was likewise translated under the same conditions. Hence, the currently available evidence for the 70S *tip* of leaderless mRNAs is either rather indirect or controversial.

In this study, we demonstrate the proficiency of stable non-dissociated 70S ribosomes in *in vitro* translation of leaderless mRNAs. Moreover, we report that leaderless mRNA translation continues under conditions where the prevalence of 70S ribosomes is mimicked *in vivo*, and where translation of bulk mRNA ceases. These studies provide definite evidence for a third *tip* in bacteria, wherein translation initiation of leaderless mRNAs is accomplished by 70S monosomes.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E.coli* strains MC1061 [F^- *araD139 Δ(ara-leu)7679 galE15 galK16 Δ(lac)X74 hsdR2 mcrA mcrB1 rpsL* (Sm^r)], LJ14 (MC1061 *frr14* (Ts) (21) and RL425 (*metB* HfrP4X λ^- *rpsB*^{ts} (32) have been described. Plasmid pRB381*c*I harbors a translational fusion in which the first 63 codons of the λ *c*I mRNA are abutted to the eighth codon of the *lacZ* gene (12).

Toeprinting

Plasmid pUH100 (33) served as a source for generating PCR templates suitable for *in vitro* transcription of *ompAΔ117* mRNA with T7 RNA polymerase. The PCR was performed with primer V8 (5′-GGGCTCTAGAGTAATACGACTCACTATAGATGATAACGAGGCGCAAAAATG-3′) containing a T7 promoter and with primer *Ava*II (33). The T7-RNA polymerase directed transcription generated a 131 nt long mRNA (Figure 1B), which was used for the toeprinting studies as specified by Hartz *et al*. (27). As described previously (34), the 5′ end-labelled primer was annealed to a region downstream of the authentic start codon of *ompAΔ117* mRNA. The molar ratio of ribosomes:tRNA:mRNA was 1:4:0.01.

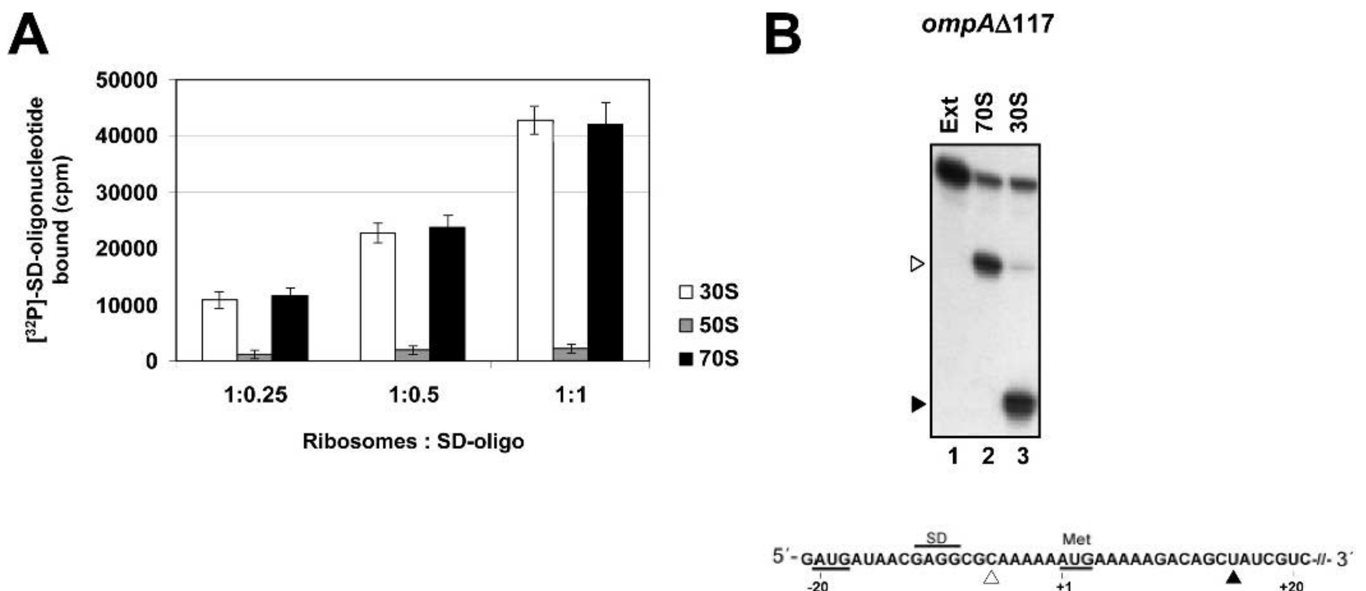

Figure 1. Lack of 70S translation initiation complex formation at an internal start codon despite the accessibility of the 70S anti-SD sequence. (**A**) Binding of the [32P]-labelled SD-oligonucleotide to 30S and 50S subunits, respectively, and to 70S ribosomes. The molar ratios between ribosomal particles or ribosomes and the SD-oligonucleotide used in the filter binding assay are indicated. Binding of the oligonucleotide to 30S subunits, 50S subunits and 70S ribosomes is represented by white, gray and black bars, respectively. The X-axis represents c.p.m. values. The experiment was performed in triplicate. The error bars represent standard deviations. (**B**) Toeprinting analysis on *ompAΔ117* mRNA with 30S subunits and 70S ribosomes. Lane 1, primer extension in the absence of ribosomes and $tRNA_f^{Met}$. Lane 2, toeprinting with 70S ribosomes in the presence of $tRNA_f^{Met}$. Lane 3, toeprinting with 30S subunits in the presence of $tRNA_f^{Met}$. The molar ratio of ribosomes:tRNA:mRNA was 1:4:0.01. The signal corresponding to translation initiation complex formation at the 5′ terminal AUG and at the downstream authentic internal start codon of *ompA* mRNA is indicated by an open and a closed arrowhead, respectively. The sequence of the 5′-proximal part and of the initial coding region of *ompAΔ117* mRNA is shown below the autoradiograph. The 5′-terminal AUG, the SD sequence as well as the corresponding downstream authentic start codon of *ompA* mRNA are indicated by bars. The arrowheads indicate the position of the toeprint signal obtained with the 5′-terminal AUG and the internal AUG.

Filter binding assays

Filter binding assays were performed using a Schleicher and Schuell SRC 072/0 Minifold II Slot Blot apparatus. The binding reactions were performed under the following conditions. 30S and 50S subunits, respectively, or 70S ribosomes were incubated with the 5′ end [^{32}P]-labelled SD-oligonucleotide 5′-UAAGGAGGUG-3′ at different molar ratios (Figure 1A) in a final volume of 100 µl 1× VD buffer (10 mM Tris–HCl, pH 7.4, 60 mM NH_4Cl, 10 mM MgOAc, 6 mM β-mercaptoethanol) at 37°C for 10 min before the mixture was added to the filtration apparatus. The nitrocellulose filter was equilibrated in 1× VD buffer. It should be noted that under these buffer conditions no dissociation of 70S ribosomes was observed (see Figure 2A). After loading of the samples under vacuum the slots were washed with 20 vol of VD buffer. After drying,

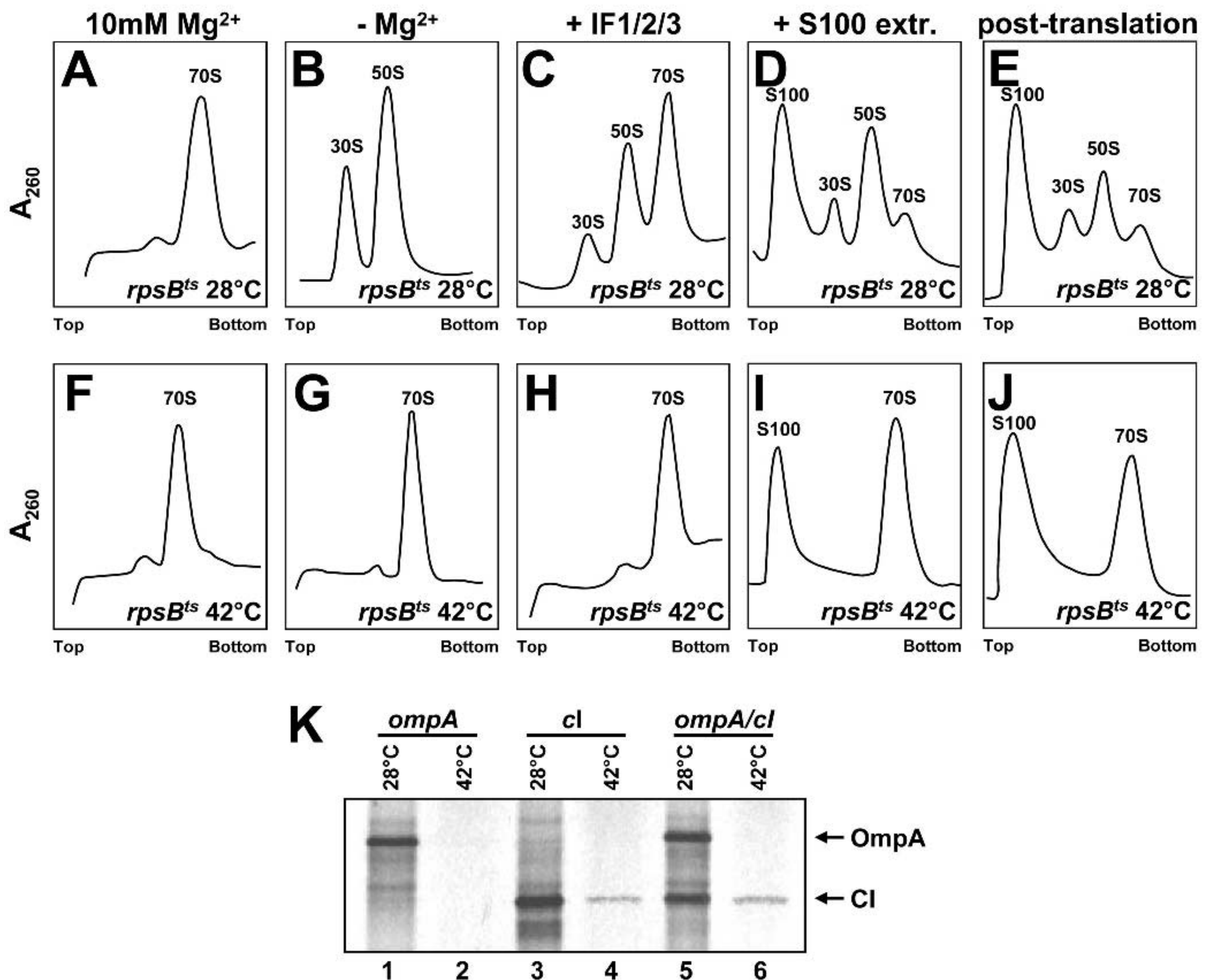

Figure 2. S1/S2-deficient 70S ribosomes derived from *E.coli* strain RL425(*rpsB*^{ts}) at the non-permissive temperature are stable and proficient in translation of leaderless λ *c*I mRNA. (**A** and **F**) Sedimentation profile (10–30% sucrose gradient) in the presence of 10 mM Mg^{2+} (1× VD buffer) of purified 70S ribosomes from strain RL425 grown at 28 and 42°C, respectively. (**B** and **G**) Sedimentation profile (10–30% sucrose gradient) in the absence of Mg^{2+} of purified 70S ribosomes from strain RL425 grown at 28 and 42°C, respectively, upon dialysis in 1× VD buffer without Mg^{2+}. (**C** and **H**) Sedimentation profile (10–30% sucrose gradient) in the presence of 10 mM Mg^{2+} (1× VD buffer) of purified 70S ribosomes from strain RL425 grown at 28 and 42°C, respectively, upon treatment with a 2-fold molar excess of IFs. (**D** and **I**) Sedimentation profile (10–30% sucrose gradient) in the presence of 10 mM Mg^{2+} (1× VD buffer) of purified 70S ribosomes from strain RL425 grown at 28 and 42°C, respectively, upon incubation with an S100 extract. (**E** and **J**) As described in Materials and methods, the ribosome sedimentation profiles were determined 40 min upon *in vitro* translation of *c*I mRNA with 70S(28°C) ribosomes (E) and with 70S(42°C) ribosomes (**J**), respectively. The gradients were centrifuged for 75 min at 195 000 *g* in a Beckman SW 50.1 rotor and analyzed by measuring the A_{260} using an ISCO UA-6 spectrophotometer. The peaks corresponding to 30S, 50S, 70S particles and to the S100 extract are indicated. (**K**) *in vitro* translation of *ompA* (lanes 1, 2, 5 and 6) and *c*I mRNA (lanes 3, 4, 5 and 6) using purified 70S ribosomes from strain RL425 grown at 28°C (lane 1, 3 and 5) and purified 70S(42°C) ribosomes from strain RL425 upon shift for 40 min to 42°C (lanes 2, 4 and 6). 15 µl of Mix A (see Materials and Methods) were mixed with 5 µl S100 extract and 5 pmol 70S ribosomes were added. The reactions were started by addition of 5 pmol of *ompA* mRNA (lanes 1 and 2), *c*I mRNA (lanes 3 and 4), or 5 pmol of both, *c*I and *ompA*, mRNA (lanes 5 and 6). The reaction mixtures were incubated at 28°C for 45 min. The proteins were separated on a 12.5% SDS–polyacrylamide gel and the labelled proteins were visualized using a PhosphorImager. The positions of the CI and OmpA proteins in the autoradiogram are indicated by arrows.

the filter was exposed to a Molecular Dynamics PhosphorImager screen for quantification of the radioactivity bound to the ribosomes.

Preparation of ribosomes and ribosome profiles

Wild-type 30S and 70S ribosomes devoid of initiation factors were prepared from *E.coli* strain MRE600 essentially as described (35). The ribosomes from strain RL425 were prepared likewise. To test the stability of 70S ribosomes prepared from strain RL425 they were (i) either dialyzed against 1× VD buffer in the absence of MgOAc, or (ii) incubated for 30 min at 37°C in 1× VD buffer containing 10 mM Mg^{2+} in the presence of a 2-fold molar excess of all three IFs or (iii) in the presence of S100 extract in the same concentration as used in the *in vitro* translation assays, respectively, and then analyzed on a 10–30% sucrose gradient prepared in 1× VD buffer containing no Mg^{2+} (i, Figure 2B and G) and containing 10 mM Mg^{2+} (ii, Figure 2C and H; iii, Figure 2D and I), respectively. The gradients were centrifuged for 75 min at 195 000 *g* in a Beckman SW 50.1 rotor and analyzed by measuring the A_{260} using an ISCO UA-6 spectrophotometer. The ribosomal profiles upon *in vitro* translation with 70S(28°C) and 70S(42°C) ribosomes derived from strain RL425 were determined as described below.

The ribosomal profiles of strain MC1061 and LJ14 were basically determined as described by Flessel *et al*. (36). At an OD_{600} of 1, a 25 ml sample of the respective culture was withdrawn and chloramphenicol (Cam) was added to a final concentration of 100 µg/ml. Then, the culture was chilled on ice, and the cells were collected by centrifugation at 5000 *g* for 5 min. The pellet was resuspended in 4.5 ml of buffer 1 (500 mM sucrose, 100 mM Tris pH 8, 100 mM NaCl, 100 µM Cam), and upon addition of EDTA and lysozyme to a final concentration of 10 mM and 100 µg/ml, respectively, the cells were incubated for 10 min on ice. Subsequently, 10 mM $MgSO_4$ was added and centrifugation at 5000 *g* was performed for 5 min. The pellet was resuspended in 450 µl of lysis buffer (10 mM Tris pH 7.6, 10 mM $MgSO_4$, 50 mM NH_4Cl, 1 mM DTT, 50 µg/ml DNase I, 100 µg/ml Cam), and the cells were disrupted by adding 25 µl of 5% Brij58. Cell debris was removed by centrifugation for 10 min at 10 000 *g*. The supernatant was layered onto a 10–30% sucrose gradient prepared in 10 mM Tris pH 7.6, 10 mM $MgSO_4$, 50 mM NH_4Cl and 1 mM DTT.

Crosslinking of 70S ribosomes

The 70S ribosomes prepared from strain MRE600 were dialyzed against a buffer containing 20 mM Bicine, 10 mM $MgCl_2$, 10 mM KCl, 2 mM β-mercaptoethanol (pH 9) (37). Solid dimethylsuberimidate (Me_2Sub) was neutralized with an equivalent amount of 1 N KOH, and the ribosomes were added immediately. The final Me_2Sub concentration was 10 mM. Crosslinking was performed at 4°C for 6 h and stopped by the addition of 0.1 vol of 1 M Tris pH 6.7. The ribosomes were recovered by centrifugation in a TLA100.3 rotor at 39 000 r.p.m. for 20 h and resuspended in 1× VD buffer. The crosslinking of the ribosomes was verified by centrifugation on a 10–30% sucrose gradient prepared in 1× VD buffer in the presence of 1 mM MgOAc (Figure 3A). The *c*ross*l*inked 70S *r*ibosomes (70SCLR) were purified by collecting the respective fractions of these gradients. The purity and stability of the 70SCLR were tested upon dialysis of the ribosomes against 1× VD buffer lacking MgOAc, and then analyzed on a 10–30% sucrose gradient prepared in 1× VD buffer in the absence of Mg^{2+} (Figure 3B). In addition, the 70SCLR were incubated for 30 min at 37°C in 1× VD buffer in the presence of S100 extract in the same concentration as used in the *in vitro* translation assays. Upon this treatment the 70SCLR were analyzed on a 10–30% sucrose gradient prepared in 1× VD buffer (Figure 3C). The presence of ribosomal proteins S1 and S2 on 70SCLR ribosomes was determined by applying 5 pmol of either purified 70S wild type or 70SCLR (Figure 3B) to a Schleicher and Schuell SRC 072/0 Minifold II Slot Blot apparatus. Ribosomal proteins S1 and S2 were detected with anti-S1 and anti-S2 antibodies as described previously (38).

Monitoring CI-LacZ synthesis

E.coli strains MC1061 and LJ14 harboring plasmid pRB381*c*I were grown in M9 minimal medium at 28°C. At an OD_{600} of 0.5, the cultures were divided. One half was shifted to 42°C, whereas the other was further incubated at 28°C. At 30 min after the shift 100 µl aliquots were withdrawn. Pulse labelling was carried out by addition of 1 µl of [^{35}S]-methionine (10 mCi/ml), and by further incubation for 1 min at 37°C. The reactions were stopped by addition of an equal volume of 10% TCA followed by centrifugation. The cell pellets were washed once with 90% acetone, dried under vacuum for 5 min, resuspended in 25 µl Laemmli buffer (39), and boiled for 3 min prior to loading onto a 10% SDS–polyacrylamide gel. For the different OD_{600} values the same amount of total cellular protein was separated. The gels were dried and exposed to an X-ray film.

In vitro translation assays

The *in vitro* translation of *c*I and *ompA* mRNAs with *E.coli* S100 extracts was essentially performed as described (13). To 15 µl of Mix A (16.6 mM MgOAc, 80 mM NH_4Cl, 30 mM Tris–HCl pH 7.7, 3.3 mM DTT, 1.6 mg/ml of *E.coli* tRNA, 0.2 mM citrovorum, 16.6 mM KCl, 0.33 mM amino acids (-lys), 66.6 µM [^{14}C]-lys, 3.3 mM ATP, 0.66 mM GTP, 16.6 mM phosphoenolpyruvate and 0.04 mg/ml of pyruvate kinase) either 5 µl S30 extract or 5 µl S100 extract and 5 pmol 70S ribosomes were added. The reactions were started by addition of 5 pmol of mRNA and incubated at the temperatures specified in the figure legends. The reactions were stopped after 45 min by addition of Laemmli buffer (39), and the labelled proteins were separated on 12.5% SDS–polyacrylamide gels. The gels were dried under vacuum and exposed to a Molecular Dynamics PhosphorImager screen for quantification. To test whether 70S monosomes persist upon *in vitro* translation with 70S(42°C) ribosomes derived from strain RL425, the *in vitro* translation assay corresponding to Figure 2K, lane 4, was 10-fold scaled up. After 40 min, 10% of the translation mix was resolved on a 12.5% SDS–polyacrylamide gel to confirm translation of *c*I mRNA. At the same time, 90% of the translation mix was layered on a 10–30% sucrose gradient prepared in 1× VD buffer, and the ribosome profile (see Figure 2J) was analyzed as described above. As a control, the profile of the 70S(28°C) ribosomes

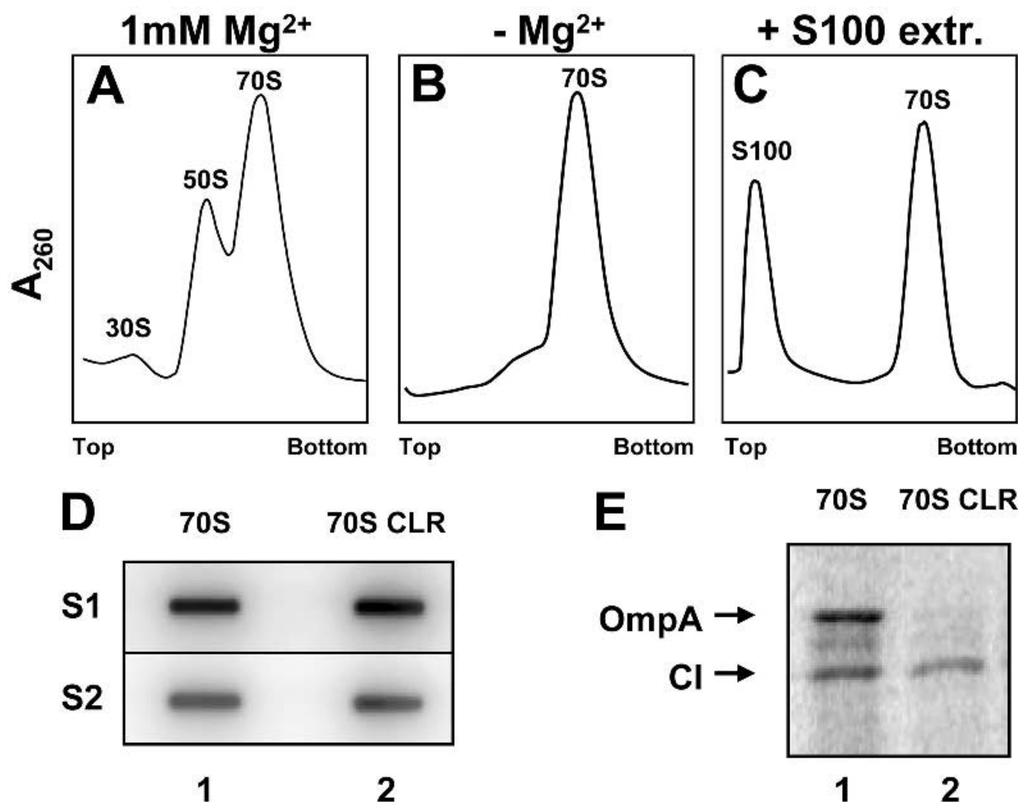

Figure 3. *In vitro* translation of the leaderless *c*I mRNA and *ompA* mRNA with crosslinked 70S ribosomes. (**A**) Sucrose gradient profiles (10–30%) of 70S ribosomes upon crosslinking with Me_2Sub in the presence of 1 mM Mg^{2+}. Peaks representing 30S, 50S and 70S particles are indicated. (**B**) The crosslinked 70S ribosomes were purified from the sucrose gradient shown in panel A, and tested for their stability on a second sucrose gradient in the absence of Mg^{2+} upon dialysis against 1× VD buffer lacking MgOAc. (**C**) The purified crosslinked 70S ribosomes were incubated with S100 extract in the same concentration as used for *in vitro* translation and their sedimentation profile was then analyzed in the presence of 10 mM Mg^{2+}. (**D**) Ribosomal proteins S1 and S2 are retained on crosslinked 70S ribosomes (70SCLR). As described in Materials and Methods, 5 pmol of either 70S wild-type ribosomes or 70S(CLR) ribosomes were immobilized on a nitrocellulose filter, which was then probed with anti-S1 and anti-S2 antibodies, respectively. (**E**) Concomitant *in vitro* translation of *ompA* and *c*I mRNA with wild-type 70S ribosomes (lane 1) and with 70SCLR (lane 2). To 15 µl of Mix A, 5 pmol of either 70S ribosomes or 70SCLR, respectively, and 5 µl of S100 extract were added. The reactions were started by the addition of 5 pmol of each mRNA and incubated at 30°C for 45 min. The proteins were separated on a 12.5% SDS–polyacrylamide gel and the labelled proteins were visualized using a PhosphorImager. The positions of the CI and OmpA proteins in the autoradiograph are indicated by arrows.

used in the *in vitro* translation assay corresponding to Figure 2K, lane 3, was likewise determined upon *in vitro* translation (see Figure 2E).

RESULTS

70S initiation complex formation occurs exclusively at 5′-terminal start codons

Balakin *et al*. (28) have shown that 70S ribosomes form a translation initiation complex at the 5′ terminal start codon of leaderless λ *c*I mRNA, whereas a 70S initiation complex was not formed at an internal start codon spanning nucleotides 68–70. Since AUG_{68-70} of λ *c*I mRNA does not represent a canonical translational start (40), we have previously tested whether 70S translation initiation complex formation occurs exclusively at 5′ terminal AUGs or whether 70S initiation also applies to internal translational starts. Under conditions where 70S ribosomes do not dissociate, they failed to form an initiation complex at the canonical *r*ibosome *b*inding *s*ite (*rbs*) of *E.coli ompA* mRNA and on phage T4 gene *32* mRNA (41).

Balakin *et al*. (28) have suggested that the anti-SD is not accessible in 70S ribosomes, and therefore not available for the SD–anti-SD interaction. We therefore first examined whether the inaccessibility of the anti-SD in 70S ribosomes could account for the failure of 70S particles to form a translation initiation complex at an internal *rbs*. As shown in Figure 1A, the RNA SD-oligonucleotide 5′-UAAGGAGGUG-3′ complementary to the anti-SD bound almost equally well to 30S and 70S ribosomes, whereas only negligible binding to the 50S subunit was observed. It should be noted that the 70S ribosomes were stable under these assay conditions and did not to dissociate into their subunits (see Figure 2A). Thus, the lack of translation initiation complex formation at an internal *rbs* by 70S ribosomes cannot be attributed to the inaccessibility of the anti-SD in these particles. Rather, 70S initiation appeared to require a 5′ proximal position of the start codon.

To address this question, we studied 70S initiation complex formation on *ompA*Δ*117* mRNA (Figure 1B), which contains a 5′ terminal AUG codon, and downstream the canonical *rbs* of *ompA* mRNA. This model mRNA is suited to analyze conditions which affect either 5′ terminal or internal translation initiation complex formation since the ribosomal choice of

either AUG start codon is mutually exclusive (13,42). As shown in Figure 1B, lane 2, 70S ribosomes formed a translation initiation complex only at the 5′ terminal AUG, whereas 30S subunits generated a ternary complex almost exclusively at the internal canonical *rbs* (Figure 1B, lane 3). Thus, 70S ribosomes preferentially form a translation initiation complex at 5′ terminal starts which might be attributed to topological constraints of the 70S mRNA track rather than to an inaccessibility of their anti-SD.

Selective translation of a leaderless mRNA by particularly stable 70S monosomes derived from an *E.coli rpsB* mutant

We have recently shown that a leaderless *c*I-*lacZ* mRNA was selectively translated at the non-permissive temperature in the *E.coli rpsB*^{ts} strain RL425, which carries a temperature sensitive mutation in the gene encoding ribosomal protein S2 (32). At the elevated temperature the RL425 ribosomes were found to be deficient in both, ribosomal proteins S1 and S2 (38). During a further analysis of these S1/S2-deficient 70S particles, we noticed that they were particularly stable. As shown in Figure 2, in contrast to RL425-70S ribosomes isolated at 28°C [70S(28°C)] (Figure 2A–E), the 70S particles isolated at 42°C [70S(42°C)] (Figure 2F–J) did not dissociate in the absence of Mg^{2+} cations (Figure 2G). Moreover, the addition of all three initiation factors IF1, IF2 and IF3 in a 2-fold molar excess over 70S(42°C) ribosomes did likewise not result in dissociation (Figure 2H), whereas the control 70S(28°C) ribosomes dissociated substantially in the presence of the factors (Figure 2C).

Studying translation initiation by 70S ribosomes *in vitro* is complicated by the fact that cellular extracts are used, which contain initiation factors. Upon addition of 70S ribosomes to an *E.coli* S100 translation mix, the majority of the 70S ribosomes most likely dissociates, and then translation initiation commences with 30S subunits (Figure 2D; 43). Given the results shown in Figure 2F–H, we inticipated that the presence of initiation factors in S100 extracts would not lead to a dissociation of the 70S(42°C) monosomes. To test this, both the 70S(28°C) and 70S(42°C) ribosomes were incubated with the S100 extract for 40 min. The subsequent sedimentation profile revealed that a substantial amount of the 70S(28°C) ribosomes had dissociated after incubation with the S100 extract (Figure 2D), whereas the mutant 70S(42°C) ribosomes were retained quantitatively as 70S tight couples (Figure 2I). To test whether the 70S(42°C) ribosomes can accomplish translation initiation of a leaderless mRNA but not that of an mRNA with an internal canonical *rbs*, *in vitro* translation extracts were used, which contained either 70S(28°C) or 70S(42°C) ribosomes. Both extracts were programmed with *E.coli ompA* mRNA, the leaderless *c*I mRNA or both, and the *in vitro* translation reactions were carried out for 40 min at 28°C. As mentioned above, 70S ribosomes did not form a translation initiation complex on *ompA* mRNA *in vitro* (41). Consistent with this observation, 70S(42°C) ribosomes failed to translate *ompA* mRNA (Figure 2K, lanes 2 and 6). However, these 70S(42°C) monosomes translated the leaderless *c*I mRNA (Figure 2K, lanes 4 and 6). To test whether the 70S(42°C) ribosomes still persisted after translation, ribosome sedimentation profiles were determined upon *in vitro* translation of *c*I mRNA with 70S(28°C) ribosomes (Figure 2E) and with 70S(42°C) ribosomes (Figure 2J), respectively. As shown in Figure 2J, the 70S(42°C) ribosomes did not dissociate after the translation reaction. Taken together, the selective translation of a leaderless mRNA *in vivo* in strain RL425 at 42°C (38) and the observed *in vitro* translation of *c*I mRNA by the 70S(42°C) tight couples supported the hypothesis that translation initiation of leaderless mRNAs can be accomplished by non-dissociated 70S ribosomes.

In vitro translation of leaderless mRNAs by crosslinked 70S ribosomes

In contrast to leaderless mRNA (38), translation of canonical mRNA in *E.coli* requires ribosomal protein S1 (34,44). Thus, the lack of translation of the canonical *ompA* mRNA by 70S(42°C) tight couples (Figure 2K) cannot be solely attributed to their particular stability but also to the absence of the ribosomal proteins S1 and S2. Therefore, we sought for a means to verify the data shown in Figure 2K with ribosomes containing the full complement of ribosomal proteins. More than 30 years ago, Slobin (37) has crosslinked the ribosomal subunits with dimethylsuberimidate to test whether a 30S-$tRNA_f^{Met}$–mRNA complex is an obligatory intermediate in protein synthesis. These crosslinked 70S ribosomes were functional in *in vitro* translation of poly(U), whereas they were incompetent in phage f2 mRNA translation. Therefore, the author concluded, that dissociation of the ribosome into subunits is mandatory for initiation of protein synthesis on natural mRNAs. Given that poly(U) resembles leaderless mRNAs in that it lacks ribosomal recruitment signals and has a 5′ terminal start codon, we tested whether 70SCLR are likewise functional in translation of the natural leaderless *c*I mRNA. Upon crosslinking of the subunits, ~70% of the ribosomes were found to be covalently joined and did not dissociate into their subunits in the presence of 1 mM Mg^{2+} (Figure 3A). These 70SCLR were purified by sucrose gradient centrifugation and tested for their stability in the absence of Mg^{2+} cations as well as in the presence of an S100 extract. As shown in Figure 3B and C, the 70SCLR monosomes remained stable under both conditions. In addition, the 70SCLR contained both ribosomal proteins, S1 and S2 (Figure 3D). When 70SCLR were added to the *in vitro* translation assay, only the leaderless *c*I mRNA was translated, whereas the 70SCLR failed to translate *ompA* mRNA (Figure 3E, lane 2). This result clearly demonstrated the proficiency of non-dissociated 70S ribosomes to translate leaderless mRNA.

Preferential translation of a leaderless mRNA in an *E.coli frr*^{ts} mutant *in vivo* correlates with the prevalence of 70S ribosomes

The ribosome recycling factor in concert with elongation factor G (EF-G) and GTP is known to catalyze the fourth step in protein synthesis, namely the disassembly of the post-termination complex of ribosome, mRNA and tRNA in order to supply free ribosomal subunits for the next cycle of translation (23,24,45,46). Upon inactivation of RRF at 42°C in the *E.coli frr*^{ts} mutant LJ14, the ribosome remained on the mRNA after termination of translation and initiated random translation downstream of the stop codon (21). In other experiments it was observed that under these conditions ribosomes are released from the 3′end of mRNA as 70S monosomes (G. Hirokawa and A. Kaji, unpublished work). We therefore

reasoned that this would create a situation where wild-type 70S ribosomes are prevalent and would give us the opportunity to examine whether this correlates with a preferential translation of a leaderless mRNA when compared to canonical transcripts.

To test this idea, the ribosomal profile of the *frr*^{ts} mutant strain LJ14 was first analyzed under permissive and non-permissive conditions. Even at the permissive temperature (28°C) strain LJ14 contained higher levels of 70S monosomes than the isogenic wild-type strain MC1061 at either 28 or 42°C (Figure 4A and B). Upon shift of strain LJ14 to the non-permissive temperature the peaks corresponding to the 30S and 50S subunits were nearly absent and 70S ribosomes were predominant in the cell (Figure 4C). Next, we assessed whether the translation yield of a leaderless mRNA correlated with the determined high levels of 70S monosomes in strain LJ14. The strains MC1061(*frr*$^{+}$) and LJ14 (*frr*^{ts}) were transformed with plasmid pRB381*c*I harboring a chimeric *c*I-*lacZ* gene. The *c*I-*lacZ* gene, which starts with the A of the initiating codon of the *c*I gene (12) was used for the ease of identification of the encoded fusion protein upon separation of total cellular proteins in SDS–polyacrylamide gels. Both *E.coli* strains were grown at 28°C in minimal medium and then either kept at 28°C or shifted to 42°C for 40 min. The cells were then pulse labelled for 1 min with [^{35}S]-methionine and total proteins of equal amounts of cells were separated on a 12% SDS–polyacrylamide gel. As shown in Figure 4D, even at 28°C, the relative translation of the leaderless *c*I-*lacZ* gene was approximately 3-fold higher in strain LJ14 when compared to the wild-type strain MC1061 (Figure 4D, lanes 1 and 2). Upon temperature shift of strain LJ14 to 42°C translation of bulk mRNA was greatly diminished, whereas translation of the leaderless mRNA was hardly affected when compared to that obtained at the same temperature in the isogenic wild-type strain (Figure 4D, lanes 3 and 4). Thus, conditions that created the prevalence of 70S ribosomes *in vivo* clearly correlated with a preferential translation of the leaderless mRNA.

Finally, we performed *in vitro* translation assays with leaderless *c*I mRNA and *ompA* mRNA using an S30 extract prepared from the *frr*^{ts} mutant strain LJ14 at 28°C and 42°C, respectively. The *in vitro* translation assays were performed at 28°C and programmed with equal molar amounts of the leaderless *c*I mRNA and the canonical *ompA* mRNA, respectively. As shown in Figure 4E, lanes 4 and 5, the leaderless *c*I mRNA was translated with comparable efficiency with both S30 extracts. However, translation of *ompA* mRNA was

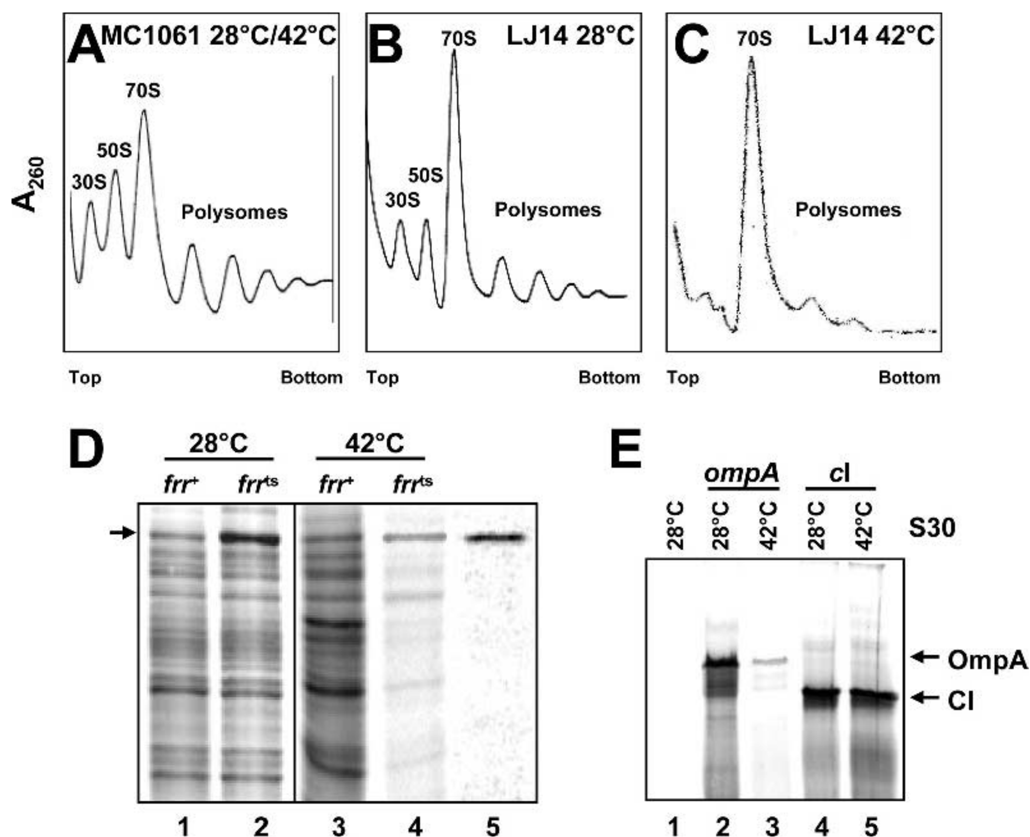

Figure 4. Preferential translation of the leaderless *c*I-*lacZ* mRNA in the *frr*^{ts} strain LJ14. (**A**) The sedimentation profiles (10–30% sucrose gradient) of ribosomes from strain MC1061 (*frr*$^{+}$) grown at 28°C and 42°C, respectively, were found to be identical. (**B** and **C**) Sedimentation profiles of ribosomes from strain LJ14 (*frr*^{ts}) grown at 28°C (B) and 42°C (C), respectively. The peaks representing 30S, 50S and 70S particles and polysome fractions are indicated. (**D**) *De novo* synthesis of the leaderless CIΦlacZ fusion protein in strain MC1061 (lanes 1 and 3) and LJ14 (lanes 2 and 4) at 28 and 42°C, respectively. Lane 5, CIΦLacZ protein synthesized *in vitro* using a S30 extract. The arrow shows the position of the CIΦlacZ protein in the autoradiogram of the 12.5% SDS–polyacrylamide gel. (**E**) *In vitro* translation with an S30 extract prepared from strain LJ14 at 28°C (lanes 1, 2 and 4) or at 42°C (lane 3 and 5) programmed with equimolar concentrations of either *ompA* mRNA (lane 2 and 3) or *cI* mRNA (lane 4 and 5). Lane 1, no mRNA was added to the *in vitro* translation assay (control). The translation reaction was performed for 45 min at 28°C, and the synthesized proteins were separated on a 12.5% SDS–polyacrylamide gel. The positions of the CI and OmpA proteins in the autoradiograph are indicated by arrows.

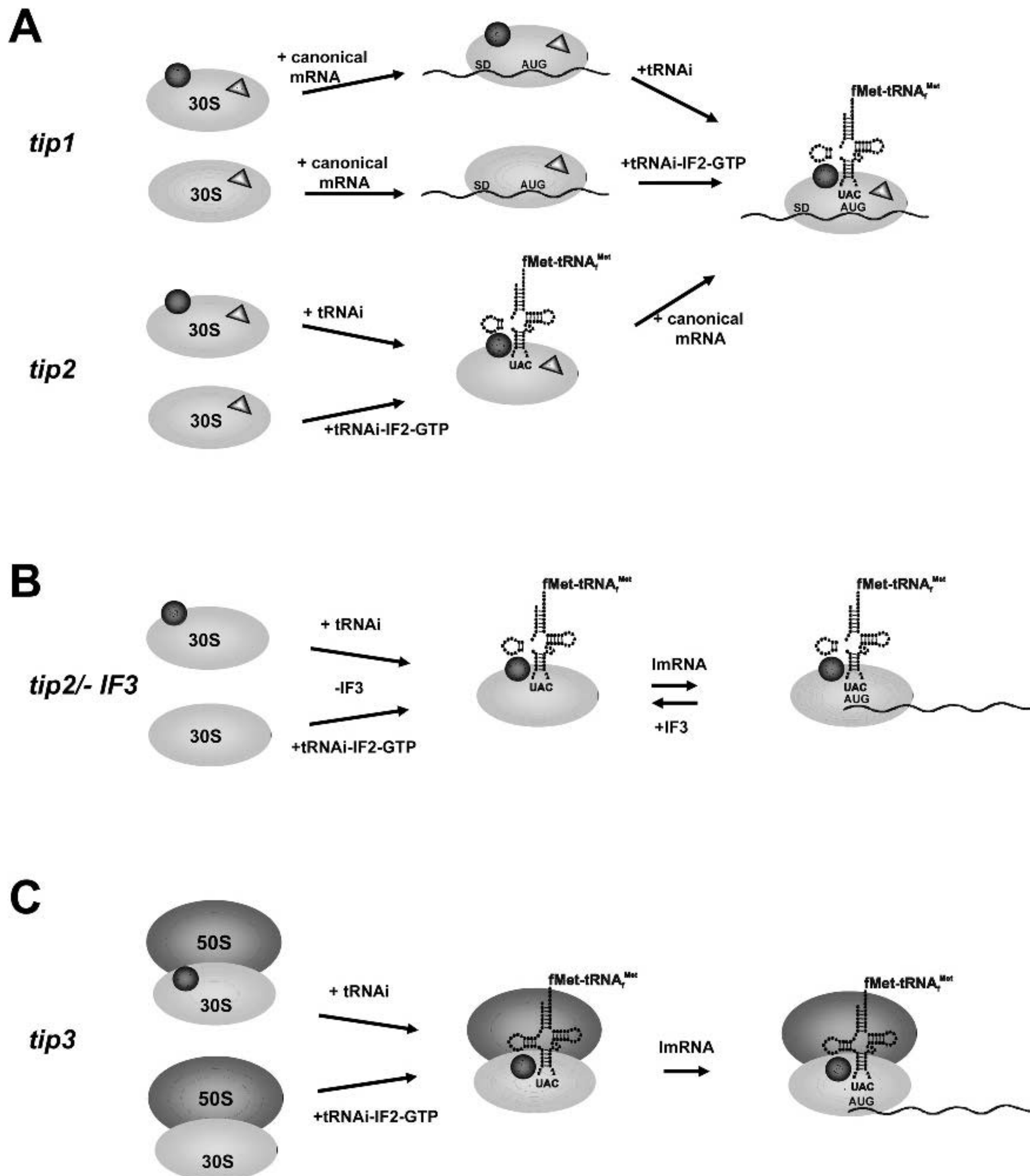

Figure 5. Translation initiation pathways for canonical and leaderless mRNAs in bacteria. (**A**) Recruitment of a 30S subunit through the SD–anti-SD interaction in the absence of P-site bound fMet-tRNA$_f^{Met}$ (RNAi) (*tip1*) and through a 30S-RNAi–IF2 complex (*tip2*) by a canonical prokaryotic mRNA, respectively. The translation initiation factors IF2 and IF3 are symbolized by a circle and by a triangle, respectively. IF1 is omitted. (**B**) Recruitment of a 30S-tRNAi–IF2 complex to a 5′ terminal start codon of a leaderless mRNA (lm RNA) in the absence of IF3 (*tip2*/-IF3) and rRNA–mRNA interactions (12). Since the mRNA and the 30S subunit are tethered solely by the start codon–anti-codon interaction, the conformational dynamics imposed by IF3 (58) is believed to destabilize the ternary complex. (**C**) Translation initiation of a leaderless mRNA by a 70S-tRNAi–IF2 complex (*tip*3). In either pathway (*tip1*, *tip2*, *tip2*/-IF3 and *tip3*), IF2 and RNAi may either bind independently to the 30S subunit (4) and to the 70S ribosome, respectively, or the tRNAi–IF2–GTP ternary complex may bind directly to the ribosome (7).

drastically reduced with the S30 extract prepared at 42°C when compared with the S30 extract prepared from cells at 28°C (Figure 4E, lanes 2 and 3).

DISCUSSION

During logarithmic growth of *E.coli*, leaderless mRNAs are poorly translated (13). Given that under these physiological conditions 70S ribosomes dissociate after disassembly from mRNA (25), leaderless mRNAs must most likely compete with canonical mRNAs for 30S subunits. For the following reasons leaderless mRNAs are poor substrates for 30S subunits. So far, no ribosomal recruitment signals other than the start codon have been identified on leaderless mRNAs (14). Instrumental for ribosome recruitment to these mRNAs is the interaction of the start codon with the anticodon of initiator tRNA bound to the 30S subunit (13). The stronger tethering of initiator tRNA to 70S ribosomes when compared to 30S

subunits (47) can therefore readily explain the intrinsically low stability of 30S ternary complexes on 5′ terminal AUGs as well as the preference of 30S subunits for a canonical *rbs* (see Figure 1B; 13,28,29). A number of studies have shown that IF3 discriminates (28,29,40) against 30S initiation complex formation on leaderless mRNAs *in vitro*, and that their translational efficiency is negatively correlated with the concentration of IF3 *in vivo* (40,48,49). These observations cumulated in the hypothesis that translation initiation of leaderless mRNA with 30S subunits can only be accomplished by a 30S-tRNAi-IF2 complex in the absence of IF3 (14,29,49; see Figure 5B).

In spite of the prevailing view that *de novo* translation initiation events in bacteria obligatorily require free 30S ribosomes (see Figure 5A), more than a decade ago Balakin *et al*. (28) have put forward the idea that translation initiation of leaderless mRNA commences with 70S monosomes. In this study, we have made use of stable S1/S2-deficient 70S ribosomes and of crosslinked 70S ribosomes, and have shown that these ribosomes do not dissociate under the *in vitro* conditions used for translation (Figure 2I and J, and Figure 3C). Translation of *c*I mRNA by these 70S ribosomes demonstrated for the first time the proficiency of intact 70S monosomes to initiate translation of a naturally occurring leaderless mRNA. In contrast, the failure of these ribosomes to translate the canonical *ompA* mRNA corroborated the *in vitro* toeprinting results in that 70S initiation requires a 5′ terminal start codon (Figure 1B). The ability of 70S ribosomes to form initiation complexes at 5′ terminal start codons but not at internal initiation sites might be explained by topological constraints of the mRNA track which is situated at the subunit interface. From the crystal structure of the ribosome (50,51) it appears that entry of the mRNA into the template channel of the 70S monomers requires its 5′ end to be pulled through the channel between both subunits. Thus, it seems conceivable that a 5′ terminal AUG can find its way through the tunnel until it is locked in the ribosomal P-site. The same may be less probable for an internal AUG preceded by RNA sequences that can form secondary structures. Further support for this idea comes from studies showing that the 5′ proximal position of the start codon is important for the *in vivo* translational efficiency of leaderless mRNAs (52).

By viewing poly(U) as a quasi-leaderless mRNA these results can now explain why Slobin (37) observed poly(U) translation but not phage f2 mRNA translation with crosslinked 70S ribosomes, the latter of which comprises only internal ribosome binding sites. Likewise, we have observed poly(U) translation with the S30 extract derived from the frr^{ts} strain LJ14 grown at 42°C, when 70S ribosomes are predominant (Figure 4C), whereas translation of phage MS2 RNA was hardly discernable (A. Kaji, unpublished work). It seems therefore conceivable that earlier in evolution a stable proto-ribosome-tRNA complex simply initiated translation at the 5′ terminal codon of single-stranded RNA polynucleotides, and that the contemporary leaderless mRNAs represent remnants of ancestral RNAs which are nowadays equipped with a canonical start codon.

Translation of the leaderless *c*I-*lacZ* mRNA was observed in strain LJ14 at the non-permissive temperature (Figure 4D), which provides the first *in vivo* correlation between preferential translation of a leaderless mRNA and the prevalence of 70S ribosomes. 70S monosomes are known to be prevalent under adverse conditions, like carbon source downshifts, stationary phase and slow growth, and during cold adaptation (53–55). As shown in Figure 5B and C, the two *tips* with IF3-deficient 30S subunits and with intact 70S ribosomes would ensure a steady translation of leaderless mRNAs under normal physiological conditions as well as at the extremes of physiology. In this way, a constant translation of the leaderless λ *c*I as well as of the Tn*1721 tetR* (56) repressor mRNAs would, for instance, ensure silencing of both accessory genetic elements. Several leaderless mRNAs from *Streptomyces* species encode proteins that confer resistance to antibiotics, which, although structurally diverse, have the ribosome as a common target (57). Being leaderless could therefore provide a means for translation of these mRNAs to counteract the respective antibiotic under a variety of physiological conditions, including those where 70S ribosomes are prevalent.

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