70S-scanning initiation is a novel and frequent initiation mode of ribosomal translation in bacteria

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According to the standard model of bacterial translation initiation, the small ribosomal 30S subunit binds to the initiation site of an mRNA with the help of three initiation factors (IF1–IF3). Here, we describe a novel type of initiation termed "70S-scanning initiation," where the 70S ribosome does not necessarily dissociate after translation of a cistron, but rather scans to the initiation site of the downstream cistron. We detailed the mechanism of 70S-scanning initiation by designing unique monocistronic and polycistronic mRNAs harboring translation reporters, and by reconstituting systems to characterize each distinct mode of initiation. Results show that 70S scanning is triggered by fMet-tRNA and does not require energy; the Shine–Dalgarno sequence is an essential recognition element of the initiation site. IF1 and IF3 requirements for the various initiation modes were assessed by the formation of productive initiation complexes leading to synthesis of active proteins. IF3 is essential and IF1 is highly stimulating for the 70S-scanning mode. The task of IF1 appears to be the prevention of untimely interference by ternary aminoacyl (aa)-tRNA•elongation factor thermo unstable (EF-Tu)•GTP complexes. Evidence indicates that at least 50% of bacterial initiation events use the 70S-scanning mode, underscoring the relative importance of this translation initiation mechanism.

protein synthesis | ribosomal functions | translational initiation | 30S-binding initiation | 70S-scanning initiation

It is textbook knowledge that 30S subunits initiate protein synthesis in bacteria; they recognize the initiation site of the mRNA composed of the Shine–Dalgarno (SD) sequence, the AUG codon, and fMet-tRNA, together with three initiation factors (IFs) forming the 30S initiation complex (30SIC). Association of the large 50S subunit triggers the release of the IFs, leading to the 70S initiation complex (70SIC) that enters the elongation phase of translation (reviewed in 1). We term this initiation path the "30S-binding mode" of bacterial initiation. After elongation and termination, it is thought that the ribosome dissociates into its subunits, thus providing 30S subunits for the next round of initiation.

The functional role of IF2 is well defined. It can bind directly to the 30S, providing a docking site for fMet-tRNA (2), but it can also enter the 30S subunit as ternary complex fMet-tRNA•IF2•GTP (3). Both IF2 and IF3 are essential for viability. IF3 has a binding site at the 30S interface (4), which explains its antiassociation effect (5, 6), as well as its role in dissociation of the terminating 70S ribosome (7). However, the in vivo concentration of IF3 is 100-fold less (8) than required for full dissociation of 70S in vitro (4). Evidence for the presence of IF3 on 70S ribosomes was reported (9), indicating that the functional spectrum of IF3 is possibly not restricted to an antiassociation effect. Both IF3 and IF2 are also responsible for the fidelity of decoding the initiation AUG by fMet-tRNA$_f^{Met}$ at the P site of 30S subunits (10).

IF1 is universal (11) and essential for viability (12). It is the smallest factor, with 72 amino acid residues in *Escherichia coli*, and binds to the decoding center at the ribosomal A site (13). Several functions have been described, including stimulation of the formation of the 30SIC and subunit association (14). Interference with the binding of ternary complexes aminoacyl (aa)-tRNA•elongation factor thermo unstable (EF-Tu)•GTP to 30S subunits has also been suggested (15). Omitting IF1 in 30S-binding tests decreased the accuracy of fMet-tRNA selection over the elongator Phe-tRNA about 60-fold, which was suggested to account for the essential nature of IF1 (16). All three factors are thought to dissociate upon 50S arrival or shortly thereafter (1). IF1 is required for proper initiator-tRNA selection on 70S along with IF2 and IF3, in contrast to the 30SIC, where IF2 and IF3 provide tRNA selection (17).

In addition to the 30S-binding initiation, a second initiation mode exists that has a niche existence in bacteria: Leaderless mRNA (lmRNA) contains an initiator AUG codon within the first 5 nt at the 5′-end, and thus does not contain an SD sequence. This initiation mode uses 70S ribosomes with the special feature that the ribosomal proteins S1 and S2 are not required, which are otherwise important for the 30S-binding mode (18). Initiation of lmRNA can even occur in the absence of all IFs (19, 20). Additional information about lmRNAs is provided in *SI Appendix, Introduction*.

Significance

Until now, two initiation modes for bacterial translation have been described: (*i*) the standard 30S-binding mode, where the small ribosomal subunit selects the initiation site on an mRNA with the help of three initiation factors (IFs), and (*ii*) the rare initiation of leaderless mRNAs, which are mRNAs carrying the initiation AUG within the first 5 nt at the 5′-end. The existence of a third "70S-scanning" mode for bacterial initiation was conjectured in past decades but has remained experimentally unproven. Here, we demonstrate the existence of a 70S-scanning mode of initiation and characterize its mechanistic features. The three initiation modes demonstrate specific patterns of requirements for IF1 and IF3.

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The existence of a third initiation mode, *viz*. a 70S type of bacterial initiation, has been conjectured several times previously (21–23), although no in-depth mechanistic evidence has verified this mode thus far. For example:

i) The formylation of the initiator Met-tRNA$_f^{Met}$ in bacteria was interpreted as an indication of a 70S initiation mode (22). Indeed, only the anticodon loop of a tRNA and a part of the anticodon stem interact with the 30S subunit (24, 25), leaving the fMet residue as a substrate for the peptidyltransferase center on the large subunit within the 70S ribosome.

ii) When an AUG codon without a preceding SD sequence follows a stop codon within a distance of <20 nt, a mutational study unexpectedly revealed that efficient protein synthesis can be initiated in vivo at this AUG codon. The interpretation was that ribosomes were sliding down from the stop codon of the preceding cistron, although it was not analyzed whether 70S ribosomes or 30S subunits were involved in sliding or whether factors were required (26). Further evidence for a 70S type of initiation is described in *SI Appendix, Introduction* and concerns both studies of translational coupling and a consideration of the fact that more than 75% of the intercistronic distances are shorter than 30 nt, which is too short to allow an independent termination of cistron n and initiation of downstream cistron n + 1 (*SI Appendix*, Fig. S1 *A* and *B*).

Here, we demonstrate that there is an additional and frequent initiation mode that we term "70S-scanning initiation." The 70S ribosomes, rather than the 30S subunits, scan the sequence surrounding the termination signal for the presence of an SD sequence after termination. Furthermore, we show that the requirement of IF1 and IF3 for the three initiation modes (30S binding, 70S scanning, and initiation of lmRNAs) is distinct for each mode.

Results

We first assessed the sucrose-density gradient A_{260} profiles of *E. coli* lysates, and performed Western blot analysis with antibodies against IF1 and IF3 across gradient fractions (*SI Appendix, Results*). The results demonstrate that both IFs are present on 30S ribosomal subunits, and, surprisingly, also on 70S ribosomes and disomes (polysomes; *SI Appendix*, Fig. S2 *A* and *B*). These findings may possibly reflect 70SICs containing IF1 and IF3 that are more frequent than anticipated by the generally held view, where the IFs leave the ribosome upon 50S association, forming 70S ribosomes.

First Circumstantial Evidence for a Scanning Mechanism: Expression of Renilla and Firefly Luciferase from a Bicistronic mRNA. The first pilot experiment was performed in a coupled transcription/translation assay, where we assessed the extent to which the expression of a second cistron of a bicistronic mRNA depends on the expression of the first one. We adapted the dual-luciferase assay using Renilla luciferase (Rluc) and firefly luciferase (Fluc), which require different sets of reaction partners for their chemiluminescence, and thus allow both enzymes to be measured independently with high precision (27).

The bicistronic mRNA in Fig. 1*A* contains a 5′-UTR and an intercistronic region (IR) of 73 nt free of secondary structures. An optimal SD region for 30S-binding initiation precedes both cistrons. To block translation of one or the other cistron specifically and a possible scanning over the IR, we designed antisense oligo-DNAs specifically targeting Rluc, Fluc, and the middle of the IR (anti-Rluc, anti-Fluc, and anti-IR, respectively) because DNA/RNA helix structures severely impede ribosomal elongation rates (28), and thus the translation of a cistron. The mRNA was transcribed and translated in RTS lysate (Roche; *SI Appendix*), and luminescence was normalized to 100%. Hybridization of an oligo-DNA did not impair the stability of the synthesized mRNA (*SI Appendix*, Fig. S3*A*). Further controls

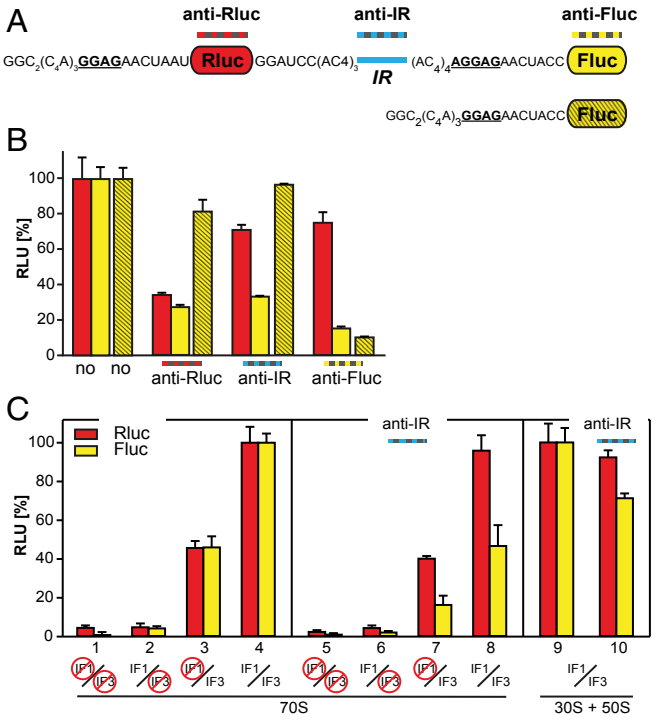

Fig. 1. Expression of bicistronic mRNAs. (*A*) Scheme of the mRNAs used in *B* and *C*, with SD sequences underlined. The bicistronic mRNA codes for Rluc and Fluc and the monocistronic mRNA code for Fluc are shown. Short antisense-DNA of 20–30 nt hybridizes specifically to the Rluc cistron (anti-Rluc), the IR (anti-IR), and the Fluc cistron (anti-Fluc). The 5′-UTRs and the IR were free of secondary structures. (*B*) Expression of the mRNAs shown in a coupled transcription/translation lysate system (RTS lysate; Roche). Hatched bars indicate control expression from the monocistronic mRNA coding for Fluc. (*C*) Expression of the bicistronic luciferase mRNA in *A* in the PURE system, with IF1 and IF3 when indicated. Expression by 70S reassociated ribosomes (*Left* and *Middle*) and by 30S plus 50S (*Right*) is shown. Anti-IR was present in the experiments (*Middle*, lanes 5–8; *Right*, lane 10). Red bars, relative amounts of Rluc; yellow bars, relative amounts of Fluc. RLU, relative light units.

with monocistronic mRNA coding for Rluc or Fluc demonstrated that both anti-Rluc and anti-Fluc blocked expression of their corresponding cistron but exhibited low effects (<20%), if any at all, on the other cistron due to an unavoidable low sequence similarity with the target mRNA. Most importantly, the anti-IR did not block Fluc expression at all (Fig. 1*B*, hatched columns and *SI Appendix*, Table S1).

Rluc (Fig. 1*B*, red bars) is reduced with the addition of any antisense DNA; a slight reduction of about 25% is seen with anti-IR and anti-Fluc, and a strong reduction of about 70% is seen in the presence of anti-Rluc as expected (Fig. 1*B*). Surprisingly, anti-Rluc provokes the same strong reduction of the second cistron Fluc (Fig. 1*B*, yellow bars), whereas blocking the second cistron affects the first one much less. Most interestingly, blocking a possible scanning with anti-IR reduces translation of the second cistron comparably to blocking the first cistron. Thus, blocking translation of the first cistron by anti-Rluc or preventing ribosomal scanning by anti-IR dramatically impairs the expression of the second cistron. We note that neither anti-Rluc nor anti-Fluc completely blocks the expression of the targeted cistrons Rluc and Fluc, respectively. It follows that the antisense DNAs bind to a major fraction, but not to all of the bicistronic mRNAs. This interpretation is most likely also valid for anti-IR, suggesting that the 70% reduction of Fluc is related to 70% of the mRNA hybridized with anti-IR preventing 70S scanning,

whereas the 30% unblocked mRNA would still allow 70S scanning. However, if we assume a quantitative binding of anti-IR to the IR, an alternative conclusion would be possible, namely, that at least 70% of the initiation of the second cistron occurs via a scanning initiation mode, whereas the remaining 30% would be subjected to a recycling depending on the ribosomal recycling factor (RRF) and the elongation factor G (EF-G), providing 30S subunits for initiating the second cistron. Furthermore, it is unlikely that a scanning ribosome can dislodge the hybridized anti-IR from the intercistronic sequence.

These results prompted us to analyze the translation of the same but now purified mRNA under more defined and well-controlled conditions of a highly defined translation system, the Protein synthesis Using Recombinant Elements (PURE) translation system (*Materials and Methods*). The PURE system used here and in some of the following experiments contains highly purified components, including RRF and EF-G (29, 30); the latter reference contains a precise description of the components and their concentrations, except that our PURE system lacked IF1 and IF3, which we only added when indicated. Furthermore, we diminished the total Mg^{2+} concentration from the usual 13–8.5 mM. The 70S dissociation and subunit association depend on free Mg^{2+}; our modified PURE system contains 2 mM ATP and GTP each, which bind about 1–1.5 mM Mg^{2+} per mM NTP (31), yielding a free Mg^{2+} concentration of about 2.5 mM, which is very near to in vivo conditions (32). In the ionic milieu of our modified PURE system, we observed an extremely slow equilibrium rate between 70S ribosomes and the subunits: The 70S ribosomes did not dissociate at up to 120 min of incubation in the presence of GTP and ATP (*SI Appendix*, Fig. S4*A*). Ribosomal subunits did not associate within 15 min, and poorly after 30 min, whereas the majority associated after 120 min (*SI Appendix*, Fig. S4*B*). In the following, we will demonstrate stark differences in translation after the addition of ribosomal subunits or 70S ribosomes, indicating that the initiation complexes 30SIC and 70SIC are formed within 15 min, during which the association or dissociation state of vacant ribosomes did not change. We further note that all ribosomes and ribosomal subunits used in the in vitro experiments reported here were derived from one and the same preparative batch (*Materials and Methods*).

In the presence of 70S ribosomes and IF1 alone, no expression of either cistron was observed, whereas with IF3, a substantial expression of both cistrons occurred (Fig. 1*C*, *Left*, lanes 2 and 3, respectively). IF3-dependent expression was strongly stimulated by IF1 (Fig. 1*C*, lane 4). When the same experiment was performed in the presence of anti-IR, preventing possible scanning, the expression of the second cistron Fluc was reduced by a factor of 2 (Fig. 1*C*, lane 4 vs. 8). The fact that anti-IR did not completely block Fluc expression can be explained by two alternative scenarios as mentioned above for a comparable case shown in Fig. 1*B*. The experiments in Fig. 1*C* indicate that 70S scanning depends on the presence of IF3.

Surprisingly high expression was observed with 30S plus 50S subunits with and without anti-IR (Fig. 1*C*, *Right*), although free ribosomal subunits could not associate at a free Mg^{2+} concentration of about 2.5 mM within 15 min and only poorly within 30 min (*SI Appendix*, Fig. S4*B*). It follows that 30SIC can easily associate with 50S subunits to form 70S ribosomes, in contrast to empty, nonprogrammed 30S (nonenzymatic conditions), which require activation energy of 79 kJ/mol or 19 kcal/mol for the association with 50S subunits (33). The 30S subunits can easily overcome the presence of anti-IR, because the mRNA is present in a groove of isolated 30S subunits. Therefore, 30S can bind to internal initiation sites, whereas the mRNA is located in a tunnel of 30S within a 70S ribosome, preventing direct binding to internal initiation sites (34, 35). Fig. 1*C* further demonstrates that ribosomes and ribosomal subunits derived from the same preparation, also used in *SI Appendix*, Fig. S4 and in the following experiments, are active in translation.

IF3 Is Essential for Initiating lmRNA, but IF1 Is Not Involved. We have seen that there is a slow equilibrium between vacant 70S and subunits in the PURE milieu in the absence of IFs, tRNAs, and mRNA. Therefore, it should be possible to design mRNAs that can be exclusively initiated and translated by either 70S ribosomes or ribosomal subunits, and thus unequivocally to assess the initiation dependence on IF1 and IF3. We began with the analysis of the translation of lmRNA, which can be initiated by 70S ribosomes (18, 20).

Fig. 2*A* shows our lmRNA construct for the expression of Rluc. An lmRNA is defined by an initiator-AUG codon within the first 5 nt at the 5′-end, and thus lacks an SD sequence. The lmRNA starts with GG, followed by the initiation AUG of Rluc. In the absence of both IF1 and IF3, as well as in the presence of only IF1, lmRNA is not expressed. In contrast, full expression is observed in the presence of only IF3, whereas the addition of IF1 did not potentiate this effect. We not only confirm that lmRNA can be initiated by 70S ribosomes in agreement with Moll et al. (18) and Udagawa et al. (20), but we also show that ribosomal subunits cannot initiate lmRNA (Fig. 2*A*, *Middle*). Furthermore, IF3 was thought to inhibit initiation of 70S ribosomes due to its 70S-dissociation activity (20, 36), whereas we find that IF3 is essential for lmRNA translation, although IF1 is not involved.

Under artificial in vitro conditions, such as a large excess of both mRNA and fMet-tRNA, it is known that a 70SIC complex can be formed nonenzymatically (i.e., mRNA, 70S ribosomes, and fMet-tRNA were incubated without any factor; e.g., ref. 37). We formed a 70SIC nonenzymatically before adding the complex to the PURE system. Rluc synthesis, although reduced, was observed in the absence of both IF1 and IF3 (Fig. 2*A*, *Right*). We will use this nonenzymatic initiation in a later experiment.

30S Subunits Can Bind Directly to an Initiation Site, Whereas 70S Ribosomes Cannot. Next, we designed an mRNA that should be translated exclusively by ribosomal subunits, rather than by 70S ribosomes. We exploit the fact that the 70S-entrance pore for an mRNA bounded by S3, S4, and S5 does not allow the passage of dsRNA (38).

The designed mRNA shown in Fig. 2*B* contains (*i*) a 54-nt-long 5′-UTR, where a possible scanning is blocked by an antisense oligo-DNA covering the mRNA from the third to 22nd nucleotide (anti–5′-UTR), and (*ii*) an SD sequence in front of the cistron coding for Fluc (the sequence is shown in Fig. 1*A*). We prevented the formation of 70S runoff ribosomes, which would make the interpretation ambiguous, by fusing the Fluc sequence to a gene section of the secM gene that was linked by a sequence coding for Gly_4Ser to pose no constrains on the Fluc folding. The *secM* gene fragment codes for a peptide that stalls the translating ribosome (39), and thus prevents its recycling. Consequently, every translating ribosome will undergo only one initiation event. Controls indicated that the synthesized [^{35}S]-labeled protein was exclusively present as peptidyl-tRNA (*SI Appendix*, Fig. S3*B*).

Fig. 2*B* shows that in the presence of the anti–5′-UTR, productive initiation occurs exclusively with free 30S + 50S subunits, whereas 70S ribosomes cannot initiate the Fluc cistron at all. This observation allowed us to assess unequivocally the requirements of IF1 and IF3 for the 30S-binding initiation. The 30S-binding initiation generates only background activity of Fluc in the absence of IF1 and IF3, whereas in the presence of either IF1 or IF3, considerable activity of around 20% is observed. Full activity is seen only in the presence of both factors, indicating a strong cooperativity. It follows that 30S-binding initiation can occur directly at internal initiation sites, whereas 70S ribosomes cannot but instead have to scan to the initiation site. In the

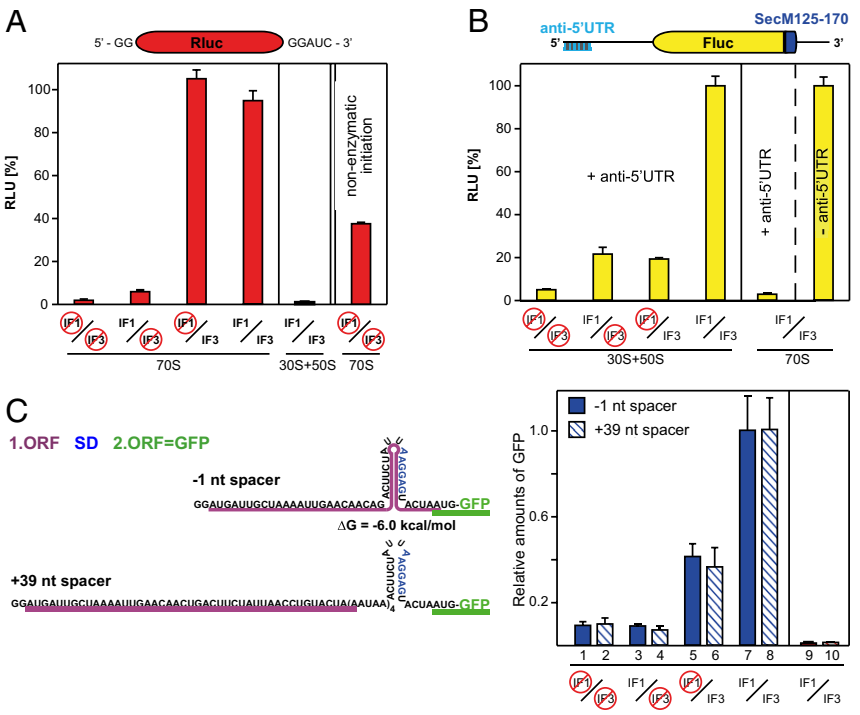

Fig. 2. Analyses in the PURE system. (*A*) IF1/IF3-dependent expression of an lmRNA coding for Rluc (explanation is provided in the main text, and methods of preparation are provided in *SI Appendix*). (*B*) IF1/IF3-dependent expression of a monocistronic mRNA coding for Fluc, which can be initiated exclusively by the 30S-binding mode as long as a secondary structure blocks the 5′-UTR for 70S ribosomes (details are provided in the main text). (*C*, *Left*) Bicistronic mRNAs with a leaderless first cistron and a GFP-coding sequence as the second cistron. The mRNAs were designed to block 30S-binding initiation (SD sequence hidden in a secondary structure) and only allow 70S-scanning initiation in the PURE system. (*C*, *Right*) Expression with IF1 and IF3 when indicated. The hatched bars represent synthesized GFP from the bicistronic mRNA with a prolonged IR of 39 nt. Activities are exclusively observed in the presence of 70S ribosomes in contrast to 30S and 50S subunits.

absence of the anti–5′-UTR, 70S ribosomes initiate and translate the Fluc as efficiently as the ribosomal subunits in the presence of the oligo-DNA. The 70S ribosomes can now bind to the 5′-end of the mRNA and scan downward to the initiation site of Fluc.

We conclude that (*i*) for optimal 30S-binding initiation, both IF1 and IF3 are required, although either factor alone can productively initiate the mRNA, and (*ii*) monocistronic mRNA can be initiated by a 70S-scanning mode.

Design of a Bicistronic mRNA That Can Be Exclusively Initiated by 70S Ribosomes. Given our initial findings regarding the unique characteristics of 70S-scanning initiation, we next designed an mRNA with two ORFs (Fig. 2*C*, *Left*; "−1-nt spacer"), where canonical 30S-binding initiation should not be possible due to the following two features:

i) The first short ORF is an lmRNA, which can be initiated only by 70S ribosomes (Fig. 2*A*, *Left* and *Middle*). The 70SIC at the first ORF was formed nonenzymatically, so that IF1, IF2, and IF3 are not required to translate this ORF (Fig. 2*A*, *Right*).

ii) The SD sequence of the second cistron GFP is hidden in a short hairpin with a stability of ΔG = −6.0 kcal/mol at 30 °C, which sequesters the SD sequence of the GFP cistron; 30 °C was also the incubation temperature during translation. Furthermore, the stop codon of the first ORF overlaps with the initiation AUG of the second cistron (GFP), mimicking the L29-S17 transition found within the S10-operon [mRNA with the −1-nt spacer (40)].

The 70S ribosomes translated GFP, as measured by GFP-band intensity in an SDS gel due to [^{35}S]Met incorporation (Fig. 2*C*, lane 7). In the absence of both IF1 and IF3 or only IF3, very low amounts of GFP were found (Fig. 2*C*, lanes 1 and 3). In contrast, low but substantial GFP amounts were detected with IF3 alone, which increased two- to threefold upon addition of IF1 (Fig. 2*C*, lanes 5 and 7, respectively). Because addition of 30S and 50S subunits did not show any activity (Fig. 2*C*, lane 9), these results indicate that one and the same 70S ribosome translates the first ORF and the following GFP cistron.

Does the apparent 70S-type initiation of the second cistron GFP require the overlapping junction of the two cistrons? To test this hypothesis, the same mRNA, but now with a spacer of 39 nt between the two cistrons, was constructed (Fig. 2*C*, *Lower Left*; mRNA with a +39-nt spacer). This spacer is long enough so that a ribosome, which terminates translation of the first cistron, cannot melt the secondary structure of the downstream SD sequence. The results are identical: GFP synthesis depends on the presence of IF3, and IF1 strongly stimulates the IF3-dependent expression (Fig. 2*C*, hatched bars). Again, 30S plus 50S subunits were not able to translate the GFP cistron at all (Fig. 2*C*, lane 10), thus indicating that 30S scanning is absent. Because 30S subunits cannot initiate the GFP cistron, the 70S ribosome must scan downward to the GFP initiation site after terminating the translation of the short upstream ORF. Hence, scanning 70S, rather than binding 30S, can melt the secondary structure hiding the SD sequence. In summary, we conclude that IF3 is essential for 70S scanning and that IF1 strongly stimulates the efficiency.

70S Scanning Analysis with a Minimal Model mRNA: fMet-tRNA$_f^{Met}$ Alone Can Trigger Scanning. We next constructed a minimal system for scanning, where the first cistron fragment can program a post-termination complex with a deacylated tRNA^{Phe} in the P site (codon UUC) and a stop codon UAA at the A site. The downstream cistron fragment consists of an initiation site with a SD sequence, followed by an AUG start codon and the Lys codon AAA. The 70S position on the mRNA was assessed using the toe-printing method (Fig. 3*A*; mRNA 1).

Deacylated tRNA^{Phe} fixes the 1-UUC codon at the P site of a 70S ribosome. Surprisingly, addition of fMet-tRNA in the absence of any factor or energy is able to shift ∼70% of the ribosomes to the downstream 1-AUG, suggesting that the 1-AUG signal appears at the expense of the 1-UUC signal (Fig. 3*A*, lanes 1 and 2). This surprising result led us to analyze 70S scanning in depth using the minimal system.

70S Scanning Analysis with a Minimal Model mRNA: A Rigorous Analysis. Next, we rigorously test by three different approaches whether or not the posttermination 70S complex indeed scans

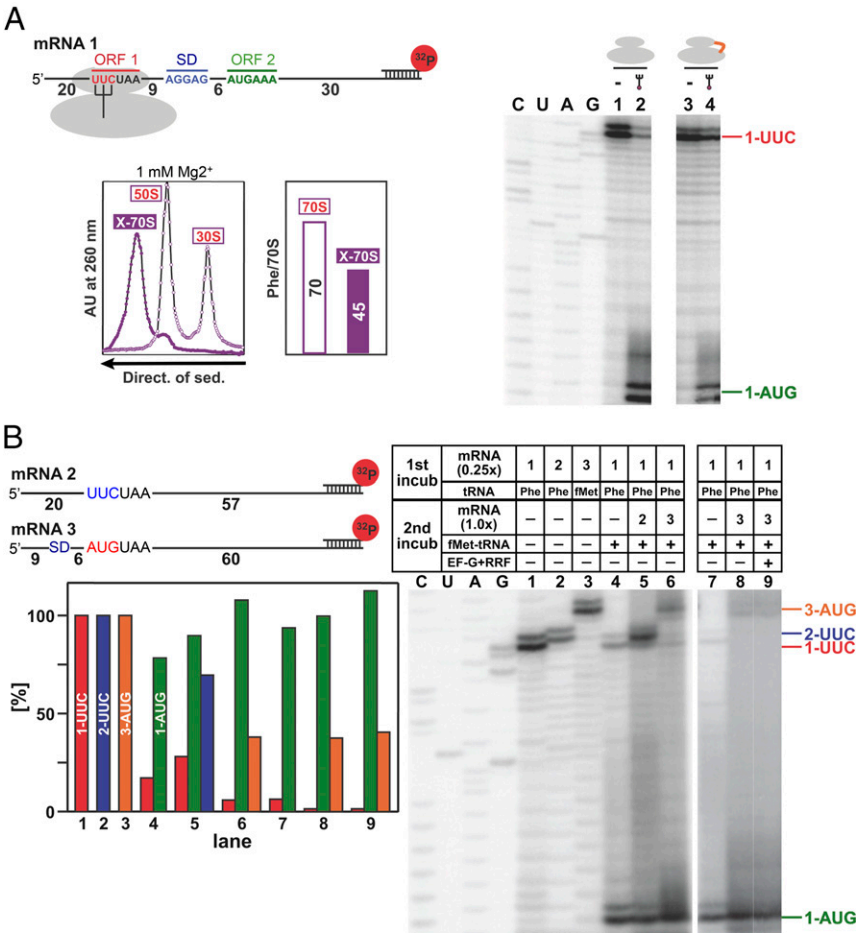

Fig. 3. Scanning of 70S between a termination and a downstream initiation site (toe-printing). (*A*) Structure of the mRNA 1; the numbers indicate the number of nucleotides. The gel shows toe-printing signals from ORF1 (1-UUC) and ORF2 (1-AUG). As shown in lanes 1 and 2, posttermination complexes were constructed with 70S, mRNA, and deacylated $tRNA^{Phe}$, and fMet-tRNA was added for lane 2; the system did not contain factors. Lanes 3 and 4 are the same as lanes 1 and 2, respectively, but with X-70S ribosomes [no dissociation at 1 mM Mg^{2+}, 60% activity of standard ribosomes in poly(Phe) synthesis (*Lower Left*)]. AU, relative absorption units. (*B*) mRNA 2 contains only ORF1; mRNA 3 contains only ORF2, including the SD sequence. 1-UUC and 1-AUG bands were obtained from mRNA 1, 2-UUC, and 3-AUG from mRNA 2 and mRNA 3, respectively. The first incubation was with with 70S ribosomes and the indicated mRNA (molar ratio of mRNA/70S = 0.25) and $tRNA^{Phe}$ or $tRNA^{fMet}$; in a second incubation, fMet-tRNA (gel, lanes 4–6) and mRNA 2 and mRNA 3 were added (gel, lanes 5 and 6, respectively; molar ratio of mRNA/70S = 1). Lanes 7 and 8 are as lanes 4 and 6, respectively. Lane 9 is as lane 6, but in the presence of RRF, EF-G, and GTP. (*Lower Left*) Bands of the toe-printing signals were scanned, and the relative intensities are shown. Red bars, intensities corresponding to the 1-UUC bands; green bars, intensities corresponding to the 1-AUG bands. The numbers at the *x* axis represent the lane numbers of the gel. All lanes shown in *A* and *B* were derived from the same gel.

downward to the initiation site of the second ORF fragment upon fMet-tRNA binding or, alternatively, dissociates and rebinds to the initiation site.

In the first test, we used cross-linked 70S (X-70S) ribosomes (with dimethyl-suberimidate as the cross-linking agent and purification by sucrose-gradient centrifugation; details are provided in *SI Appendix*). X-70S cannot dissociate, whereas reassociated 70S ribosomes quantitatively dissociate at 1 mM Mg^{2+} (Fig. 3*A*, *Lower Left*). The X-70S ribosomes are able to incorporate, on average, 45 Phe per 70S in a poly(U)-dependent poly(Phe) system, which is about 60% of the efficiency of untreated ribosomes (Fig. 3*A*, *Lower Right*). Also, X-70S can form a posttermination complex, and, again, the addition of $fMet\text{-}tRNA_f^{Met}$ triggers a downshift with an efficiency of about 50% (gel picture in Fig. 3*A*, lanes 3 and 4, respectively), corresponding to the activity in the poly(Phe) assay. The nonspecific cross-linking procedure likely establishes several cross-links between the subunits of one ribosome; thus, X-70S ribosomes should not be able to open the mRNA tunnel required for dissociation from an mRNA. However, a single cross-link per 70S ribosome would allow for separation at the subunit interface, possibly opening the tunnel, as shown with active 70S ribosomes containing covalently tethered 16S and 23S rRNAs (41).

Second, in addition to this strong indication for 70S scanning, we tested a possible release of the mRNA from posttermination complexes and rebinding to the downstream ORF2 by a chasing experiment. To this end, mRNA 2 and mRNA 3 were designed (Fig. 3*B*) for use in conjunction with mRNA 1. As an important control with non–X-70S ribosomes, a posttermination complex forms on mRNA 1, giving the toe-printing signal 1-UUC (Fig. 3*B*, *Right*, lane 1). After adding fMet-tRNA, the downstream signal 1-AUG is seen (Fig. 3*B*, lane 4) as in Fig. 3*A*. The mRNA 2 and mRNA 3 give the toe-printing signals 2-UUC and 3-AUG in the presence of the corresponding cognate tRNAs as expected (Fig. 3*B*, lanes 2 and 3, respectively).

Next, we constructed a posttermination complex as in lane 1 and then added mRNA 2 or mRNA 3, together with fMet-tRNA, for a second incubation. The mRNA 2 and mRNA 3 were added in a 4 M excess over the posttermination complex, corresponding to a stoichiometric amount with respect to the total 70S. The expectation was that when 70S ribosomes fall off the mRNA 1 upon addition of fMet-tRNA, the presence of an excess of mRNA 2 or mRNA 3 will sample the ribosomes before they can bind to the downstream initiation signal. This scenario will substantially weaken the 1-AUG signal. Assuming a release of the 70S ribosomes upon fMet-tRNA addition, we can estimate that the 1-AUG signal would be weakened about 10-fold (details of the estimation, together with *SI Appendix*, Fig. S5, are given in *SI Appendix*).

However, we did not see any weakening of the 1-AUG signal (Fig. 3*B*, *Lower Left*; compare the 1-AUG band in lane 4 with the 1-AUG bands in lanes 5 and 6, and the corresponding green bars representing the scanned band intensities). Even the presence of EF-G•GTP and RRF in addition to an excess of mRNA 3 does not weaken the 1-AUG signal (Fig. 3*B*; compare green bars in lanes 8 and 9). These two factors were suggested to be involved in the release and dissociation of 70S ribosomes after termination (7). Likewise, addition of IF1, IF2, and IF3 did not influence the fMet-tRNA–induced effect (*SI Appendix*, Fig. S6).

Taken together, 70S scanning occurs rather than reaching the 1-AUG codon via dissociation and reassociation, and 70S scanning does not require energy-rich compounds such as GTP.

70S Scanning Analysis with a Minimal Model mRNA: SD Selects the Landing Codon. Scanning can be triggered in our model system not only by fMet-tRNA$_f^{Met}$ but also by Met-tRNA$_f^{Met}$ and deacylated tRNA$_f^{Met}$. In *SI Appendix*, Fig. S7*A*, we demonstrate that even an elongator His-tRNA^{His} (anticodon GUG) in the absence of initiation and elongation factors can trigger scanning. Interestingly, the mRNA contains five cognate CAC His codons between the UUC codon (red) and AUG codon (green) that are precisely complementary to the anticodon of the tRNA^{His} (*SI Appendix*, Fig. S7*A*, *Bottom*). None of the five cognate codons was selected by the scanning 70S ribosome, but rather the near-cognate wobble codon CAU following the SD sequence with an optimal spacer of 5 nt.

If the SD sequence plays an important role for selecting the landing codon of the 70S-scanning ribosome, removal of the SD sequence of mRNA 1 should substantially weaken the landing signal; in fact, this expectation was fulfilled: Without the SD sequence, addition of tRNA^{His} did not result in a CAU band at ORF2. Likewise, in the presence of fMet-tRNA, no 1-AUG band appeared (*SI Appendix*, Fig. S7*B*). Remarkably, the 1-UUC band was at least as strong as in the control lane 1 (*SI Appendix*, Fig. S7*B*) without triggering tRNAs (tRNA^{His} or fMet-tRNA), because one might expect that a scanning 70S leaves the UUC position, thus weakening the UUC band. A possible explanation is as follows: In the presence of an SD sequence, a triggering tRNA (e.g., fMet-tRNA) can fix the scanning 70S at the cognate AUG codon. In this situation, the upstream UUC cannot be occupied by a second 70S•tRNA^{Phe} complex coming from the 5′-end, because a 70S ribosome covers at least 15 nt upstream and downstream of a P-site codon on the mRNA (42). In this way, a distance of more than 30 nt between the 1-UUC and 1-AUG codons is required for binding a second 70S at the 1-UUC codon, whereas the corresponding distance in mRNA 1 is only 23 nt (Fig. 3*A*). In contrast, a scanning ribosome is not fixed at the downstream AUG in the absence of SD, having 53 nt until the primer site, thus allowing the binding of a second 70S•tRNA^{Phe} complex to the 1-UUC codon.

These results demonstrate the decisive importance of the SD sequence, which selects the landing codon of the downstream cistron for a 70S-scanning ribosome.

IF1 Specifically Reduces Occupation of the A Site. We saw that IF1 strongly stimulates the expression of GFP from the second cistron of a bicistronic mRNA via 70S scanning (Fig. 2*C*). It is known that IF1 binds to the decoding center at the A site (13); therefore, its function during 70S scanning might be to prevent premature pseudoinitiation by ternary aa-tRNA•EF-Tu•GTP complexes. Such pseudoinitiation occurs during standard poly(Phe) assays, where synthesis starts via binding of a Phe-tRNA•EF-Tu•GTP complex to poly(U) programmed 70S ribosomes. *SI Appendix*, Fig. S8 shows that IF1 can indeed reduce the binding of ternary Phe-tRNA•EF-Tu•GTP complexes to the A site (blue columns), rather than the arrival of Phe-tRNA at the P site (yellow columns). The latter point is notable, because IF1 at the A-site decoding center does not impede tRNA passage to the P site of empty ribosomes as far as we can measure with our methods. Thus, IF1 shields the decoding center against premature entry of an elongating ternary complex during the scanning process. We note that the molar ratio of IF1/70S was 10:1 in the last experiment, and thus larger than in the other experiments.

IF1 Deprivation in Vivo More Strongly Inhibits the 70S Scanning Mode than the 30S Binding Initiation. Here, we make use of an *E. coli* strain Ec(IF1$^-$)/pAraIF1, where the *infA* gene encoding IF1 has been deleted from the chromosome. The essential IF1 is encoded on the pAraIF1 plasmid under the control of an arabinose-inducible promoter. IF1 synthesis occurs in the presence of arabinose and is suppressed in the presence of glucose.

The ability to modulate IF1 levels in vivo allows for an analysis of how IF1 affects expression of the second cistron of the luciferase mRNA shown in Fig. 1*A*. Reducing IF1 concentration in the presence of glucose by about 75%, down to 25% of the WT level (discussed below), dramatically reduces expression of the second cistron down to 20%, whereas the effect on the first cistron was much weaker (~70% activity; *SI Appendix*, Fig. S9, *Left*). The expression bias was not caused by a difference in the sugars, because a control experiment using *E. coli* strain MG1655 containing a WT IF1 gene on the chromosome showed an even stronger expression of both cistrons in the presence of glucose (*SI Appendix*, Fig. S9, *Right*), although the expression of the second cistron was slightly less than the expression of the first one. We conclude that the expression of the second cistron depends on the presence of IF1 much more than the expression of the first cistron does.

We have seen that 70S ribosomes can initiate a monocistronic mRNA via the 70S scanning mode in vitro (Fig. 2*B*, *Right*; 70S control without the oligo-DNA anti–5′-UTR). Therefore, we next sought to compare the in vivo effects of IF1 deprivation on the expression of monocistronic mRNAs. To this end, we constructed two mRNAs coding for GFP (Fig. 4*A*). The first one has an unstructured 5′-UTR of 49 nt (mRNA-unstr) allowing for both 30S-binding and 70S-scanning initiation. The second one is identical except that it has a strong secondary structure with $\Delta G = -25.1$ kcal/mol at 25 °C (mRNA-str), which should be initiated only by 30S subunits. The reason is that a scanning 70S cannot melt a secondary structure of a comparable stability (−28 kcal/mol at 30 °C; anti-IR in Fig. 1*A* and *SI Appendix*), in contrast to one of −6 kcal/mol (Fig. 2*C*).

Plasmids carrying one of the two GFP constructs downstream of a tac promoter were transformed into both the Ec(IF1$^-$)/pAraIF1 and WT strains. Cells were grown in glucose [IF1 deprivation in the strain Ec(IF1$^-$)/pAraIF1 in contrast to WT], and GFP expression was induced for 2 h at 25 °C to stabilize the secondary structure of mRNA-str. Western blots were performed with S-30 lysates probed for GFP, IF1, and IF3 and, as a ribosome reference, against the ribosomal protein S7. Both the relative amounts of GFP and the IF1/IF3 ratio in WT cells were set to 100%. The amount of IF3 did not change during IF1 deprivation (*SI Appendix*, Fig. S10). Importantly, during the expression of GFP from the structured mRNA in WT and mutant cells Ec(IF1$^-$)/pAraIF1, for example, the only changed parameter was the in vivo concentration of IF1. Therefore, the different GFP amounts seen in WT and mutant cells can be directly related to the difference in IF1 concentration in vivo.

In WT cells, the mRNA secondary structure (only 30S-binding mode) reduced the relative GFP expression to 55% (Fig. 4*A*, *Left*, green bars; relative IF1 level at 100%, shown as a violet bar), suggesting that 70S-scanning initiation accounts for about 45% of the initiation events. In mutant cells, the relative IF1 level was reduced to 30 ± 10% (Fig. 4*A*, *Right*, violet bar), and the GFP expression from both mRNAs was about the same in both cases (35%). Thus, 30S initiation alone (mRNA-str) at low IF1 amounts (Fig. 4*A*, *Right*) is comparable to initiation by both 30S binding and 70S scanning (mRNA-unstr). This observation suggests that the initiation mode of 70S scanning in vivo is more sensitive to IF1 deprivation than the 30S-binding mode, whereas, in vitro, both modes are strongly stimulated by IF1. Further, monocistronic mRNAs can be initiated by the 70S-scanning mode provided that the 5′-UTR does not contain a strong secondary structure.

The low dependence of the 30S initiation mode in IF1 in vivo was surprising, which prompted us to interrogate this phenomenon further with bicistronic luciferase mRNAs, one of which

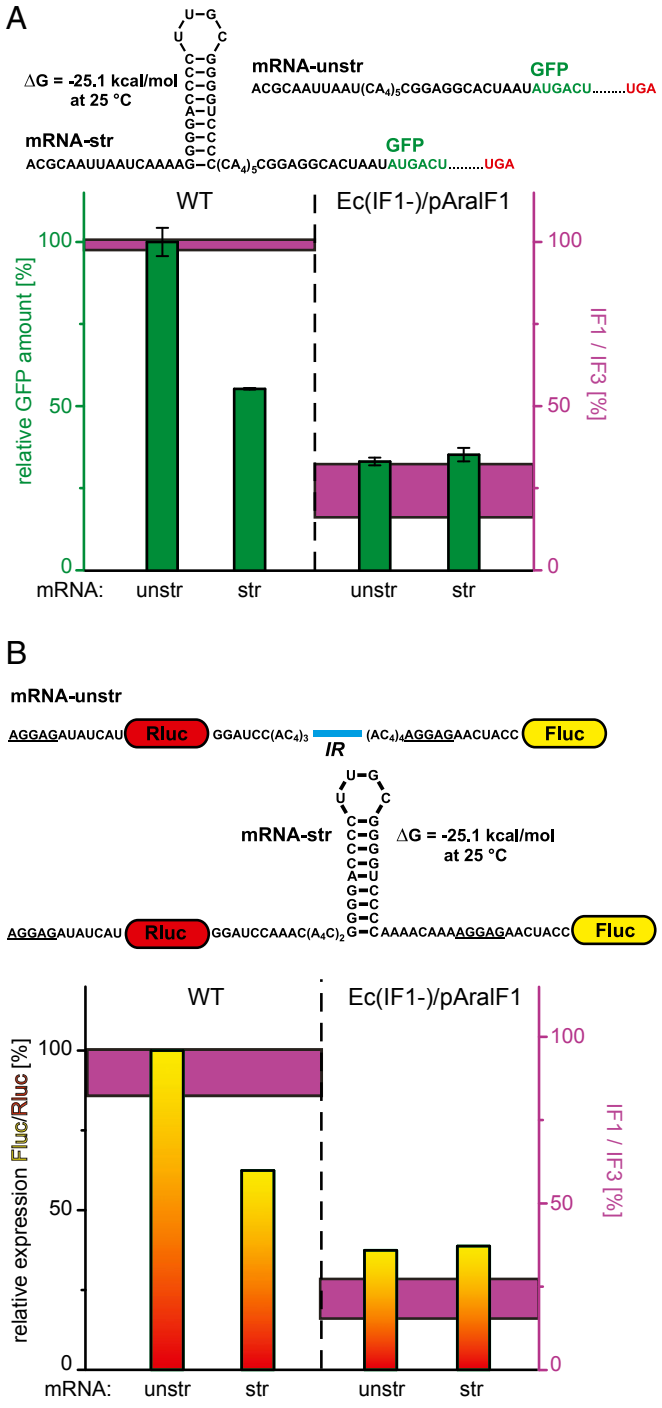

Fig. 4. In vivo expression under IF1 deprivation from mRNAs with and without secondary structures. (*A*) In vivo expression of GFP (green bars) from monocistronic mRNAs without and with a secondary structure in the 5′-UTR (mRNA-unstr and mRNA-str, respectively) under normal (WT cells) and IF1-deprived conditions [Ec(IF1$^-$)/pAraIF1] grown in the presence of glucose. The relative IF1/IF3 ratios are given as horizontal violet beams. The IF3 amount was the same in all strains and conditions (*SI Appendix*, Fig. S10). (*B*) In vivo expression of Rluc and Fluc from bicistronic mRNAs with and without a secondary structure in the IR in WT cells (normal IF1 amounts) and in the mutant [Ec(IF1$^-$)/pAraIF1, IF1-deprived conditions]; both strains were grown in glucose. The expression of both mRNAs in WT and mutant cells was assessed by a Northern blot test using [^{32}P]anti-Fluc DNA and was found to be about the same in all cases.

contained a secondary structure in the IR in front of Fluc (Fig. 4*B*). The inserted stem-loop was the same as in the monocistronic GFP-mRNAs above, where its position in the IR excluded any interference with termination of the upstream Rluc or with the 30S-binding initiation of the downstream Fluc cistron. The growth conditions were identical in WT and mutant strains, and the only changed parameter in vivo during expression of one of the mRNAs was the IF1 concentration. A key feature of this experiment is that the ratio of Fluc/Rluc expression reliably reflects the relative Fluc amount, independent of the lysate input for the determination of luciferase activity.

The results correspond well to the results of the GFP experiment. In WT cells with normal IF1 amounts, the stem-loop in front of Fluc reduces its expression to 60% (i.e., about 40% of the initiation of the second cistron coding for Fluc is caused by 70-scanning ribosomes). In contrast, at relatively low IF1 amounts (22 ± 7% of WT), the secondary structure hardly affects the relative expression of Fluc; without and with the stem-loop, the relative Fluc amounts are 37% and 39%, respectively. Therefore, low IF1 concentrations severely impair the 70S-scanning mode in contrast to the 30S-binding initiation (i.e., the 30S-binding mode of initiation clearly depends less on IF1 than 70S-scanning initiation).

Discussion

The idea that a ribosome dissociates after every translation of a cistron to supply 30S subunits for initiation dates back to 1968, when Kaempfer (43) demonstrated an intensive subunit exchange between heavy and light ribosomes. A convincing point was that known translation inhibitors could block subunit exchange. However, the experimental method raises some questions: (*i*) 18 amino acids were added, which is not enough for protein synthesis, and (*ii*) sonication leading to a breakdown of polysomes into 70S monosomes containing mRNA fragments did not significantly reduce the subunit exchange. Most short mRNA fragments do not contain a stop codon, and thus do not allow for orderly termination.

Nevertheless, the 30S-binding mode of initiation is well documented (1) but poses several paradoxes, as described in the Introduction. The 70S-scanning mode first postulated in 1966 (44) can resolve these contradictions. Our experiments suggest that 70S ribosomes do not necessarily dissociate after termination, but rather scan the mRNA around the stop codon searching for a nearby SD sequence. We do not know whether and when the 70S ribosome dissociates and leaves the mRNA during an RRF/EF-G•GTP–dependent recycling process, as described below.

Three observations indicate that it is not the 30S subunit, but rather the 70S ribosome, that scans the surrounding nucleotides of the last stop codon for an SD sequence:

i) The bicistronic mRNA used in Fig. 2*C* was designed to prevent 30S initiation, and, indeed, a 30S-binding mode of initiation was not observed (lanes 9 and 10), in contrast to a 70S-dependent initiation causing a strong translation of the second GFP cistron requiring 70S scanning.

ii) Robust 70S scanning from a posttermination complex was triggered by fMet-tRNA$_f^{Met}$ and was not affected by the presence of an excess of competing mRNAs, which would sample the ribosomes after dissociation (Fig. 3*B*; compare the 1-AUG bands of lane 4 with the 1-AUG bands of lanes 5 and 6). Even the presence of both an excess of competing mRNA 3 and RRF+EF-G•GTP did not diminish the strength of the 1-AUG band (Fig. 3*B*, lanes 8 and 9). RRF+EF-G•GTP is thought to be required for dissociating a posttermination 70S complex. The latter results are particularly interesting, because they suggest that 70S dissociation (recycling) is not an obligatory phase after termination of the translation of a cistron.

iii) Finally, scanning worked equally well using X-70S ribosomes (Fig. 3*A*, compare lanes 2 and 4). We conclude that the 70S-scanning initiation represents an important alternative initiation mode complementing the 30S-binding initiation. The 70S scanning also seems to work upstream in a few cases (in 7% of all IRs of *E. coli*, where 70S ribosomes after translation of a cistron should move upstream for limited distances of 1, 2, and 4 nt; *SI Appendix*, Fig. S1*B*), but occurs preferentially downstream as predicted from in vivo evidence (21).

A surprising result was that fMet-tRNA$_f^{Met}$ can trigger 70S scanning in the absence of factors (Fig. 3*A*). There is a significant free pool of this charged tRNA in the cell, from where it can be selected by ribosomal particles containing IF2 (45). The formyl blockage of the α-amino group stabilizes the ester bond (46), an important prerequisite for the significant $t_{1/2}$ of an aminoacylated tRNA in the cytosol. In contrast, elongator tRNAs only have a negligible free pool. Most of the elongator tRNAs are fully aminoacylated as long as no amino acid starvation occurs (47, 48) and are complexed with EF-Tu•GTP, which protects the labile ester bond (49). Deacylated tRNA also will not interfere, because the vast majority is bound to ribosomes and synthetases. Therefore, our observation that His-tRNA can also trigger 70S scanning (*SI Appendix*, Fig. S7*A*) indicates a principal feature, but likely has no importance in vivo. However, fMet-tRNA is not sufficient to promote scanning in a complete system containing all of the translational factors, but IF3 is an absolute requirement, strongly supported by IF1 (Fig. 2*C*).

IF1 binds preferentially to 70S ribosomes and polysomes, and less than 30% binds to 30S subunits (*SI Appendix*, Fig. S2*B*). Polysomes obviously contain a significant fraction of scanning/initiating 70S ribosomes. The strong effects of IF1 on 70S scanning (Figs. 1*C*, 2*C*, and 4 *A* and *B*) are probably due to preventing entry of ternary complexes (*SI Appendix*, Fig. S8) before the scanning 70S has reached the adjacent initiation site.

Another surprise was the observation that IF3 can bind to both 30S subunits and 70S ribosomes, where up to 20% of the IF3 was found on 70S disomes and trisomes (*SI Appendix*, Fig. S2*A*). The fact that IF3 is essential for the 70S-scanning initiation (Fig. 2*C*) does not necessarily contradict its well-documented antiassociation activity (5), where IF3 was thought to bind exclusively to the 30S subunit. Both foot-printing studies and X-ray analysis demonstrated its binding site at the interface of the small subunit (4, 50). The foot-printing studies were done with 30S subunits and not tried with 70S ribosomes. As mentioned in the Introduction, evidence for IF3 presence on 70S ribosomes was reported (9). The overlapping binding sites on 30S and 70S (9) could be reconciled with a distinct binding region, whereas the binding site derived from a crystallographic study could not, because the C-terminal domain of IF3 was assigned to the upper end of the shoulder on the solvent side of 30S subunits of *Thermus thermophilus* (51).

Functional IF3 studies revealed that this factor stabilizes dissociated ribosomal subunits in the presence of RRF and EF-G (52, 53), using high concentrations of IF3 (90 and 20 molar excess over 70S ribosomes, respectively). The IF3 concentrations used in our work were below 0.6 μM (in an IF3/70S molar ratio of 1–1.5; concentration of 70S ribosomes was 0.4–0.5 μM), near to the in vivo molar ratio of ∼0.2 for all three IFs (54). Even at a concentration of 4.6 μM, IF3 could not induce 70S dissociation in a polyamine buffer similar to the buffer used here (55). Thus, the IF3 effects observed here are unlikely to result from IF3-dependent 70S splitting into subunits, because (*i*) we used a low IF3/70S ratio of 1–1.5 (as was the corresponding ratio for IF1) and (*ii*) even a 10 molar excess of IF3 over 70S ribosomes under our conditions could not induce dissociation.

We conclude that in addition to stabilizing 30S subunits and forming the 30SIC complex, IF3 has a second important function for the 70S-scanning process: IF3 keeps 70S ribosomes scanning competent. The first function of IF3 is related to the accuracy of initiation, because IF3 increases the dissociation rate of non-canonical 30SIC (56), a function that is restricted to 30SIC rather than to 70SIC (17), leaving IF3's important role for 70S scanning as its main second function.

Our results reveal distinct patterns of IF1 and IF3 contributions for the three initiation modes observed in *E. coli*, which are shown in Fig. 5, together with the following likely scenario for

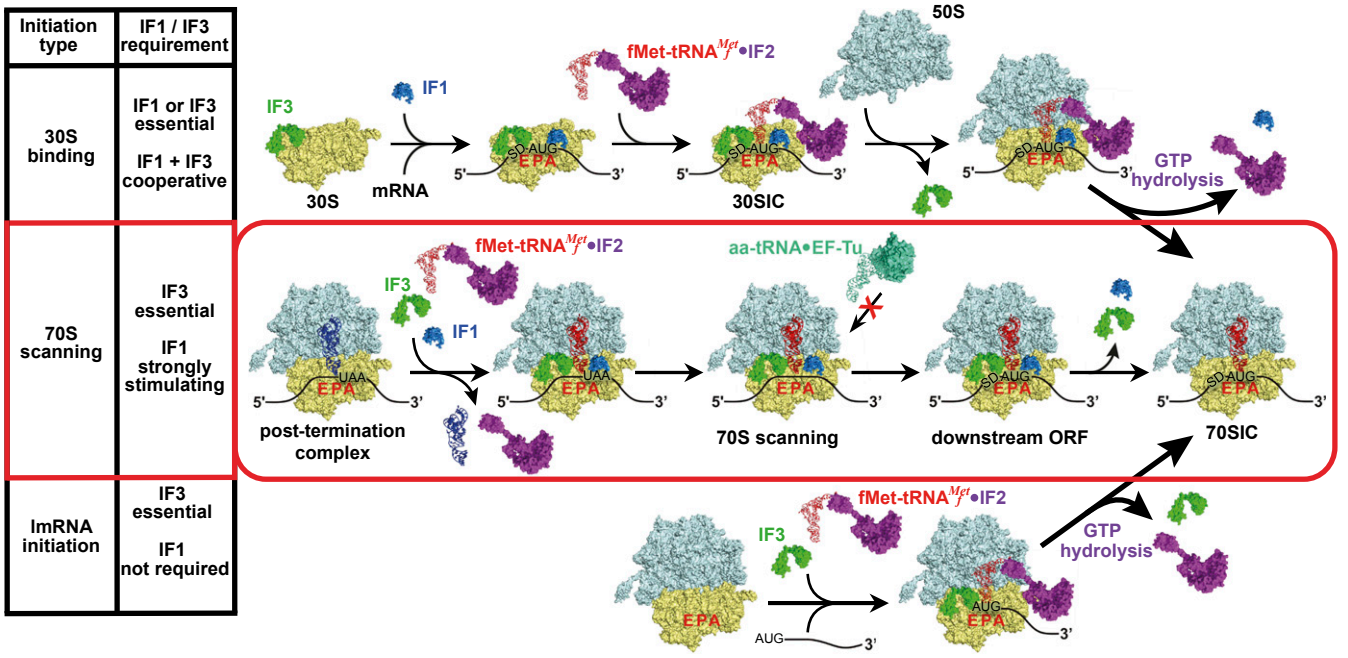

Fig. 5. Sketch of the three initiation modes of *E. coli* bacteria with their requirements for IF1 and IF3. Note that the in vivo experiments (Fig. 4) suggest a low dependence of the 30S-binding initiation on IF1 in contrast to the 70S-scanning initiation. aa-tRNA, aminoacyl-tRNA.

70S-scanning initiation. When a stop codon enters the A site, the class I termination factor RF1 or RF2 triggers the hydrolysis of the peptidyl-tRNA at the P site. Then, RF1 or RF2 dissociates from the ribosome with the help of RF3 (57). In the next step, the factors IF1, IF2, and IF3, together with fMet-tRNA, trigger 70S scanning. IF3 keeps the 70S ribosome scanning competent with the help of IF1. The latter factor additionally prevents a deleterious entry of ternary aa-tRNA•EF-Tu•GTP complexes, which would interrupt the scanning process before the adjacent SD would be found. It may be that IF1 also prevents RRF binding, because the IF1 binding site at the decoding region actually overlaps with domain II of RRF and domain IV of EF-G (58, 59), and would therefore prevent RRF and EF-G from splitting 70S into subunits. The existence of the 70S-scanning initiation questions the idea that the RRF- and EF-G–dependent recycling phase is an obligatory process between termination and initiation (60).

Because 70S scanning does not require energy-rich compounds (Fig. 3*A*), we assume that the 70S complex moves according to unidimensional diffusion along the mRNA until the next SD sequence. If a postterminating 70S ribosome does not find an fMet-tRNA or, alternatively, the scanning 70S does not encounter an SD sequence, the factors RRF and EF-G might take over and trigger the release of 70S from the mRNA, perhaps accompanied by dissociation into the ribosomal subunits (7). We do not yet know what makes the scanning 70S ribosome susceptible to RRF and EF-G. Our in vivo results (Fig. 4 *A* and *B*) detail the participation of IF1 and IF3 in the various initiation modes as described in Fig. 5, where IF1 seems to be of particular importance for the 70S-scanning mode rather than for the 30S-binding initiation.

Interestingly, monocistronic mRNA, and therefore also the first cistron of a polycistronic mRNA, can be initiated by the 70S-scanning mechanism provided that the 5′-UTR has no or weak secondary structures (Figs. 2*B* and 4 *A* and *B*). In contrast to the 30S-binding mode, the fact that 70S-scanning initiation is abolished in the presence of a strong secondary structure in the 5′-UTR (Fig. 2*B*; anti–5′-UTR) reflects a structural peculiarity of the mRNA location on the 30S subunit: in a groove in isolated 30S subunits and a tunnel in 70S ribosomes (34, 35). The inability of 70S ribosomes to initiate at internal initiation sites has been demonstrated (18, 19). Thus, 30S subunits can initiate at any initiation site as long as the SD sequence is accessible, whereas 70S ribosomes must thread the mRNA from the 5′-end. Scanning 70S ribosomes are blocked by strong secondary structures with a stability of at least −20 kcal/mol. In this respect, we emphasize a crucial point of our in vitro experiments: We analyzed the productive formation of initiation complexes (i.e., successful formation of an initiation complex was tested via the synthesis of the corresponding protein; Figs. 1 and 2). However, when we tested 70S binding in the presence of fMet-tRNA to the second cistron of a bicistronic mRNA similar to the mRNA shown in Fig. 1*A*, we detected bound 70S ribosomes that could not form a productive 70S complex. A similar case was previously reported by Takahashi et al. (61), where an anti-UTR present on the 5′-end of an mRNA did not prevent 70S binding, but a corresponding construct (Fig. 2*B*) completely blocked 70S-dependent translation in contrast to 30S + 50S subunits. It follows that simple 70S binding to an mRNA does not necessarily represent a physiological step toward a productive initiation complex. A striking exception is the initiations at lmRNA, which could form productive initiation complexes when bound to the 5′-end of the lmRNA nonenzymatically in the presence of fMet-tRNA (Fig. 2*A*). Obviously, it is not a problem to thread the 5′-end of an lmRNA into the mRNA tunnel of 70S ribosomes in the absence of IFs.

The length distribution of the 5′-UTRs of the mRNAs in *E. coli* has a median of 37 nt, and 5′-UTRs not longer than 37 nt contain secondary structures with a stability of less than $\Delta G = -5$ kcal/mol on average (*SI Appendix*, Fig. S11 *A* and *B*). Because 70S-scanning ribosomes easily resolve secondary structures with $\Delta G = -6$ kcal/mol (Fig. 2*C*), it is clear that many of the monocistronic mRNAs might also use the 70S-scanning mode for initiation. Our results suggest that 70S scanning is a frequent initiation mode in bacteria, and possibly also in archaea because they have operon structures similar to the corresponding bacterial ones. In eukaryotes (humans and mice), translation of approximately half of the transcripts are regulated by short upstream ORFs (uORFs) (62, 63) and translation of the downstream ORF requires reinitiation. Recently, it was shown that after a termination event, 40S subunits, and probably also 80S ribosomes, could scan along the mRNA downstream or upstream to the next AUG position (64). Ribosome profiling methodology also revealed the possibility that 80S ribosomes scan downstream after translating a cistron in yeast (65). These observations suggest that the scanning mode of 70S or 80S ribosomes might be a universal ribosomal feature of the ribosomal translation process.

Materials and Methods

Buffers. The buffer $H_{20}M_6K_{30}SH_4$ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 6 mM $Mg(Ac)_2$, 30 mM K(Ac), 4 mM β-mercaptoethanol] was used. The standard buffer used for functional tests was $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 4.5 mM $Mg(Ac)_2$, 150 mM KAc, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine]. The dissociation buffer used was $H_{20}M_1N_{200}SH_4$ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 1 mM $MgCl_2$, 200 mM KCl, 4 mM 2-mercaptoethanol].

Large-Scale Isolation of Ribosomal Subunits and Reassociated 70S Ribosomes (Tight Couples). Ribosomes were isolated from the *E. coli* strain Can/20-12E lacking five RNases, including RNase I (66). Up to several hundred grams of log-phase cells were ordered from Kalju Vanatalu (private entrepreneur, Tallinn, Estonia; a stem culture of the cells to be propagated has to be sent to the supplier). The cells were washed with $H_{20}M_6K_{30}SH_4$. Cell rupture was performed with an M-110L microfluidizer (pressure = 17,000 psi, 4 °C; Microfluidics), and the membranes and cell debris were removed by low-speed centrifugation (10 min at 15,000 × *g*, 4 °C) yielding the S30 lysate. Crude ribosomes (mostly 70S) were pelleted by ultracentrifugation (17 h at 40,000 × *g*, 4 °C) and resuspended in $H_{20}M_6K_{30}SH_4$. Tightly coupled ribosomes withstand these conditions, whereas loosely coupled ribosomes dissociate into subunits. Tightly coupled ribosomes are functionally competent in contrast to the loosely coupled ones. The 70S ribosomes were isolated via zonal centrifugation (Beckman Ti-15; 6–40% sucrose gradient made in $H_{20}M_6K_{30}SH_4$, 5,000–8,000 A_{260} units per run, 16 h at 21,000 rpm). The 70S containing fractions were collected, and the ribosomes were pelleted (these 70S still contain tRNAs and mRNA fragments), resuspended in dissociation buffer $H_{20}M_1N_{200}SH_4$, and again subjected to a zonal run, but this time in $H_{20}M_1N_{200}SH_4$. Fractions with 30S and 50S subunits were collected and pelleted, resuspended in $H_{20}M_6K_{30}SH_4$, aliquotized, shock-frozen, and stored at −80 °C. From a fraction of the isolated 30S and 50S subunits, reassociated 70S ribosomes were prepared according to the method of Blaha et al. (33). All ribosomes and ribosomal subunits used in this study were derived from one and the same preparation, yielding several thousands A_{260} units of subunits and 70S ribosomes, which were stored in small aliquots at −80 °C. The A_{260} units of 70S, 50S, and 30S correspond to 24 pmol, 36 pmol, and 72 pmol, respectively.

Modified PURE System. The system was provided by one of the authors (T.U.), as well as the purified His-tagged IF1 and IF3. The expression in the PURE system was performed according to the method of Shimizu et al. (30), with the following modifications. The final concentration of ribosomes in the reaction was 0.5 μM. The amount of translation factors was accordingly reduced; our modified PURE system lacked IF1, IF3, or both factors if their presence was not indicated. T7 polymerase, as well as CTP and UTP, were omitted from the reaction mixture, and GTP and ATP were present at 2 mM each. The final Mg^{2+} concentration was decreased to 8.5 mM. Note that the free Mg^{2+} concentration in our PURE system was about 2.5 mM in the presence ATP and GTP (2 mM each), which binds about 1–1.5 mM Mg^{2+} per 1 mM NTP (31). This ionic milieu is near to the in vivo conditions (32).

Additional methods are provided in *SI Appendix*.

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SI Appendix

70S-Scanning Initiation is a Novel and Frequent Initiation Mode of Ribosomal Translation in Bacteria

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Introduction

Additional information about lmRNAs: According to the definition of lmRNAs (AUG codon within the first 5 nucleotides at the 5'-end of the lmRNA) three lmRNAs have been identified in the in the *E. coli* genome, *viz*. *racR*, *ymfK* and *rhlB* (1); only RhlB, an ATP-dependent RNA helicase, was reported to be essential for viability in some genetic backgrounds (2). lmRNAs can be formed by the stress-induced toxin-antitoxin module *mazEF* (3). In many archaeal prokaryotes the majority of mRNAs is leaderless (4).

Further evidence of a 70S-type of initiation: Translational coupling of ribosomal-protein synthesis means that a blockage of usually the first cistron also blocks translation of all following cistrons. Translation of the second cistron depends on that of the first one and requires an SD sequence (5). It was thought that translation of cistron A opens the initiation site of the following cistron B, which is hidden in a secondary structure (6); the assumption was that one and the same 70S ribosome translates all cistrons of an mRNA thus importantly contributing to the stoichiometric synthesis of ribosomal proteins, although transcriptional polarity and downstream degradation could counteract translational coupling.

The preceding argument is backed up by an analysis of the length of intercistronic distances of *E. coli* mRNAs: On average we find 3.3 cistrons per polycistronic mRNA (**Figure S1A**); more than 75% of the intercistronic distances are shorter than 30 nt (**Figure S1B**). This is even more pronounced, when we consider the mRNAs derived from the two largest operons S10 and Spc, which code for 11 and 12 cistrons (mainly ribosomal proteins) with an average intercistronic length of 12.2 and 14 nt, respectively. Since both 30S subunits and 70S ribosomes cover 16-18 nt downstream and 18-20 nt upstream of the P-site codon (7), a terminating 70S ribosome will clash with a simultaneously initiating 30S subunit on the following cistron. If a 70S ribosome always must leave the mRNA before a 30S subunit can initiate, it is conceivable that secondary structures hiding the initiation site could form before the 30S binds, thus compromising the stoichiometric synthesis of the ribosomal proteins. Here again a scanning 70S ribosome/30S subunit would solve the problem. Recently, it could be demonstrated that *E. coli* cells containing 70S ribosomes with tethered subunits (and thus not able to dissociate) were viable (8).

Results

Ribosomal binding targets of IF1 and IF3

To identify the ribosomal particles, which bind IF1 and IF3 in the bacterial cell, we prepared *E. coli* lysates under near *in vivo* ionic conditions and analyzed the ribosomal profile of a sucrose gradient by Western blots using antibodies against IF3 and IF1 (**Figures S2A and B, respectively**). The two initiation factors bound to ribosomal particles show conspicuously distinct patterns. Most of IF3 was bound to ribosomal particles, about 50% was found on 30S subunits, and to our surprise about 25% on 70S ribosomes and disomes (polysomes). The majority of IF1 was present in the supernatant and might have been lost from ribosomal particles during centrifugation, but we reproducibly observed that not more than 30% of the bound IF1 was found on 30S subunits, the remaining were associated with 70S ribosomes and even with polysomes. 30SIC in the polysome fraction comparable to the “halfmeres” occasionally seen in sucrose-gradient profiles of eukaryotic lysate, when initiation is hampered or in the presence of the antibiotic cycloheximide (9), are not known in prokaryotes. Therefore, these observations cannot be easily reconciled with the generally accepted view that IF1 and IF3 leave the 30S initiation complex upon

50S association. Obviously, the functional horizon of these initiation factors is wider than anticipated, and might include initiation on 70S ribosomes.

Materials and methods

Bioinformatic Analyses

Intercistronic distances: Intercistronic distances were taken from the annotation of operons provided by the RegulonDB resource, version 6.7 (10). In cases of overlapping transcription units we selected the longest contiguous one. RNA genes and transposons were removed from the analysis.

Length and folding energies of 5'-UTRs: A comprehensive screen of transcription start sites was conducted by (11). We retrieved their measurements, which employed a Rapid Amplification of 5' complementary DNA ends (5' RACE) protocol, from RegulonDB (10). The most upstream transcription start site was selected for genes found with multiple transcription-start sites. Folding energies were calculated with UNAFold, Version 3.5 with standard parameters for RNAs at 37 °C (12).

The presence of IF1 and IF3 on ribosomal particles in sucrose-gradient profiles of S-30 extracts from *E. coli* cells (Figures S2A and B)

E. coli CAN20-12E cells were grown in LB medium at 37 °C to an OD_{600} = 0.5. Cells were fast cooled in pre-chilled bottles containing ice (100 g of ice / 100 ml of culture) and pelleted at 5,000 rpm / 10 min / 4°C. Pellets were resuspended in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ containing lysozyme (f.c. 0.4 mg/ml) and shock frozen in liquid nitrogen. Samples were thawed on ice in the cold room and centrifuged at 10,000 rpm for 5 min to separate supernatant from cell debris. 10 AU_{260} were loaded onto a 10-30% sucrose gradient prepared in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. Centrifugation was carried out at 18,000 rpm for 18 h using an SW40 rotor. The gradient was pumped out from bottom to top and the A_{260} was measured to obtain the polysome profile.

Proteins in the collected fractions of the gradient were TCA precipitated and analyzed by SDS-PAGE and immunoblot using IF1 and IF3 specific polyclonal antibodies from rabbit with secondary antibodies from goat (ECL anti-rabbit IgG HRP-linked F(ab')2 Fragment from goat; GE Healthcare). The bands were quantified densitometrically using the software ImageQuant. After Western blotting the pmol

amounts were calculated by means of the pixel numbers of reference bands in the same gel corresponding to known amounts of purified IF1 and IF3.

Sucrose gradients of 70S ribosomes and ribosomal subunits (Figure S4)

20 pmol of re-associated 70S ribosomes or 30S plus 50S subunits (20 pmol each) were incubated at 30 °C for 15, 30 or 120 min in PURE system reaction buffer ($H_{20}M_{8.5}K_{100}Spd_2Spm_{0.05}SH_4$) including 2 mM of ATP and GTP each. Samples were loaded on to a 10-30% sucrose gradient prepared in $H_{20}M_6K_{30}SH_4$, which does not promote association of isolated ribosomal subunits or dissociation of tightly coupled 70S ribosomes (13). The sucrose gradient was overlayed with 200 µl of a 5% sucrose cushion prepared in PURE system reaction buffer including ATP and GTP as mentioned before. Centrifugation was carried out at 24,000 rpm for 20 h at 4 °C using an SW40 rotor. The gradients were pumped out from bottom to top and the A_{260} was measured to obtain the ribosome profile.

Construction of the monocistronic mRNAs (Figures 2A and B)

(i) Construction of the leaderless mRNA coding for Renilla luciferase (lmRluc). The gene coding for Rluc was amplified from pRL-TK (Promega) using the primers lmRluc-fw and Rluc-rev and cloned *via Bgl*II and *Bam*HI into pET23c (Novagen). To prevent a possible initiation on an internal AUG (Met14) *via* 30S binding the Arg11 codon AGG as part of the preceding SD sequence was changed to CGU according to the QuickChange® Site-Directed Mutagenesis protocol (Stratagene) using the primers Rluc-mutSD-fw and Rluc-mutSD-rev. Oligonucleotides used: lmRluc-fw (5'TTTTTAGATCT**TAATACGACTCACTATA**GGATGACTTCGAAAGTTTATGATCC AG–3'), Rluc-rev (5'–AAAAAGGATCCTTATTGTTCATTTTTGAGAACTCGC–3'), Rluc-mutSD-fw (5'GATCCAGAACAACGTAAACGGATGATAACTG–3'), Rluc-mutSD-rev (5'CAGTTATCATCCGTTTACGTTGTTCTGGATC–3'). Endonuclease cleavage sites are underlined. The T7 promoter sequence is shown in bold.

(ii) Construction of the monocistronic mRNA coding for Firefly luciferase (Fluc-stal) enabling exclusively 30S binding initiation. The plasmid pET23c IR-F was obtained from pET23c R-IR-F (see next section) by PCR amplification and religation using the primers 5'-IR-Fluc-fw and T7-rev. Fusion of Fluc with the stalling sequence of SecM was performed by amplification of the gene coding for Fluc with the primers T7-up and Fluc-rev, using pET23c IR-F as template, and amplification of the 3'-end of SecM

with the primers SecM-stal and T7-term from an already existing plasmid. The PCR products were phosphorylated followed by digestion with BglII and HindIII, respectively, and ligated with the corresponding prepared vector pET23c. Oligonucleotides used: 5'-IR-Fluc-fw (5'GCAGGATCGAGCGCAGACTG–3'), T7-rev (5'CTATAGTGAGTCGTATTAAGATCTCGG–3'), T7-up (5'–ACGCTGCCCGAGATCTCGATCC–3'), Fluc-rev (5'–CACGGCGATCTTTCCGCCCTTCTTG–3'), SecM-stal (5'GGTGGTGGTGGTTCTCTGCTGACCCAGGAAGGCACG–3'), T7-term (5'GCTAGTTATTGCTCAGCGG–3'). Endonuclease cleavage sites are underlined.

Transcription of the genes was performed *in vitro* using T7 RiboMAX^{TM} Express (Promega) with pET23c lmRluc (linearized with *Bam*HI) or pET23c Fluc-stal (linearized with *Hind*III) as template. Transcribed RNAs were purified *via* gelfiltration and ethanol precipitation.

Translation of the luciferase mRNAs in the PURE system (Figures 1C, 2A and B) was carried out as follows: A reaction mixture containing *in vitro* transcribed mRNA (where indicated pre-hybridized with anti-IR (cagtctgcgctcgatcctgc), molar ratio to mRNA = 3), all amino acids, tRNAs, synthetases and factors except IF1 and IF3 was preincubated for 5 min at 30 °C and aliquotized. Where indicated IF1 and/or IF3 were added to a final concentration of 0.45 µM each. After addition of 70S ribosomes or 30S and 50S subunits protein synthesis was carried out for 2 h at 30 °C. Kinetics at 30 °C revealed a linear synthesis of luciferase for at least 3 h.

For the translation of lmRluc initiated non-enzymatically (Figure 2A, right panel) re-associated 70S ribosomes were preincubated with a two-fold excess of *in vitro* transcribed mRNA and a two-fold excess of f[^{3}H]Met-tRNA$_f^{Met}$ for 15 min at 30 °C in binding buffer $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ (20 mM Hepes-KOH pH 7.6 (adjusted at 0°C), 4.5 mM $Mg(Ac)_2$, 150 mM KAc, 4 mM β-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine) and then added to the reaction mix. 3 µl of reactions were taken for measuring luciferase activity by Dual-Glo Luciferase Assay System (Promega), the chemiluminescence was measured in a Centro Microplate-Luminometer LB 960 (Berthold Technologies).

Construction and tests of the bi-cistronic mRNAs *in vitro* (Figures 1, 2C and 3)

(i) Construction and expression in vitro *of the bi-cistronic mRNA coding for Renilla and firefly luciferase (Figure 1)*: Cloning experiments were performed following standard protocols (14). The genes coding for Renilla (Rluc) and firefly luciferase (Fluc) were amplified from pRL-TK and pGL3-Control (Promega) using the primers Rluc-fw and Rluc-rev or Fluc-fw and Fluc-rev, respectively. Primers Rluc-rev and Fluc-fw were 5'-phosphorylated prior to amplification. The two genes were ligated with each other leading to a bi-cistronic operon under control of a T7 promoter and cloned *via Bgl*II and *Eco*RI into pET23c (Novagen). The plasmid was named pET23c R-F. The intercistronic region (IR) was introduced as a double stranded oligonucleotide providing 5'-overhangs compatible to *Bam*HI and *Nco*I restriction sites *via* ligation into pET23c R-F, which was accordingly digested. Oligonucleotides used: Rluc-fw (5'GAAGATCT**TAATACGACTCACTATA**GGCCCCCCACCCCACCCCACCCCAGG AGAACTAATATGACTTCGAAAGTTTATGATCCAGAACAAAGG–3'), Rluc-rev (5'AAAAAGGATCCTTATTGTTCATTTTTGAGAACTCGC–3'), Fluc-fw (5'AAAAAAGGAGAACTACCATGGAAGACGCCAAAAAC–3'), Fluc-rev (5'TTTTTGAATTCTTACACGGCGATCTTTCCGC–3'), IR-sense (5'GATCCACCCCACCCCACCCCGCAGGATCGAGCGCAGACUGACCCCACCCCA CCCCACCCCAGGAGAACTAC–3'), IR-antisense (5'CATGGTAGTTCTCCTGGGGTGGGGTGGGGTGGGGTCAGTCTGCGCTCGATC CTGCGGGGTGGGGTGGGGTG–3'). Endonuclease cleavage sites are underlined. The T7 promoter sequence is shown in bold. Corresponding monocistronic reporter plasmids were obtained by PCR amplification and religation deleting either 'IR-F' or 'R-IR' from pET23c R-IR-F.

The operon coding for the two luciferases was expressed in a coupled transcription/translation system (RTS, Roche; Figure 1B) as described (15), except that each reaction volume of 10 µl contained 1 µl of 250 ng plasmid solutions (pET23c R or F or R-IR-F) and 1 µl of antisense oligoDNA: 14 pmol anti-Rluc (caaacaagcaccccaatcatggccgacaaa) or 70 pmol anti-IR (cagtctgcgctcgatcctgc) or 28 pmol anti-Fluc (ggaaacgaacaccacggtaggctgcgaaat). The anti-Rluc was complementary to the region 346 to 375 of the Rluc mRNA, which had a total length of 936 nt. After 20 min at 30 °C, 1 µl of reaction was taken for measuring dual

luciferase activity by Dual-Glo Luciferase Assay System (Promega), the chemiluminescence was measured in a Centro Microplate-Luminometer LB 960 (Berthold Technologies). The stability of the mRNA/anti-IR hybrid was calculated to -32 kcal/mol according to (16) in the presence of 1 M NaCl and estimated to have a value of -28 kcal/mol at 30 °C and 150 mM monovalent salts as in our *in vitro* systems according to http://dinamelt.bioinfo.rpi.edu/twostate.php. The RNA stability was checked by Northern-blotting using [^{32}P]-labeled anti-Fluc directed against bi-cistronic luciferase mRNA. The RTS mixture of 40 μl contained non-labelled 56 pmol anti-Rluc (against the first cistron), and during incubation at 30 °C 10 μl were withdrawn at various times (5, 10, 20 min), phenolized and followed by an ethanol precipitation. Each pellet was resuspended in 10 μl water, 4 μl of which was loaded on an agarose gel. For identifying the luciferase mRNA [^{32}P]-labeled anti-Fluc (hybridized against the second cistron) was used.

The background and 100% values in the experiment shown in Figure 1B (RTS) are reported in the legend of **Table S1**.

The background and 100% values (background subtracted) in the experiment shown in Figure 1C (PURE system) were as follows. Rluc / Fluc values In the presence of 70S: 100% corresponding to 123,880 / 90,810 relative light units (RLU) and background values to 1,310 / 1,590; in the presence of 30S+50S: 100% values 470,040 / 150,360 RLU and background values 1,450 / 1,500 RLU.

(ii) *Bi-cistronic mRNA with a short ORF1 followed by an ORF2 coding for GFP for expression in the PURE system (Figure 2C)*: The gene coding for GFP was amplified using the primers -1nt-fw or +39nt-fw in combination with GFP-rev leading to a short leaderless open reading frame upstream of GFP overlapping by one nucleotide or with a 39 nucleotide long spacer. A T7 promoter sequence was attached to the constructs in a second amplification step using the primers T7-fw and GFP-rev. The resulting fragments were cloned via BglII and EcoRI into pET23c. Oligonucleotides used: -1nt-fw (5'GGATGAATGCTAAAATTGAACAACTGACTTCTATTAAGGAGTACTAATGAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCC–3'), +39nt-fw (5'GGATGAATGCTAAAATTGAACAACTGACTTCTATTAACCTGTACTAAATAAAATAAAATAAAATAAACTTCTATTAAGGAGTACTAATGAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTG–3'), GFP-rev (5'CCCGAATTCTTATTTGTATAGTTCATCCATGCCATGTGTAATCC–3'), T7-fw

(5'CCCCAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGATGAATGCTA AAATTGAACAACTGAC–3'). Endonuclease cleavage sites are underlined. The T7 promoter sequence is shown in bold. The sequence of the leaderless open reading frame is indicated in italic.

Transcription of the genes was performed *in vitro* using T7 RiboMAXTM Express (Promega) with the newly constructed plasmids (linearized with EcoRI) as template. Transcribed RNAs were purified via gelfiltration and ethanol precipitation.

8 pmol of re-associated 70S ribosomes or 30S subunits were preincubated with a four-fold excess of *in vitro* transcribed mRNA and a two-fold excess of f[^{3}H]Met-tRNA $^{Met}_{f}$ for 15 min at 30 °C in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ to allow formation of initiation complexes. Aliquots of 8 µl were withdrawn and added to 12 µl preincubated (5 min / 30 °C) reaction mix lacking IF1 and IF3. Where indicated IF1 and/or IF3 were added to a final concentration of 0.6 µM and protein synthesis was carried out for 1 h at 30 °C. In case of 30S-initiation complexes equivalent amounts of 50S subunits were added to the reaction. For quantification of the reporter protein (in our case GFP) the incorporation of [^{35}S]-Met was measured. Samples were separated *via* SDS-PAGE and the gels were exposed on phosphoimager plates for 14-16 h. The amount of radiolabeled product was quantified using the Image Quant software. The background and 100% values (background subtracted) in the experiments shown in Figure 2C were as follows. –1 nt / +39 nt spacer: 100% (1.0) corresponding to $1.05x10^5$ / $1.12x10^5$ pixel and background values to $6.3x10^3$ / $6.6x10^3$ pixel, respectively.

(iii) A minimal bi-cistronic mRNA consisting of an ORF1 fragment with a termination site (UUC-UAA) followed by an ORF2 fragment with an initiation site containing an SD sequence and an AUG-AAA for toeprinting experiments (Figure 3): The following mRNAs were used: mRNA 1:

gggacacacacacacacaca***uucuaa***cacacacac**<u>aggag</u>**accacc***augaaa*cacacacacacacac acacacacacacacag<u>cacaucagauaguaacgag</u>; it contains a Shine-Dalgarno sequence (bold underlined) and codes for Fstop and MK (bold italic) and primer binding site (underlined).

mRNA 2:

gggacacacacacacacaca***uucuaa***cacacacacacacacaccaccaccaccacacacacacacaca cacacacacacacacg<u>cacaucagauaguaacgag</u>.

mRNA 3:

gggacacac**aggag**accacc***augaaa***cacacacacacacacaccaccaccacacacacacacacac acacacacacacacacacgcacaucagauaguaacgag.

mRNA 4 (Figure S7B):

gggacacacacacacacaca***uucuaa***cacacacacacaccaccacc***augaaa***cacacacacacaca cacacacacacacagcacaucagauaguaacgag.

The mRNAs were annealed to a [^{32}P]-5'-end-labeled primer (underlined) as described in (17) and then used to program ribosome complexes. Only in Figure S7B Cy5-labeled primer (Jena Bioscience GmbH) was used. Briefly, 80 pmol 70S or X-70S were incubated with 20 pmol mRNA 1-4 annealed with the primer and 400 pmol $tRNA^{Phe}$ in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. Aliquots of the reaction mixture with 5 pmol 70S were incubated, and when indicated in Figures 3 and S7, in the presence of 5 pmol fMet-$tRNA_f^{Met}$ or $tRNA^{His}$, 5 pmol mRNA 2 or 3, 4 pmol EF-G plus 1.5 mM GTP, and 5 pmol RRF in $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$. The factor mix was preincubated 5 min at 37 °C, the total reaction mix was incubated at 37 °C for 10 min. Toeprinting reaction and resolving of product at sequence gels followed (18).

Construction of the crosslinked 70S ribosomes (X-70S; Figure 3A)

To create ribosomes unable to dissociate, the compound dimethyl-suberimidate (DMS) was used according to Moll et. al.(19) with modifications. A typical reaction producing cross-linked ribosomes was performed in $H_{20}M_6K_{30}SH_4$. The buffer was supplemented with DMS to 10 mM final concentration and the pH adjusted to 8.5 – 9. Ribosomes were diluted in this buffer yielding a concentration of 0.3 µM. Cross-linking was allowed for 2.5 hours at 30 °C and stopped by the addition of 0.1 volume of 1 M Tris-HCl, pH 6.7. Next, the sample was dialyzed against 100 volumes of $Tris_{10}N_{60}M_{10}SH_4$ for 45 min. To purify the non-dissociable 70S fraction from not cross-linked ribosomes, the sample was placed on a 10-30 % sucrose gradient in $H_{20}M_1N_{60}SH_4$ and centrifuged. The dissociation resistant fraction was collected, pelleted and dissolved in $H_{20}M_6N_{30}SH_4$. A_{260} was measured, the sample aliquotized and stored at -80 °C. An analytical sucrose gradient centrifugation (SW 60 rotor at 45,000 rpm for 2 h 15 min) was performed under low magnesium concentration (1 mM), where native and cross-linked ribosomes were compared in their dissociation behavior.

Functional test of the crosslinked 70S ribosomes (X-70S; Figure 3A)

Activity test of X-70S ribosomes via poly(Phe) synthesis: Under standard conditions (15 µl of reaction mix) the assay was performed in the buffer system $H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$. The reaction was carried out by incubating 25 µg of poly(U), 0.33 µM of 70S ribosomes (or 0.6 – 1 µM of ribosomes) as well as 100 µM [^{14}C]Phe (10 dpm/pmol), 3 mM ATP, 1.5 mM GTP, 5 mM acetyl-phosphate, 2 µM $tRNA^{Phe}$ and an optimal amount of S-100 preparation. The reaction mix was kept at 37 °C for 30 min. Aliquots were withdrawn and the synthesis stopped by addition of 2 ml 10% TCA and a drop of 1% BSA. The mix was incubated at 90 °C for 15 min, the samples cooled to 0 °C and filtered through glass filters. The filters were washed 3-times with 5% TCA and twice with 5 ml of diethylether/ethanol (1:1). The radioactivity was measured in a liquid scintillation counter. The toeprinting results indicated an active fraction of 60% of the X-70S as compared to non-modified ribosomes, kinetics of Phe incorporation revealed that the X-70S were about two- to three-times slower than wildtype ribosomes. From these data we estimated that the Phe incorporation rate of X-70S was 50 to 80% of that of wildtype ribosomes.

Estimation of the chasing effects with mRNAs 2 and 3 (lanes 5, 6, 8 and 9 in Figure 3B)

In Figure S5 we show the complete toeprinting gel of Figure 3A; the molar ratio of (mRNA 1) to ribosomes was 0.25:1. The intensities of the bands of the 1-UUC signal (lanes 1 and 3) are mainly caused by the ribosome bound mRNA, all the bands above the 1-UUC signal until the end of the gel are due to non-bound mRNA. From both scanning values we calculate the bound fraction of mRNA 1 to (25±5)% corresponding to <7.5% of the ribosomes carrying mRNA 1 (the bands below the 1-UUC signal - with the exception of the free primer at the bottom of the gel - are distributed between bound and non-bound mRNA 1 with the same fraction ratio). The same result was obtained from lane 1 of Figure 3B. A direct measurement of the ribosome-bound mRNA in the presence of a ten-molar excess of an [^{32}P]-labeled mRNA over ribosomes demonstrated that 60% of the ribosomes carried an mRNA (20), also in ref. 17 the mRNA did not contain an SD motif as the ORF 1 from mRNA 1, the experiment was performed under corresponding conditions. Interpolation to one molar excess of mRNAs 2 or 3 over ribosomes corresponding to lanes 5, 6, 8 and 9 of Figure 3B indicates that not more than 30% of the ribosomes carry an

mRNA, leaving 70% of the mRNAs 2 or 3 for chasing. If the 70S bound to ORF 1 of mRNA 1 had to dissociate in order to reach the 1-AUG position on mRNA1, the 1-AUG signal would be reduced about 10-fold.

Construction of the *E. coli* strain Ec(IF1-)/pAraIF1 and expression of luciferase mRNAs *in vivo* (Figures 4 and S9)

We started with an *E. coli* strain deprived of the *infA* gene, PMF1A/pRK04, a kind gift of Dr. Leif Isaksson (University of Stockholm), that has been described elsewhere (21). The plasmid contained an ampicillin-resistance marker and the IF1 gene after the natural promoter; for the sake of simplicity we call this strain Ec(IF1-)/pIF1. We wanted to replace the plasmid by one that contains IF1 after the inducible AraB-promoter. To this end the *infA* gene was cloned under the control of the AraB-promoter into the vector pSSC12-C using *KpnI* and *XhoI* restriction sites; this plasmid carried genes mediating resistance against kanamycin (Kan) and chloramphenicol (Cm). The plasmid was then transformed into Ec(IF1-)/pIF1 and grown on LB-plates containing the selective compounds Kan, Cm and arabinose (Ara) yielding a strain with two plasmids. Hereafter the strain was inoculated in M9-medium and grown for 6 days at 37 °C. Several dilutions of the cell suspension were prepared and replica plating performed on agar containing LB/Kan/Cm/Ara or LB/ampicillin (Amp)/glucose (Glu). Several clones that had shown growth on both media were picked and used for another round of selection to force rejection of the original plasmid from the cell. These clones were replicated twice on LB/Kan/Cm/Ara. Subsequently we carried on with a small sub-set of colonies that was grown in M9/Kan/Cm/Ara medium for 12 days at 37 °C. During this period several samples were drawn, diluted and replica plated again until growth was no longer observed on M9/Glu/Amp plates. Clones with such behavior were termed Ec(IF1-)/pAraIF1 and used for some experiments in this study. PCR controls demonstrated that the strain contained only the desired plasmid.

Expression in vivo of the monocistronic mRNA-unstr and mRNA-str and the corresponding bi-cistronic luciferase mRNAs (Figure 4): The gene coding for GFP was mounted behind an either unstructured 5'-UTR (mRNA-unstr) or a 5'-UTR with a strong secondary structure of -25.1 kcal/mol at 25 °C (mRNA-str). The PCR fragments were cloned using the restriction endonucleases *Nde*I and *Sal*I into the vector pFLAG-CTS (Sigma Aldrich), in which the provided origin of replication

(pBR322) was replaced against the origin p15A to enable the compatibility of the vector in the strain Ec(IF1-)/pAraIF1.

For expression of GFP E. coli cells (wildtype MG1655 and Ec(IF1-)/pAraIF1 containing either the plasmid coding for mRNA-str or mRNA-unstr described in Figure 4A) were grown overnight at 37 °C in LB medium (arabinose 0.2%, ampicillin 25 mg/L, the Ec(IF1-)/pAraIF1 strain contained in addition kanamycin 15 mg/L, and chloramphenicol 10 mg/L). Cells were washed once with LB medium without arabinose and diluted with LB medium (glucose 0.5% and ampicillin 25 mg/L for all, chloramphenicol 10 mg/L and kanamycin 15 mg/L for Ec(IF1-)/pAraIF1 only) to achieve an OD_{600} shown below for time 0 and incubated at 30 °C. IPTG (1 mM) was added at the OD_{600} shown below, incubation was continued for 2 hours at 25 °C in order to stabilize the secondary structure of mRNA-str. The cells were harvested, washed with buffer $H_{20}Mg_6K_{30}$ resuspended in the same buffer to obtain 1.5 g per ml and broken with the Microfluidizer (Model M-110L; Microfluidics Corporation, Newton MA; two strokes with 18k psi at 4 °C). A low speed centrifugation yielded S-30 lysate. Aliquots of the S-30 were mixed with SDS-sample buffer (60 mM Tris•HCl, pH 6.8, 200 mM β-mercaptoethanol, 10% glycerol, 2% SDS and 0.05% bromophenol blue) and proteins were denatured during an incubation at 95 °C for 5 minutes and subjected to a PAGE. A 17.5% polyacrylamide gel was used for the determination of the relative amounts of IF1 and IF3, a 12.5% polyacrylamide gel for determination of GFP. In the Western blots antibodies against IF1, IF3, GFP and ribosomal protein S7 were used (see Figure S10; the band corresponding to S7 was used for normalization the input per lane). All four samples were loaded onto a 12.5% polyacrylamide gel for the analysis of GFP and S7, and onto a 17.5% polyacrylamide gel for IF1 and IF3. Western blot for GFP and S7: Both GFP and S7 have similar molecular weights (GFP 22 kDa, S7 20.5 kDa). After transfer to the membrane anti-GFP was first added, and a picture was taken from the GFP bands. Then the membrane was washed 3 times for 15 min with PBS-tween buffer, before anti-S7 was added. Western blot for IF1 and IF3: After transfer the membrane was cut and the section containing IF1 was exposed to anti-IF1 and that with IF3 exposed to anti-IF3 (IF1 8 kDa, IF3 20 kDa; polyclonal rabbit antibodies; secondary antibodies ECL anti-rabbit IgG HRP-linked F(ab')2 Fragment (from goat; GE Healthcare, a former Amersham Bioscience; NA9340-1ML). The reason for this procedure was that anti-IF3 reacted with a side-band near to the IF1 band.

After subtracting the background, 100% values corresponded to $3.68x10^5$ pixel/$0.89x10^5$ pixel for IF1/IF3 (WT, mRNA-unstr) and $3.4x10^5$ pixel/$0.85x10^5$ pixel for IF1/IF3 (WT, mRNA-str), and $1.55x10^5$ pixel for GFP.

OD_{600} of the four cultures at different times

Time (h)	WT-unstr (OD_{600})	WT-str (OD_{600})	Time (h)	Ec(IF1-)/ pAraIF1-unstr (OD_{600})	Ec(IF1-)/ pAraIF1-str (OD_{600})
0	0.005	0.005	0	0.04	0.04
4 (IPTG)	0.206	0.176	6 (IPTG)	0.300	0.345
6	0.761	0.485	8	0.386	0.414

WT-unstr and WT-str are *E. coli* wildtype strains containing either the plasmid coding for mRNA-unstr or mRNA-str, respectively; similarly the strains Ec(IF-)/pAraIF1-unstr or Ec(IF-)/pAraIF1-str contain the plasmid for mRNA-unstr and mRNA-str, respectively.

Renilla and Firefly luciferase genes (Figure 4B) were PCR amplified from pET23c R-IR-F and cloned into the vector pFLAG-CTS p15A ori using the restriction sites *Nde*I and *Eco*RI. The intercistronic region (IR) was cut out by *Bam*HI and *Nco*I and the residual construct annealed with and ligated to DNA oligos (second-sense and second-antisense), which could form the required secondary structure. second-sense (5'gatccaaacaaaacaaaacggggacccttgcggggtccccaaaacaaaaggagaactac-3'), second-antisense (5'catggtagttctccttttgttttggggaccccgcaaggggtccccgttttgttttgtttg-3').

Wildtype and Ec(IF1-)/pAraIF1 were transformed with the plasmid containing bi-cistronic luciferase operon with or without the secondary structure in the intercistronic region shown in Figure 4B. The cells were grown overnight, pelleted and washed and resuspend as described for the monocistronic mRNAs yielding the OD_{600} indicated below. Growth continued at 30 °C and at the cell OD_{600} given below IPTG (3 mM) was added and incubated now at 25 °C to stabilize the secondary structure of the corresponding bi-cistronic mRNA *in vivo* for about one generation. Cells were harvested at the OD_{600} shown below and broken in the presence of lysozyme (0.4 mg/ml) and DNase (RNase free) *via* two freeze-and-thaw cycles. The S-30

supernatant was analyzed for luciferase activity (see above) and treated for Western analyses (IF1, IF3, S7) as described for the supernatant containing the monocistronic mRNAs (Figure 4A).

The OD_{600} of the four cultures at different times.

	Time (h)	WT-str	WT-unstr	Time (h)	Ec (IF1-)/ pAraIF1-str	Ec (IF1-)/ pAraIF1-unstr
OD_{600}	0	0.04	0.04	0	0.04	0.04
	2 (IPTG)	0.274	0.328	3 (IPTG)	0.208	0.145
	3	0.586	0.591	9	0.416	0.284

IF1 effects on ternary complex binding (Figure S8)

5 pmol of 70S ribosomes were incubated in the presence and absence of 10 pmol $tRNA^{Phe}$ and 50 pmol IF1 as well as 20 µg poly(U) mRNA for 5 min at 37 °C (volume 12.5 µl); such a $tRNA^{Phe}$ excess fills mainly the P site (22). [^{14}C]Phe-$tRNA^{Phe}$ (2.5 pmol) was incubated at 37 °C for 5 min with EF-Tu (7.5 pmol) in the presence of PK (1.5 µg), PEP (5 mM) and GTP (2 mM) (volume 12.5 µl) before addition to the ribosome aliquot. Binding was allowed for up to 60 sec at 25 °C. The reaction in the aliquots withdrawn at various times was stopped by adding 2 ml of cold $H_{20}M_{4.5}K_{150}SH_4Spd_2Spm_{0.05}$ buffer and the binding was assessed by nitrocellulose filtration.

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Table S1

Relative activities of the Renilla (Rluc) and firefly luciferase (Fluc) in the RTS system (Figure 1B).

	Rluc %		Fluc %	
	mono	bi	mono	bi
anti-Rluc	38±1.9	34±1.3	81±6.7	27±1.3
anti-IR	82±3.5	71±2.9	96±0.6	33±0.5
anti-Fluc	72±2.4	75±4.6	10±0.5	15±1.1

100% corresponds to 410.4 x 10^3 relative light units (RLU, Rluc) and 1884 x 10^3 RLU (Fluc), the backgrounds were 9.5 x 10^3 and 6.7 x 10^3 RLU, respectively. Mono, monocistronic; bi, bi-cistronic.

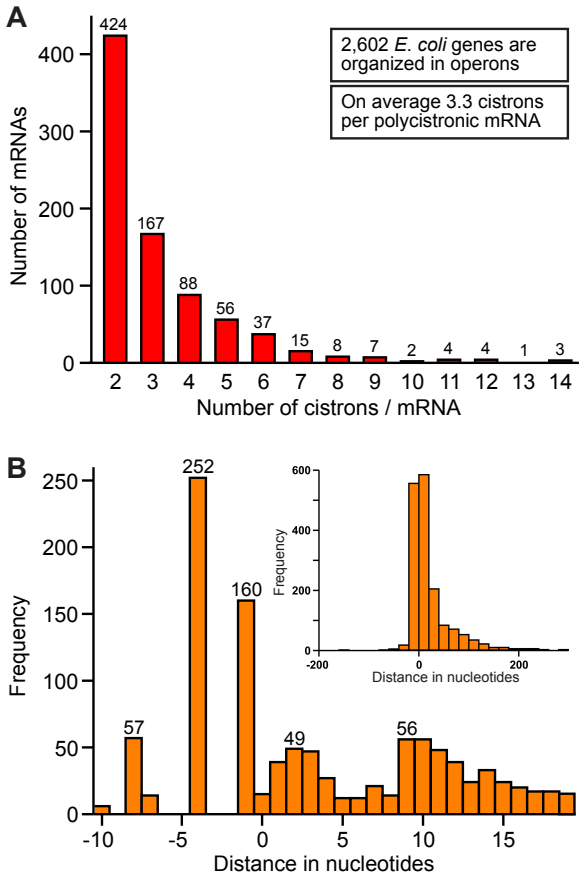

Figure S1: Polycistronic mRNAs from *E. coli.* A, frequency distribution of polycistronic mRNAs depending on the number of cistrons per mRNA. **B**, length of intercistronic distances in nucleotides (nt) from the first nucleotide following the stop codon until the nucleotide in front of the start AUG codon of cistron n+1; for example, intercistronic regions with a length of 10 nt exist 56 times in the *E. coli* genome. Negative numbers mean that the downstream cistron starts in the preceding decoding region. The insert shows the length distribution of the intercistronic regions (IR) up to a length of 300 nt.

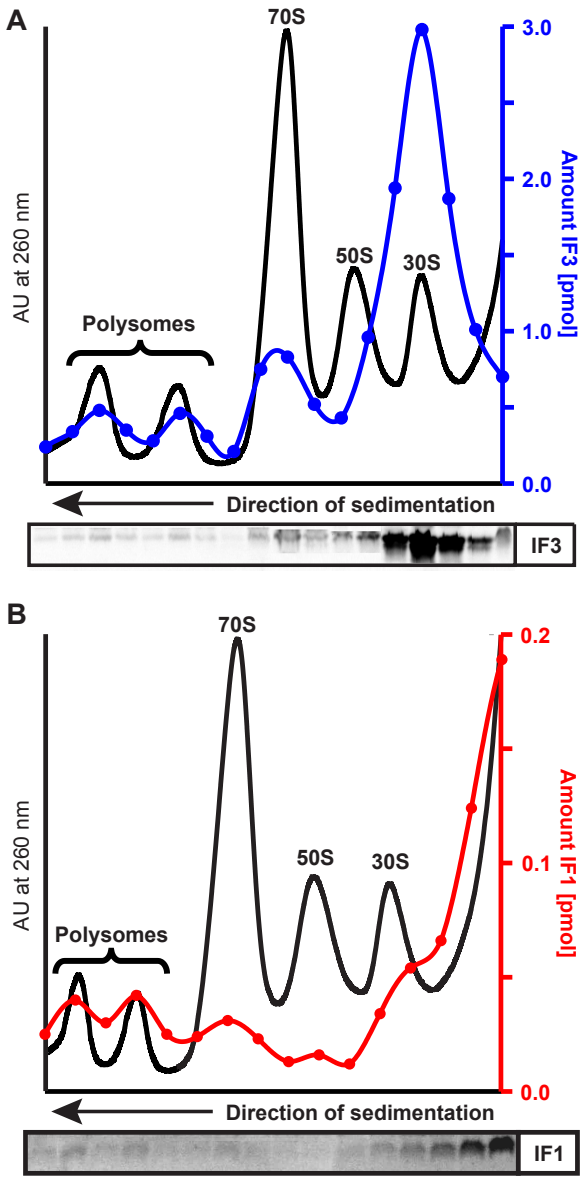

Figure S2: Sucrose gradients of lysates from *E. coli*. A, Western blots showing the presence of IF3 in a lysate A_{260} profile of a sucrose-gradient run, antibodies against S3 were taken as 30S marker. **B**, same as A but for IF1. The amounts of 30S subunits and 70S ribosomes in the peak fractions were estimated to be 4.6 and 3.1 pmol, respectively (with the help of analyses of the anti-S3 bands).

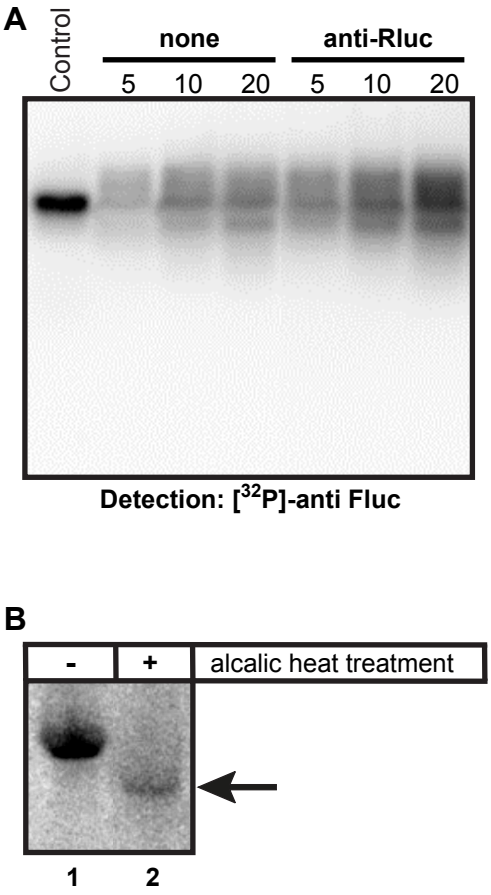

Figure S3: **Controls**. **A**, control for Figure 1B. Stability of the bi-cistronic mRNA transcribed in the RTS system is stable. The synthesized bi-cistronic mRNA coding for Renilla and firefly luciferase was analyzed by an antisense DNA specific for the firefly luciferase (anti-Fluc, second cistron). No degradation products of the mRNA were found during 20 min in the absence or presence of 14 pmol of anti-Rluc (first cistron). **B**, control for Figure 2B. The SecM fragment prevents recycling by stalling the ribosome that carries peptidyl-tRNA. Without alkalic treatment only a band from peptidyl-tRNA is seen in lane 1, with alkalic treatment a band from the peptide (arrow, lane 2).

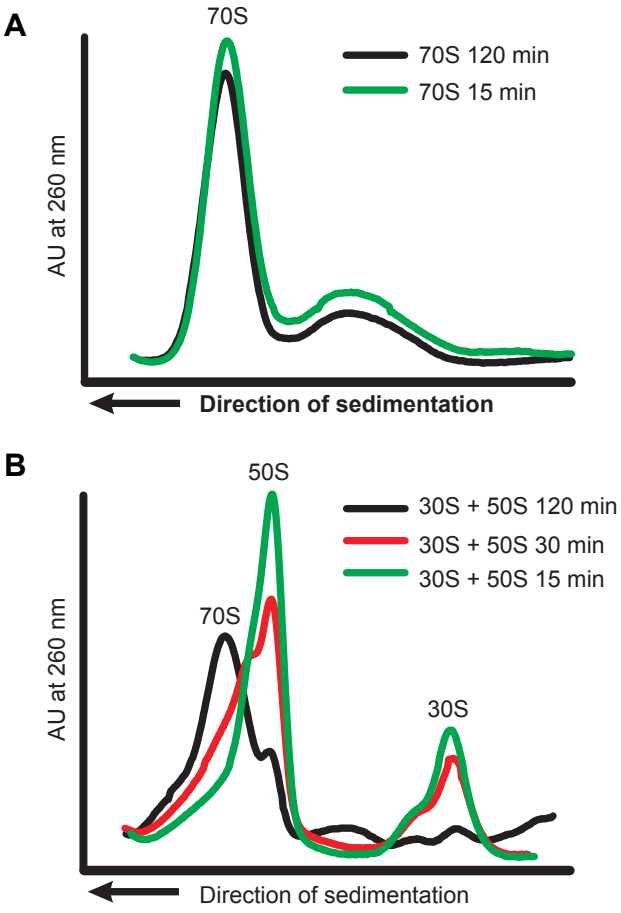

Figure S4: **Slow equilibrium between 70S ribosomes and its subunits under our PURE conditions**. Sucrose gradient profiles of **A**, re-associated 70S ribosomes, and **B**, ribosomal subunits under the ionic conditions of the PURE system including 2 mM ATP and 2 mM GTP. The particles were incubated for 15, 30 or 120 min at 30 °C before loading on a sucrose gradient (see Supporting Methods for further details; for 70S ribosomes (A) only the patterns observed after 15 and 120 min are shown). The 30S and 50S subunits used in the sucrose gradient (B) were from the same preparation batch that was used in the functional experiments shown in the other Figures.

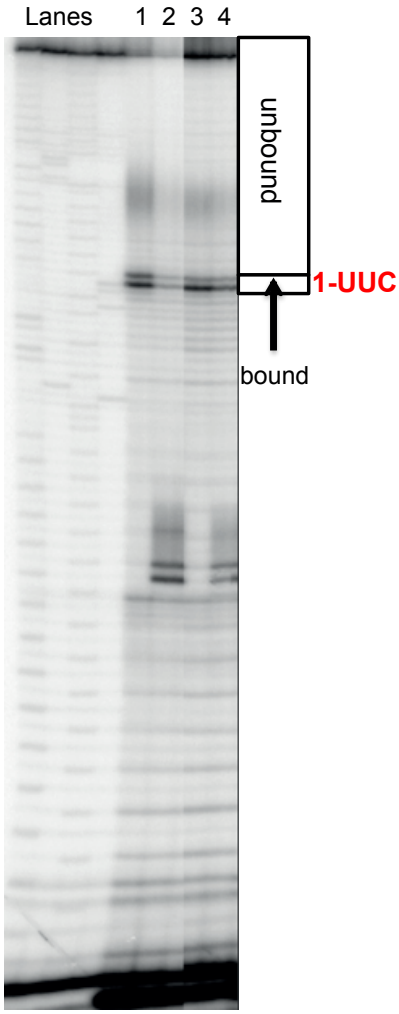

Figure S5: Estimation of the ribosome-bound and unbound fraction of mRNA 1. The complete gel of the Figure 3A, the lane numbers correspond to those in Figure 3A. The intensities of the 1-UUC bands of lanes 1 and 3 indicated by "bound" represent the bound mRNA 1, the band intensities of the region "unbound" the unbound mRNA 1. For details see SI Appendix-Materials and methods.

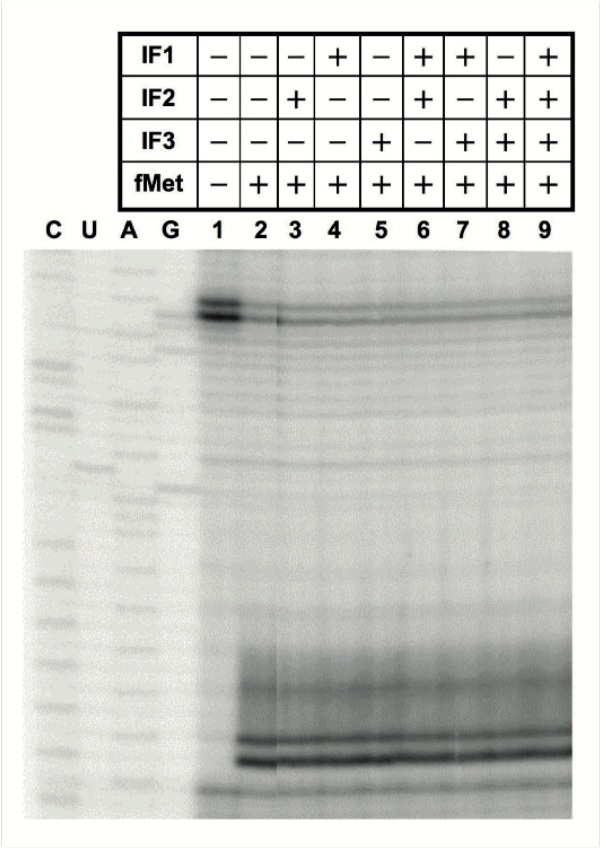

Figure S6: Effects of initiation factors on 70S scanning. The indicated initiation factors were added to the toeprinting assay shown in Figure 3A with 70S ribosomes and fMet-tRNA. The factors were added in a 1.5 molar excess over ribosomes, GTP concentration was 1.5 mM.

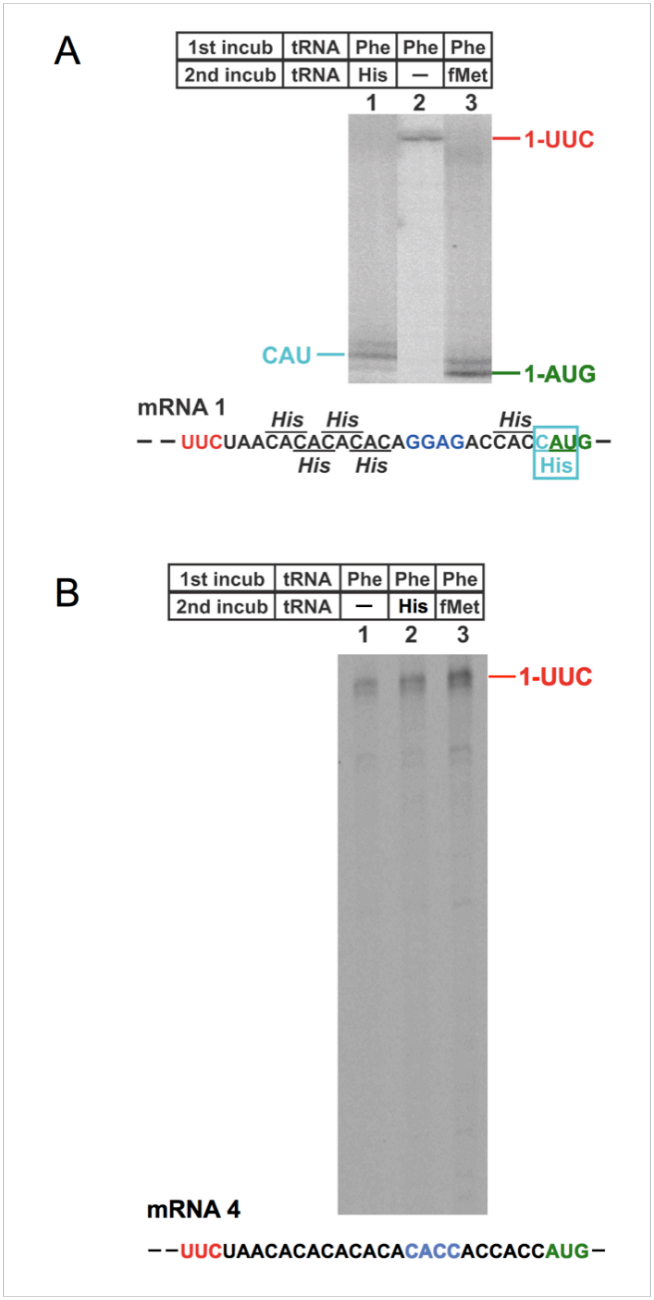

Figure S7: **Importance of the SD sequence for selecting the landing codon for the scanning 70S.** **A**, toeprinting assay after adding His-tRNA^{His} (lane 1) or fMet-tRNA (lane 3) to the post-termination complex (lane 2). **B**, without an SD sequence 70S ribosomes do not recognize the downstream initiation site, regardless whether one adds tRNA^{His} or fMet-tRNA. All lanes shown in A or B were derived from the same gel.

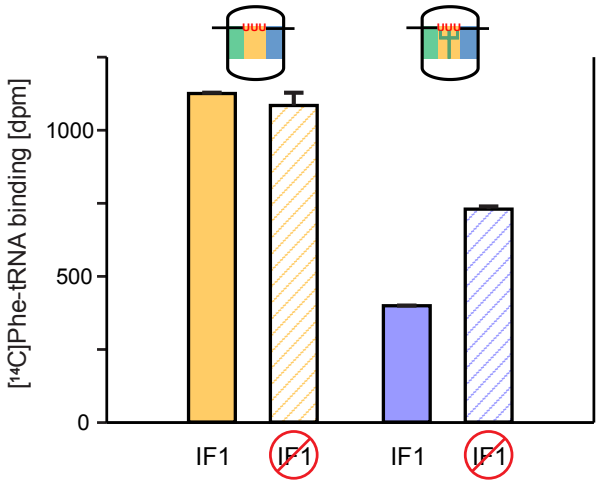

Figure S8: **IF1 effects on ternary-complex binding**. First two columns: adding the ternary complex Phe-tRNAPhe•EF-Tu•GTP to programmed 70S ribosomes. After EF-Tu dependent GTP cleavage Phe-tRNA slides into the P site at 37 °C in the absence of EF-G (yellow; (20)). Last two columns: the ternary complex Phe-tRNAPhe•EF-Tu•GTP is bound to the A site (blue) of programmed 70S ribosomes after filling the P site with tRNA^{Phe}.

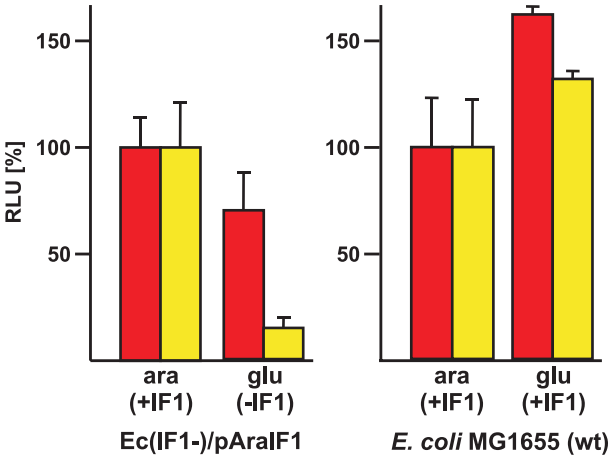

Figure S9: ***In vivo*** **expression of the bi-cistronic luciferase mRNA shown in Figure 1A.** Left panel, expression of Renilla and firefly luciferase (red and yellow columns, respectively) at normal and low IF1 amounts (+IF1 and –IF1, respectively; strain Ec(IF1$^-$)/pAraIF1). Right panel, control expression of the same mRNA in a wild type strain MG1655 containing a chromosomal IF1 gene.

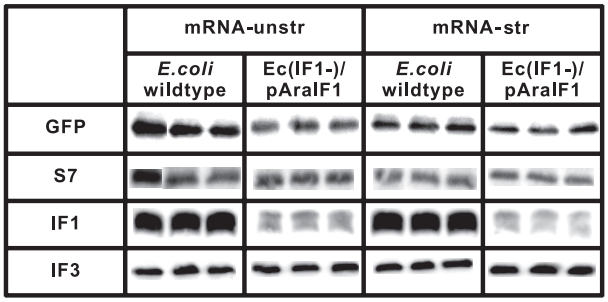

Figure S10: **Western blots of the S-30 lysates from *E. coli* wildtype and Ec(IF1$^-$) /pAraIF1 cells used for the quantification of GFP, IF1 and IF3 (Figure 4).** The expression level of IF1 did not seem to influence the expression of the ribosomal protein S7. Therefore we used the S7 band for normalizing the input. The GFP and S7 bands were derived from one and the same gel as were the bands of IF1 and IF3.

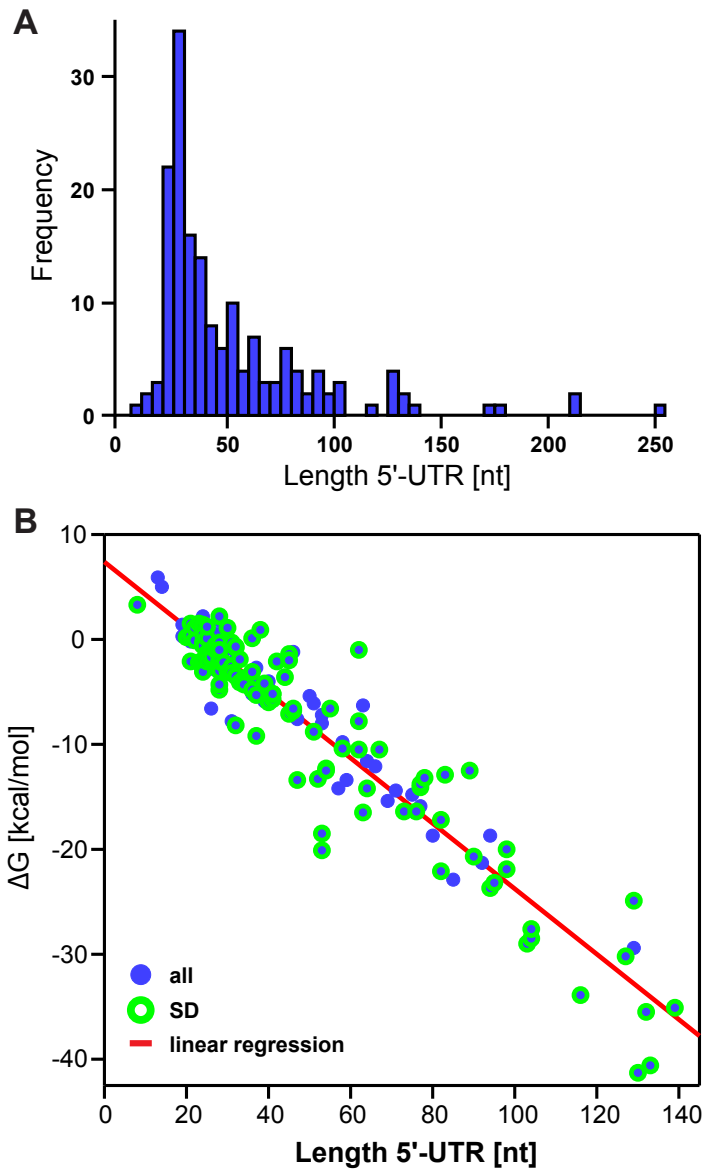

Figure S11: **Parameters of 5'-UTRs of mRNAs in *E. coli***. **A**, frequency distribution of 5'-UTRs depending on their length. **B**, folding energies of 5'-UTRs depending on their length. Dots surrounded by a green circle indicate the presence of an SD sequence in front of the initiation start codon.