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.

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, 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 ofImRNA can even occur in the absence of all IFs (19, 20). Additional information aboutImRNAs is provided in SI Appendix, Introduction.


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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 formation of the initiator Met-tRNA\textsubscript{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 Met 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 ImRNAs) is distinct for each mode.

Results

We first assessed the sucrose-density gradient A\textsubscript{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. 1A 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 of 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. S3A). Further controls 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. 1B, hatched columns and SI Appendix, Table S1).

Rluc (Fig. 1B, 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. 1B). Surprisingly, anti-Rluc provokes the same strong reduction of the second cistron Fluc (Fig. 1B, 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.

**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 reassocciated 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.
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. S4A). Ribosomal subunits did not associate within 15 min, and poorly after 30 min, whereas the majority associated after 120 min (SI Appendix, Fig. S4B). 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. 1C, Left, lanes 2 and 3, respectively). IF3-dependent expression was strongly stimulated by IF1 (Fig. 1C, 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. 1C, 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. 1B. The experiments in Fig. 1C indicate that 70S scanning depends on the presence of IF3.

Surprisingly high expression was observed with 30S plus 50S subunits and without anti-IR (Fig. 1C, 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. S4B). 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. 1C 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 IF3, 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. 2A 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. 2A, Middle). Furthermore, IF3 was thought to inhibit initiation of 70S ribosomes due to its 70S-association 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 Met-tRNA, it is known that a 70SIC complex can be formed nonenzymatically (i.e., mRNA, 70S ribosomes, and Met-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. 2A, 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. 2B 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. 1D). We prevented the formation of 70S runoff ribosomes, which would make the interpretation ambiguous, by fusing the Fluc gene to a sequence gene of the secM gene which was linked by a sequence coding for GlySer 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 [\(^{55}\)S]-labeled protein was exclusively present as peptidyl-tRNA (SI Appendix, Fig. S3B).

Fig. 2B shows that in the presence of the anti-5′-UTR, productive initiation occurs exclusively with free 30S plus 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
non-enzymatic IF3

Next, we rigorously test by three different approaches Left A and Left Lower in the P site (co-

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2. ORF = GFP

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Analyses in the PURE system. (Left 3′ A 2. ORF = GFP can be initiated by a 70S-scanning mode. )

left 3′ A 2. ORF = GFP

resulting in an SDS gel due to [35S]Met incorporation (Fig. 2C, 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. 2A, Right).

1. ORF SD 2. ORF = GFP

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 [35S]Met incorporation (Fig. 2C, lane 7). In the absence of both IF1 and IF3 or only IF3, very low amounts of GFP were found (Fig. 2C, 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. 2C, lanes 5 and 7, respectively). Because addition of 30S and 50S subunits did not show any activity (Fig. 2C, lane 9), these results indicate that one and the same 70S ribosome translates the first ORF and the following GFP cistron.

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. 2C, 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. 2A, 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. 2A, 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)].

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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. 2C, 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. 2C; hatched bars). Again, 30S plus 50S subunits were not able to translate the GFP cistron at all (Fig. 2C, 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-Phe 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. 3A; mRNA 1).

Decacylated 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. 3A, 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...
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. 3A, 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. 3A, Lower Right). Also, X-70S can form a posttermination complex, and, again, the addition of fMet-tRNA\(^{Met}\) triggers a downshift with an efficiency of about 50% (gel picture in Fig. 3A, 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. 3B) 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. 3B, Right, lane 1). After adding fMet-tRNA, the downstream signal 1-AUG is seen (Fig. 3B, lane 4) as in Fig. 3A. 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. 3B, 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 would 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. 3B, 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. 3B; 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 (Met-tRNA)\(^{\text{Met}}\) but also by Met-tRNA\(^{\text{Met}}\) and deacylated tRNA\(^{\text{Met}}\). In *SI Appendix, Fig. S7A,* we demonstrate that even an elongator His-tRNA\(^{\text{H}}\) (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\(^{\text{H}}\) (*SI Appendix, Fig. S7A, 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\(^{\text{G}}\) did not result in a CAU band at ORF2. Likewise, in the presence of fMet-tRNA, no 1-AUG band appeared (*SI Appendix, Fig. S7B*). Remarkably, the 1-UUC band was at least as strong as in the control lane 1 (*SI Appendix, Fig. S7B*) without triggering tRNAs (tRNA\(^{\text{H}}\) or Met-tRNA\(^{\text{H}}\)) 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\(^{\text{Phe}}\) 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. 3A). 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\(^{\text{Phe}}\) 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. 2C). 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\(^{\text{Phe}}\)•EF-Tu•GTP. Such pseudoinitiation occurs during standard poly(Phe) assays, where synthesis starts via binding of a Phe-tRNA\(^{\text{Phe}}\)•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\(^{\text{Phe}}\)•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. 1A. 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 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. 2B, 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. 4A). 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 ∆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. 1A and *SI Appendix*), in contrast to one of ~6 kcal/mol (Fig. 2C).

Plasmids carrying one of the two GFP constructs downstream of a tac promoter were transformed into both 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 amount of GFP and the EF-Tu/GTP ratio of IF1 were reduced by 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. 4A, 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. 4A, 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. 4A, 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
contained a secondary structure in the IR in front of Fluc (Fig. 4B). 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. 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 70S-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 interference with termination of the upstream Rluc or with the 30S-binding initiation of the downstream Fluc cistron. 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.

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**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 expression of both mRNAs in WT and mutant cells 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 [32P]anti-Fluc DNA and was found to be about the same in all cases.
Finally, scanning worked equally well using X-70S ribosomes (Fig. 3A, 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. S1B), but occurs preferentially downstream as predicted from in vivo evidence (21).

A surprising result was that fMet-tRNA<sup>Met</sup> can trigger 70S scanning in the absence of factors (Fig. 3A). There is a significant free pool of this charged tRNA in the cell, from which it can be selected by ribosomal particles containing IF2 (45). The formyl blockade of the α-amino group stabilizes the ester bond (46), an important prerequisite for the significant <i>t</i><sub>1/2</sub> of an aminoacylated tRNA in the cytosol. In contrast, 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. S7A) indicates a principal feature, necessary for the 70S-scanning process, that IF3 is essential for the 70S-scanning initiation (Fig. 2C).

IF1 binds preferentially to 70S ribosomes and polysomes, and less than 30% binds to 30S subunits (SI Appendix, Fig. S2B). Polysomes obviously contain a significant fraction of scanning/initiating 70S ribosomes. The strong effects of IF1 on 70S scanning (Figs. 1A, 2C, and 4A 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. S2C). The fact that IF3 is essential for the 70S-scanning initiation (Fig. 2C) 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 <i>Thermus thermophilus</i> (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

### Table: Initiation Types and IF1/IF3 Requirements

<table>
<thead>
<tr>
<th>Initiation Type</th>
<th>IF1/IF3 Requirement</th>
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<tbody>
<tr>
<td>30S Binding</td>
<td>IF1 or IF3 essential</td>
</tr>
<tr>
<td>70S Scanning</td>
<td>IF3 essential</td>
</tr>
<tr>
<td>ImRNA Initiation</td>
<td>IF3 essential</td>
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![Fig. 5](image-url)
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 ribosome 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 RFF binding, because the IF1 binding site at the decoding region actually overlaps with domain II of RFF and domain IV of EF-G (58, 59), and would therefore prevent RFF 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. 3A), 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 RFF 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 scanning 70S ribosomes susceptible to RFF and EF-G.

In our in vivo results (Fig. 4A and B) we 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 weak secondary structures (Figs. 2B and 4A 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. 2B; 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 can only initiate at the 5′-end of the mRNA. When 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. 1A, 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. 2B) 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 initiation at LmRNA, which could form productive initiation complexes when bound to the 5′-end of the LmRNA non-enzymatically in the presence of fMet-tRNA (Fig. 2A). 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 ΔG = −5 kcal/mol on average (SI Appendix, Fig. S11A and B). Because 70S-scanning ribosomes easily resolve secondary structures with ΔG = −6 kcal/mol (Fig. 2C), 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 is 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 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 H₂MgX₂SH₄₂₀ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 6 mM Mg(Ac)₂, 30 mM K(AC₄), 4 mM β-mercaptoethanol] was used. The standard buffer used for functional tests was H₂MgX₂K₄SH₄₂₀sp₅ₛ₇₅α₃ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 150 mM Mg(Ac)₂, 170 mM KAc, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine]. The dissociation buffer used was H₂MgX₂N₂SH₂₀ [20 mM Hepes-KOH (pH 7.6) at 0 °C, 1 mM MgCl₂, 200 mM KCl, 4 mM β-mercaptoethanol].

**Large-Scale Isolation of Ribosomal Subunits and Reassociated 70S Ribosomes (Tight Couples).** Ribosomes were isolated from the E. coli strain Can20/12E lacking fMet-tRNAs, including tRNAs (66). Up to several thousand A₆₀₀ units of hybridized ribosomes were harvested and the cells with H₂MgX₂SH₄₂₀. 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. Cytosol ribosomes (Tightly couples) were isolated by centrifugation (17 h at 40,000 × g, 4 °C) and resuspended in H₂MgX₂SH₄₂₀. Tightly coupled ribosomes withstand these conditions, whereas loosely coupled ribosomes dissociate into subunits. Tighty 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 H₂MgX₂SH₄₂₀, 5,000–8,000 A₆₀₀ units per run, 1 h at 21,000 rpm). The 70S containing fractions were collected, and the ribosomes were pelleted through an 18%/35% sucrose gradient (4 °C) and resuspended in H₂MgX₂SH₄₂₀. Fractions with 30S and 50S subunits were collected and pelleted, resuspended in H₂MgX₂SH₄₂₀, aliquotized, shock-frozen, and stored at −80 °C. From a fraction of the isolated 30S and 50S subunits, resolated 70S ribosomes were prepared according to the method of Blaha et al. (33). All ribosomes and ribosomal subunits used in the following experiments were derived from this preparation, yielding several thousands A₆₀₀ units of subunits and 70S ribosomes, which were stored in small aliquots at −80 °C. The A₆₀₀ 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 Mg²⁺ concentration was decreased to 8.5 mM. Note that the free Mg²⁺ 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 μM Mg²⁺ 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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